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Session I: Proteomics I

Montag, 03.03.2014: 10:50 - 12:30, OSZ H1

In-depth characterization of oxidatively carbonylated proteins in human plasma using targeted mass spectrometry

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Carbonylation is universally accepted as a biomarker of oxidative stress and thus one of the most widely studied non-enzymatic oxidative protein modifications. So far only a few methods are available to identify carbonylated proteins by mass spectrometry. Reproducible, in-depth identification of carbonylation sites in proteins remains a challenging task due to the very low modification degrees and the wide variety of possible carbonylation products. This is especially true for complex samples, such as human plasma samples. Here, we report a new strategy for a reproducible in-depth characterization of the carbonylated human plasma proteome, which relies on affinity chromatography, targeted mass spectrometry and specific bioinformatic tools.

Experimenteller Teil

Carbonylated proteins in plasma samples of lean individuals (control) and obese patients with and without type 2 diabetes mellitus (T2DM) were derivatized with *O*-(biotinylcarbazoylmethyl) hydroxylamine, digested with trypsin, enriched by biotin-avidin affinity chromatography, and analyzed by nanoUPLC-ESI-LTQ-Orbitrap-MS using data dependent acquisition (DDA). Feature detection (*m/z* ratio, charge state and retention time) and alignment across the initial LC-MS experiments were performed with the software tool SuperHirn. Inclusion lists containing potentially modified peptides were built (Prequips platform) for the following targeted LC-MS/MS analysis. The data set was analyzed by Sequest to reveal the carbonylated peptide sequence including the oxidized amino acid residue.

Ergebnisse und Diskussion

Using an MS-centric workflow a total of 158 unique carbonylated proteins were identified, of which 52 proteins were detected in plasma samples of all three groups, whereas 18 and 36 unique carbonylated proteins were detected only in plasma samples from obese (Ob) patients without and with T2DM (Ob/T2DM), respectively. A total of 53% of the modifications resulted from low molecular weight reactive (di-)carbonyls, 26% from Michael addition of α,β-unsaturated aldehydes and 21% from direct oxidation of the amino acid residues. The majority of carbonylated proteins originated from liver, plasma, platelet and endothelium. Carbonylated proteins identified in the plasma of Ob and Ob/T2DM patients were mainly involved in cell adhesion, signaling, angiogenesis and cytoskeletal remodeling thus linking them to common Ob- and Ob/T2DM-specific complications, such as endothelial dysfunction, cytoskeletal and extracellular matrix remodeling.

Neue Aspekte

Molecular identities of carbonylated proteins were characterized in human plasma of diabetic and non-diabetic obese patients using targeted mass spectrometry.

Combining high content with high throughput in proteomics using data independent acquisition and targeted data analysis

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Current mass spectrometric approaches can be broadly categorized as data dependent (DDA) (e.g. shotgun-MS) or targeted proteomics technologies (e.g. LC-MRM). Recently, a variant of targeted proteomics, based on data independent acquisition (DIA) or SWATH-MS [1], has been introduced that promises to combine the high content aspect of shotgun proteomics with the quantitative qualities of targeted proteomics. Conceptually, DIA in combination with targeted data analysis shares many aspects with MRM: Quantification is done on MS/MS signals and spectral libraries akin to MRM-assays are used.

These features, comprehensiveness of acquisition and targeted signal processing, promise to solve the missing data problem that severely limits the application of shotgun proteomics for protein quantification across multiple samples.

Experimenteller Teil

To generate a "Gold Standard Sample Set", known dilutions of three protein mixes were spiked into a constant HEK293 human cell lysate background. A comprehensive spectral library for the HEK293 cell lysate and spike in proteins was generated. The sample series was measured in DIA and DDA modes on an AB Sciex 5600 (DIA) and Thermo Q Exactive (DIA and DDA) instruments. The data was analyzed with the MaxQuant analysis software for DDA and with Spectronaut™ for the DIA SWATH-MS.

Plasma samples of 14 healthy individuals were collected monthly over a time period of 6 months. DIA data was acquired and aligned with Spectronaut. Protein profiles were extracted within and between individuals.

Ergebnisse und Diskussion

Full peptide profile analysis of DDA/shotgun-MS and DIA/SWATH-MS analysis of the background proteome in the gold-standard set showed that the number of full profiles in DIA/SWATH-MS surpasses the numbers of DDA/shotgun-MS with increasing sample number (DIA: 14572 in 1 run; 8921 in 24 runs; DDA 16423 in 1 run; 4448 in 24 runs). The SWATH-MS profiles showed lower variance as compared to the shotgun-MS profiles (Median CV 9.1% vs. 12.8%).

In the longitudinal plasma data set 1500 peptides were profiled across 86 samples. Analysis of inter and intra-personal protein expression revealed 3 classes of proteins: Stable across time and individual, stable across time but different between individuals, unstable expression across time and individual.

We have shown with a synthetic and a clinically relevant dataset that DIA allows protein quantification across multiple samples and large number of proteins. With increasing number of samples the DIA significantly outperforms DDA for protein profiling. Furthermore, the targeted data analysis approach provides a straightforward solution to gap-free multi-run alignment.

We report for the first time a direct comparison of peptide quantification precision using DIA/SWATH-MS and DDA/shotgun-MS. Furthermore, we profiled 86 longitudinal plasma samples using DIA and identified interpersonal differences in the plasma proteome that are stable over time.

Referenzen

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Neue Aspekte

Global comparison of quantification performance between data dependent and data independent acquisition on a high mass accuracy trap instrument (Q-Exactive).

Perfect timing: fragment ion mobility based performance increase on a qTOF instrument

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The field of proteomics is, to a large extent, still driven by advances in technology enabling the analysis of ever more complex proteomes at ever decreasing time and sample quantities. Even though modern mass spectrometers have reached a high level of sensitivity enabled by improved ionization techniques and exquisitely sensitive detectors, the duty-cycle of oa-Q-TOF instruments is still somewhat inefficient across a wide m/z range. In this study, we show that the MS/MS duty cycle (and sensitivity) of a oa-qTOF instrument equipped with a travelling wave ion mobility device can be greatly improved by synchronizing the fragment ion mobility times with the pusher pulsing of the oa-TOF.

Experimenteller Teil

All experiments were performed on a SYNAPT G2-S*i* quadrupole TOF mass spectrometer equipped with a traveling wave ion mobility device and coupled online to a nanoAcquity UPLC system (Waters, Manchester, UK). Complex proteome digests were analyzed by data dependent acquisition (DDA) of MS/MS spectra either with or without fragment ion - oa-TOF synchronization enabled (termed high-definition or HD-DDA). Dilution series and replicates were used to assess the merits of HD-DDA for sensitivity, speed and proteome coverage. Optimized methods were applied to the analysis of human tissue proteomes, phosphopeptides enriched from complex digests and the interaction of the HDAC inhibitor SAHA with human proteins. Protein identification and quantification was performed using Mascot (Matrix Science).

Ergebnisse und Diskussion

Applying ion mobility to peptide fragment ions generated by collision induced dissociation (CID) leads a) to the separation of fragment ions of different m/z values into different mobility drift time bins and b) the physical concentration of particular fragment ions into a particular mobility drift time bin. These effects can be exploited to improve the TOF duty cycle by using the temporal separation of ions and synchronizing the drift time of ions of a given mass with the pusher that injects ions orthogonally into the TOF. This leads to an increase in sensitivity, with all ions across the m/z range being sampled efficiently at close to 100% duty cycle compared to an asynchronous pusher were ca.75% of all ions are lost.

Infusion experiments of single peptides revealed an increase in MS/MS sensitivity of 15x for HD-DDA over normal DDA. Dilution experiments using HeLa digests (10-1,000 ng on column) showed improvements in detection limits of one order of magnitude. In addition, the overall number of identified proteins was improved about 2-fold for every dilution and similar observations were made at the peptide level. The enhanced MS/MS signal not only lead to higher identification scores, it also allowed operation of HD-DDA at higher speed than possible for DDA (7.5 Hz vs 5.3 Hz).

We highlight the utility and competitiveness of HD-DDA in proteomics by a number of examples. HD-DDA enabled the identification of close to 4,000 in a single 6 hour LC-MS/MS run. Analysis of eight human tissue samples by GeLC-MS/MS (12 gel cuts, 2 h LC-MS/MS each), identified >10,000 proteins. IMAC enrichment of phosphopeptides from A431 cells lead to the identification of approx. 8,000 phosphorylation sites in 5 hours of measurement time and TMT-based quantification allowed the selectivity and dose response profiling of the HDAC inhibitor Trichostatin-A in a chemoproteomic experiment.

Neue Aspekte

Investigation of a new ion mobility enhanced DDA acquistion method on a qadrupole TOF instrument.

Integrating database search and de novo sequencing to improve the peptide identification

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A key step in shotgun proteomics is peptide identification. There are two complementary approaches for the analysis of LC-MS/MS spectra: database search and de novo sequencing. A protein sequence database search is prioritized for database peptides and modified peptides when a database is available. De novo sequencing is the only option for novel or homologous peptides which are not in a database. However, unlike target-decoy validation for database searching, de novo sequencing lacks a validation approach. Here we describe a workflow integrating database search and de novo sequencing, in which database peptides are used to validate de novo peptides. Thus, the accuracy of de novo peptides can be estimated. The workflow maximized the peptide identification.

Experimenteller Teil

1. A local confidence score was assigned to each residue of the de novo peptides to indicate how likely a residue is correctly sequenced.
2. Let **T1** be the set of MS/MS spectra. Perform de novo sequencing and database search with **T1**.
3. Let **T2** be the set of the spectra identified by database search with 1% of FDR. A de novo peptide in T2 was validated with the database peptide at residue level. The local confidence score distributions were plotted for de novo residues that agree/disagree with database residues.
4. For the de novo peptidesin **T3** = **T1** –**T2**, their score distributions of correct and incorrect residues were estimated with validated distributions in Step 3.

Ergebnisse und Diskussion

Three LC-MS/MS data sets were used in the evaluation. The three datasets contain 8031, 5152, 58159 MS/MS spectra, acquired from Ion Trap/ETD, Ion Trap/CID, and Orbitrap/HCD, respectively.

Database search was performed by consensus search (PEAKS DB [1] + MASCOT [2] + X!Tandem [3]) for each data set. Database peptide assignments with 1% false discovery rate were considered confident.

De novo sequencing was performed with PEAKS De Novo [4] for each data set. A residue local confidence score was computed by combining multiple scoring features for the amino acid residues in a *de novo* sequence.

A *de novo* sequence can be validated by comparison to a database peptide if the spectrum can be confidently assigned (FDR < 1%) in the protein database search. For this subset of *de novo* sequences that can be validated, local confidence score distributions can be plotted for the *de novo* residues that agree/disagree with database peptides. The de novo sequencing results were considered correct where they agreed with database peptide sequences, incorrect otherwise.

For the MS/MS spectra without confident database assignment, their *de novo* peptides cannot be validated directly by database peptides. However, their score distributions of correct and incorrect residues can be estimated with maximal likelihood by using the distributions of validated residues as models. Then, a local confidence score threshold can be determined to filter low confidence residues. Average local confidence was used to filter de novo sequences with a residue error rate of less than 15%. The confident de novo peptides were exported with confident peptides by database search.

Compared to database search alone, 5%, 7%, and 6% more peptide identifications were obtained with this workflow for the Ion Trap/ETD, Ion Trap/CID, and Orbitrap/HCD data sets, respectively. By manual validation with protein BLAST, 90% of the exported de novo peptides are significant.

Referenzen

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Neue Aspekte

A novel approach for the validation of de novo sequencing results.

New aspect of the Maillard reaction: mass spectrometric study of protein glycation and glycoxidation under environmental stress conditions in plants

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Protein glycation is a common posttranslational modification, formed by reaction of reducing sugars (aldoses and ketoses) with amino groups. Resulting Amadori and Heyns compounds undergo further oxidative degradation, yielding advanced glycation end-products (AGEs). These compounds are readily formed during food thermal treatment. The absorbed food AGEs interact with macrophage and endothelial receptors for AGEs (RAGEs), triggering pro-inflammatory response resulting in atherosclerotic changes.

Enhanced protein glycation can not only occur upon cooking but already during the life time of crop plants, and this might increase the negative effects of related meals. In this context, it is important to know, if glycoxidation is increased in plants affected by environmental stresses, especially if accompanied by improper agricultural conditions or pollution with heavy metals.

Experimenteller Teil

The model of light stress (8 h day/16 h night, 700 µmol photons/$m^{-2}s^{-1}$, 14 days) was established with *Arabidopsis thaliana*, while the model of cadmium stress was established with *Brassica napus* (16 h day/8 h night, 150 µmol photons/$m^{-2}s^{-1}$, 7 days) using 0, 30, 300 and 1000 µmol/L Cd^{2+} in full liquid cultural medium. The leaves and roots were harvested before stress application and distinct time points throughout the stress period. Soluble proteins were isolated, digested with trypsin and the digests were analyzed by LC x LC-LIT-Orbitrap-MS/MS, using boronic acid affinity chromatography or HILIC as the first dimension and RP-nanoUPLC as the second one. Modified peptides identified by database search peptides were quantified by label-free nanoUPLC-ESI-Orbitrap-MS approach.

Ergebnisse und Diskussion

Under light and cadmium stress *A. thaliana* and *B. napus* plants displayed rich patterns of glycated and glycoxidated proteins, representing mostly enzymes and regulatory molecules. For plants, this information, as well as exact modification sites, is reported here, to the best of our knowledge, for the first time. The majority of peptides annotated with Xcorr ≥ 2.0 could be clearly identified by characteristic tandem mass spectrometric patterns. For both types of environmental stress, under unstressed conditions the modification patterns were dominated by N^{ε}-carboxymethyllysine (CML), methylglyoxal-derived hydroimidazolone (MGH) and argpyrimidine. However, glyoxal-derived hydroimidazolone (Glarg)-modified peptides were more abundant in *A. thaliana* plants. Low concentrations of Cd(II) resulted in detectable oxidative stress after 7 days of treatment. The severity of stress (as assessed by the panel of biochemical markers) correlated with the increase in total leaf Cd(II) concentration. It was also accompanied with an increase in abundance of AGE-modified peptides and accumulation of oxidative modifications. Moreover, the number of identified peptides and corresponding modification sites was increased under stress conditions. Remarkably, for plants, the information on modified proteins and on distinct modification sites is reported here for the first time.

Neue Aspekte

Plant protein glycation and glycoxidation patterns in absence and presence of environmental stress are characterized for the first time.

Session II: Lipid- u. Kohlenhydrat-Analytik I

Montag, 03.03.2014: 10:50 - 12:30, OSZ H4

Novel Approach on Clinical Diagnostic of Lysosomal Storage Disease in DBSs by Fluorimetry and MRM-MS using Coumarin Derivates based Substrates

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The loss of lysosomal enzyme activity is a characteristic of lysosomal storage diseases (LSDs), a group of mostly genetic metabolism desorders (e.g., mucopolysaccharidosis, mucolipidoses, Fabry's Disease; Gaucher's Disease etc.). The enzyme substrates can no longer be processed and are accumulating in the lysosome causing severe disease symptoms leading to multiple organ failure and finally death. For several LSDs treatment is available in the form of replacement therapy with high success if the therapy is started in time, making the diagnosis of crucial importance. State of the art now in LSDs diagnostic employs in first stage enzymatic determinations by fluorimetry based in 4-methylumbelliferone substrates or by mass spectrometry (in few cases) based on different compounds with a terminal sugar moiety.

Experimenteller Teil

Here we describe highly specific and sensitive diagnostics on dry blood spots (DBS) for (i), molecular determinations of LSDs, particularly muco-polysaccaridoses and muco-lipidoses, by simultaneous fluorimetric and mass spectrometric analysis using newly developed, identical substrates and standard derivatives; (ii), clinical diagnostics of LSDs by multiplex- MS-MRM analysis using specific fragmentations of substrates and multiply-^{13}C-labelled standard derivatives; (iii), the clinical validation of the new methods with DBS samples, as shown for patients with established LSDs

Ergebnisse und Diskussion

A typical substrate for the fluorimetric measurement of a lysosomal enzyme activity is a molecule with 2 different parts bound covalently: a group recognized by the target enzyme (e.g. sugar, lipid) and 4-umbelliferone. The product of the enzymatic reaction is always 4-methyl umbelliferone. In our tests, the enzymatic activity levels in DBSs were determinate by fluorimetry or multiple reactions monitoring mass spectrometry in the presence of an internal standard (4 – methyl-umbelliferone 1,2,3,4, C13) showing a good statistical correlation in singles assays. Example from the 18 singles assays perfected for both fluorimetry and MRM_MS on umbelliferone derivatives performed in our group: mucopolysaccharidosis 1 and 2, Fabry Disease etc. Further more, we developed duplex and triplex assays for the diagnosis of different LSDs from the muccopolysacharidoses family using modifies substrates based on different coumarin derivates obtained through Pechman condensation. The new umbelliferone derivatives were created by substituting groups in position 4, 5 and 6 of the alpha-hydroxy-coumarin molecule with others e.g. 4propyl-umbelliferone, 4- ethyl-umbelliferone; 5-ethoxy- umbelliferone etc. Their MS/MS spectra showed different ion patterns, with the exception of neutral loss of CO and CO2, which is translated in a good compatibility in a multiplex experiment. An example of multiplex assay based on novel umbelliferone derivatives for MPS6, MPS2 and MPS 4b. In conclusion, by measuring umbelliferone derivatives as products of enzymatic reactions either by fluorimetry or mass spectrometry, the proposed substrates have the advantage of possibility of direct result correlation with other laboratories.

Referenzen

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Neue Aspekte

Clinical diagnosis of LSDs using the same substrate for fluorimetry and MRM-MS techniques

A simple MALDI MS-based method to identify ether lipids in complex lipid mixtures of spermatozoa

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There is increasing evidence that MALDI MS is a useful method of lipid research [1]: MALDI MS is fast, sensitive, tolerates sample impurities and provides simple mass spectra because singly charged ions are nearly exclusively detectable. The considerable robustness of the MALDI ionization process paves the way for applying selected auxiliary agents, for instance, salts such as CsCl that help to overcome assignment problems of different lipid species in complex lipid mixtures [2]. 2,4-dinitrophenylhydrazine (DNPH) is a classical derivatization agent and a "reactive" matrix that helps to convert aldehdyes into derivatization products. We will demonstrate a simple approach to monitor the presence of plasmalogens (alkenyl-ether phosholipids) in spermatozoa extracts without the need of using highly sophisticated MS equipment.

Experimenteller Teil

All chemicals, solvents, the applied MALDI matrices (9-aminoacridine (9-AA) and 2,5-dihydroxybenzoic acid (DHB)) and a 0.2 M DNPH solution (in phosphoric acid) were obtained from Sigma-Aldrich. Phospholipid standards were from AVANTI Polar Lipids and used as supplied. TLC/MALDI analysis was performed as recently described [3,4].

DHB was either used as 0.5 M solution in methanol or as a solution of 100 mg/ml in acetonitrile/water (1:1, v/v), while 9-AA was used in a concentration of 10 mg/ml in isopropanol/acetonitrile (60/40, v/v)) [5]. Lipid extracts from spermatozoa were obtained according to the Bligh & Dyer method and the spectra recorded as previously described [1]. Derivatization with DNPH was performed either in solution or directly on a TLC plate.

Ergebnisse und Diskussion

Spermatozoa cells contain in addition to common diacyl (phospho)lipids also significant amounts of ether lipids. Alkenyl-ether lipids (plasmalogens) are of particular interest because they are considered as natural antioxidants because the alkenyl-ether linkage has a much higher reactivity with reactive oxygen species (ROS) than the olefinic residues within the fatty acyl chains. In combination with a complex fatty acyl pattern, the unequivocal identification of plasmalogens is a challenging task - in particular if only a simple TOF device without MS/MS capacity is available. Fortunately, plasmalogens can be easily identified by simple chemical derivatization and without the need of sophisticated MS equipment: plasmalogens are extremely sensitive against traces of acids that convert the plasmalogen into a lyso lipid (lacking the ether residue) and the corresponding aldehyde. The lyso lipid can be easily identified while the simultaneously generated aldehyde is not detectable under conditions of high vacuum. We will show that the derivatization of the generated aldehydes by DNPH is a convenient method to overcome this problem. The derivatization products are best detected as negative ions and this helps to minimize interferences with bulk phospholipids such as PC. Samples of boar spermatozoa will be used to illustrate the power of this derivatization. Of course, the DNPH derivatization may be also performed directly on a TLC plate and gives - subsequent to MALDI MS - direct information about the contribution of plasmalogens to the individual lipid classes. Finally, it will be shown that the applied highly acidic conditions are very helpful to overcome overlap problems between different adducts and lipids with different fatty acyl compositions. It is concluded that this is a very simple and convenient approach that can be used on all MALDI mass spectrometers.

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Neue Aspekte

A simple method to identify plasmalogens in spermatozoa lipid extracts was developed and is also applicable to other physiologically-relevant samples.

OxoLipidomics - new analytical platform for profiling of lipid oxidation and nitration products

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Lipids are crucial in maintaining cell and tissue structures, metabolism and signaling. Recent advances in MS-based lipidomics revealed an extremely high complexity of mammalian lipidomes with a large number of species still to be discovered. Furthermore, it appears that *in vivo* modifications of lipids are significant for organism homeostasis and play a key role in the pathophysiology of several human disorders. Thus, oxidation and nitration of lipids can induce diseases, such as atherosclerosis, obesity, inflammation and autoimmune diseases, and contribute to their development. New analytical strategies are required to reveal the modified lipidome and its effects on the pathophysiology. Here we present new MS-based analytical platforms for modified lipids profiling in cellular and human plasma lipidomes.

Experimenteller Teil

Five different MS-based methods were developed to access oxidized and nitrosylated lipids in cells and human plasma samples. The lipids and their modified versions were relatively quantified by a shotgun approach with implemented gas-phase fractionation (GPF) in data-dependent acquisition (DDA) on an ESI-LTQ-Orbitrap-MS. Nitrated and oxidized fatty acids (FA) before and after phospholipid saponification were quantified by optimized multiple-reaction monitoring (MRM) on an ESI-QTRAP-MS. Oxysterol enrichment, separation and quantification relied on C_{18}-RPC-ESI-IT-MS. Identification and relative quantification of low and high molecular weight carbonylated lipid peroxidation products derivatized with 7-(diethylamino)coumarin-3-carbohydrazide (CHH) was accomplished by both shotgun and LC-MS.

Ergebnisse und Diskussion

The new analytical techniques were applied to several cell models of oxidative and nitrosative stress. The influence of NO, a known physiological vasodilator with different concentration-dependent effects, on the lipidome was investigated on primary cardiomyocytes. A new MRM-method was used to determine the formation kinetics of nitrated FAs after 15, 30, 70 min and 16 h of nitrosative stress induction. The levels of nitro-, dinitro-oleic acid and nitro-docosahexaenoic acid (DHA) significantly increased after 30 min, whereas oxidatively modified FAs, such as oxo-oleic and hydroxy-nitro-DHA acids were elevated 70 min after NO-stress induction. FA nitration was correlated to the generation of different eiscosanoids, which quantities increased gradually over time. For instance, 5-F_{2t}-isoprosatne and 5-epi-F_{2t}-isoprostane, formed by oxidation of arachidonic acid, were elevated 4.4 times after 16 h in comparison to control, indicating the increase in oxidative modifications over nitration with the incubation time. Furthermore, different low and high molecular weight carbonylated lipids, known to be highly reactive towards nucleophilic substrates, were identified using shotgun lipidomics. Several newly formed PL-bound aldehydes, generated by oxygen addition or oxidative cleavage of unsaturated FA, were present exclusively after 16 hours of incubation. Among them were, for instance, 1-palmitoyl-2-(9-oxo-nonanoyl)-glycerophosphatidylcholine and 1-palmitoyl-2-(7-oxo-heptanoyl)-glycerophosphatidylcholine, two main products of PL-esterified linoleic acid oxidative cleavage. These results represent the first lipidome-wide profiling of oxidative and nitrosative modifications in cellular lipidome and demonstrate the rapid increase of FA nitration (30 min) followed by elevated oxidative FA and lipid modifications after longer incubation times. Results were additionally correlated with several biological parameters. Perinuclear mitochondrial clustering, for example, was observed and correlated to increased nitrosative lipid modification degrees. Furthermore, our analytical platform "OxoLipidomics" was used to profile human plasma samples obtained from patients with obesity and type II diabetes.

Neue Aspekte

New MS-based OxoLipidomics platform was developed to identify new modified lipids and quantify them in cellular and plasma lipidomes.

Why so Negative? – Ion Mobility-Mass Spectrometry of Negatively Charged Complex Carbohydrates

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Carbohydrates are of great importance for a variety of biological functions. They can, for example, be linked to proteins as post-translational modifications or exist as free oligosaccharides in complex biological matrices such as milk. Oligosaccharides consist of monosaccharide building blocks, which often exhibit an identical atomic composition and mass. Moreover, each building block has multiple reactive sites at which new glycosidic bonds can be formed. As a consequence a variety of structurally different isomers can be expected for the resulting oligomers. This, in combination with the fact that the stereochemistry can vary at each linkage, makes carbohydrates extraordinarily complex and difficult to analyze using established techniques.[1]

Experimenteller Teil

A promising approach to provide an additional separation step for carbohydrate analysis is ion mobility-mass spectrometry (IM-MS). After ionization of the samples the ions travel through a gas-filled cell aided by an electric field and are separated according to their shape and size, allowing the differentiation of isomers. While the drift time of an ion depends on the underlying instrument conditions, the collision cross section (CCS) is a molecular property that can be compared and calculated theoretically. As a result, there is a huge potential to implement carbohydrate CCSs into databases as an additional search criterion for structural assignments. Due to the inhomogeneous travelling wave field utilized in most of the commercially available instruments, however, CCS estimation requires careful calibration.

Ergebnisse und Diskussion

Recently, we reported a calibration protocol using sodiated, positively charged *N*-glycans which were released from well-characterized, commercially available glycoproteins (e.g., fetuin, ovalbumin, ribonuclease B) using an easy to follow procedure.[2] The underlying absolute CCSs of these glycans and their resulting fragments were measured in He and N_2 using a modified drift tube Synapt IM-MS instrument. To complement the data, we report here CCSs of negatively charged glycans and polysaccharides. The negative ion mode can be especially helpful for carbohydrate analysis, since an increased amount of cross-ring cleavages leads to much more informative fragmentation spectra.

Furthermore, we investigated the impact of adduct formation and ion polarity on the separation of different isobaric carbohydrate mixtures.[3] Experiments on a series of isobaric milk sugars for example revealed, that species, which can neither be distinguished by LC-MS nor tandem MS, can be separated with baseline resolution using IM-MS. However, for a successful separation, the ionization mode turned out to be crucial. This can be explained on basis of their underlying gas-phase structure. The conformation of sodiated carbohydrates, for example, was shown to be largely dominated by coordination of the metal ion, which in turn leads to only very subtle CCS differences in IM-MS. Deprotonated ions on the other hand, retain structural characteristics of each individual sugar. As a consequence their CCS differ considerably, which enable an efficient separation via IM-MS.

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Neue Aspekte

Analysis of complex carbohydrates in negative ion mode using ion mobility-mass spectrometry.

Glycan and glycopeptide profiling of biopharmaceuticals

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Glycosylation is among the most complex post-translational modifications (PTM) undergone by proteins. Because glycans are assembled from various, partly isomeric monosaccharides linked in multiple regio- and stereospecific ways, their analysis is extremely challenging. However, many research endeavours and industrial applications rely on rapid and efficient profiling of PTMs. MS features high selectivity and broad applicability. Thus, in combination with efficient sample preparation and separation techniques, MS enables increasingly detailed glycosylation profiling.

Glycosylation profiling is important in the development and production of biopharmaceuticals, such as therapeutic antibodies and growth hormones. Specific differences in glycosylation can have a significant effect on pharmaceutical properties, such as plasma half-life and efficacy. Therefore, detailed MS based glycan profiling is gaining importance for the development and quality control of functional glycosylation.

Experimenteller Teil

At the CPM, we are currently using mainly three MS based methods for glycosylation analysis: For immunoglobulin G (IgG) F_C glycosylation, 1) an LC–ESI-MS method [1] and 2) a MALDI-MS method [2], and 3) a MALDI-MS method for PNGase F released N-glycan profiling. The MALDI methods include a solid phase extraction in a pipet tip format for glycan or glycopeptide enrichment. Both hydrophilic interaction (2, 3) and reversed-phase stationary phases (3) are employed. Released glycans are also modified by ethyl esterification of sialic acid groups for stabilization and resolution of sialic acid linkage (α2-3 vs α2-6). Using Herceptin, an IgG1 therapeutic monoclonal antibody, these methods were compared to multiple chromatographic and spectrometric methods.

Ergebnisse und Diskussion

Overall, the glycosylation profiles obtained for Herceptin with the different methods were well comparable. For example, the value of the relative abundance of the main glycoform varied with a relative standard deviation below 5% and a maximum deviation of 8% between the 12 methods. Consequently, the biases within each method seem to be small. One important source of bias and a major distinctive factor between the methods is sensitivity. Of the three in-house methods, the released glycan method was the most sensitive. While we generally handled a 0.2% cut-off value (relative abundance), this method hinted at bisecting species at a level just below 0.1%. Though all three in-house methods require little hands-on time, the MALDI methods are more laborious, due to the SPE purification and sample spotting steps. However, the LC–MS method requires significantly more instrument time (13 min vs. 10 s) which gives the MALDI methods a cost advantage, especially for larger sample numbers.

The derivatization by ethyl esterification presents an efficient way to stabilize sialic acids and thereby avoid the fragmentation of sialic acid containing glycans which is otherwise observed in MALDI-TOF-MS with reflectron mass analysis. Consequently, sialic acid containing glycans are no longer underestimated by the signal loss resulting from the formation of metastable ions. In addition, the ethyl esterification is selective for α2-6 linked sialic acids. α2-3 linked sialic acids are also stabilized, but in the form of lactones. The resulting mass difference of 46.04 Da allows easy distinction without the need for fragmentation.

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Neue Aspekte

We present the results of an extensive method comparison for glycosylation analysis and a novel derivatization method for sialic acids.

Session III: Instrumentelle Entwicklungen I

Montag, 03.03.2014: 10:50 - 12:30, OSZ H5

High Resolution Ion Mobility-TOF MS for the analysis of natural products and complex mixtures

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The analysis of natural products represents a major analytical challenge because of their high chemodiversity. Typically, fast LC-MS and direct infusion ultra-high resolution MS are used to investigate this type of samples but usually fail to resolve multiple isomers. Alternatively, ion mobility (IM) can be employed for separation of isomeric species without increasing cycle times and with minimal added experimental complexity. So far, IM has been used mainly as a filter and not as separation dimension due to limited resolution. We have compared the performance of a high resolution IM coupled to TOF-MS for the analysis of natural products such as flavonoids to a standard UHPLC-TOFMS approach. Additionally, the applicability of IM for the fingerprinting of complex samples such as whiskey and wine is demonstrated.

Experimenteller Teil

All measurements were carried out on a Tofwerk IMS-TOF-MS. The system comprises an ESI source, a 10cm desolvation tube, a 20cm drift tube (both made from resistive glass) and a Tofwerk HTOF TOF-MS. Desolvation and drift tube were kept at atmospheric pressure and 150°C with nitrogen as the buffer gas. Ion mobility separation was carried out at a field strength of ca. 400V/cm. Complex samples were either analysed directly by dilution with an appropriate solvent or following a quick enrichment procedure using liquid-liquid or solid phase extraction. PCA and linear discriminant analysis were carried for sample discrimination after post-processing of the raw IM-TOF-MS data.

Ergebnisse und Diskussion

High resolution IM-MS and rapid UHPLC-MS were investigated for analysis of closely related isomeric flavonoids and their glycosides. On a time scale of a few minutes, the flavonoid aglycones were all separated by ion mobility, but not by UHPLC. The glycosides were better resolved by IMMS but not completely separated by both methods. The ion mobility resolving power was routinely >150, indicating that the system provides sufficient resolution for separation of isomeric natural products even in complex samples. Therefore, IM-MS was applied to the analysis of plant extracts, whiskey and wine, resulting in the detection of hundreds of different features each. Many of these features originated from isobaric ions (isomeres) and were only separated in the ion mobility dimension. Yet, these isomeres can have a strong impact on the sample characteristics. For example, the ratio of quercus lactone isomers (important ingredients in the aroma) varied between different whiskeys; similar observations were made for phenolic compounds such as isomeric flavonoids in wine. This was achieved by multiplexed IM-MS using Hadamard transform, improving ion transmission over 200 times and S/N ratios 10 times compared to conventional pulsed mode. In combination with post-processing of the raw data, ion mobility resolutions >200 can be reached even in these real-world samples as required for reliable analysis of complex mixtures. In conclusion, we demonstrate the potential of IM-MS for the analysis of isomeric natural products. Additionally, fast and direct analysis of complex mixtures can routinely be achieved by IM-MS, allowing for fingerprinting and sample discrimination as required for quality and fraud control.

Neue Aspekte

High-resolution IM-MS applied to natural product analysis and fingerprinting of complex mixtures.

New Insight into Highly Complex Samples by Ultrahigh-Resolution Mass Spectrometry

Arnd Ingendoh, Jens Fuchser, Matthias Witt, Goekhan Baykut

Bruker Daltonik GmbH, Deutschland

FTMS provides high mass resolution and accuracy and is applied for highly complex samples like in metabolomics, fulvic acid mixtures or direct tissue analysis. However, FTMS performance was tightly connected to high magnetic fields and thus to high cost. Recently, a new type of an FTMS cell was introduced providing mass resolutions of 40 million even at the moderate magnetic field strength of 7T. This not only enables the analysis of crude mixtures with several 1000s compounds without the need of any front-end separation, but also elucidates the isotopic fine structure of components, leading to an unambiguous determination of elemental compositions. Performance values and several applications like biomarker discovery and identification in metabolomics and MALDI imaging will be discussed.

Experimenteller Teil

The new FTMS cell results as well in a high mass accuracy with an RSD in the range of several 10 ppb and a much increased dynamic range compared to previous analyzer cells. This was proven in a metabolomics study of myxobacteria which represent an important source of novel natural products with a wide range of biological activities. Currently, successfully established methods are based on LC/MS requiring about 20 min analysis times per sample. The analysis of several 1000 myxobacterial strain isolates as well as numerous genetic knock out mutant strains means then a serious time bottleneck. Here, several metabolite extracts from genetic knock-out mutants were analyzed by ultra-high resolution, high-throughput FTMS in direct infusion, requiring only 1 min/sample.

Ergebnisse und Diskussion

In a MALDI Imaging study, mass spectra from frozen rat testis sections were acquired in the mass range of m/z 500-900 at a laser focus of 10 µm and a step size of 5 µm.

With a broadband mass resolution of > 200k in the m/z range of 700-900 and a mass accuracy < 1 ppm, elemental compositions of several phospholipids could be identified. E.g., a co-localized pair of ions with a 3 mDa mass distance at m/z 808 was determined as $[C44H84NO8P]^{Na+}$ and $[C46H82NO8P]^{H+}$ and their structures were tentatively assigned as PC 36:2 and PC 38:5.

Neue Aspekte

A new FTMS cell was introduced providing mass resolutions of 40 million even at the magnetic field strength of 7T

Technische Weiterentwicklungen der Orbitrap Plattfrom und deren Anwendung für die Charakterisierung von Biopharmazeutika

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Orbitrap-basierte Massenspektrometer wurden seit der Markteinfühung im Jahr 2005 stetig weiterentwickelt, wodurch die Charakterisierung von einer Vielzahl auch anspruchsvoller Analyten wie z. B. von Biopharmazeutika nicht nur ermöglicht sondern auch deutlich verbessert wurde. Biopharmazeutika sind in den meisten Fällen strukturell anspruchsvoll Moleküle, deren umfassende Charakterisierung sowohl eine Herausforderung darstellen als auch die Verwendung einer Vielzahl und Kombination verschiedener massenspektrometrischer Methoden erforderlich machen, die sich mit der qualitativen und quantitativen Analyse von intakten Proteinen und deren Modifizierungen sowohl auf Protein- als auch Peptidebene befassen.

Die Analyse intakter Proteine und speziell grosser Proteine wie z.B. intakten Antikörpern oder gar von Proteinkomplexen mit Hilfe der Orbitrap Plattfrom hat in den letzten wenigen Jahren rasante Fortschritte gemacht, die erst durch technische Weiterentwicklungen ermöglicht wurden.

Experimenteller Teil

Verschiedene biopharmazeutische Proteine mit klinischer Relevanz wurden sowohl intakt als auch nach chemischer Reduktion an dem neuen Q Exactive Plus Benchtop Quadrupol Orbitrap Massenspektrometer analysiert, um die Anwendbarkeit und die Vorteile der technischen Neuerungen demonstrieren zu können: den Verbesserungen in der Hardware der Ionenoptik sowie die Möglichkeiten zur Verwendung von erhöhter Auflösung von 280.000 (FWHM) sowie der Möglichkeit zur Druckregulierung zum verbesserten Einfangen, Stabilisieren und Detektieren von intakten, hochgeladenen Spezies.

Ergebnisse und Diskussion

Die Präsentation ist fokussiert auf die Vorstellung der technischen Neuerungen, die die signifikanten Verbesserungen der Geräte-Performance bewirken, sowie die Diskussion von Ergebnissen der Analyse von anspruchsvollen biopharmazeutischen Proben sowohl auf intakter Ebene sowie nach top-down Fragmentierung.

Neue Aspekte

Implementierung von technischen Weiterentwicklungen zur verbesserten Analyse von intakten Proteinen und Proteinfragmenten mit erhöhter Auflösung und Sensitivität.

Synchronising a unique MS geometry with T-wave ion mobility separations for ultra-sensitive, targeted MS/MS and improved compound identification / quantification

Alistair Wallace[1], Nick Tomczyk[1], Mike McCullagh[1], Steve Preece[1], Sabine Lange[2], Marc Kipping[2], Steven Pringle[1], Jason Wildgoose[1], Kevin Giles[1]

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Ion mobility spectrometry (IMS) coupled with mass spectrometry (MS) has grown dramatically due to its ability to provide differentiation of molecules on the basis of size, shape and charge, as well as mass. Here we report results from the use of a unique orthogonal acceleration time-of-flight (oa-Tof) MS instrument with high-efficiency Travelling-wave ion mobility separations.

Here we report the use of ion mobility separation which can be used to deliver increased analytical sensitivity and specificity for discovery and targeted quantitation (through synchronization with Tof-MS), reduce the occurrence of false positive and negative identification in screening applications (with routine CCS measurements), the detection of protomer formation, and how that can lead to development of more reliable and specific targeted quantitative methods.

Experimenteller Teil

Protein identification experiments used an enzymatic digest of a complex protein mixture. Injections were made onto a UPLC BEH-C_{18} nanoACQUITY column. A water/acetonitrile (0.1% Formic Acid) gradient was applied and data was acquired in MS^E, $HDMS^E$ and HD-DDA modes and processed using ProteinLynxGlobalSERVER v3.0.

Quantitation experiments used common drug like molecules. These were injected on to ACQUITY UPLC columns. Rapid gradients were applied and data was acquired in MS, MRM and HD-MRM modes and processed using Targetlynx.

CCS screening assay was based on the analysis of an EU-RL proficiency test. 5µl were injected on to UPLC BEH-C_{18} column. A water/acetonitrile (0.1% Formic Acid) gradient was applied and data was acquired in $HDMS^E$ mode and processed using UNIFI CCS Research Edition.

Ergebnisse und Diskussion

The demands for performance increases for the analysis of complex protein mixtures and digests is constantly increasing with respect to number of identifications and confidence in those protein identifications. The novel use of ion-mobility on the SYNAPT G2-S*i* in both $HDMS^E$ and HD-DDA provide significant advances in the number of proteins identified and quality of fragment ion spectra from even the least intense precursors. We show that the separation of ions in the IMS cell can both increase sensitivity and impart selectivity.

The drive for lower limits of detection (LOD) and quantitation (LOQ) in even the most complex matrices has driven users to investigate novel modes of acquisition for quantitation including the use of high resolution mass spectrometers. We show here that by making use of ion mobility separations in MRM and HD-MRM experiments we can significantly improve both sensitivity and selectivity of these experiments and drive the LOD and LOQ levels ever lower.

UPLC $HDMS^E$ (CCS) data was initially acquired for a series of pesticide solvent standard mixtures. The collision coss section (CCS) values generated were entered into a scientific library within a new scientific information system (UNIFI CCS Research Edition) and used as a confirmatory parameter to increase confidence in identification, as well as reduce false positive and negative identifications. The use of CCS enabled the initial specificity of applied screening parameters to be reduced.

Neue Aspekte

Improved sensitivity, specificity and selectivity through the novel use of travelling wave ion mobility with Time of Flight mass spectrometry.

NON-TARGET SCREENING IN FOOD AND ANIMAL FEED BY UHPLC-HR-Q-TOF MS AS ALTERNATIVE FOR THE CONVENTIONAL TARGET SCREENING APPROACH

Jens Lütjohann[1], Jens Jacobsen[2], Frank Schreiber[1], Stefan Neubauer[1], Jürgen Kuballa[1], Eckard Jantzen[1]

[1]Galab Laboratories GmbH, Bergedorf, Deutschland; [2]Waters GmbH, Deutschland;

Presently the prevailing method for determining pesticides, Vet drugs and other legislative relevant contaminants in food and animal feedstock is a targeted approach using LC/MS/MS or GC/MS/MS. The definitions of methodologies and its target compounds are mainly based on the juridical requirements as well as customer requests on known residuals of which relevance is mostly based on historical experiences. New active substances, contaminants and substances, not in the mainstream of public awareness, are often overlooked hitherto the inherent methodology of targeted analytical technologies. In an increasingly globalised World with foodstuff and packaging materials from a multitude of suppliers and origins, a targeted screening will inherently risk of omitting potential unwanted and toxic contaminants.

Experimenteller Teil

The experimental goal was to find a sensitive non-targeted screening methodology for determination of pesticides, Vet Drugs and other contaminants in Food and Animal feedstock using high resolution UHPLC and QToF MS technology. After intensive investigations on routine screening equipment in the high resolution mass spectrometry range, the selection was made on a UHPLC QToF (Acquity UPLC and Xevo G2-S QToF, Waters Corporation). I was tested if in the same sample run, unknowns can also be isolated and possibly identified using compound-specific characteristics, such as accurate mass, the identification of adducts, fragmentation patterns, retention time and isotope ratios.

Ergebnisse und Diskussion

The validation of this non-targeted screening method was carried out using the current SANCO document 12495/2011 and also the Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines (Community References Laboratories Residues) and was successful for more than 600 selected pesticides, 200 Vet Drugs and 250 other contaminants of relevance. The SDLs were in the range from 1 to 10 µg/kg. This novel approach in analytical work will in the near future replace the tandem MS systems, which will be continued in the usage for confirmatory analytical work.

Neue Aspekte

Non targeted screening with high resolution data combined in one software platform and all fragment data collection.

Session IV: Organische MS I

Montag, 03.03.2014: 15:30 - 16:30, OSZ H4

Assessment of the genotoxicity of naturally occurring ingredients of plant foodstuffs using isotope-dilution UPLC-ESI-MS/MS

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Nutrition plays an important role in the development of certain types of cancer. Foods may contain ingredients that potentially initiate tumorigenesis via induction of DNA damage reflecting genotoxicity. Besides carcinogenic constituents of processed food, *e.g.* benzo[*a*]pyrene and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), several secondary plant metabolites were reported to form DNA-reactive products after metabolic activation and, therefore, pose a significant genotoxic or even carcinogenic risk. Here, we report on the determination of DNA adducts formed by 1-methoxy-3-indolylmethyl (1-MIM) glucosinolate, present in *Brassica* vegetables (*e.g.* broccoli and cabbage) or methyleugenol (ME), occurring in many herbs and spices (*e.g.* basil and pimento). Mass spectrometric methods were developed for adduct identification and quantification and subsequently applied to *in vitro* and animal studies and to human samples.

Experimenteller Teil

We used an ACQUITY-UPLC system equipped with a Xevo TQ MS (Waters) for our investigations. Reaction products of metabolically activated 1-MIM glucosinolate or ME with cell-free DNA and 2'-deoxynucleosides (dN) were identified by MS/MS experiments and NMR spectroscopy. Then, we synthesized stable isotopic labelled standards of the predominant adducts detected. These adduct standards were utilized for accurate quantification in several biological systems (bacteria, mammalian cells, various organs of mice, human urine and liver samples) using three mass transitions for multiple reaction monitoring (MRM) of each adduct. Adducts were detected as adducted dN. Therefore, enzymatic hydrolysis of modified DNA and subsequent sample purification using solid phase extraction, 1-butanol extraction or protein precipitation were optimized.

Ergebnisse und Diskussion

After metabolic activation 1-MIM glucosinolate and ME form adducts with exocyclic amino groups of DNA nucleobases. Using product ion scans supported by NMR spectroscopic data the main adducts were identified as N^2-(1-MIM)-2'-deoxyguanosine (dG), N^6-(1-MIM)-2'-deoxyadenosine (dA) and N^2-(*trans*-methylisoeugenol-3'-yl)-dG, N^6-(*trans*-methylisoeugenol-3'-yl)-dA. We have synthesized stable-isotopic labelled analogues of these adducts and developed isotope-dilution MRM methods for their quantification by UPLC-ESI(+)-MS/MS [1,2]. For quantitative liberation of these adducts from genomic DNA, similar amounts of DNA hydrolyzing enzymes were required [3]. Upon reasonable enrichment of 1-MIM or ME adducts, LODs in genomic DNA between 0.1 and 10 adducts per 10^8 dN were determined [1,5]. Accurate quantification was assured using two qualifier transitions in addition to the quantifier for each adduct. Recovery, inter- and intraday variation and linearity of detection were excellent for the adducts studied. We were able to detect dG and dA adducts in bacterial and mammalian cells and various tissues of mice in a rather constant ratio of 3 after treatment with 1-MIM glucosinolate [1,3,4] or 50 after application of ME [2,3]. In mice, we clearly detected DNA adducts of both substances after application of doses relevant for the human exposure. Indeed, we were able to detect ME DNA adducts in 29 of 30 human liver samples [5]. This was the first demonstration of DNA adducts formed by a xenobiotic in human liver using UPLC-ESI-MS/MS. Moreover, N^2-(1-MIM)-dG and N^6-(1-MIM)-dA were detected in human urine after consumption of broccoli, indicating the suitability of our methods to different matrices. Our data show that isotope-dilution UPLC-ESI-MS/MS is a selective, sensitive, robust and reproducible method for quantification of DNA adducts originating from genotoxic plant products. Therefore, it serves as a helpful tool for the toxicological risk assessment of such compounds.

Referenzen

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Neue Aspekte

Assessment of *in vitro* and *in vivo* genotoxicity of 1-methoxy-3-indolylmethyl glucosinolate and methyleugenol via DNA adduct analysis by isotope-dilution UPLC-ESI-MS/MS.

Preventing Wine Spoilage: Rapid Screen & Quantification of Off-flavor Phenolics using Ambient Ionization coupled with High Resolution MS/MS

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A major source of wine spoilage worldwide is due to the yeast *Brettanomyces bruxellensis*, which is responsible for significant economic loss in the wine industry[1]. Wines exhibiting a "Brett" character include medicinal and barnyard odors that are attributed to two volatile phenols, 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG)[2]. When 4-EP and 4-EG are at levels less than 400 µg/L they contribute positively to the sensory complexity of the wine giving it a spicy, smoky and leather aroma. However, when levels are greater than 620 µg/L, the "Brett" character may become too strong and the wine contains off-flavor notes and is often no longer marketable. With constant monitoring for 4-EP and 4-EG, winemakers can control production of these compounds and prevent economic loss.

Experimenteller Teil

Direct Analysis in Real Time (DART) ambient ion source was coupled to a high resolution accurate mass (HRAM) quadrupole mass spectrometer for targeted full-scan MS/MS analysis of 4-EP and 4-EG directly from wine samples. The DART source is unique in that samples, in their native state, can be directly introduced into the sampling region and directly ionized for MS detection. For quantitative measurements, internal standards, 4-ethylphenol-d4 and 4-ethylguaiacol-d5 (CDN Isotopes), were spiked into the samples at 500 µg/L. The targeted MS/MS experiment yielded detection limits in the very low ppb range by combining sample concentration with sorptive stir bars (PDMS Twister). Sample concentration onto the sorptive stir bars was carried out overnight, but shown to be complete in 30 minutes.

Ergebnisse und Diskussion

More than 20 wine samples were subjected to both GC-MS and DART-MS/MS analysis for comparison between the two methods. Sample analysis time using the DART ionization approach was 3 minutes per sample employing a slow heater ramp to thermally desorb the analytes directly from the Twister sorptive stir bar surface. The phenolic compounds 4-EP and 4-EG were detected as low as 10 µg/L from the wine samples, well below the critical level of 620 µg/L threshold for wine spoilage. The results of the DART-MS/MS method for monitoring 4-EP and 4-EG compared favorably with the GC-MS method where the DART approach is very easy to implement and a much faster and less labor intensive analysis than GC-MS for rapid screening and quantitation, with the use of internal standards. The direct DART method employing the stress free analyte concentration method using the simple sorptive Twister stir bar spinning protocol allows the analyst to prepare samples overnight without time consuming interactions with the samples. The concentration set-up takes less than 10 minutes to set-up: aliquot 2 mL of wine per vial, introduce a stir bar into each vial and begin the stirring, 30 minutes up to overnight (12 hours). The simple and short analysis time with DART ambient ionization, consisting of 3 minutes analysis time per sorptive stir bar sample, compared with a typical GC-MS run of 20-30 minutes, allows for many samples to be analyzed in high throughput, which is a key step forward to increase representative sampling of wine batches and for results in real time enabling better control of the wine production to prevent product loss due to spoilage.

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Neue Aspekte

Ultra-fast, direct method to quantitatively monitor phenolic off-flavor compounds in wine to prevent product spoilage during fermentation.

Seeking polyfunctionally cross-linked elastin peptides

Christoph Schräder[1], Christian Schmelzer[1], Wolfgang Höhenwarter[2], Reinhard Neubert[1], Andrea Heinz[1]

[1]Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany; [2]Leibniz Institute for Plant Biochemistry, Halle (Saale), Germany

Elastin is a vital extracellular protein that provides elasticity to various connective tissues such as blood vessels or lung [1]. It is formed through cross-linking of its precursor tropoelastin resulting in flexible macromolecules with characteristic bi- and polyfunctional cross-links whose exact pattern is still unclear [2]. Elastin is deposited for life and does not undergo significant turnover in healthy tissues. However, pathological conditions, including COPD and atherosclerosis, are associated with its irreversible destruction [3]. Thus, there is a strong need for biomarkers to assess the progression of such severe diseases. In this study, mass spectrometric techniques were developed to get insight into the fragmentation behavior of elastin cross-links to eventually enable the identification of cross-linked elastin peptides in biological samples.

Experimenteller Teil

MS/MS and pseudo MS^3 CID fragment spectra of the polyfunctional cross-links desmosine (DES) and isodesmosine (IDE) were obtained on an ESI-QqTOF instrument and for mass accuracy less than 3 ppm HCD fragment spectra on an ESI-LTQ-Orbitrap instrument, respectively. Fragment spectra were analyzed and characteristic fragment ions were compared to those of peptides from enzymatic digests of human elastin. A set of fragment spectra of linear peptides derived from an enzymatic digest of human tropoelastin, which does not contain cross-linked species, was measured by nanoHPLC-nanoESI-QqTOF MS/MS and used as negative control.

Ergebnisse und Diskussion

It was found that CID of DES and IDE leads to the formation of the same fragment ions differing only in their relative intensities. In particular, unspecific losses of CO and NH_3 groups and breaks of bonds at the pyridinium nitrogen were observed. Further breakdown leads to the formation of fragments of the molecular formula C_xH_yN suggesting that the pyridinium core itself remains intact even at elevated energies.

On the basis of this knowledge, cross-linked peptides showing these characteristic fragment ions in addition to immonium ions revealing the presence of K, A, F and P residues were targeted and identified. Elevated collision energies are necessary since DES/IDE are tetrafunctional amino acids and thus several bonds have to be broken simultaneously to release these marker ions.

Based on these findings, an algorithm was developed for the rapid identification of DES/IDE cross-linked peptides in biological samples such as blood plasma and urine as well as in enzymatic digests of mature elastin obtained from tissue biopsies. Since DES/IDE are important biomarkers for breakdown of elastin resulting from lung matrix injury in COPD, identification of the peptide bound forms of these cross-links in body fluids can significantly improve diagnosis and further understanding of elastin's breakdown processes. Moreover, the identification of cross-linked peptides in digests of elastin contributes to the long-awaited elucidation of elastin's cross-linking pattern.

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Neue Aspekte

A fast and versatile method was developed to identify DES-containing peptides, which serve as biomarkers for elastin degradation

Session V: Proteomics II

Montag, 03.03.2014: 15:30 - 16:30, OSZ HS 5

Absolute Quantification with the Q Exactive: Direct infusion vs. HPLC

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Absolute quantification of proteins becomes more and more important in proteome research. In the classical approach for absolute quantification (AQUA [1]) isotopically labeled counterparts of relevant endogenous peptides are added to the sample as internal standards followed by targeted MS-analysis e.g. multiple reaction monitoring (MRM) on a triple quadrupole instrument.

Experimenteller Teil

In order to avoid critical steps for successful absolute protein quantification such as unwanted sample loss as well as circumventing laborious method optimization for MRM analysis in our project we were interested in

1. simplifying and shortening the analyses by omitting an HPLC-based separation step. This was done by direct infusion of a complex peptide sample.

2. optimizing an alternative targeted MS-approach using the Q Exactive hybrid quadrupole-orbitrap mass spectrometer called targeted single ion monitoring (tSIM).

Ergebnisse und Diskussion

For our study different cytochrome P450 enzymes – membrane proteins of the endoplasmatic reticulum which are relevant for drug metabolism in the liver - [2] were overexpressed under different conditions in *E. coli*. Cells were lysed and separated into a membrane and a soluble fraction. Heavy labeled peptides were added and digested along with the protein samples.

For absolute quantification the samples were measured via direct infusion/MS in tSIM mode [3] as well as via HPLC/MS in tSIM mode. Resulting data were analyzed using the Qual Browser of the Xcalibur software.

Our results show high linearity in both direct infusion/MS and HPLC/MS approaches in the range of 2 fmol – 2 pmol. With both methods similar results were obtained in terms of absolute protein quantification demonstrating the power of our newly developed direct infusion tSIM approach for quantification of high abundant/overexpressed proteins in complex samples.

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Neue Aspekte

With our study we could demonstrate that direct infusion-tSIM is suitable for absolute quantification.

When is Mass Spectrometry Combined with Affinity Approaches Essential? A Case Study of Tyrosine Nitration in Proteins

Brindusa - Alina Petre[1,2], Claudia Andrieş[1,2], Mihaela Stumbaum[2], Bogdan Bernevic[2], Martina Ulrich[3], Gerd Doering[3], Gabi Drochioiu[1], Michael Przybylski[2]

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Oxidative modification of proteins may cause substantial biochemical as well as pathophysiological changes, both by chemical reactions and specific enzymatic pathways. In particular, nitration of Tyrosine residues has been associated to pathophysiological effects in proteins related to neurodegeneration, atherosclerosis, and broncho-alveolar diseases [1,2]. While immuno-analytical methods suffer from low detection specificity of antibodies, mass spectrometric methods for identification of Tyrosine nitrations are hampered by low stabilities and levels of modification, and by possible changes of structure introduced by the nitro group. High resolution FTICR mass spectrometry using electrospray in combination with immuno-affinity procedures have been developed as powerful tools for unequivocal identification of Tyr-modifications in proteins which is hampered, by low levels of modifications [3].

Experimenteller Teil

The proteolytic affinity extraction-MS approach using a 3-NT antibody column was employed for the identification of nitration sites in human eosinophils.The Eosinophil cationic protein and Eosinophil-derived neurotoxin were digested in solution and the mixtures applied to the column. After 2 hrs incubation, washing steps were performed to remove non-binding peptides, the bound peptides were eluted and analysed by nano-ESI-FTICR- MS. A direct online combination of bioaffinity and MS, was employed for the simultaneous characterization and quantification of the interaction of nitrated peptides with anti-3NT-antibody. The online affinity-MS approach utilized a surface-acoustic wave biosensor [4], with the anti-3NT antibody immobilized on a chip surface. Following association of peptides, dissociation was performed using an acidic buffer into the interface for ESI-MS analysis.

Ergebnisse und Diskussion

To enable the unequivocal identification of specific 3-NT residues in two eosinophil proteins (ECP and EDN) purified from eosinophil granules of patients with eosinophilia a proteolytic affinity-MS extraction ("PROFINEX"- MS) approach was developed. EDN was cysteine- carbamidomethylated and then digested in solution with thermolysin, used instead of trypsin due to the low number of Arg and Lys residues in the N-terminal part of the protein. The mixture of proteolytic peptides produced was submitted to the affinity column, incubated for 2 h, and the supernatant non-binding peptides removed by washing and analyzed by MALDI-TOF-MS as a control. The remaining antibody-peptide complex was then dissociated at slightly acidic conditions, and the elution fraction analyzed by high resolution FTICR- mass spectrometry. The ESI-FTICR-MS revealed a single Tyr-peptide, EDN (^{29}VINNY(NO_2)QRRCKNQNTF^{43}) with a monoisotopic mass corresponding to a nitration at Tyr^{33} with a mass increment of 103 Da (45 Da for the Tyr^{33}-nitro group, 58 Da for Cys^{37}-carbamidomethyl). Accessible surface areas were calculated using the Surface Racer program. The spatial orientation was compared for all tyrosine residues in EDN and showed that the Tyr^{33} residue has the highest surface accessibility (100.8 Å^2), compared with other tyrosines. These results showed that the affinity differences between the nitrated ECP peptides correlate with a specific sequence environment of the tyrosine residues. The SAW-MS biosensor results showed that the affinity of the nitrated ECP peptide was completely abolished upon replacing the positively charged residues Arg^{28}, Arg^{34}, Arg^{36}, and Lys^{38} by alanine, thus confirming the importance of cationic amino acids in the vicinity to the nitration site for affinity binding. Affinity determinations of the nitrated peptide in comparison to the intact ECP protein using the SAW biosensor provided dissociation constants of approximately 6 nM for the nitrated peptide ECP (24–41), and 28 nM for the intact ECP.

Referenzen

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Neue Aspekte

The PROFINEX-MS approach may be used as a high specific and sensitive tool for the molecular identification of other PTM's.

Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics

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Mass spectrometry (MS) is an essential tool for large-scale proteomic analyses. In addition to classical data-dependent acquisition (DDA) workflows for protein identification, data-independent acquisition (DIA) schemes are increasingly used. These do not suffer from inherent limitations of DDA approaches, such as irreproducible precursor ion selection and under-sampling. Recently, traveling wave based ion mobility separation (IMS) has been introduced into commercially available high-resolution instruments, providing an additional dimension of separation without increasing acquisition time, thereby improving overall system peak capacity while concomitantly reducing chimeric and composite interference. However, this significant increase in system peak capacity so far failed to deliver deeper proteome coverage. Here, we present an optimized IMS-enhanced DIA-based discovery proteomics workflow, capable of identifying over 4400 proteins from HeLa tryptic digest in triplicate runs.

Experimenteller Teil

HeLa cell lysate (20 µg) was digested using a modified FASP protocol. Peptides (20 - 300 ng) were separated by 1D-nanoUPLC on a 75µmx250mm HSS-T3 column and analyzed on a Waters Synapt G2-S instrument in data-independent acquisition mode with and without ion-mobility separation. Standard ramped and optimized collision energy profiles were evaluated. Rawdata processing and database searching was performed in PLGS3.0, searching UniProtKB/Swissprot (human reference proteome) and IPIv3.87 human databases. Results were parsed from PLGS and imported into a MySQL database using ISOQuant software followed by subsequent processing including retention time alignment, clustering and TOP3 based quantification. False discovery rate was limited to <1% on both peptide and protein level. Only proteins with at least two assigned peptides were reported.

Ergebnisse und Diskussion

We present an optimized discovery proteomics workflow based on ion mobility separation enhanced DIA (1). Ion-mobility provides an additional dimension of separation, leading to a 100% increase of identified proteins as compared to DIA experiments without ion mobility. Our novel acquisition method using optimized collision energy profiles further increased the number of identified proteins by 47% as compared to standard ion mobility-based LC-MS workflows. Data processing through the ISOQuant software increased identified proteins by >20% and – more importantly – the percentage of proteins identified in three out of three replicates from <50% to >97%. Using a 180 min nanoUPLC gradient, our IMS-DIA-based workflow enabled the identification of 4428 protein groups and >47,000 unique peptide sequences from triplicate 180 min gradient runs of only 300 ng of HeLa tryptic digest (UniprotKB/Swissprot database, at <1% FDR, requiring 2 peptides per protein). Analyzing the rate of identified unique peptides/s (averaged for each 10 s RT window and over three technical replicates), we demonstrate maximal peptide identification rates of >13 unique peptides/s, thereby exceeding the theoretical maximum for HCD workflows on current generation Orbitrap instruments. Concludingly, even utilizing significantly lower on-column loads, these results exceeded DDA-based data obtained from a Q-Exactive instrument using identical nanoUPLC conditions and on column loads, demonstrating the high sensitivity of the optimized workflow. Additionally, high precision and accuracy of DIA-based label-free quantification were verified using a hybrid proteome sample. Our data demonstrate that IMS-based DIA acquisition on fully commercially available, current generation instrumentation allows exceeding levels of performance compared to DDA-based workflows on Q-Exactive instruments, regarding sensitivity, number of identified proteins and peptide identification rates. We anticipate that future improvements in both IMS and mass resolution capabilities will increase system peak capacity and concomitantly also the number of identifiable proteins and peptides in DIA based proteomics.

Referenzen

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Neue Aspekte

Improved peptide fractionation efficiency and deep proteome coverage using ion-mobility based data-independent acquisition with drift time-specific collision energies.

Session VI: Proteomics III

Dienstag, 04.03.2014: 14:45 - 16:45, OSZ HS 1

A mass-spectrometry based hybrid method for the structural characterisation of protein complexes

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Emerging methods in structural mass spectrometry (MS) can pose an exciting alternative to classical methods for the determination of the structure of protein complexes, due to their low sample requirements and comparatively high measuring speed [1,2,3,4]. This is especially true if a complex remains refractory to high resolution methods, e. g. due to the transient nature of the macromolecular assembly. Native MS has been used to characterize the composition, stoichiometry and connectivity of protein complexes. Chemical cross-linking coupled to MS (CXMS) provides protein-protein interaction data with peptide resolution, supplemented with distance information on spatial proximity of residues in the native protein structure [3,4]. Ion mobility can provide additional information on shape and topology.

Experimenteller Teil

Here, we incorporate for the first time native MS, ion mobility MS and CXMS datasets in one integrated workflow to generate restraints for subsequent structure determination of multi protein complexes. Our approach incorporates label free quantification, native MS, ion mobiliy and CXMS datasets with modelling approaches in one single workflow to generate restraints for subsequent structure determination of multi protein complexes. The various MS based data are combined into a weighed scoring scheme capable of ranking models according to their violation of the restraints derived from the experimental data. We trained our approach on a set of protein complexes with known high resolution structures before validating it on a protein assembly of high biological interest.

Ergebnisse und Diskussion

We show that using this purely MS based approach we can not only generate reliable subunit interaction maps of unknown intact complexes but when combined with high resolution subunit information, even generate 3 dimensional pseudo-atomic models. We present a generic method that integrates structural data derived from different mass spectrometric (MS) techniques with a modelling strategy for the structural characterization of protein assemblies. It differs from other approaches by its ability to generate orthogonal datasets from the same sample and to computationally integrate the MS data generated with different levels of resolution and information content. The method capitalizes on the fact that the experimental techniques require similar quality and amounts of protein complex, exhibit comparatively high measuring speed and tolerate heterogeneous sample environments. We demonstrate accuracy and confidence levels of the method by assembling near-native models for three hetero-complexes with known structures and use it to characterize the intact proteasomal lid and an assembly intermediate thereof. As this approach is in principle applicable to a wide range of protein complexes, we expect this integrated technology to become an important component of the structural biology toolbox.

Referenzen

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Neue Aspekte

The method highlights a novel combination of approaches that overcomes the limitations in each technique when used in isolation.

Incorporation of 13C-labeled p-Benzoylphenylalanine and Mass Spectrometry for Photo Cross-Linking of Protein/Peptide Complexes

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Photo-affinity labeling is a powerful tool to study protein-protein interactions. Photo-activatable amino acids, such as benzoylphenylalanine (Bpa), can be introduced by synthetic incorporation into a peptide or by direct incorporation into a protein [1]. Bpa reacts via a biradical mechanism after UV-A activation, but a mass spectrometric identification of cross-linking products after photo-reaction is challenging due to the lack of an indicative mass label. To facilitate the identification of cross-links, $^{13}C_6$-labeled Bpa was incorporated into binding peptides from skeletal muscle myosin light chain kinase (skMLCK, M13 peptide) [2] and from retinal guanylyl cyclase (retGC peptide) [3] to map their interactions with calmodulin (CaM) and the guanylyl cyclase activating protein-2 (GCAP-2).

Experimenteller Teil

$^{13}C_6$-labeled Bpa was synthesized from $^{13}C_6$-labeled benzene and was synthetically incorporated into the M13 peptide replacing Trp-4 and into a retGC peptide replacing Tyr-7. For interaction studies with CaM and GCAP-2, equal amounts of non-labeled and $^{13}C_6$-labeled Bpa-peptides were incubated with their binding proteins with and without Ca^{2+} and irradiated with UV-A light. Complexes were analyzed intact and in a top-down fashion by ETD fragmentation using *offline*-nanoESI-Orbitrap-MS. Reaction mixtures were separated by SDS-PAGE and gel bands representing the complex were excised and digested with trypsin/GluC or trypsin/AspN. Obtained peptide mixtures were separated by nanoHPLC (Dionex) and analyzed by mass spectrometry (LTQ-OrbitrapXL, Thermo Fisher Scientific). Potential cross-linking products were identified with StavroX and screened for characteristic isotope patterns, based on ^{13}C-labeling.

Ergebnisse und Diskussion

Two protein/peptide complexes were investigated by photo-affinity labeling in combination with mass spectrometry. The complexes under investigation included the CaM/M13 peptide and the GCAP-2/retGC peptide complex. For this, non-labeled and $^{13}C_6$-labeled Bpa were incorporated at position Trp-4 (M13 peptide) and Tyr-7 (retGC peptide), respectively. After separation by one-dimensional gel-electrophoresis, gel bands revealed that complexes were created between M13 peptide and CaM and between the retGC peptide and GCAP-2. The Bpa-peptides were exclusively identified in the excised gel bands of the complex. Detailed MS/MS analyses of the peptide mixtures revealed a single cross-linking site between the Bpa-peptide of retGC and Met-64 of GCAP-2, both in the calcium-free and calcium-bound state. This indicates a specific reaction of Bpa.

Several cross-linking sites were identified between the M13 peptide and CaM pointing to the known antiparallel binding mode [4]. A few cross-linked products point to a parallel binding mode, but they were confirmed as a minor species by gel-electrophoresis and relative quantification. Additional ETD fragmentation of the intact CaM/M13 peptide complex confirmed only the antiparallel binding mode.

Cross-linking products were validated based on their isotope patterns within the mass spectra and comparable CID-MS/MS fragments of non-labeled and $^{13}C_6$-labeled Bpa-peptides. An exclusive identification of cross-links by assigning fragment ions would be highly questionable as fragmentation of Bpa cross-linking products usually results in neutral losses of the precursor. ^{13}C-labeling of Bpa significantly improved the reliability of cross-link identification both on the MS level by their characteristic isotope patterns and in MS/MS spectra by their characteristic mass shifts.

Referenzen

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Neue Aspekte

$^{13}C_6$-labeled benzoylphenylalanine for improved reliability of cross-link identification

Epitope Peptide Validation by Affinity Mass Spectrometry.

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Autoimmune diseases affect millions of people. Current diagnosis relies on the use of full length autoantigens which only detect a portion of patients. Moreover, patients suffering from different diseases can carry autoantibodies against one and the same antigen. It is therefore suggested to use epitope peptides instead of full length antigens as diagnostic probes. Currently, peptide-chip microarrays use overlapping synthetic peptides (typically 15mers *e.g.* with a frame-shift of 6 residues), that cover the full-length autoantigen proteins[1]. Yet, to improve cost effectiveness, the reduction to informative "epitope peptides" is desired. Disease-specific epitope peptides, however, need to be identified and validated which can only be achieved by highly efficient epitope mapping methods that can determine epitope - antibody reactivities at fmol levels.

Experimenteller Teil

The epitope extraction procedure was performed in 96-deep-well plates using three columns in parallel (one for each antibody and one for the buffer control) with a programmable Rainin E4 XLS 8-channel electronic pipette, equipped with 200 µL PureSpeed Protein Tips. After intial equilibration of the protein A/G resin, antibodies of choice were captured. The peptide mixtures were then administered. The nonspecifically bound peptides were sequentially washed away and the still bound peptides were eluted and analysed using MALDI TOF-MS (Bruker Reflex III) using 2,5-dihydroxybenzoic acid as matrix. Synthetic peptides comprising the epitope and point mutations, respectively, were used for paratope blocking and epitope competition experiments. Secondary structures of synthetic peptides were investigated using nano-ESI IMS-MS (Synapt G2-S) and CD spectroscopy.

Ergebnisse und Diskussion

Here, we describe the development of the epitope mapping approach with protein A / protein G resins that are embedded in pipette tips and are used in combination with an electronic programmable multi-channel pipette for epitope extraction[2]. The advantage of this system is the simple one-step generation of miniaturized custom-made affinity columns combined with programmable reproducible chromatography that can be instantly applied for mapping epitopes on large (auto)antigen proteins. We developed the protein A / protein G-based epitope mapping procedure using a monoclonal anti-His tag antibody together with the His-tag containing recombinant human autoantigen glucose-6-phosphate isomerase. Using this approach, the C-terminal His-tag epitope peptide (^{550}QQREARVQLEHHHHHH^{565}, $[M+H]^+$ 2079.02 Da) was identified as specifically interacting with the antibody. The procedure was then tested successfully with a polyclonal antibody against autoantigen TRIM21 which had already been determined to detect an assembled epitope[3]. The epitope peptide "E**L**EKD**E**RE**Q**LRI**L**GE" of recombinant human TRIM21 was captured by the polyclonal anti-TRIM21 antibody and identified by its protonated molecular ion with m/z 3498.82 by MALDI-TOF-MS analysis using as low as 100 fmol of the antibody and 200 fmol of the TRIM21 peptide mix, obtained by LysC digestion.

In addition to epitope identification, the influence of epitope structure modifications on antibody binding specificities were studied in detail with synthetic peptides by exchanging amino acid residues at specific positions. Charge state comparison and/or ion mobility analysis of eluted epitope peptides enabled to investigate higher order structures. The epitope peptide of the TRIM21 autoantigen that is recognized by the polyclonal antibody was determined as an assembled "L-E-Q-L" motif on one and the same side of an α-helix. Secondary structure determination by CD spectroscopy and structure modeling are in accordance with the mass spectrometric results and the antigenic behavior of the epitope peptide variants from the full-length autoantigen.

Referenzen

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Neue Aspekte

Miniaturized Antibody-Decorated Affinity Columns

Observing global structural changes of an ion channel during its gating by ion mobility mass spectrometry

Albert Konijnenberg[1], Duygu Yilmaz[2], Helgi Ingólfsson[2], Anna Dimitrova[2], Siewert Marrink[2], Armağan Koçer[2], Frank Sobott[1]

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Mechanosensitive ion channels are sensors probing membrane tension in all species. Despite their importance in many cell functions, their gating mechanism remains to be elucidated. Developing a novel approach for releasing membrane proteins from their detergents in the gas phase using native ion mobility-mass spectrometry (IM-MS), we could detect for the first time i) the native mass, hence the oligomeric state, of the mechanosensitive channel of large conductance (MscL); ii) the global structural changes during gating; and iii) functioning of MscL in the absence of a lipid bilayer. We triggered gating using heteropentameric MscLs and followed the resulting conformational changes by monitoring the collision cross section with IM-MS. Our findings will allow studying native structure of many other membrane proteins.

Experimenteller Teil

MscL homo- and heteropentamers (0-5 cystein mutants) were separated by chromatofocussing and solubilised in different detergent at 2x critical micelle concentration, with varying amounts of MTSET drug added. Proteins at 5-20 mM were recorded in positive ion mode on a commercially available traveling wave ion mobility mass spectrometer, Synapt G2 HDMS (Waters) with 32k quadrupole and settings optimized for transmission of large complexes.

Simulations of MscL gating were performed using the Martini coarse-grain model. The topology of MscL was derived from the crystal structure of the closed state Tb-MscL and the channel was solvated in 562 CG 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipids and around 20k CG water beads. Continuous wave EPR measurements were performed using a MiniScope benchtop X-band EPR spectrometer.

Ergebnisse und Diskussion

In this talk we report on the ability to directly observe the global structural changes of a mechanosensitive ion channel during its gating by using a new mass spectroscopy approach that combines native mass spectrometry and ion mobility. We studied the mechanosensitive channel of large conductance, MscL, from *E. coli*.

Our strategy has been the following: (1) we identified a class of detergents and ion mobility mass spectroscopy conditions that reduce the amount of activation energy for removing membrane protein-bound detergent molecules during measurements; (2) we determined the native mass of the ion channel and its heteropentameric versions in their closed form; (3) we triggered the opening of the ion channel into different sub-open states by chemically charging increasing number of hydrophobic pore residues of its heteropentameric versions; (4) we measured the global structural changes of MscL by measuring the collision cross section area of each sub-open state; (5) we verified our findings with EPR spectroscopy and molecular dynamics simulations.

To the best of our knowledge this is the first experimental demonstration of (1) direct observation of the global structural changes that a mechanosensitive ion channel undergoes during its gating; (2) functioning of this membrane protein in the absence of a lipid bilayer; and (3) the importance of the hydrogen bond forming capacity of a detergent for its use in native mass spectroscopy. We believe that our findings will enable the study of native structures of many other membrane proteins, and will be of great interest to scientists engaged in ion channel structure and function as well as native spectroscopy of membrane proteins.

Neue Aspekte

Use of a novel detergent for integral membrane protein analysis (monitoring ion channel gating) in native IM-MS

Combining non-covalent MS with chemical crosslinking: mechanistic insights into heat shock complexes.

Nina Morgner[1], Carla Schmidt[2], Ima-obong I. Ebong[2], Carol V. Robinson[2]

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Protein folding in cells is regulated by networks of chaperones including heat shock protein 40 (Hsp40), which controls complex formation between Hsp70 and client proteins, prior to interaction with the Hsp90 complex, which assembles in parallel. The client has to be transferred from the Hsp70 onto the Hsp90 complex. This processes need to be well regulated.

We investigated the Hsp70 and the Hsp90 cycles in interaction with a client protein - the glucocorticoid receptor (GR).

Experimenteller Teil

Non-covalent mass spectrometry is ideally suited to investigate the development of multicomponent complexes over time. All participating complexes can be monitored simultaneously in real time without need for labelling or uncertainty about the oligomerization state.

To gain structural information about the thus formed complexes we applied chemical cross-linking methods to the most dominant complexes formed during the chaperone assembly, as the complex initiating the client transfer from Hsp70 to Hsp90. Performance of BS3-crosslinking as well as comparative crosslinking in combination with non-covalent MS is particularly powerful since not only the protein stoichiometry but also the interaction interfaces and protein orientation can be defined.

Ergebnisse und Diskussion

Here we present a detailed analysis of the Hsp70/90 system with its co-chaperones and the client GR.

With non-covalent MS we followed the assembly of the Hsp70 and the Hsp90 cycle and then investigated the joining of the two cycles, evolving to a dominating $(Hsp90)_2$ $(Hop)_1$ $(Hsp70)_2$ $(GR)_1$ complex. Chemical cross-linking allowed to establish protein proximities and composition with regard to each other.

Combining both methods we found that Hsp40 not only catalysed binding of the client onto Hsp70, but as well Hsp70 self-dimerization in an antiparallel fashion. We could show that this antiparallel dimer exists in an equilibrium between different conformational states and is stabilised by a phosphorylation site, identified as a hot spot for phosphorylation in 11 eukaryotic species.

We propose that in the context of the Hsp70/40/GR chaperone cycle this dimerization plays a key role - one copy of Hsp70 binds to $Hsp90_2$ and Hop while the other Hsp70 binds to a client, facilitating client binding onto the $(Hsp90_2)(Hop)$ complex via dimerization of these two Hsp70 subunits to form the prevalent $(Hsp90)_2$ $(Hop)_1$ $(Hsp70)_2$ $(GR)_1$ complex. Cross-linking locates the client in close proximity to $Hsp90_2$, in preparation for transfer onto the Hsp90 dimer where folding and maturation of client proteins takes place.

Overall from these results we propose that Hsp70 antiparallel dimerization, mediated by catalytic amounts of Hsp40 and stabilised by phosphorylation, facilitates client transfer from Hsp70 to Hsp90.

Neue Aspekte

Combination of non-covalent MS and chemical cross-linking allows identification of key complexes on interaction pathways, their components and functional roles.

Session VII: Imaging

Dienstag, 04.03.2014:14:45 - 16:45, OSZ H4

Accurate mass MALDI imaging at 25 µm pixel size for proteins after on-tissue digestion

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The direct detection and identification of intact proteins in MALDI imaging remains a challenging task due to limited sensitivity and mass range. Here we present new approaches for on-tissue tryptic digestion of proteins. We focus on optimizing the spatial resolution and reliability of peptide identification.

Experimenteller Teil

Trypsin solution was deposited in several cycles on tissue with a spraying device. An atmospheric pressure matrix assisted laser desorption (AP-MALDI) ion source coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific GmbH, Bremen) was used for imaging experiments. Tryptic peptides were identified by matching imaged m/z peaks to peptides which were identified in complementary LC-MS/MS measurements of an adjacent tissue sections. All MS measurements were based on accurate mass (< 3 ppm RMS).

Ergebnisse und Diskussion

A coronal mouse brain section was measured at 50 µm pixel size. Peptide peaks were detected on tissue with a mass resolution of R=80000 (@m/z700). This measurement reveals detailed histological structures such as the ependyma (consisting of a single cell layer) which is clearly defined by several identified peptides. Highly reliable information about protein distribution was also obtained for clinical human tissue originating from brain and gastric cancer biopsies. This data is used to investigate intratumor heterogeneity on a molecular level. A whole body section of an infant mouse was imaged at 50 µm pixel size. Strategies for handling and processing this 40 GB data set by conversion to the common data format imzML will be discussed.

A coronal mouse brain section was imaged at a pixel size of 25 µm. The resulting ion images of tryptic peptides showed excellent correlation with myelin and H&E staining. Peptide peaks were detected on tissue with a mass resolution of R=40000 (@m/z700). The sensitivity could be significantly improved compared to previous experiments and about 150 tryptic peptides which show a clear spatial distribution were identified.

Initial results for formalin-fixed paraffin-embedded tissue will be discussed. Different protocols for deparaffinization and antigen retrieval were evaluated. Tryptic peptides were detected with accurate mass at 50 µm pixel size in mouse brain tissue.

The optimization of on-tissue digestion will be continued in the framework of a comparison study of the EU-funded COST Action (European Cooperation in Science and Technology) „Mass Spectrometry Imaging: New Tools for Healthcare Research" (BM1104) which will be briefly discussed.

Neue Aspekte

Increased spatial resolution, analysis of FFPE tissue and strategies for large data sets for MS imaging of tryptic peptides.

Laser Ablation-Based Bioimaging with Simultaneous Elemental and Molecular MS: Towards Spatially Resolved Speciation Analysis

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Speciation analysis covers the determination of metals or other heteroelements in their various oxidation states and binding forms. This is based on the fact that the properties of and the effects caused by the element species are not caused by the element itself, but by its particular chemical form. The determination of a sum concentration of this element is therefore not sufficient to estimate any effects from a selected elemental species. Traditionally, speciation analysis is carried out by a liquid or gas phase separation technique with complementary molecular and elemental mass spectrometry for analyte identification and quantification.

Experimenteller Teil

To add spatially-resolved information on the elemental distribution, which is particularly important for the analysis of heteroatom-containing drugs in animal and human tissues, laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) has been introduced in recent years. While low limits of detection are achieved using this technique, molecular information on the analytes is not available. MALDI-MS, however, is able to provide many answers in this field, but its complementary use to LA-ICP-MS requires different sample preparation techniques. Furthermore, these two methods cannot be used simultaneously for the analysis of the same sample.

Ergebnisse und Diskussion

The first simultaneous elemental and molecular mass spectrometric imaging approach based on laser ablation sampling has been developed. A 213 nm frequency-quintupled Nd:YAG laser is used to ablate material from solid samples. The generated aerosol is transported by a carrier gas, which consists of nitrogen or argon and may contain helium as additive. The gas flow is separated in a T-piece into two streams, which are directed into the ICP-MS and the atmospheric pressure chemical ionization (APCI)-MS, respectively. This way, elemental and molecular information is gathered in parallel.[1,2]

For tissue slices with a thickness between 3 and 10 µm, quantitative ablation can be achieved, which is important to apply quantification strategies for ICP-MS based on external, matrix-matched element standards. LA-APCI-MS provides the complementary molecular information on the detected species. A spatial resolution of 25 µm was obtained, and although higher resolutions down to 4 µm may be achieved with currently available instrumentation, it has to be considered that the ablated amount of analyte is decreasing with increasing resolution. Based on this approach, application methods have been developed for several solid samples including tissue slices of animal and human origin, tablets, thin layer chromatography cards and dried droplets.[1,2] Target analytes were pharmaceuticals (e.g. cisplatin, paracetamol) and histological staining agents (e.g. eosin, hemalum) as well as endogenous molecules such as thyroxin. To show the distribution of the target analyte, images for the non-fragmented pseudomolecular ions $[MH]^+$ obtained by LA-APCI-MS and for the heteroatoms observed by LA-ICP-MS were created. Exemplarily, the staining agent Eosin was examined in tissue slices of mouse organs. In LA-APCI-MS, the Eosin molecules were detected as $[MH]^+$ ions with a deviation of less than 4 ppm between the determined and calculated mass-to-charge ratio. In LA-ICP-MS, the Eosin distribution is reflected by the observed bromine.

Neue Aspekte

The first simultaneous elemental and molecular MS bioimaging method for pharmaceuticals and organics in tissue slices is presented.

MALDI-MS-Imaging with a Synapt G2-S Operated at Elevated Pressure: Improving the Analytical Sensitivity and the Lateral Resolution to ~10 µm

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Mass spectrometers from the Waters Synapt-G2-HDMS family are widely employed for MALDI-MS-Imaging. These instruments are operated at relatively low pressures of ~0.3mbar in the intermediate pressure ion source and (before introduction of the G2-Si-model) with a relatively low lateral resolution of a few ten µm. Here, we demonstrate that upon elevating the source pressure to about 0.7mbar an increase in analyte ion abundances by up to a factor of 10 can be achieved. A dithranol matrix was used that provides a particularly efficient generation of lipid ions in the negative ion mode. Using an external low-diffraction N_2-laser instead of the Nd:YAG-laser of the G2-S enabled recording the distribution of glyco- and phospholipids in brain tissue with a resolution of ~10µm.

Experimenteller Teil

Dithranol matrix was dissolved in $CHCl_3$:MeOH (2:1) to ensure an efficient extraction of (glyco-) lipids from mouse brain tissue slices of 15-20 µm thickness. A pneumatic spray system [1] was used to produce uniform matrix layers with crystal sizes of a few µm. The ion source of the Synapt G2-S instrument (Waters) was amended with electromagnetic valves and pressure gauges that are controlled by custom-made software [2], as well as apertures that confined the pressure regions. This allows adjusting the pressure in the sample region between 0.05-4 mbar. A second modification allows to use a system of external lasers in addition to the default Nd:YAG laser. Here, we show results obtained with two low-diffraction N_2 and Nd:YAG lasers.

Ergebnisse und Diskussion

Elevating the ion source pressure above the default value of ~0.3 mbar significantly increased both dithranol matrix and lipid analyte ion signals. For example, an increase in the analyte ion abundances by about a factor of 10 was found at optimal pressure values of 0.7 mbar. An improved ion transport due to laminar gas flow as well as an enhanced collisional cooling [3] and focusing could contribute to the signal increase. Both, the particular uniform coverage of the tissue slices with the microcrystalline dithranol matrix and the high efficiency of this matrix for the generation of glyco- and phospholipid ions (including sulfatides and gangliosides) in the negative ion mode lend themselves to perform MALDI-MSI with close to cellular resolution. Making use of this potential, however, required changes in the laser beam focusing of the used Synapt G2-S instrument in order to produce equivalently small spot sizes.

Using the expanded beams (2-3 times) of a low-diffraction N_2-laser (MNL-100, Lasertechnik Berlin) and/or a Nd:YAG-laser (Flare PQ UV 1000-30, Innolight/Coherent) and replacing the standard focusing lens by one with a smaller focal length allowed to record the distribution of glyco- and phospholipids from brain tissue slices with a true lateral resolution of about 10 µm (i.e., essentially without oversampling). Based on the distribution of these endogenous lipids and by a comparison with H&E-stained slices (prepared after washing off the matrix), various small-sized brain areas with dimensions in the low 10 µm-range could be differentiated.

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Neue Aspekte

First report on MALDI-MS Imaging with close to cellular resolution using a Synapt G2-S mass spectrometer.

High-Resolution Tandem Mass Spectrometry Imaging

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High spatial resolution MALDI mass spectrometry imaging (MSI) has become a valuable histological tool providing molecular and topological information on various types of biomolecules (1,2). High mass resolution allows to differentiate thousands of different compounds in complex biological samples. High mass accuracy furthermore allows to identify many of these compounds based on elemental composition. The major part of compounds in biological tissue, however, exhibits a large number of isomeric structures with identical elemental compositions, requiring MS/MS fragmentation for structural elucidation. To structurally identify isobaric components of a tissue section, MS/MS analysis has to be performed under imaging conditions, whereas offline fragment ion analysis of tissue homogenates by ESI MS/MS does not allow to assign isomers to their individual topological distributions.

Experimenteller Teil

Tissue sections of 10-20 µm thickness were prepared with a microcryotome. For positive ion measurements 2,5 dihydroxybenzoic acid (DHB) was homogeneously deposited by means of a high-resolution matrix-preparation robot (SMALDIPrep, TransMIT GmbH, Giessen, Germany). A high-resolution atmospheric-pressure MALDI ion source (AP-SMALDI10, TransMIT GmbH, Giessen) was used for imaging (1). The source was coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific GmbH, Bremen), set to a mass resolving power between 50,000 and 140,000 at *m/z* = 200. Internal calibration was performed using a matrix ion signal as a lock mass, resulting in a mass accuracy of typically better than 3 ppm. Ion fragmentation during imaging measurements was performed by collisional activation in the HCD cell of the mass spectrometer.

Ergebnisse und Diskussion

Images were generated with a bin width of ±5 ppm. The software package 'MIRION' was used to generate mass images from raw files generated by the mass spectrometer. Matrix cluster peaks were used for internal calibration and ions generated by 30 laser pulses per spot were accumulated prior to detection. Images with a pixel size of 10 to 25 µm were formed by high resolution scanning. Optical images of samples were taken before and after measurements with a light microscope. Post-measurement optical images were obtained after washing and staining, if tissue quality allowed.

MS/MS data of plant, insect and mammalian samples were acquired in imaging mode (MS^2I), resulting in high-resolution images of fragment ions. High quality topological structures were obtained for fragment ions of moderate to high signal intensities. Due to the limited resolution in precursor ion selection of most modern mass spectrometers, ion fragmentation can typically not be restricted to only one precursor ion. The high quality of fragment ion images derived from such measurements, however, in most cases allowed to unambiguously attribute fragment ions to their precursor ions, based on their identical distribution images.

It was found that MS^2 imaging is routinely possible with almost the same quality and sensitivity as MS imaging. MS^2I-assisted structure analysis, as described above, has been found to be a powerful tool for analysis of complex samples under insufficient precursor selection conditions.

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Neue Aspekte

High resolution MALDI MS/MS imaging was employed to elucidate isomeric structures and to assign isobaric precursor ions.

Localization of secondary metabolites involved in flavonoid biosynthesis during fruit development in strawberry

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Flavonoids are important secondary metabolites in strawberry (*Fragaria × ananassa*), since they accomplish a wide variety of physiological functions and are valuable for the human health. For example the bright red anthocyanin in ripe fruits attracts herbivores for seed dispersal. However, the localization of secondary metabolites within the fruit tissue is still unknown. As a result matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MSI) was employed to shed light into the spatial distribution of flavonoids during fruit development.

Experimenteller Teil

The octoploid strawberry cultivar 'Senga Sengana' was used. Fruits of two ripening stages were collected from wild-type as well as two transgenic lines with impaired flavonoid pathway and analyzed by MALDI-MSI. Strawberry sections were cut on a cryostat and transferred to conductive ITO-coated glass slides, dried at room temperature, and stored in a desiccator for 30 minutes. Subsequent the MALDI matrix THAP was applied onto the tissue sections using an air-brush device. Mass spectra were immediately acquired using an UltrafleXtreme (Bruker Daltonics, Germany), operating in reflectron negative mode. A *m/z* range of 0 to 1,500 was analyzed, accumulating 3,000 laser shots per spot. Data sequence preparation, MS acquisition and visualization was performed using the FlexImaging and FlexControl software.

Ergebnisse und Diskussion

Initially, the method was validated with reference compounds to confirm the suitability of the matrix. Targeted analysis using diagnostic ions of selected flavonoids showed that putative flavonoids were either localized at the outer edges of the tissue (primarily anthocyanins) or evenly distributed within the whole tissue slice. Some presumed flavonoids actually showed differences in the localization upon the ripening stage. The transgenic lines varied in the signal intensities of certain ions concluding that the related compounds are involved in the flavonoid biosynthesis pathway. The MALDI-MSI data could only partly be confirmed by LC-ESI-MS analysis of strawberry extracts obtained from the transgenic lines [1].

For the identification of the secondary metabolites MALDI-MS^2 analysis was performed on all major signals and compared with the data obtained by LC-ESI-MS^2 [1]. However, the identification was challenging since no separation of the different compounds bearing identical molar masses prior to fragmentation was possible and therefore mixed spectra were generated by MALDI-MS^2. This is the first time that MALDI-MSI was employed to reveal the distribution of flavonoids during fruit development in strawberry.

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Neue Aspekte

MALDI-MSI of strawberries

Session VIII: Organische MS + Grundlagen

Dienstag, 04.03.2014:14:45 - 16:45, OSZ H5

Separation of charge isomers of small zwitterions by differential ion mobility spectrometry–tandem mass spectrometry

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This presentation focuses on the gas-phase separation of charge isomers of zwitterionic quinolone antibiotics formed by electrospray ionization (ESI). ESI-LC-MS/MS analyses of quinolone drugs reported in the literature show inconsistent product ion spectra and erratic MRM results, which, among other things, seem to vary with composition and pH of the HPLC mobile phase. We hypothesize that these inconsistencies are the result of charge isomers formed during ESI, which under collision-induced dissociation conditions generate different product ion mass spectra. Herein, we investigated the species formed during ESI by differential ion mobility spectrometry (DMS) combined with tandem mass spectrometry.

Experimenteller Teil

ESI-DMS-MS/MS experiments were performed on an AB Sciex QTRAP quadrupole linear ion trap (QqLIT) instrument, which was equipped with a SelexIon differential ion mobility device between ESI source and entrance quadrupole q0. Different quinolone molecules in the MW range between 300 and 350 Da were examined as well as several volatile solvents to enhance separation in the DMS device. Gaussian computational calculations were performed to determine the proton affinities of the different functional groups in the molecules.

Ergebnisse und Diskussion

4-Quinolone drugs share a common 4-oxo-1,4-dihydroquinoline skeleton; while all members of this antibiotic class posses an acidic carboxyl group, several important variants also have a basic moiety (*e.g.* a piperazinyl group) in their structure, thus making them zwitterionic compounds. While in solution the site of protonation at low pH is clearly defined (*viz.* at the basic group, where protonation at low pH is only possible. The experimental dissociation constant, p*K*, of the basic group is several orders of magnitude higher than the carboxyl group), several important product ions seen in the CID spectra cannot be explained by fragmentation of this particular $[M+H]^+$ species. We hypothesize that the $[M+H]^+$ ions formed by ESI, with the proton initially residing at the piperazinyl group, isomerize during the transfer from liquid to gas-phase during the ESI process by solvent mediated CI, and a mixture of $[M+H]^+$ ions then enters the gas phase. If this was true, the CID spectra would represent composite CID spectra of several $[M+H]^+$ charge isomers. This theory was first investigated by computational calculations of proton affinities. Interestingly, the theoretical results indicate that in fact the acid moiety exhibits a slightly higher proton affinity in the gas phase than the piperazinyl group, thus reversing the order of basicity in the solution phase. This property readily allows proton retention at both locations within the molecule in the gas phase. Subsequently, we used differential ion mobility spectrometry to probe the $[M+H]^+$ species generated by ESI. These experiments yielded two major species, the structures of which we could unambiguously identify by their entirely different CID spectra. The presentations describes aspects of the DMS separation such as the use of chemical modifiers to enhance resolution and differential experiments with acidic quinolones that highlight how strongly charge location influences selectivity for small molecules in DMS.

Neue Aspekte

Separation of ESI generated charge isomers of small drug molecules by differential ion mobility prior to tandem mass spectrometry analysis

Simulation of the Oxidative Drug Metabolism by Electrochemistry/(Liquid Chromatography/)Mass Spectrometry using a Microfluidic Electrochemical Cell

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Electrochemical (EC) oxidation has proven to be a valuable tool in the simulation of oxidative drug metabolism. Online coupling to mass spectrometry (MS) allows the subsequent detection and identification of the generated oxidation products. In order to detect even short-lived compounds, such as radical cations, the transfer time between EC and MS has to be kept to a minimum. In this presentation, we present an approach for the electrochemical generation of oxidative metabolites using a microfluidic electrochemical cell with an integrated electrospray ionization (ESI) needle in order to achieve a short transit time between EC and MS detection

Experimenteller Teil

The hyphenation of an electrochemical cell to electrospray ionization mass spectrometry (ESI-MS) enables the immediate detection of the generated oxidation products. Application of a potential ramp from 0 - 1200 mV and the presentation of the data in form of three-dimensional mass voltammograms provides a broad overview on the generated oxidation products. The electrochemical oxidation was performed using a microfluidic electrochemical cell with an integrated ESI needle, which allowed the rapid detection of generated oxidation products. Furthermore, an LC system was integrated into the set-up in order to investigate the reactivity of the generated reactive intermediates towards endogenous peptides and proteins, thus allowing the estimation of toxic side effects.

Ergebnisse und Diskussion

The oxidative metabolism of several pharmaceutically relevant compounds was investigated using a new microfluidic electrochemical cell with an integrated ESI needle. The main advantages of this set-up are the segregated channels of the working and the counter electrode, assuring the avoidance of follow-up reactions of the generated oxidation products as well as the short transfer time between EC and MS detection, which enables the determination of short-lived species.

The phase I metabolism of the three drugs amodiaquine (AQ), clozapine (CLZ) and chlorpromazine (CPZ) was simulated electrochemically. In case of AQ and CLZ, the main metabolites, which are known to be highly reactive towards biomolecules, were successfully generated by means of EC. Furthermore, oxidation of CPZ allows the detection of the respective sulfoxide and of a short-lived radical cation, which cannot be detected using the established commercial thin-layer cell due to the extended transfer time between oxidation and MS detection.

Additionally, acetaminophen (APAP) was oxidized on chip and a solution of the endogenous nucleophile glutathione (GSH) was directly added to the effluent of the cell, thus allowing the subsequent generation of adducts with the reactive metabolite of APAP. Hereby, the phase II metabolism, which includes conjugation reactions of metabolites with e.g. GSH, was successfully simulated. In addition to that, different proteins containing free cysteine moieties were allowed to react with the electrochemically generated reactive metabolites. In these experiments, an online HPLC separation was integrated between EC and MS. Protein adducts of different metabolites were identified, thus giving information about their reactivity towards larger biomolecules which are also present in the human body.

Neue Aspekte

Electrochemical oxidation on chip with an integrated ESI needle allows the rapid MS detection even of short-lived metabolites.

Development and application of mass spectrometric methods for the investigation of organocatalytic reactions

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In the last few years organocatalysis has emerged as a new catalytic method based on metalfree organic molecules. In many cases these often small compounds give rise to extremely high enantioselectivity. The catalysts can be easily synthesized in both enantiomerically pure forms and are often more stable than enzymes or other bioorganic catalysts. The mechanistic details of many organocatalytic reactions are still not fully understood. The knowledge of the reaction mechanism on the other hand is important to optimize the reaction conditions toward higher reaction efficiency.

This work presents some examples of studies about organocatalytic reactions. The aim of this work was to understand and follow all chemical transformations of these reactions.

Experimenteller Teil

ESI-MS and APCI-MS data were acquired using a Thermo TSQ Quantum Ultra AM triple quadrupole mass spectrometer (Thermo Scientific, Dreieich, Germany) equipped with an APCI and an ESI source which were controlled by Xcalibur software. High resolution MS data were aqcuirred using an LTQ-Orbitrap Elite mass spectrometer (Thermo Scientific, Bremen, Germany).

Additional specific techniques such as isotopic labeling, ion/molecule reaction in the collision cell of the MS triple quadrupole analyzer were applied to characterize of the reactive reaction intermediates and the determination of the reaction pathways of the product(s) and side product(s).

Ergebnisse und Diskussion

Structural elucidation of organic compounds is commonly achieved by analytical techniques such as NMR-, UV/Vis- or IR-spectroscopy. The information gained from these methods is essential for the characterization of the reactive intermediates and thus the reaction mechanism investigation. These spectroscopic methods however have enormous limitations when the compounds of interest are existent only in low concentrations or have very short life times.

Mass spectrometry is the method of choice for the analysis of rapid and complex catalytic reactions. Because mass spectrometry is generally a very fast and sensitive technique, it is capable of investigating compounds with short life times and in low concentrations. In addition it is also possible to characterize different analytes at the same time without separation and, with MS/MS experiments, to perform structural elucidation of each of these directly from the reaction solution.

High resolution MS can be used to obtain the elemental composition of the unknown analyte(s), which can give further insight into the studied mechanisms and the compounds that are involved.

Here, details of complex organocatalytic reactions are presented and formation pathways will be described.

Neue Aspekte

Mass spectrometry as method of choise to investigate organocatalytic reaction mechanisms

TLC-MS: a combination of two powerful analytical methods

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Coupling TLC (thin-layer chromatography) with mass spectrometric detection is a current hot topic of analytical (bio)chemistry [1]. The individual methods are well established, widely used and appreciated particularly for their stability, reproducibility and sensitivity. Nevertheless, there are still serious limitations to overcome. TLC is obviously strong in seperating complex samples mixtures, however, it is limited by the availability of reference substances and staining methods and/or the UV activity of the analytes. In contrast, MS delivers excellent classification of substances by their mass, but leads to an overflow of information in analytical mixtures that may be hardly manageable. Therefore, TLC/MS combination is a very attractive couple even if some further methodological improvements are necessary.

Experimenteller Teil

TLC-MALDI experiments were performed using HPTLC plates with aluminum backs and different silica layer thicknesses. Subsequent to chromatographic separation of selected mixtures, the entire TLC plates were dipped into a solution of 2,5-dihydroxybenzoic acid (DHB, 100mg/ml) dissolved in acetonitrile/water (50:50, v/v) [2]. TLC plates were mounted onto a specially designed adapter target and automatically scanned using TLC-MALDI software developed by Bruker Daltonics. Spectra were recorded in the positive ion reflectron mode using an Autoflex instrument.

Elution-based experiments were performed using the TLC-MS interface from Camag to extract zones from the TLC plate and transfer them online into the MS.

Ergebnisse und Diskussion

The traditional method [3] of combining the power of TLC separation with mass spectrometric characterization is based on the re-extraction of the sample from the TLC plate. This approach has the advantage that basically all MS methods can be used. However, there are also the disadvantages that (a) extraction losses may occur and (b) compounds with very similar Rf values can be hardly differentiated. In contrast, desorption methods such as MALDI easily overcome the resolution problem because the resolution is determined by the MALDI laser spot size. Nevertheless, MALDI uses normally a small organic molecule as matrix and this matrix leads to a significant background which is a problem for smaller molecules.

It will be shown that the intensity of the matrix background signals (for instance m/z 551.0 in the case of positive ion mode spectra recorded in the presence of DHB) can be significantly reduced and a sensitivity gain can be achieved if the silica gel layer thickness is decreased from 200 to 100 µm. This will be illustrated by using selected lipid mixtures with the focus on phosphatidylcholines (PC) and phosphatidylethanolamines (PE) which are abundant in biological samples and present in nearly all samples from a biological origin [4].

Additionally, it will be shown that the silica gel layer thickness has a significant impact on the quality of the achievable mass spectra when re-elution and different ionization techniques (such as ESI) are used: improved detection limits, increasing sensitivity and improved S/N ratios can be obtained when "thinner" TLC layers are used. Therefore, applications for pharmaceutical ingredients, cosmetic actives and other molecules of biological relevance will be presented.

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Neue Aspekte

It was demonstrated that the silica layer thickness has an extreme impact on the quality of the related mass spectra.

Phthalate screening of toys using DESI MS

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Phthalates are still the most frequently reported hazards found in children toys[1]. High exposure of infants and toddlers with phthalates are associated with a serious hormonal disruption, especially in male children[2]. Six phthalates are currently restricted by the EU to a limit of 0.1 percent of weight in plastic toys[3]. Routine analysis methods involve homogenization, extraction and GCMS analysis[4] and are thus not suitable for screening. Current screening methods based on FTIR lack the ability to distinguish restricted phthalates from their permitted isomeric alternatives. Here we explore the possibility to utilize desorption electrospray ionization (DESI) and a portable mass spectrometer (MS) as a screening system for phthalates.

Experimenteller Teil

Analysis of phthalate standards and toys was carried out using a custom-built DESI ion source that was mounted onto a motorized 2D stage. Motorized stage was controlled with Servo Design Kit hardware and software. Standards solutions were deposited on PE filters for DESI MS analysis. Toys were analyzed with no further sample treatment. Mass spectra were acquired using an Orbitrap Exactive instrument (Thermo Fisher Scientific Inc., Bremen, Germany). Standard solutions were additionally measured with a portable mass spectrometer Mini 11[5] (Aston Labs, Purdue University, USA) and a LTQ FTICR instrument (Thermo Fisher Scientific Inc., Bremen, Germany).

Ergebnisse und Diskussion

Nine phthalate standards including the six restricted phthalates (BBP, DNOP, DINP, DEHP, DIDP, DBP) and two isomeric substitutes (DIOP, DIBP) were analyzed with DESI MS. Determined limits of detection (LOD) were found to meet limits given by legislation. Background phthalate signal of the instrumental setup however had a strong impact on determined LODs. Accordingly a background reduction could improve LODs of the method considerably in the future. In addition a puppet containing DEHP was analyzed with DESI MS. The phthalate was readily detected on the sample surface without any sample preparation demonstrating the potential of DESI as an ionization method for an on-site screening system. In parallel a multi-reaction monitoring method was set up for reliable identification of isomeric phthalates allowing immediately the unambiguous identification of 5 out of 6 restricted phthalates without any chromatographic separation. Furthermore determined LODs of the phthalate at a portable mass spectrometer were found to meet limits of legislation enabling its planned use for on-site analysis. Given these first encouraging results, future research will focus on the development of a quantitative DESI method for phthalates and the coupling of DESI with the portable mass spectrometer.

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Neue Aspekte

DESI MS based method for screening of phthalates in toys

Session IX: Proteomics IV

Mittwoch, 05.03.2014:10:30 - 13:00, OSZ H1

Structure and Function of Eukaryotic Translation Initiation Factor 2 and its Nucleotide Exchange Factor - Insights from Mass Spectrometry

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Protein synthesis in eukaryotes is a highly regulated process and its initiation requires the interplay of at least ten initiation factors (eIFs). eIF2 delivers methionyl initiatior tRNA to the ribosome and thus starts translation initiation[1]. This requires hydrolysis of GTP and subsequent nucleotide exchange by eIF2B. We combined mass spectrometry (MS) and chemical cross-linking to study eIF2, its nucleotide exchange factor eIF2B and their interactions. MS of intact complexes reveals their stoichiometries and stable interaction modules, while chemical cross-linking gives insights into protein-protein interactions on the atomic level. Using a comparative cross-linking strategy we describe tRNA binding and its effect on eIF2 flexibility. Location of identified phosphosites within the interaction interfaces suggests their role in complex stability and ligand binding.

Experimenteller Teil

eIF2 and eIF2B complexes were overexpressed in *Saccharomyces cerevisiae* and affinity purified applying either a His- or FLAG-tag strategy. Mass spectra of intact protein complexes were acquired on a Q-Star XL mass spectrometer (MDS Sciex) modified for high masses[2]. Chemical cross-linking was performed using deuterated (d4) and non-deuterated (d0) BS3-cross-linker. Cross-linked proteins were digested with trypsin and generated peptides were analysed by LC-MS/MS on a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). Potential crosslinks were identified using Massmatrix web server[3] and were validated by inspection of the MS and MS/MS spectra. Homology models of protein subunits were obtained from MODELLER and SWISS-MODEL web servers. Phosphosites were identified by LC-MS/MS and database searching after enrichment of phosphopeptides with TiO_2.

Ergebnisse und Diskussion

eIF2 is a hetero-trimeric complex comprised of a, b and g subunits. Binding of initiator tRNA leads to formation of the ternary complex (TC), which delivers the tRNA to the ribosome to start translation initiation. eIF2B then exchanges GDP for GTP to recycle eIF2 for a new round of translation initiation. We combined MS and homology modelling to study the structural arrangement of these protein complexes in detail.

Mass spectra of intact eIF2 confirmed the presence of one copy of each subunit and further revealed weak associations with the b subunit in free eIF2. We used chemical cross-linking to map protein-protein interactions within eIF2. Projecting cross-links onto homology models showed that eIF2 is highly flexible in solution. tRNA-binding was then studied by MS and cross-linking and revealed stoichiometric binding of tRNA to eIF2. However, it was difficult to draw conclusions from this cross-linking experiment since only few inter-protein cross-links were obtained. We therefore performed comparative cross-linking utilizing BS3-d4 and BS3-d0 cross-linkers; we cross-linked eIF2 and the TC side by side using BS3-d0 and BS3-d4, respectively, and pooled the two solutions in a 1:1 ratio. Intensities of the cross-linked peptides reflect changes in cross-linking intensities within eIF2 and the TC. Using this comparative strategy we found that the binding of tRNA to form the TC induces a more rigid structure than free eIF2. We also analysed phosphorylation sites within eIF2. Their location in homology models suggests their role in complex stability and ligand binding.

We also studied the pentameric nucleotide exchange factor eIF2B and its sub-complexes. Surprisingly, MS spectra of the intact complex showed the sole presence of a dimer of eIF2B-pentamers rather than the expected pentameric complex. Applying cross-linking we identify interactions between eIF2 and eIF2B allowing us to propose a multistep mechanism for nucleotide exchange.

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Neue Aspekte

Structural arrangement within free and tRNA-bound eIF2 and its complex formation with nucleotide exchange factor eIF2B.

Incorporation of Photoactivatable Amino Acids for Cross-Linking of Protein-Protein Complexes studied by Mass Spectrometry

Knut Kölbel[1], Philip Lössl[2], Dirk Tänzler[1], Andrea Sinz[1];

Chemical cross-linking, combined with high-resolution mass spectrometry (MS) and computational modeling, has been established as a strategy to elucidate three-dimensional structures of proteins, protein-ligand or protein-protein complexes [1]. Recently, this approach was augmented by photo-cross-linking of deliberately incorporated UV-activatable residues [2].

Pull-down assays, commonly used as screening method for interacting proteins, can yield large amounts of questionable or false positives – especially if sensitive MS methods are applied for detection. Recently, diazirine-labeled photoamino acids have been developed to covalently link protein complexes within living cells [3]. Although designed without MS analysis in mind, they proved particularly suited for obtaining structural information of protein complexes using distance constrains from cross-linked products.

Experimenteller Teil

Proteins (nidogen-1 and laminin-γ1 short arm variant) were recombinantly produced in HEK293 EBNA cells. Labeling was achieved by adding photo-leucine and photo-methionine into medium depleted of leucine and methionine (both ThermoFisher Scientific). Extracts of non-transfected HEK293 cells served as prey. Mixtures of purified proteins and, if expedient, cell extracts were subjected to UV-A irradiation in a home-built device, proteolytically digested, and analyzed by LC/nanoESI-MS/MS (Ultimate 3000 nanoHPLC (Ultimate 3000, Dionex coupled to an LTQ-Orbitrap XL hybrid mass spectrometer, ThermoFisher Scientific equipped with a nanoelectrospray ionization source, Proxeon). Potential cross-linked products were identified using the in-house software StavroX [4] and verified by manual inspection of the spectra.

Ergebnisse und Diskussion

The photo-reactive amino acids photo-leucine and -methionine were incorporated into the extracellular matrix proteins nidogen-1 and laminin-γ1 (short arm variant). Both proteins were purified by affinity chromatography and incorporation of photoamino acids was ascertained by MS/MS. Assuming unaltered ionization efficiencies compared to the unlabeled tryptic peptides, incorporation yields varied between ca. 1% (photo-leucine) and 65% (photo-methionine).

Concordant structural information of the monomeric forms of both proteins as well as of their heterodimer was obtained by 1) chemical cross-linking (BS^2G) of unlabeled proteins and 2) photo-cross-linking of labeled proteins, subsequent SDS-PAGE, in-gel digestion, and LC/nanoESI-MS/MS. These results served as input for structural modeling of the nidogen-/laminin-γ1 heterodimer. The model of the complex was then verified by results from in-solution digested photo-cross-linking products of purified labeled nidogen-1 and crude laminin-γ1 solutions.

Identified intramolecular interactions in nidogen-1 and computational modeling revealed an alternative, more globular, fold of nidogen-1 compared to the previously assumed elongated structure. Moreover, novel interaction sites in nidogen-1 and laminin-γ1 were identified, which both agree and disagree with the canonical pattern.

In order to identify novel interaction partners, purified labeled nidogen-1 and crude HEK293 cell extracts were used as bait and as prey, respectively. After the photo-reaction, the proteins were precipitated and potential binding partners were enriched by denaturing IMAC via the covalently attached His6-tagged nidogen-1. Despite denaturing conditions being applied, false positives were detected, even in the negative control. However, two potential binding partners, desmoplakin and desmoglein-1, were identified in three independent experiments and were found to be specifically enriched (i.e. not occurring in negative control samples).

Finally, results from a previous study of cross-linkable synthetic peptides [5] and >100 fragment ion spectra of tryptic peptides (cross-linking products and linear peptides) were evaluated to elucidate the influence of labeling and photo-cross-linking upon collisionally induced dissociation.

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Neue Aspekte

Photo-amino acids were incorporated into proteins to study three-dimensional structures of proteins and protein complexes by MS.

Investigation of protein–RNA interactions by UV induced cross-linking, high resolution mass spectrometry and automated database search

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Mass spectrometry is increasingly often applied to the investigation of protein–RNA interactions. In combination with UV induced cross-linking, MS is powerful in identifying proteins interacting directly with RNA. We have developed enrichment strategies and an automated data analysis workflow to identify cross-linking sites not only on the protein but rather on the peptide or even amino acid level. Our strategy can be applied to protein–RNA complexes of a great variety in composition, as it allows unbiased searches against entire proteomes. Furthermore, it allows additional data analysis in terms of sequence determination (i.e., of known and hitherto unknown RNA-binding motifs) and location of protein–RNA interaction sites in tertiary and quaternary structures of protein–RNA complexes.

Experimenteller Teil

Yeast and human (pre-)mRNA complexes were isolated and cross-linked by UV irradiation at 254 nm. Sample preparation combined hydrolysis, size exclusion and C18 chromatography, and titanium dioxide solid phase extraction. Cross-linked peptide–RNA oligonucleotides were analyzed on an Orbitrap Velos with HCD fragmentation. The high quality product ion scans are suitable for automated identification of the cross-linked peptide. Our data analysis workflow is based in the OpenMS environment and initially includes several filtering steps to exclude spectra of noncross-linked species. Precursor mass variants are then generated for putative cross-links and the resulting data is searched with OMSSA against the yeast or human UniProt database. For precursor variant generation, various nucleotide compositions and cross-linking chemistries can be taken into consideration.

Ergebnisse und Diskussion

Yeast protein–RNA complexes were isolated by TAP-tag purification with a tagged cap-binding protein Cbp20; human complexes were assembled on a pre-mRNA containing MS2 loops for isolation with the MS2–MBP fusion protein. From the resulting MS data, spectra corresponding to residual noncross-linked peptides (confidently identified with FDR < 1%) were filtered. XICs of remaining precursors were compared with those of a non-irradiated control samples; fragment spectra of precursors that appeared in both samples with comparable intensities were removed. After data reduction, masses of oligonucleotides (1–4 nt; neutral loss of H_2O, HPO_3 or H_3PO_4; at least one U in sequence) were subtracted from the remaining experimental precursor masses. The resulting precursor mass variants, together with the experimental fragment information, were subjected to database search with OMSSA. For each spectrum, the best-scoring peptide-to-spectrum match was retained and the cross-linked oligonucleotide derived from the mass difference between the corresponding precursor mass variant and the experimental precursor mass.

In yeast, we identified 184 peptide–oligonucleotides heteroconjugates corresponding to 64 unique protein regions in 49 proteins. In 39 regions, the cross-linking site could be narrowed down to a single amino acid. In human, 189 cross-links in 60 protein regions of 35 proteins were found; in half of the regions the cross-linked amino acid was identified. The vast majority of human proteins contained known RNA-binding motifs such as RRMs and KH domains. In contrast, eight yeast proteins correspond to metabolic enzymes without well-established RNA binding function. These findings demonstrate the high specificity of UV induced cross-linking and mass spectrometry as well as their capability to identify hitherto unknown RNA binding proteins, which in principle allows for the recognition of novel RNA binding motifs.

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Neue Aspekte

An automated data analysis workflow allows identification of peptides cross-linked to RNA by UV irradiation in searches against entire proteomes.

MALDI-ISD and POROS R2 protein nLC a promising combination

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Top-down proteomics is still a minor track in the field of protein mass spectrometry. The main focus in the proteomics field remains on bottom up strategies based on Trypsin proteolysis. These workflows are well established and have a broad acceptance throughout the proteomics community. In this work we demonstrate that there is room for MALDI based Top-down proteomics for well-defined sub-proteomes. MALDI ISD in combination with a high resolution MALDI Orbitrap instrument and powerful protein chromatography based on POROS R2 particles is well suited for N- and C-Terminal sequencing of different complex I samples from Yarrowia lipolytica. In combination with intact mass measurement the information can be used for functional analysis of different proteins.

Experimenteller Teil

Protein chromatography is done on a Dionex Ultimate (Thermo Fischer, Bremen) system with in-house self-packed POROS R2 (Life Technologies) columns using a Sunchrome column-packing-system (Sunchrome, Friedrichsdorf). The nLC eluted proteins were either spotted on a MALDI Orbitrap (Thermo Fischer, Bremen) target and mixed with a saturated 1,5-DAN solution in 50% ACN and 0,1% TFA (Sigma Aldrich) for MALDI ISD or on a MALDI STR (ABSciex, Darmstadt) target and mixed with 30 mg/ml sDHB in 30% ACN and 0,1% TFA for MALDI TOF intact mass measurement. For data processing an in-house software tool was used before submitting the MALDI-ISD .mgf files to Mascot server (Mascot Matrix science) with a custom made database.

Ergebnisse und Diskussion

Method validation of the HPLC setup included sensitivity, reproducibility, peak capacity and resistance towards different detergents of protein containing solutions. The peak capacity was determined using standard protein mixtures with 50ng/protein which is the required amount for sufficient ISD fragmentation on the MALDI Orbitrap and resulted in a peak capacity of ≈130 for a 60min gradient (Neue, 2005). Reproducibility was determined with a mean peak deviation of 0,21 min in triplicate analysis and the resistance towards different detergents for the POROS R2 material was already examined in earlier work (Gorka, et al. 2012). Different Complex I samples from Yarrowia lipolytica were analyzed using the established workflow. The combination of intact mass measurement with N- and C-Terminal sequencing is used for the determination of possible post translational modifications of the ACPM1 proteins from complex I. The knowledge of the N- and C-Terminus in combination with the intact mass leads to a deeper insight of the possible ACPM1 modifications with a high confidence. Comparing the MALDI Orbitrap data and expanding the limited mass range by using a MALDI TOF instrument results in a more complete sequencing data. This also shows the limitations of both instruments like reduced mass range of the MALDI Orbitrap and limited resolution and sensitivity of the MALDI-TOF. The data obtained from the complex I samples including tagged ACPM1 proteins and the extracted peripheral arm leads to the conclusion that ACPM1 proteins show different activities depending on their origin. This information can be used for the determination of ACPM1 in the mitochondrial segment. Future experiments will be carried out aiming for a more detailed view on the function of ACPM1 in the mitochondrial complex I based on the MALDI Top-down results presented in this study.

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Neue Aspekte

Protein-nLC MALDI Top-down approach with improved sensitivity and a user friendly data management.

Species determination of Culicoides biting midges via peptide profiling using matrix-assisted laser desorption ionization mass spectrometry

Katrin R. Uhlmann[1], Sebastian Gibb[2], Stefan Kalkhof[1], Uriel Arroyo-Abad[3], Claudia Schulz[4], Bernd Hoffmann[4], Francesca Stubbins[5], Simon Carpenter[5], Martin Beer[4], Martin von Bergen[1,6], Ralph Feltens[1]

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Culicoides biting midges are vectors of bluetongue and Schmallenberg viruses that inflict large-scale disease epidemics in ruminant livestock in Europe. Methods based on morphological characteristics and sequencing of genetic markers are most commonly employed to monitor and differentiate *Culicoides* to species level. Proteomic methods, however, are also increasingly being used as an alternative method of identification. These techniques have the potential to be rapid and may also offer advantages over DNA-based techniques.

Experimenteller Teil

Proteins extracted from 7 *Culicoides* species were digested and resulting peptides purified. Peptide mass fingerprint (PMF) spectra were recorded using MALDI-TOF-MS and peak patterns analysed in R using the MALDIquant R package. Additionally, offline LC-MALDI-TOF-MS/MS was applied to determine the identity of peptide peaks in one exemplary MALDI spectrum that was obtained using an unfractionated extract.

Ergebnisse und Diskussion

We showed that the majority of *Culicoides* species reproducibly yielded mass spectra with peak patterns that were suitable for classification. Although there is basically no sequence information concerning *Culicoides* proteins available, we were able to determine the identity of 28 peptide peaks observed in one MALDI spectrum in a mass range from 1.1 kDa to 3.1 kDa via offline LC-MALDI-TOF-MS/MS. All identified peptides were conserved between *Culicoides* and other dipteran species and derived from one of five highly abundant proteins.

Shotgun mass mapping by MALDI-TOF-MS has been shown to be compatible with morphological and genetic identification of specimens and therefore offers a rapid, highly sensitive and inexpensive alternative for accurate identification of *Culicoides* biting midges collected in the field. The future availability of complete *Culicoides* genomes may enable a more stringent detection based on species-specific peptide sequence information.

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Neue Aspekte

First use of peptide peak patterns for *Culicoides* species classification obtained by MALDI-TOF-MS and peptide identification using LC-MALDI-MS/MS and LC-ESI-MS/MS.

Session X: Lipid- u. Kohlenhydrat-Analytik II

Mittwoch, 05.03.2014:10:30 - 13:00

MS Imaging of Metabolites During Barley Grain Development – What We can Learn About Biological Processes.

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The first two weeks in a barley kernels life are characterized by complex physiological events resulting in tremendous structural changes [1]. Thus, also the chemical composition of the different grain parts is considered to reflect these changes. Using an untargeted MALDI MS *Imaging* (MSI) approach we aim to detect candidate compounds that affect specific developmental processes in barley kernels.

The presentation will hence introduce our approach [2] to use the MALDI MSI technique in a plant physiological context for: i) the visualization of grain developmental steps on a metabolite level, and ii) the selection of candidate compounds for targeted analyses and integration into the physiological context of grain developmental processes.

Experimenteller Teil

MALDI MSI was carried out using longitudinal and cross sections of barley grains from different developmental stages [2, 3]. The data were analyzed by multivariate statistics to generalize the stage specific metabolite patterns. Candidate *m/z* values were selected by comparison of MSI runs from different developmental stages and by evaluating developmental changes by using the DHB matrix as an internal standard. Identification of *m/z* values constitutes a major challenge. MS/MS analyses as well as on tissue digestion helped to classify many of the compounds. Targeted analyses regarding further identification and quantification using dissected seed material were performed by means of LC-MS, GC-MS and LC coupled to electrochemical detection. Furthermore, expression analyses by qRT PCR validated the obtained metabolic data.

Ergebnisse und Diskussion

The investigation of characteristic developmental stages of barley grains by MALDI MSI revealed highly tissue specific patterns of particular metabolites. Data mining by multivariate statistics reflected the typical developmental proceedings. In general, the specificity of molecules in the endosperm and in the nutrient transfer region increased from the prestorage stage to the storage stage, whereas the abundances of molecules in the pericarp decreased.

The endosperm, which is the main storage organ, constitutes specific phospholipid distributions during the storage stage of barley grain development. By means of MALDI MSI increasing gradients of particular lipids towards the periphery were obtained. These accumulations are suggested to reflect characteristics of the starch-lipid complex formation.

The specific oligosaccharide distribution patterns during the storage stage revealed an accumulation of linear type fructans around the endospermal cavity. This grain part belongs to the nutrient transfer region that realizes the massive transport events of assimilates towards the endosperm. In contrast, branched type fructans accumulate during the prestorage stage. Transcript analyses exhibited specific expression patterns for genes from fructan metabolism that correspond to the profiles of the individual fructans. Their differential patterns during grain development suggest particular functions concerning a transient carbon partitioning and a relation to stress responses, such as membrane stabilization and ROS detoxification.

Referenzen

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Neue Aspekte

Application of MALDI MSI in plant physiology - Detection of candidate compounds that affect developmental processes.

Influence of fatty acid composition on glycation and glycoxidation of phosphatidylethanolamines

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Formation and accumulation of advanced glycation end product (AGEs) appear to correlate to several diseases, such as diabetes mellitus, atherosclerosis and inflammation. Whereas AGE-modified proteins and peptides are well characterized, lipid-AGE-adducts have been less investigated. At elevated concentrations (hyperglycemia) glucose can react with amino groups of phosphatidylethanolamines (PE) and phosphatidylserines (PS) to form Schiff bases or Amadori products, which can be further degraded to various AGEs. As this degradation may depend on the fatty acid (FA) present in an amino-phospholipid, we have studied glycoxidation for four PEs.

Experimenteller Teil

Dipalmitoyl- (DPPE), palmitoyl-oleoyl- (POPE), palmitoyl-linoleoyl- (PLPE) or palmitoyl-arachidonoyl-phosphatidylethanolamine (PAPE; all 1.2 mmol/L) were incubated with glucose (5 mmol/L) in methanol at 100°C for 30 min (1200 µL) and dried under the nitrogen. Each glycated PE (1 mmol/L) were dissolved in ammonium bicarbonate (5 mmol/L) and oxidized with $Fe(II)SO_4$ (80 µmol/L) and H_2O_2 (50 mmol/L) for 2 h. Samples were extracted with a mixture of methanol and chloroform (1:1, v:v; 500 µL) and analyzed by shotgun lipidomics using data-dependent acquisition on an ESI-LTQ-Orbitrap-MS.

Ergebnisse und Diskussion

High resolution mass spectrometry (MS) combined with MS^n allowed identifying several types of PE species: (i) unmodified, (ii) oxidized, (iii) glycated, (iv) glycoxidized, (v) glycated with oxidized FA and (vi) glycoxidized with oxidized FA. The number and type of modification depended clearly on the PE-FA-composition. Glycated products were detected for all PE species, but number of oxidized products increased with the number of double bonds in the FA. Thus, polyunsaturated FA yielded many PE that were glycated and simultaneously oxidized at FA. i.e. 8, 44 and 130 different species were detected for POPE, PLPE and PAPE, respectively. DPPE and POPE yielded four glycoxidized PEs with unmodified FA, whereas glycoxidation and FA-oxidation was characteristic for PLPE and PAPE (13 and 21 products, respectively). Among the detected PE-bound AGEs dominated carboxymethyl-PE, carboxyethyl-PE and glucuronic acid- PE.

The CID fragment ion spectra of modified PEs displayed specific signals for the modified head group and the oxidized FA-moieties that can be used for their MRM-quantification.

Neue Aspekte

High resolution MS and MS^n reveal the high complexity of glycated and glycoxidized-PE species and allowed to deduce their structures.

Ecdysteroidome of developing Drosophila

Oksana Lavrynenko, Jonathan Rodenfels, Suzanne Eaton, Andrej Shevchenko
MPI CBG, Deutschland

Ecdysteroids are key regulators of *Drosophila* development: changes in ecdysteroid concentration trigger moulting and successful progression of larvae through the developmental cycle. A developing larvae contains picograms of various ecdysteroids together with milligram of proteins and lipids.Therefore, the complexity of the ecdysteroidome – the full complement of ecdysteroids, their conjugate forms and catabolytes - are poorly characterized because of the paucity of specific and quantitative analytical methods.

Experimenteller Teil

Ecdysteroids were recovered by cold methanol extraction followed by hexane partioning and SPE clean-up of extracts. to Extracts were analyzed by LC-MS/MS in MRM mode on Agilent 1200 LC system coupled to TSQ Vantage triple quadrupole mass spectrometer via HESI ion source for the quantification of known hormones. For the discovery of novel hormones extracts were injected into Agilent 1200 LC system coupled to a Q Exactive tandem mass spectrometer via robotic TriVersa ion source.

Ergebnisse und Diskussion

We designed a generic LC-MS/MS method pipeline for the quantitative profiling of known and discovery of new ecdysteroids in *Drosophila.* All major known hormones: ecdysone, 20-hydroxyecdysone and makisterone A were quantified by MRM. with the limit of detection of 5 pg that suffices quantifying hormones in a single animal. For the first time we obtained the quantitative profile of endogenous hormones through the entire developmental cycle from embryos to adults with the time resolution of 4 hours.

In parallel, we developed the unbiased high resolution screening method for the discovery of ecdysteroids, which identified several novel molecules. Their chemical structure was confirmed by tandem mass spectrometry and Girard derivatization. Novel hormones were quantified using targeted SIM on a Q Exactive instrument. Taken together, the complete ecdysteroidome profile exemplified quantitative changes in the abundance of precursors, active hormones and major metabolites spanning the entire developmental cycle. Our work contributed an important resource for the field of *Drosophila* developmental biology.

Furthermore, we also established the link between dietary sterols and endogenous ecdysteroids that provided insight into the molecular mechanisms of *Drosophila* response to nutritional and environmental challenges.

Neue Aspekte

Complete ecdysteroidome of the developing *Drosophila*

Is the Lysophosphatidylcholine Content of human Spermatozoa and Erythrocytes a potential Biomarker of Fertility? A MALDI MS Study

Kristin Zschörnig[1], Susanne Pyttel[2], Ariane Nimptsch[1], Uwe Paasch[2], Jürgen Schiller[1]

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Obesity (i.e. a body mass index (BMI) higher than 30) is an increasing worldwide problem and seems to favour the development of many diseases such as cancer [1]. There is also considerable evidence that obesity is accompanied by inflammatory processes, i.e. an excessive generation of reactive oxygen species (ROS). Obesity and ROS generation does also affect the probability of offspring: human spermatozoa are characterized by a significant content of highly unsaturated phospholipids which makes them very sensitive to oxidation [2]. Although the oxidation behaviour of lipids is complex, we have previously shown that lysophosphatidylcholine (LPC) is a reliable marker of lipid oxidation [3]. We will show here that MALDI-MS is a simple method to determine the cellular LPC contents.

Experimenteller Teil

All chemicals, solvents, and the applied MALDI matrices (9-aminoacridine (9-AA) and 2,5-dihydroxybenzoic acid (DHB)) were obtained in the highest commercially available purity from Sigma-Aldrich (Taufkirchen, Germany). Phospholipid standards (to study lipid oxidation on a model level) were from AVANTI Polar Lipids (Alabaster, AL, USA) and used as supplied.

DHB was either used as 0.5 M solution in methanol or as a solution of 100 mg/ml in acetonitrile/water (1:1, *v/v*), while 9-AA was used in a concentration of 10 mg/ml in isopropanol/acetonitrile (60/40, *v/v*)) [4]. Lipid extracts from spermatozoa were obtained according to the Bligh & Dyer method. Positive ion MALDI spectra were recorded on an Autoflex workstation (Bruker Daltonics, Bremen) in the reflector mode.

Ergebnisse und Diskussion

The membrane of human sperm has a complex architecture, which is particularly characterized by a unique fatty acyl composition of the related phospholipids: the significant content of highly unsaturated fatty acyl residues (particularly docosahexaneoic acid (22:6)) is essential for the fertilization process, i.e. the successful fusion of the sperm with the female oocyte. Unfortunately, highly unsaturated fatty acids are also very sensitive to oxidation by ROS. Therefore, oxidative stress may be one reason why the spermatozoa of obese men (BMI > 30 kg/m^2) have a reduced fertilizing ability.

Human sperm as well as erythrocytes (from the blood) were isolated from donors differing in their BMI. The organic (chloroform / methanol) extracts of both cells were directly analyzed by (normally positive ion) MALDI-TOF MS for their lipid compositions. There were two remarkable results: first, the PC/LPC ratio decreases with increasing BMI, i.e. obese donors possess an enhanced LPC content. The MS data also correlate with established clinical markers of sperm quality such as the mobility of the sperm and, thus, the LPC content seems to represent a promising disease marker. Second, the PC/LPC ratios determined in the sperm extracts correlate with the PC/LPC values determined in the extracts of the erythrocytes. These results suggest that a decreased PC/LPC ratio is not only a measure of regional or organ-related stress but rather characteristic of the "oxidative status" of the whole organism. Furthermore, the increased contribution of LPC can be easily determined by analyzing the lipid composition of erythrocytes, whereas the analysis of sperm (that are more difficult to obtain) is not absolutely necessary. We are currently testing to which extent the LPC concentration is also affected by contributions of the enzyme phosphilase A_2 the activity of which is also enhanced under inflammatory conditions.

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Neue Aspekte

LPC (that can be easily determined by MALDI MS) has been established as a potential biomarker of human fertility.

Lipidomics Study of Human Lung Tissues

Torsten Goldmann[1,2], Verena Scholz[1], Helga Lüthje[1], Julia Müller[1], Bernhard Schmitt[1], Dominik Schwudke[1,2]
[1]Forschungszentrum Borstel, Deutschland; [2]Airway Research Center North (ARCN), German Center for Lung Research (DZL)

The lung is the barrier organ, which, besides the skin, has the utmost contact to the environment. Within several seconds new air is inhaled for the gas exchange with which bacteria, aerosols, toxic substances and allergens get in contact with the epithelial tissues. Relatively little is known about the lung lipidome and how it influences the maintenance of the barrier functions and gas exchange. We started to study the lipid composition of clinical samples covering human bronchial, alveolar and cancer tissues using the lipidomics screen approach (1).

Experimenteller Teil

Histological and clinical characterized alveolar, cancer and bronchial tissue biopsies were homogenized and subsequently extracted with a methyl tert-butyl ether / methanol based extraction method (2). Lipidomics screens were performed using an Apex Qe FTICR-MS mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a TriVersa NanoMate robot (Advion, Ithaca, USA) accessing quantitative information of approximately 200 membrane and neutral lipids (1). Positive as well negative ion mode ESI was utilized enabling quantitation of PC, LPC, PE, SM, TAG, Chol-FA, PG, PS and PI (3).

Ergebnisse und Diskussion

Between the different tissues, complex lipid profile changes were observed which we start to associate with age, gender and pathology. As an evaluation of our approach we tested if cancer tissues could be differentiated from healthy surrounding alveolar tissue of the same patient. Hierarchical clustering of quantitative lipid profiles enabled the unambiguous separation of the cancer and alveolar tissues biopsies from the control tissue independent of age and gender. Moreover our results indicated that the quantity of typical surfactant lipids in alveolar tissues could directly be used for such association. Here we observed that specifically PC 32:0 and PC 30:0 is decreased in abundance reflecting a lowering of surfactant in the squamous-cell carcinoma biopsies. This pilot study is a starting point to investigate the influence of lipid metabolism in the lung and associated diseases.

Referenzen

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Neue Aspekte

First lipidomics screen of human lung tissues.

Session XI: Instrumentelle Entwicklungen II

Mittwoch, 05.03.2014:10:30 - 13:00, OSZ H5

Breaking the NanoLC-MS Throughput Barrier

Joerg Niebel
MS Wil GmbH, Schweiz

Nanospray is an essential tool in high-sensitivity mass spectrometry, but limited robustness, reproducibility, and ease-of-use have historically challenged the adoption of nanospray in quantitative applications. Recent trends toward MS-based biomarker quantitation have placed strict requirements on the analytical performance of nanobore LC-MS. Nanospray MS and nanobore LC-MS both rely heavily on nanospray source hardware for successful experiments. Nanospray source hardware has matured from simple homemade devices to sophisticated, application-specific instrumentation featuring stage automation, thermal control, and high-resolution imaging. Many of these enhanced features provide robustness (automated tip rinsing, automated emitter change), throughput (multi-channel workflows), ease of use (multi-chip systems) or experimental flexibility. Here we present novel nanospray source solutions which delivers enhanced features of stage automation, multi-channel operation and thermal control.

Experimenteller Teil

Liquid Chromatograph: Eksigent Ultra Nano LC – 3 Channel
• Channel 1: loading pump, 1 µL/min, 2% Acetonitrile
• Channel 2: gradient elution pump, 300 nL/min, 2-30% acetonitrile
• Channel 3: washing pump, 1 µL/min, 80% acetonitrile
Autosampler: CTC Leap
• Nano-rotor injection valve, 1 µL inj. volume
ESI Source: New Objective PicoSlide source with three columns.
• PicoChips: 75 µm ID x 10 cm, C18 (Reprosil 3 µm)
• Custom Valco nano-switching valve
• Stage and valve acontrolled by custom Digital PicoView software on host PC
Mass Spectrometer: Thermo Scientific LTQ
• Operated in full-scan mode (300-1200 m/z)
• ESI voltage: 2.1 kV, 180º C inlet temperature

Ergebnisse und Diskussion

• Multiplexed operation improves MS duty cycle to be greater than 95%

• Highly efficient, long injection times are fully supported, eliminating the need for inefficient trapping columns.

• The PicoChip format enables easy multiplexed operation and rapid column switching.

• A novel valve rotor design simplifies the plumbing and provides a linear flow path between the pump and column.

Referenzen

[1] http://www.newobjective.com/downloads/posters/ASMS13_PicoSlide.pdf

Neue Aspekte

Eliminate Injection times delays and gradient delay
Remove system flow-rate-to-volume ratio restrictions
Gain duty cycle > 95% through multiplexed operation

Select-eV: The next generation of ion source technology

G. Horner, Leonhard Pollack, S. Koschinski, L. McGregor, S. Smith, N. Bukowski
Markes International GmbH, Deutschland

Until now, electron ionisation has not been considered a suitable technology for soft ionisation. The space-charge built by free electrons emitted from the filament shields the potential field between filament and ion chamber according to the Child-Langmuir Law, resulting in a poor electron density beam and low ion flux. By optimisation of the electric fields in the electron gun (e-gun) it has been possible to overcome the space-charge limitation at ionisation energies as low as 10eV.

Experimenteller Teil

This presentation will introduce Select-eV through the characterisation of a variety of compounds (alkanes, aromatic compounds, PCBs, fragrance compounds, etc.) at low ionisation energies, comparing them with their equivalent 70eV spectra.

Ergebnisse und Diskussion

Select-eV, a ground-breaking development in electron ionisation, solves this problem through the ability to switch between hard and soft electron ionisation with no inherent loss in sensitivity. In contrast to chemical ionisation, electron ionisation is a general ionisation technique which can be applied to almost every vaporised substance. Therefore, the possibility to generate a low energy electron beam creates a universal soft ionisation technique.

Select-eV offers a wide, tuneable range of ionisation energies without the requirement for source switching or additional reagent gases. The use of soft electron ionisation enhances the intensity of molecular and structurally-significant fragment ions, magnifying differences between isomeric spectra and, by consuming less instrumental dynamic range, a wider concentration range of analytes can be supported in any single analysis.

Neue Aspekte

Select-eV provides the ability to switch between hard and soft electron ionisation with no inherent loss in sensitivity.

Precision Mass Spectrometry on Short-lived Nuclides: New Methods and Results

Lutz Schweikhard[1], for the ISOLTRAP collaboration[2], and the SHIPTRAP collaboration[3]

[1]Institute of Physics, University of Greifswald, Germany; [2]ISOLDE, CERN, Geneva, Switzerland; [3]GSI, Darmstadt, Germany

High-precision mass measurements of short-lived nuclides are routinely performed by Penning trap mass spectrometry at several online facilities worldwide. The mass values provide valuable input data for the study of many fundamental questions such as the structure of atomic nuclei, the limits of the chart of nuclei with respect to the proton and neutron driplines and the region of superheavy elements, as well as the simulation of the stellar nucleosynthesis of elements. Two new techniques have been introduced recently that improve the accessibility of nuclides with respect to half-life, production rate and “contaminating” isobars.

Experimenteller Teil

The Phase-Imaging Ion-Cyclotron-Resonance (PI-ICR) method uses a position sensitive-ion detector to determine the location of an ion in the Penning trap after a well-defined excitation of the ion motion followed by an excitation-free period. This allows the free accumulation of phase of the ion motion from which the cyclotron frequency follows directly.

The Multi-Reflection Time-of-Flight (MR-ToF) technique uses two ion mirrors to extend the drift length by orders of magnitude from the one-meter dimension of the instrument up to several kilometers. Nevertheless, the total flight time of the keV ions is only a few milliseconds.

Ergebnisse und Diskussion

PI-ICR MS was introduced[1] and characterized[2] at SHIPTRAP at GSI/Darmstadt. In contrast to the conventional ToF-ICR method, where the Ion-Cylotron-Resonance frequency is determined by repeatedly monitoring the ions' Time of Flight to a detector (outside the strong magnetic trapping field) after rf-excitation and axial ejection, the non-scanning PI-ICR method provides valuable data from just a few ion counts.

In addition, its precision is about five times higher than that of (Ramsey-)ToF-ICR MS. Moreover, PI-ICR MS is not restricted by the Fourier limit since no amplitude is measured as a function of frequency, but instead the phase is determined. Thus, the resolving power exceeds that of the ToF-ICR technique by a factor of 40 under similar conditions.

PI-ICR MS proved its applicability in a measurement of the 129Xe-130Xe mass ratio with the experiment time reduced by an order of magnitude compared with the (Ramsey-)ToF-ICR technique[1]. This will allow measurements at the ppb level for nuclides with half-lives well below a second. With its superior resolving power, PI-ICR MS is especially valuable in cases where low-lying isomers have to be resolved. First applications will include the determination of Q values of (double-)beta/electron-capture decays.

ISOLTRAP at CERN/Geneva has been extended with an MR-ToF section[3] between the RFQ trap for ion bunching/cooling and its two Penning traps (for preparation and precision mass measurements). At first the MR-ToF section has been applied as a mass separator. Isolation from the orders-of-magnitude more abundant 82Rb allowed the first direct mass measurement of 82Zn[4], which is of significant interest for modeling the neutron-star crust, a proposed site of r-process nucleosynthesis.

Secondly, the MR-ToF MS was used to determine the masses of the neutron-rich 53Ca and 54Ca, cornerstones for nuclear-forces studies. This confirmed the presence of a new neutron shell at N=32 and the importance of three-body interactions[5].

Referenzen

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Neue Aspekte

With these developments the frontiers of precision mass spectrometry of short-lived nuclides is further pushed to more exotic species.

What is new in Proton-Transfer-Reaction Time-Of-Flight Mass Spectrometry?

Kostiantyn Breiev[1,2], Alfons Jordan[1], Eugen Hartungen[1], Gernot Hanel[1], Philipp Sulzer[1], Simone Jürschik[1], Matteo Lanza[1,2], Lukas Märk[1], Tilmann D. Märk[1,2]

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The introduction of Time-of-Flight (ToF) mass analyzers in Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) can be considered as a major breakthrough in PTR-MS related instrumental development in the last decade [1]. Although quadrupole mass filter based PTR-MS instrumentation have been well established in environmental chemistry [2], food and flavor analysis and many other fields of application, only PTR-ToF-MS enables the simultaneous acquisition of full mass spectra in real-time and with sufficient mass resolution to separate isobaric molecules [3]. However, depending on the application the demands regarding the analytical instrument are manifold; i.e. there is a need for a variety of specialised instrumentation, which we will discuss here in detail.

Experimenteller Teil

Details on the principle instrumental setup of a PTR-ToF-MS instrument can be found in literature (e.g. [1]). In short, a hollow cathode ion source produces H_3O^+ reagent ions with a very high purity level, which are subsequently injected into a drift tube together with air containing traces of the analytes. In the drift tube proton transfer reactions between the hydronium and all compounds possessing a higher proton affinity than that of water takes place and the resulting product ions are eventually analysed and detected in a ToF mass spectrometer. It has to be mentioned that in the latest embodiments of these instruments it is possible to switch the reagent ions from H_3O^+ to NO^+, O_2^+, Xe^+ and Kr^+, respectively [4].

Ergebnisse und Diskussion

One of the most important parameters of a trace gas analyser is the sensitivity. Sensitivity is typically measured in counts-per-second (cps) per concentration unit (ppbv). Early prototypes of PTR-ToF-MS instruments performed rather low with about 0.2 to 4 cps/ppbv, whereas the first commercially available versions achieved about 25 cps/ppbv. Continuous improvements during the past five years could increase this value to about 250 cps/ppbv for high mass resolution (about 7000 m/Δm) instruments, which seems to be the limit for the original setup described in [1]. Here we present two approaches for new setups that could improve the sensitivity further, namely a "high-pressure" drift tube, i.e. a PTR-MS drift tube that operates close to 10 mbar (instead of about 2 mbar) and a new design for the transfer region between the drift tube and the mass spectrometer. The latter one is realised by replacing the transfer lens system with a quadrupole ion guide. First preliminary data were acquired using an aromatics mix gas standard (TO-14, Restek) and showed a factor of seven gain in sensitivity for combining both measures. This means that with a high pressure drift tube and quadrupole ion guide sensitivity values over 1500 cps/ppbv could be achieved.

On the other hand there is a need for compact and rugged PTR-ToF-MS instruments. In the framework of an "Austrian Space Applications Programme" project the University of Innsbruck and IONICON developed such an instrument for being mounted in an aircraft. With a sensitivity of close to 100 cps/ppbv and a mass resolution of 1200 m/Δm this instrument is perfectly suited for NASA flight campaigns from which first data are already available [5].

We want to gratefully acknowledge financial support by the FFG, Wien, and the EC, Brussels, via the FP7 projects "PTR-TOF" (GA 218065) and "PIMMS" (GA 287382).

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Neue Aspekte

Novel instrumental developments in PTR-ToF-MS technology (from compact size to very high sensitivity) and examples for their applications are presented.

Desorption/ionization induced by neutral clusters as a matrix-free, soft, and efficient ion source for ion-trap MS of biomolecules

Markus Baur[1], Andre Portz[2], Christoph Gebhardt[3], Michael Dürr[2]

[1]Hochschule Esslingen, Deutschland; [2]Justus-Liebig-Universität Giessen, Deutschland; [3]Bruker Daltonik GmbH, Bremen, Deutschland

Desorption and ionization induced by neutral clusters (DINeC) can be employed as a very soft and matrix-free method for transferring surface-adsorbed biomolecules into the gas phase. Using neutral clusters with polar constituents such as SO_2, the impacting clusters do not only provide the energy necessary for desorption but also serve as a transient matrix in which the desorbing molecule is dissolved during the desorption process. Furthermore, shattering of the cluster during its impact on the surface leads to a rapid redistribution of the system's energy and an efficient cooling of the desorbed molecules. As a consequence, desorption and ionization of oligopeptides and smaller proteins can proceed without any fragmentation at comparably low energies of the impacting clusters [1,2].

Experimenteller Teil

Desorption/ionization was induced by neutral SO_2 clusters with a mean size of 10^3 to 10^4 molecules seeded in a pulsed He beam. With a gas mixture of 2% SO_2 in He at a stagnation pressure of 15 bar, the cluster beam is characterized by a narrow velocity distribution ≤ 1.6 km/s. The desorbed ions were accumulated in an ion trap over the whole pulse duration prior to mass spectrometric analysis. Samples were prepared by simply drop casting the respective aqueous solution of biomolecules on Si/SiO_2 substrates.

Ergebnisse und Diskussion

In this contribution, we first show how desorption/ionization induced by neutral cluster impact can be efficiently combined with ion trap mass spectrometry. Since the cluster beam is generated from a pulsed nozzle with a typical pulse duration of 0.5 to 1 millisecond, the generated ions can be accumulated in the ion trap over the whole pulse length. This leads to a very efficient use of analyte material and single pulse operation mode is possible. Furthermore, as no primary ions but neutral molecular clusters are used for the desorption and ionization process, the background intensity in the spectra is comparably low. For standard oligopeptides such as angiotensin II or bradykinin, femtomol sensitivity was achieved and no fragmentation was observed. In the case of phosphopeptides, some fragments are observed but the signal of the intact molecule $(M+H)^+$ is predominant [3].

We furthermore show the wider applicability of DINeC using a variety of different classes of biomolecules in the mass range between 200 u and 2000 u. Especially samples with a multitude of components as obtained from realistic biotechnological processes such as a tryptic digest of proteins were also successfully analyzed. Peptide mass fingerprint analysis was applied for the evaluation of the respective spectra with very good sequence coverage and protein score. The results are compared with ESI and MALDI spectra of the same samples and the characteristics of the DINeC process are discussed.

In combination with the full MS^n capabilities of the ion trap MS, structural analysis is also demonstrated.

Referenzen

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Neue Aspekte

Combination of cluster-induced desorption/ionization with ion trap MS, intact desorption of phosphopeptides, application of mass fingerprint analysis to DINeC results

Postersessions:

Mo, d.03.03.2014: 17:15-19:00, Foyer Biozentrum — Poster mit geraden Nummern
Di, d. 04.03.2014: 10:50-12:30, Foyer Biozentrum — Poster mit ungeraden Nummern

PIE: Instrumentelle Entwicklungen

PIE 1

Select-eV: Increasing dimensionality in GCxGC-TOF MS

Leonhard Pollack, S. Koschinski, G. Horner, L. McGregor, S. Smith, N. Bokowski
Markes International GmbH, Deutschland

Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GCxGC-TOF MS) offers greatly enhanced peak capacity, through the coupling of two columns of different selectivities, as well as highly sensitive detection and definitive mass spectral identification of trace-level analytes.

Despite this increased separation capacity, the identification of individual compounds in complex samples may be further complicated by weak molecular ions or when similar mass spectral characteristics are evident across entire chemical classes.

Experimenteller Teil

This poster provides an introduction to Select-eV technology, as applied to GCxGC-TOFMS analyses within a range of applications; from petrochemical analyses to environmental monitoring.

Ergebnisse und Diskussion

Select-eV is a new innovation in ion source technology which aims to solve this problem through the ability to switch between hard and soft electron ionisation with no inherent loss in sensitivity.

Neue Aspekte

Select-eV offers a wide, tuneable range of ionisation energies without the requirement for source switching or additional reagent gases.

PIE 2

Enhanced aroma profiling by GC-TOF MS with variable-energy electron ionisation

Leonhard Pollack, S. Koschinski, G. Horner, L. McGregor, S. Smith, N. Bukowski

Markes International GmbH, Deutschland

Aroma profiles, such as those for wine, contain a wide variety of components at a range of concentrations. Detection and identification of important keynote compounds with a low odour threshold and compounds responsible for off-odours is a challenging prospect.

Gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF MS) is an ideal choice for such analyses. Fast acquisition speeds, full-range spectra and low detection limits allow trace components, including adulterants, to be identified even within the most challenging of matrices. Novel data-mining software for the pairwise comparison of complex chromatograms is described, allowing such minor differences to be readily distinguished.

Experimenteller Teil

This poster describes the combination of GC-TOFMS with Select-eV ionisation and novel data mining tools for rapid and reliable aroma profiling.

Ergebnisse und Diskussion

Furthermore, Select-eV, a revolutionary variable-energy electron ionisation technology provides enhanced molecular ions and reduced fragmentation to aid speciation of challenging compounds. Select-eV enables fast and simple switching between hard and soft electron ionisation with no inherent loss in sensitivity.

Neue Aspekte

Select-eV enables fast and simple switching between hard and soft electron ionisation with no inherent loss in sensitivity.

PIE 3

High Performance Thin Layer Chromatography Mass Spectrometry using an elution-based TLC-MS interface

Hans Griesinger[1], Susanne Minarik[2], Katerina Matheis[1], Michael Schulz[2]

[1]Merck KGaA, Department of Bioanalytical Chemistry, Darmstadt, Germany; [2]Merck KGaA, Merck Millipore - Lab Essentials R&D Analytical Chromatography, Darmstadt, Germany

A straightforward way to couple thin layer chromatography (TLC) with mass spectrometry (MS) is the TLC-MS Interface from Camag. It is an elution-based, semi-automatic system to extract zones from the TLC plate and transfer them online into the MS. It is suitable for all thin layer materials and every eluent that can be sprayed in the ion source. The interface can be connected to any kind of LC-coupled mass spectrometer.

Experimenteller Teil

We show how the TLC-MS interface can be used for the development of TLC-MS applications in the areas of food & beverage, pharmaceutical ingredients, cosmetic actives and peptide & protein analysis.

- separation and identification of insulin species
- investigation of UV-filters in suncream
- analysis of steroids
- determination of caffeine in energy drinks

All experiments were performed on newly developed HPTLC plates with a reduced separation layer thickness. After chromatographic separation the analytes were extracted with acetonitrile/water (95:5, v/v) and transferred online into the MS with a flow rate of 0.2 ml/min. The ionization mode was electrospray ionization (ESI) in the positive mode.

Ergebnisse und Diskussion

Thin layer chromatography allows sample preparation and chromatographic separation in one step. This is possible because of the high sample matrix tolerance of TLC. Through the coupling of TLC with mass spectrometry (TLC-MS) substance identification is possible.

It is shown that the use of thinner TLC plates leads to improved detection limits, increased sensitivity and improved S/N ratios. The sample matrix is clearly separated from the target analytes. This leads to clean mass spectra due to a very low level of ion suppression.

Further instrument and method developments might be useful to overcome technical challenges e.g. the replacement of silica particles from the plate.

Neue Aspekte

Direct coupling of thin layer chromatography with mass spectrometry via an elution-based TLC-MS interface using newly developed thinner TLC plates.

PIE 4

Charakterisierung einer Sonic-Spray-Ionisationsquelle mittels Laser-induzierter Fluoreszenz

Carsten Warschat, Arne Stindt, Andreas Bierstedt, Ulrich Panne, Jens Riedel

Bundesanstalt für Materialforschung und -prüfung, Deutschland

Bei der *Sonic-Spray*-Ionisation (SSI) werden durch Zerstäuben einer Lösung Analytmoleküle in die Gasphase überführt und dabei ionisiert. Dabei wird ähnlich der *Elektrospray*-Ionisation (ESI) ein Aerosol gebildet, jedoch keine Spannung angelegt. Die SSI liefert ESI-ähnliche, einfach-auswertbare und reproduzierbare Spektren. Eine Vielzahl unterschiedlicher Analyten in unterschiedlichen Massenbereichen ist so analysierbar, von z.B. Aminosäuren bis hin zu Proteinen.

Um ein besseres Verständnis des Verdampfungs- und des damit verbundenen Ionisierungsprozesses zu erhalten, wurde der Spray-Kegel einer Rhodamin B Lösung mittels Laser-induzierter Fluoreszenz (LIF) genauer untersucht. Neben dem Solvatationszustand der gebildeten Ionen, ist es so auch möglich Tropfengrößen und –geschwindigkeiten innerhalb des Sprays zu bestimmen.

Experimenteller Teil

Eine geerdete Airbrush-Pistole (Sogolee HP-200, Airbrushes Equipments Co., Taiwan) wird so montiert, dass eine genaue Positionierung in x- und y-Richtung möglich ist. Die Düse befand sich im Abstand von 10 mm bei einem Versatz von 3 mm zum Einlass des Massenspektrometers (API HTOF-MS, Tofwerk, Schweiz). Stickstoff diente als Trägergas bei einem Stagnationsdruck von 2 bar.

Als Anregungsquelle für das LIF-Experiment wurde ein frequenzverdoppelter DPSS-laser (Nd:YO_4 , 1064nm, 500 µJ/Puls (532 nm), 25 ns FWHM, BLADE IR 25, Compact Laser Solutions, Deutschland) verwendet.

Eine 220 mg L^{-1} Rhodamin B Lösung wurde mittels Airbrush-Pistole zerstäubt und die Fluoreszenz mittels EOS 550D Kamera (Canon, Japan) bei einem Abbildungsmaßstab von 1 detektiert. Eine gegenüberliegende Masseelektrode diente zur Simulation des MS Einlasses.

Ergebnisse und Diskussion

Ionisierung mittels SSI ist bereits für verschiedene Stoffklassen in einen breiten Massenbereich, z.B. Aminosäuren, größere Moleküle und Peptide, beschrieben. Mit Hilfe der Airbrush-SSI ist es möglich zeitlich stabile und reproduzierbare Spektren zu erhalten. Spektren kleinerer Analyten sind von protonierten Clustern $[M_n+H]^+$ dominiert, wohingegen größere Analyten zu Mehrfachladungen, z.B. $[LYZ+_nH]^{n+}$ mit n= 5-11, tendieren.

Die Detektion der Fluoreszenz des Rhodamin B erfolgt entlang des Gasflusses, so dass ein kegelförmiges Spray abgebildet werden kann. Berechnungen werden anhand von falschfarben-Bildern durchgeführt, wobei die inverse Abel Transformation Einblicke in die räumliche Verteilung der Fluoreszenz ermöglicht. Die Lösungsmittelabhängige Verschiebung der Fluoreszenzsignale von Rhodamin B geben Aufschluss über dessen Solvatationszustand im Spray. Um das Signal des solvatisierten und des desolvatisieren Ions von einander zu trennen wird ein UV Kaltlichtspiegel vor die Kamera gesetzt, welcher Signale des desolvatisierten Ions filtert.

Es kann gezeigt werden, dass die Ionen und Moleküle im Spray unter Atmosphärendruck hauptsächlich solvatisiert vorliegen und lediglich an den Rändern ein höherer Anteil an desolvatisierten Molekülen vorhanden ist. Der Bereich mit einem Maximum an desolvatisierten Molekülen deckt sich mit empirischen Beobachtungen, dass gerade an diesen Stellen die höchsten Signalintensitäten in MS Experimenten erreicht wurden. Um weitere Erkenntnisse zu gewinnen wurden mittels zeitaufgelöster Aufnahmen eine Größenverteilung und eine mittlere Geschwindigkeit der generierten Tropfen berechnet. Es werden Tropfen gebildet, welche ein maximales Volumen von 30 pL besitzen und eine Geschwindigkeit von 20 – 40 m s^{-1}. Die Geschwindigkeit der Tropfen ist somit etwa ein viertel bis halb so hoch, wie die Strömungsgeschwindigkeit des Trägergases.

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Neue Aspekte

Erstmalige Untersuchung von Solvatationszuständen in einer Sonic-Spray-Ionenquelle mittels Laser-induzierter Fluoreszenz.

PIE 5

High repetition rate atmospheric pressure MALDI in combination with liquid matrices

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One major drawback of matrix-assisted laser desorption/ionization (MALDI)[1] is the relatively poor pulse-to-pulse reproducibility of the signal intensity. This problem is circumvented by averaging the detected ion intensity over several shots. In order to get a constant ion count, as it is desirable for the optimization of experimental parameters, the sample must be moved. This approach requires a homogenous matrix/analyte co-crystallization along the sample surface that is often not given. To circumvent this hindrance, this contribution demonstrates the combination of liquid matrices with high repetition rate lasers for atmospheric pressure MALDI (AP-MALDI)[2]. Two different kinds of liquid matrices were used in combination with both, a 15 Hz laser and a diode pumped solid state laser (DPSS) operated at 5 kHz.

Experimenteller Teil

Ultraviolet Radiation at 355 nm was generated by two different pulsed lasers as excitation source. For high repetition rate investigations, a DPSS-laser (Blade IR 25, Compact Laser Solutions GmbH, Nd:YO_4, 5 kHz, 60 µJ / pulse) was used. For comparison with higher power lasers, additional experiments were done with a frequency tripled compact flash lamp pumped Nd:YAG laser system (Minilite, Continuum, 15 Hz, 3.2 mJ / pulse).

The generated laser beam was focused perpendicular to the sample. Microscope glass slides served as disposable sample holders that were positioned directly in front of the inlet of the mass spectrometer without any desorption/ionization chamber.

All experiments were conducted using an orthogonal time-of-flight mass spectrometer (API-HTOF, Tofwerk, Thun, Switzerland) with atmospheric pressure inlet.

Ergebnisse und Diskussion

Previous studies have shown that a combination of vacuum-MALDI and DPSS-lasers with high repetition rates yields in ion detection[3]. However, MALDI at atmospheric pressure and a DPSS-laser as excitation source in combination with a liquid matrix has not been published yet. To evaluate whether the energy input of the tripled DPSS laser is sufficient, mass spectra out of the liquid DHB matrix obtained with the two individual laser systems were recorded.

Additionally, matrix properties of ionic liquids were investigated. As a representative DHBB was chosen, which also forms a more homogeneous distribution between analyte and matrix compared to its solid analogue.

Signal intensities obtained with the liquid DHB matrix are slightly higher or comparable with the spectra of DHBB. Especially the number of detected analyte containing clusters is significantly lower in case of the ionic matrix.

In conclusion the liquid DHB matrix and DHBB offer a useful alternative to their solid analogue, since they produce higher signal intensities and a higher pulse-to-pulse reproducibility. Based on this, the most important advantage of liquid MALDI samples is the relatively stable ion yield and longtime-durability, which is a result of the “self-healing” surface of the liquid sample.

An extracted ion chronogram using the liquid DHB matrix was obtained. Data acquisition took over 10 minutes at a repetition rate of 5 kHz. As consequence over 3 million laser pulses hit the sample surface at the same spot. The stable ion yield indicates that the rearrangement of the sample surface is fast enough to provide a fresh surface for each desorption step.

The selected ionic liquid instead, behaves different under identical experimental parameters. After a few seconds a loss in ion yield can be observed. Once the sample was moved the ion yield increased to its original value, followed by an immediate exponential decrease.

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Neue Aspekte

Combination of high repetition rate laser and atmospheric pressure MALDI.

PIE 6

Untersuchungen zur chemischen Ionisation bei Atmosphärendruck (APCI) in der Ionenmobilitätsspektrometrie

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In der kommerziellen Ionenmobilitäts (IM)-Spektrometrie bei Atmosphärendruck werden zur Ionisation überwiegend radioaktive Quellen (z.B. ^{63}Ni) eingesetzt. Die mittlere Energie der emittierten Elektronen beträgt 17 keV. In einer Reaktionskaskade werden zunächst Reaktantionen gebildet, die in einem weiteren Schritt die nachzuweisenden Substanzen durch Ladungstransfer ionisieren. Im positiven Nachweismodus werden in Luft $(H_2O)_nH^+$ und im negativen Nachweismodus $(H_2O)_nO_2^-$ als Reaktantionen gebildet. Im Bestreben auf radioaktive Quellen zu verzichten, wird aktuell an der Entwicklung alternativer Ionisationsquellen gearbeitet. In dieser Arbeit wurden die Ionisationsreaktionen untersucht, die in einer auf Röntgenstrahlung beruhenden Ionenquelle ablaufen.

Experimenteller Teil

Eine Ionisationskammer wurde entwickelt, in der gasförmige Analyten bei Atmosphärendruck in einem definierten Badgas (Stickstoff, synthetische Luft) ionisiert werden können. Das Gas wurde entweder getrocknet oder mit einem definiertem Gehalt an CO_2 und H_2O versetzt. Die nachzuweisenden Stoffe, wie halogenierte und nitrierte Aromaten, wurden über eine Verdampfereinheit in die Gasphase gebracht. Die Ionisationskammer ist über eine Transfereinheit zur Überführung der Ionen mit einem Ionenfallen-Massenspektrometer (Thermo LTQ XL) verbunden. Das neue APCI-Massenspektrometer erlaubt die Charakterisierung der gebildeten Ionen.

Ergebnisse und Diskussion

Die wichtigsten Reaktantionen in Stickstoff bzw. synthetischer Luft wurden jeweils im positiven und negativen Nachweismodus bestimmt. Der Einfluss geringer Konzentration von Wasser und CO_2 auf die Intensität und das Spektrum der Reaktantionen wurde ebenfalls untersucht. So treten schon bei geringer CO_2-Konzentration im unteren ppm-Bereich sehr stabile Clusterionen wie $[O_2+CO_2+H_2O]^-$ auf. Die Bildungseffizienzen dieser Primärionen werden auf der Basis thermodynamischer Betrachtungen diskutiert. Zusammen mit quantenchemischen Rechnungen kann so ein tieferes Verständnis der auftretenden Ionisationsreaktionen erreicht werden.

Aromaten mit Nitro- und Halogensubstituenten können im negativen Modus ionisiert werden, da sie in der Regel eine positive Elektronenaffinität besitzen. In Abhängigkeit von den vorliegenden Primärionen werden die Analyten durch Elektronenanlagerung, Protonenabstraktion oder Komplexbildungsbildungsreaktionen mit Primärionen ionisiert. Der störende Einfluss von CO_2 und H_2O auf den Nachweis von halogenierten und nitrierten Aromaten kann auf die Stabilität der vorliegenden Primärionenkomplexe zurückgeführt werden.

Neue Aspekte

Kopplung einer APCI-Ionisationszelle beruhend auf Röntgenstrahlung mit einem Massenspektrometer, Untersuchung der Ionisationsreaktionen

PIE 7

Poly-Anion Production in Penning and RFQ Ion Traps

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The number of electrons in a cluster affects its properties, e.g. its geometrical shape, ionization potential, polarizability, or dissociation energy, making the charge state of a cluster a crucial parameter. Poly-anionic clusters are produced by sequential electron-attachment to cluster mono-anions stored in an ion trap. The poly-anion production is investigated in Penning and linear radio-frequency quadrupole (RFQ) traps at the ClusterTrap setup [1].

Experimenteller Teil

The range of anionic charge states produced with the electron-bath technique in a Penning trap is restricted by the upper mass limit of this trap. By installation of a cylindrical Penning trap with a 12-Tesla superconducting magnet, the mass and thus cluster-size range is enhanced by a factor of 20 compared to the previously used hyperbolic 5-Tesla Penning trap. In a parallel effort, a production method of poly-anions in a RFQ-trap has been developed. To this end, an electron beam is guided through an ensemble of cluster monoanions, stored in an RFQ- trap, which is operated by 2- or 3-state digital driving voltages.

Ergebnisse und Diskussion

For first experimental tests with the 12-Tesla cylindrical Penning trap, gold cluster mono-anions Au_n^{-1}, n = 330 – 350, have been exposed to an electron bath. As a result, higher negative charge states up to hexa-anionic clusters have been observed [1]. For comparison: At comparable trapping voltages the respective cluster size limit of the previous 5-Tesla hyperbolic Penning trap was about n = 60 and the maximum gold-cluster (negative) charge state was z = -3 [2].

At the RFQ-trap, di- and tri-anionic gold clusters have been produced by exposing mono-anions to an electron beam [3]. The conventional harmonic as well as the 2-state digital trapping voltages [4] affects the electron path through the trap, and thus the electron energy during attachment. However, the 3-state digital ion trap allows time slots of zero-volt potentials to be implemented in the driving signal [5]. Thus electrons can pass through the trap unhindered and at well-defined energies. A new electron source with an energy distribution ~0.5eV is currently set up for electron-attachment studies.

In addition, both polyanion-production techniques have been combined by pre-charging clusters in the RFQ-trap, transferring the resulting dianions into the Penning trap and applying the electron-bath technique to produce higher charge states [1].

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Neue Aspekte

Hexa-anionic gold clusters have been produced in a Penning trap. Furthermore, a new production method using RFQ-traps has been developed.

PIE 8

Cryogenic RF Carpet For Exotic Nuclei: Desgin, Simulation and Measurements of Ion Optics and Electronics

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At the upcoming FAIR accelerator facilities in Darmstadt high precision experiments, including mass measurements will be performed with exotic short-lived nuclei. These nuclei are produced in highly energetic collisions at relativistic particle energies. In order to do experiments with highest accuracy the exotic nuclei have to be slowed down from relativistic energies to almost at rest. This is achieved by stopping and thermalizing the ions in a cryogenic gas-filled stopping [1]. To extract the ions from the gas, an RF carpet is used to provide a repelling force and DC field to guide the ions to the extraction nozzle. An RFQ beam line is used to transmit the extracted ions to a multiple-reflection time-of-flight mass spectrometer [2].

Experimenteller Teil

The cryogenic gas-filled stopping cell has a length of 1m and is operated at temperatures of about -200°C and pressures up to 100mbar helium. The PCB based RF carpet has a diameter of 250mm and concentric electrodes with a density of 4 electrodes per mm. An RF frequency of 6.5MHz and RF amplitudes up to 130 Vpp are used. Along the stopping volume DC fields of up to 50V/cm.

Ergebnisse und Diskussion

With the help of simulations the electronics, geometry, RF frequency and amplitude of the RF carpet have been optimized for fast transport along the RF carpet without ion losses, while minimizing the power input to the cryogenic system. The system has been successfully operated offline and online with relativistic exotic nuclei [3]. Simulations were made to determine the operation frequency of the system needed to extract the ions of the desired mass. The electronics of the RF Carpet were designed for the desired operation frequency, trying to dissipate as less heat as possible. Simulations of ion motion in the stopping cell, the electronic design of the RF Carpet and measurements are is good agreement. The extraction of ions without losses with very fast extraction times of 25 ms has been demonstrated. High accuracy mass measurements (<ppm) with the MR-TOF-MS coupled to the cryogenic stopping cell of the very short lived ^{213}Rn (19.5ms half-life) have been performed and show the unique capabilities of these devices.

Referenzen

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Neue Aspekte

RF Carpet for stopping relativistic atomic ions and operation at cryogenic tempertures.

PIE 9

An (ultra-)high resolution multiple-reflection time-of-flight mass spectrometer for applications in nuclear physics

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Nuclear masses are an important input for different fields of science, e.g. nuclear physics and astrophysics. Such high accuracy mass measurements provide key information about the structure of nuclei, for example the limits of nuclear stability, as well as the nucleosynthesis in stars. The multiple-reflection time-of-flight mass spectrometer (MR-TOF-MS) [1,2] can perform ultra-high precision mass measurements of short lived exotic nuclei at extremely low production yields (~ 1/h) at present and future accelerator facilities. These nuclei are not accessible by other methods. Additionally it can be used as an isobar separator [3] e.g. for mass-selected decay spectroscopy.

Experimenteller Teil

The MR-TOF-MS is part of the FRS Ion Catcher [4] at the heavy ion research center GSI at Darmstadt. It is a test branch for the future Low-Energy Branch (LEB) of the super-conducting fragment separator (Super-FRS) at the Facility for Antiproton and Ion Research (FAIR). In the MR-TOF-MS the ions are accumulated, cooled and injected in bunches by a digital linear Paul-trap. In the analyzer they are reflected multiple times to enlarge their flight path by orders of magnitude to enhance the resolution.

Ergebnisse und Diskussion

The MR-TOF-MS has been developed, commissioned and operated on-line as part of the FRS Ion Catcher with performing the direct mass measurement of short-lived projectile fragments, which can only be detected with low yields (few ions per hour). ^{213}Rn was the shortest lived nuclei with a half-life of 19.5 ms only. Due to the single-ion sensitivity of the MR-TOF-MS a determination of the mass of the nuclei with only 25 ions was possible. The maximum mass resolving power of the MR-TOF-MS of 600.000 at a transmission rate of 50% was achieved with an off-line Cs-source. Systematic studies showed that a systematic uncertainty down to 10^{-7} can be reached.

The MR-TOF-MS is not only suitable for mass spectrometry. It will also be used, equipped with a Bradbury-Nilsen Gate, as an isobar seperator. The performance as isobar separator was shown off-line. A separation of isobars was demonstrated even with an intensity ratio of 200:1 between the different isobars. A high ion capacity in excess of 10^6 ions per second was shown. This will help to overcome present limitations at accelerator facilities, where the abundance ratio of nuclei of interest to contamination is a problem. Due to that a fast and efficient removal of the contaminations is necessary as it can be done with the MR-TOF-MS.

This makes the MR-TOF-MS to an ideal tool to perform direkt mass measurements of very short-lived exotic nuclei and to provide an isobarically clean ion beam. On the basis of this device a mobile ultra-high-resolution MR-TOF-MS [5], equipped with an atmospheric pressure inlet (API), has been developed for in-situ applications in analytical mass spectrometry.

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Neue Aspekte

Ultra-high resolution MR-TOF-MS provides a single-ion sensitivity to measure rare short-lived exotic nuclei.

PIE 10

Microarrays combined with MALDI-TOF-MS for quantitative applications: Quantification of saquinavir from lysates of peripheral blood mononuclear cells (PBMCs)

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MALDI-MS has the reputation of not being well suitable for quantitative applications. This is due inhomogeneities of analyte/matrix co-crystallization, spot-to-spot variations or low numbers of replicates.[1,4]
Here, we demonstrate a microarray target, designed for quantitative MALDI-MS applications, which overcomes the above mentioned drawbacks. The microarray has the dimensions of a standard microscope slide and consists of parallel lanes of micro-spots.
At each lane, several (nanoliter volume) replicates of one sample are aliquoted using a sliding device.[1]This approach delivers a large number of replicates for each sample, of which the small aliquots are rapidly drying and fully consumed during MALDI-MS analysis.[1]Here we demonstrate the performance of the microarray MALDI-TOF-MS approach by quantifying the HIV protease inhibitor saquinavir from lysates of PBMCs.[2]

Experimenteller Teil

Microarray targets (Microarrys for Mass Spectrometry, MAMS) were produced as described in Pabst et al. 2013[1]. Slides were mounted onto a standard AB Sciex sample target carrier using a mask as included in the "LaserBio Labs™ Mass Spectrometry Imaging Starter Kit". Analysis was performed using an AB Sciex TOF/TOF 5800 mass spectrometer (Darmstadt, Germany). Reserpine was used as internal standard and CHCA as MALDI-matrix. All chemicals were obtained from Sigma Aldrich (Buchs Switzerland). PBMCs were purchased from ZenBio and lysed as described by van Kampen et.al.[3] Quantification was performed using an external calibration method by measuring a calibration curve between concentrations of 0.39, 3.125, 12, 5, 37,5, 75 and 100 ng/ml PBMCs as well as three separate quality control samples.[2]

Ergebnisse und Diskussion

Deposition of the six calibration samples and three quality control samples to the microarray target may be achieved by aliquoting within a few seconds. Furthermore, MALDI-MS analysis of ten replicates per data point was accomplished in approximately 1.5 minutes, which is much faster than what can be obtained by methods involving chromatographic separations.
As expected for a confident analysis, the confidence belts of the curve of best fit and calculated 95% inverse confidence limits (according *to Szajli et al.*[5]) were found to be very narrow. This is certainly a consequence of the large number of replicates (n = 60 for the calibration curve, plus 10 for the quality control sample), and the overall low standard deviations of the MALDI-MS analysis (on average 3.9 %,). The accuracy for independent quality control samples were further found to be 6.5 % (53.28 ± 2.4 ng/ml), 9 % (27.25 ± 0.84 ng/ml) and 17% (1.29 ± 0.04 ng/ml), with a relative standard deviations for the replicate measurements of 4.5 %, 3.1% and 3.3 % for the lowest concentration (1.5ng/ml).[2]
Overall, the use of microarray plates, a proper sample preparation and internal mass calibration allowed accurate, precise and confident results. Performance was even comparable to results usually obtained by LC-ESI-MS, which is traditionally considered superior to MALDI in quantitative studies.

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Neue Aspekte

Here, we report on a newly developed microarray plate, designed as a sample target for quantitative MALDI-MS applications.

PIE 11

High-throughput profiling of intracellular levels of phosphorylated metabolites in fed- batch cultures using microarrays and MALDI-TOF-MS

Robert Steinhoff[1], Marija Sarenac[1], Tobias Habicher[1], Jasmin Krismer[1], Jens Boertz[2], Fabian Wahl[2], Massimo Morbidelli[1], Martin Pabst[1], Renato Zenobi[1]

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Current mass spectrometric methods for metabolite monitoring rely usually on separation techniques like liquid- or gas chromatography. The sample throughput capability of these methods is limited, since cycle times (per sample) are in best case in the range of several minutes.[1]

The aim of this investigation is to develop a high-throughput metabolite monitoring method using MALDI-MS. The method should provide comparable or more information content alongside with a good statistical significance, compared to existing analytical techniques.

Experimenteller Teil

We monitored the metabolic profile throughout a cell culture process (hybridoma CRL-1606 cell line) for monoclonal antibody production. We adapted a short cell extraction procedure using 10^6 cells. After addition of ^{13}C labelled internal standard and the MALDI matrix 9-aminoacridine (9-AA), we directly aliquoted the mixture onto a microarray target.[2] By MALDI-MS, one sample was measured within 1 minute, by considering ten technical replicates.[3] Averaged MALDI mass profiles were corrected using a stable isotope labelled internal standard and extracted for statistical analysis.

Ergebnisse und Diskussion

We describe the development and implementation of a method for high-throughput profiling of intracellular levels of phosphorylated metabolites using MALDI-MS combined with microarrays plates. We established a MALDI time of flight mass spectrometry (TOF-MS) workflow using 9-AA as matrix. Using a microarray sample target, we were able to rapidly quantify ATP, estimate the energy charge and identify several metabolites. This allows a fast targeted approach as well as a metabolite mass profiling for principal component analysis of a larger series of batches in parallel. The MALDI based high-throughput approach may assist metabolic engineering and allows routine cell batch monitoring.[4]

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Neue Aspekte

High-throughput metabolite profiling using MALDI-MS.

PIE 12

Hochauflösende Online-LC/MS zur Charakterisierung von Erdölkomponenten

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Erdöle und davon abgeleitete fossile Ressourcen stellen eine kaum zu überschauende, komplexe Mischung verschiedenster organischer Verbindungen dar. Neben der reinen Anzahl einzelner Analyten, die leicht mehrere 10.000 übersteigen kann, stellt auch die Spannbreite der jeweils vorhandenen Isomere eine große Herausforderung an die massenspektrometrische Analyse.[1]

Mit Hilfe ultrahoch auflösender Massenspektrometrie kann eine Vielzahl isobarer Analyten leicht voneinander getrennt werden. Dennoch rückt eine vorherige Vereinfachung der Probe immer stärker in den Fokus auch massenspektrometrischer Analysen. Als ein probates Mittel haben sich flüssigchromatographische Gruppentrennungen erwiesen, mit denen etwa Kohlenwasserstoffe von thiophenischen Verbindungen zu separieren sind.[2]

Durch die Entwicklung moderner Hochfeld-Orbitrap-Geräte, mit einem Auflösungsvermögen von bis zu 960.000 (FWHM @ *m/z* 400) ist eine online-Kopplung flüssigchromatographischer Trennungen mit hochaufgelöster Massenspektrometrie ein praktikabler Ansatz.

Experimenteller Teil

Eine Erdölprobe wurde zunächst durch Ausfällen aus Heptan deasphaltiert. Die asphaltenfreie Maltenfraktion wurde dann säulenchromatographisch an Aluminiumoxid weiter in gesättigte Verbindungen, (Poly-)Aromaten und sogenannte Harze aufgetrennt (Laufmittel: Heptan - Toluol - Toluol/Methanol). Das Verhalten von zunächst nur der Aromatenfraktion und später der gesamten Maltenfraktion gegenüber einer mit Pd(II)-chlorid belegten Mercaptopropanosilicagelphase[2] wurde in einer online-LC/MS-Kopplung untersucht.

Hierzu wurde die jeweilige Probe auf das Trennsystem gegeben und bei einer Flussrate von 1.5 mL/min mit einem Gemisch aus Cyclohexan und Dichlormethan (2:1) eluiert. Dem Laufmittel wurden steigende Anteile an Isopropanol als kompetitivem Liganden zugesetzt um retardierte Analyten zu eluieren.

Die massenspektrometrische Detektion der Analyten erfolgte an einer Orbitrap Elite nach Ionisation mittels APCI/APPI bei Scanraten von 0.6 - 1.3 Hz.

Ergebnisse und Diskussion

Durch Einführung der kompakten Hochfeld-Orbitrap Generation in 2010 wurde die Auflösung, die durch diese Geräte standardmäßig erreicht werden kann auf etwa 240,000 (768 ms Transient, FWHM @ *m/z* 400) erhöht. Das uns zur Verfügung stehende Gerät kann durch einen 3s Transienten eine theoretische Auflösung von 960.000 erreichen und steht damit erstmalig in direkter Konkurrenz zu FT-ICR Geräten, die bei einer Feldstärke von 7 T Auflösungen von 1.000.000 erreichen. Im Bereich massenspektrometrischer Analysen von Erdöl ist eine besonders hohe Auflösung von Bedeutung um etwa schwefelhaltige Analyten neben schwefelfreien in deutlich unterschiedlicher Konzentration sicher erkennen zu können. Für moderne FT-MS-Methoden stellt dies insbesondere bei Analyten mit Massen ab etwa 1.000 Da ein zunehmendes Problem dar.

Durch die Kopplung einer chromatographischen Abtrennung thiophenischer Verbindungen von reinen Kohlenwasserstoffen konnte dieses Problem effektiv vermieden werden. So konnten beide Stoffgruppen im Zuge derselben Analyse auch bei niedrigeren Auflösungen und damit kürzeren Scanzeiten, wie sie für chromatographische Trennungen erwünscht sind, sicher zugeordnet werden.

Über den jeweiligen Peak der eluierenden Verbindungen hinweg konnten zudem nähere Einblicke in die Retention gewonnen werden. So war bereits bekannt, dass auf einer analogen Phase in der Fraktion der thiophenischen Verbindungen die Retention in der Reihe Thiophene < Benzothiophene < Dibenzothiophene zunimmt. Dies wird allgemein mit einer höheren π-Elektronendichte am Schwefel begründet.[3] Ein ähnliches Verhalten wurde nun auch für die Fraktion der schwefelfreien Polyaromaten beobachtet. Allgemein tritt mit zunehmender Retentionszeit eine Verschiebung hin zu niedrigeren Massen und einer größeren Anzahl an Doppelbindungsäquivalenten auf. Da die reinen Kohlenwasserstoffe nicht als Liganden für das Palladium fungieren, kann dieses Verhalten nur durch eine zunehmend schlechtere Löslichkeit im Laufmittel und/oder durch eine Interaktion mit dem teilweise noch zugänglichen Silica-Träger zurückzuführen sein.

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Neue Aspekte

Die online-Kopplung von Ligandenaustauschchromatographie an Palladiumphasen mit massenspektrometrischer Detektion erlaubt detailliertere Einblicke in das Verhalten der Trennphase.

PIE 13

Ultra-sensitive Detection of Pharmaceutical and Personal Care Products (PPCP's) in Water with an Integrated On-Line Extraction (OLE)-UHPLC-MS/MS System

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The occurrence of pharmaceutical and personal care products (PPCP's) in environment water is of growing concern due to their potential harmful effects. The conventional methods based on the "template" of EPA 1694 methods require long and pre-concentration of large volume of water sample and tedious SPE cleanup, followed by LC-tandem mass spectrometer analysis in order to achieve the low ng/L (ppt) level detection.

In this study, we introduce an UHPLC system with an integrated online extraction option coupled to triple quadrupole mass spectrometer for detection of PPCPs in water. The built-in online extraction requires no additional hardware assembly (LC pump and valve), and can readily detect 1-5 ppt of PPCPs in water by rapidly pre-concentrating 0.5 mL sample.

Experimenteller Teil

More than 15 PPCP's were studied. The study was carried out on an EVOQ Elite model triple quadrupole mass spectrometer coupled to Advance UHPLC OLE system consisting of an integrated third pump (the other two for binary gradient separation) for sample preparation and a 10-port valve. The sample was injected via the loop and loaded onto an trap column (ProtoSIL, AQ C18, 30 mm x4.6 mm x 15 µm), after cleanup with the use of third pump, the targeted PPCP's were back flushed onto the analytical column (YMC-Pack ODS-AQ, 100mm x2.0 mm, 3 µm,) for separation with a binary gradient. The EVOQ was operated in ESI mode with both positive and negative polarities depending upon the target compounds.

Ergebnisse und Diskussion

The initial method development work was focused on the optimization of polarity of the ESI source, injection volume, chromatographic conditions, and breakthrough volume of the trap column, etc. The results showed that most of the PPCP's can be detected with 1-5 ppt or better with the injection of 0.5 mL water samples, with a linear response range (R^2>0.99) up to 100-500 ppt. Replicate injections with 5 ppt level spiked in tape water demonstrate good robustness with RSD of 5-15% (n=10).

Neue Aspekte

A integrated Online Exaction UHPLC coupled to MS/MS for low ppt level detection of PPCPs in water

PIE 14

Improved Proteome Coverage by Intelligent Data-Dependent Decision Tree Logics on a Novel Tribrid Orbitrap Mass Spectrometer

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For large-scale bottom-up tandem MS (MS/MS) protein sequencing techniques, the data dependent decision tree (DDDT) concept was introduced in 2008 by Coon and co-workers [1] on a Thermo Scientific™ LTQ Orbitrap™ XL ETD instrument. The concept of using complementary types of ion fragmentation, here collision-induced dissociation (CID) and electron transfer dissociation (ETD) has now been widely accepted. This DDDT approach has now been evaluated on the novel tribrid Thermo Scientific Orbitrap Fusion™ mass spectrometer and extended for higher energy fragmentation (HCD) and the newly developed fragmentation type electron-transfer/higher-energy collision dissociation (EThCD).

Experimenteller Teil

A proteolytic digest of HeLa cells was separated by reversed-phase chromatography on an EASY-Spray column (50 cm x 75 µm ID, PepMap RSLC C18, 2 µm 100 Å) using a Thermo Scientific EASY-nLC 1000 UHPLC system. The UHPLC system was coupled to a Orbitrap Fusion instrument. For evaluation of data dependent decision tree (DDDT) parameters, the following fragmentation methods were used: HCD, ETD and EThcD.

The raw data files were processed using a prototype Thermo Scientific Protein Discoverer™ software with Sequest HT™ search engine. From these data, the probability of an MS/MS scan event resulting in a high-confident peptide spectral match (PSM) was calculated.

Ergebnisse und Diskussion

Prior to the decision tree experiments, the fragmentation method ETD was evaluated with supplemental activation energies ranging stepwise from low to high energies to optimize fragmentation efficiency for ETD and EThcD.

Based thereon, the decision tree parameters have been established and tested using HeLa, resulting in increased proteome coverage compared to the application of HCD, ETD or EThcD alone.

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Neue Aspekte

Development of data dependent decision tree parameters for Thermo Scientific Orbitrap Fusion mass spectrometer.

PIE 15

Combination of online fractionation and ETD-UHR QTOF to enhance middle-down monoclonal antibodies characterization

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The potential of mAbs for therapeutic molecules design is such that there is a constant need for enhancing methods to characterize them. Recently enzymes allowing highly specific cleavage of mAbs into subunits became available. The isotopes of these fragments can be resolved on high resolution mass spectrometers such as quadrupole time-of-flight systems. Consequently, middle-down workflows have become increasingly popular. The characterization at the subunit level makes it possible to evaluate modifications such as oxidation or sequence variants while limiting the risk of artifactual modifications due to the sample prep.

Experimenteller Teil

We describe the LCMS analysis of a commercially available monoclonal antibody with an ETD enabled UHR QTOF to characterize mAbs subunits with simultaneous collection of peaks of interest in a liquid handling device. The level of information obtained through this workflow is expanded by consecutively infusing collected peaks of interest and using electron transfer dissociation (ETD) in order to obtain useful information about the peptide sequence and localize modifications. This information is obtained without additional enzymatic digestion increasing the confidence in the results.

Ergebnisse und Diskussion

The subunits are separated by HPLC and the accurate mass obtained for each main peak allows to confidently assigning those peaks to the expected subunits. The window in between the peaks is adequate to allow collecting fractions of a good purity.

The monoisotopic mass of the fragments has been determined with the help of a isotopic pattern fitting algorithm. Oxidization, pyroGlutamation and partially reduced forms have been identified with better than 2.5ppm mass accuracy. In addition the various glycoforms of the Fc/2 are resolved allowing the relative quantitation of these variants.

The fraction will further be evaluated with ETD in order to confirm termini sequences and locate the exact position of modifications without recourse to an additional tryptic digestion.

Neue Aspekte

Sequence coverage information for mAbsubunits is obtained without requiring additional enzymatic steps increasing productivity and data quality.

PIE 16

An Improved HDX Workflow for Enhanced Separation, Digestion, and Data Analysis

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An integrated Hydrogen Deuterium Exchange Mass Spectrometry (HDX MS) workflow consists of several key components including on-line digestion, sub-ambient chromatography, MS, and dedicated informatics tool. In recent years, each component has been significantly developed to improve efficiencies of the HDX workflow.

In this study, we present our effort to systematically optimize the performance of a HDX platform by using a prototype microscale UPLC system that can operate at 15,000 PSI, an immobilized pepsin column packed with mechanically strong particles, and a dedicated informatics tool with enhanced functionality. We will demonstrate that the higher operating pressure rendered by the UPLC improves chromatographic resolution; the digestion efficiency of the pepsin BEH column is enhanced during higher pressure digestion.

Experimenteller Teil

Pepsin was immobilized onto high-pressure resistant ethylene-bridged (BEH) particles. The particles were packed into a 2.1x30 mm UPLC column and its digestion efficiency was tested with a LC system specifically designed for HDX MS analysis. Phosphorylase b, cytochrome C, and IgG were digested online under a pressure range above 8000 psi at 0, 10, or 25 °C, respectively. Peptides were chromatographically separated on a 1x50 mm column, and the eluent was directed into a high resolution Q-Tof mass spectrometer for mass analysis. A comparison of sequence coverage percentage and peptide length with and without pressure digestion was performed, as well as determination of deuterium uptake at various labeling time points. Deuterated LC/MS data were automatically processed using DynamX software.

Ergebnisse und Diskussion

Immobilization of pepsin on BEH particles was successfully accomplished. Digestion tests using the particles with immobilized pepsin confirmed that the chemical immobilization process was properly carried out and enzymatic activity of pepsin was not affected by the immobilization chemistry. The online digestion using a BEH pepsin column achieved robust and reproducible digestions of phosphorylase b, a ~95 k Da protein, with and without pressure in five replicate analyses.

An increased number of overlapping peptic peptides were generated from pressurized digestion resulting in improved coverage and redundancy score. Increasing the digestion temperature of the online pepsin column resulted in higher sequence coverage of cytochrome C from 63% at 0 °C to 100% at 25 °C. The rate of back-exchange from deuterated proteins, which is an important factor during HDX MS experiments, was carefully measured for both the high-pressure tolerant BEH pepsin column and commercially available pepsin columns. Comparable back-exchange rates were achieved with the BEH pepsin column compared to commercially available pepsin columns. An enhanced chromatographic resolution was achieved for protein digests separated in less than 10 min at 0 °C at high pressure.

With a prototype microscale UPLC system that can operate at 15,000 PSI, the complex peptic digests of IgG and phosphorlyase b were reproducibly separated at 0 °C. The data processing software improved the speed and consistency of deuterium uptake calculation for the peptides generated using the HDX platform.

A set of data interpretation/display tools such as coverage map and a heat map options were provided in the informatics tool, DynamX software 2.0, facilitating efficient data comparison. The results demonstrated that enhanced performance of the HDX platform is achieved when operated at high pressure with no compromise in deuterium recovery.

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Neue Aspekte

The results demonstrate that enhanced performance of the HDX MS Q-Tof platform is achieved with no compromise in deuterium recovery.

PIE 17

Selected Accumulation Ion Mobility Spectrometry (SAIMS) Hyphenated with a Fourier Transform Mass Spectrometer (FTMS)

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The recently introduced methods of Trapped Ion Mobility Spectrometry (TIMS) and Selected Accumulation Ion Mobility Spectrometry (SAIMS) are methods which can perform high resolution mobility separations in small device and effectively interface such separations to slow mass analyzers and processes. Here we demonstrate, for the first time, SAIMS in conjunction with a Fourier Transform Ion Cyclotron (FTMS) Mass Spectrometry. The combination of SAIMS and FTICR leads to IMS-MS spectra having both high mobility and high mass resolutions. In addition to simply providing more information – i.e. mobility in addition to mass - the peak capacity of the IMS-MS spectra is substantially improved over FTMS alone. The prototype SAIMS-FTMS was used to analyze a complex samples via both ESI and MALDI.

Experimenteller Teil

In the present work, a TIMS analyzer is incorporated into an ion funnel in an FTMS. The TIMS method may be viewed as the gas phase equivalent of the solution phase mobility technique known as Electric Field Gradient Focusing (EFGF). As in EFGF, the medium (a gas) is moving, and pushing the ions, downstream while a retarding electric field holds the ions in place. To perform SAIMS-FTMS experiments, ions of a selected mobility are accumulated on a plateau in the retarding electric field and then released to an FTICR mass analyzer. Repeating this process for a range of mobilities - each time acquiring a mass spectrum - results in an array of mass spectra h together constitute an IMS-FTMS.

Ergebnisse und Diskussion

The mobility resolution was about 100. The time required to fill and extract ions from the TIMS trap were about 600 and less than 100 ms respectively. The mobility of an ion was found to be inversely proportional to the TIMS barrier potential. And the duty cycle for the selected mobility ions was about 50% whereas the overall duty cycle was dependent on the mobility range and resolution of the experiment. Complex samples including a peptide mix (peptide calibration standard II, P/N 222570, Bruker Daltonics, Billerica, MA) were analyzed by ESI-SAIMS-FTMS. The peptide mixture, which included bradykinin (1-7), angiotensin II, angiotensin I, substance p, bombesin, renin substrate, ACTH 1-7, ACTH 18-39 and somatostatin, was diluted in 50:50 acetonitrile:water and sprayed at a flow rate of 3 ul/min. The resultant IMS-MS spectrum exhibited trend lines for the 1+ through 4+ charge states. Peak capacity in the IMS-MS spectra was improved by a factor of 50 over the MS spectra alone. In a second set of experiments, the same peptide mix was analyzed by MALDI-SAIMS-FTMS producing similar results as the 1+ ESI ions. When operating with MALDI, the duty cycle was essentially 100% for the selected mobility species because ion production was limited to the fill time of the TIMS trap.

Neue Aspekte

When operating with MALDI, the duty cycle was essentially 100% for the selected mobility species

PIE 18

Development of a New Ion Mobility-Quadrupole Time-of-Flight Mass Spectrometer for High-Resolution and High-Throughput Biological Sample Analyses

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A new uniform-field ion mobility-quadrupole time-of-flight mass spectrometer (IMS-QTOF-MS) has been designed and constructed which has excellent ion mobility resolving power and comparable mass resolution, dynamic range and sensitivity when compared to current state-of-the-art QTOF mass spectrometers. A number of innovative technologies have been implemented, including a PTFE coated segmented aluminum drift tube manifold, gold coated PCB based drift electrodes and ion funnel elements.

Ergebnisse und Diskussion

A substantial reduction in size and complexity was achieved by developing a vacuum system supported by a single scroll pump and new surface-mount control electronics. New software packages were developed for data acquisition, data processing and visualization. The current instrument consists of a low pressure uniform-field drift tube with nitrogen drift gas coupled with a modified QTOF mass spectrometer. This instrument can be operated in IMS mode as well as QTOF mode when IMS separation is not required. Ion mobility resolving powers of 50-80 (close to theoretical limits) for this instrument configuration is achieved for across a broad mass range.

PIE 19

A Cold Inlet System for the Analysis of air sensitive and volatile Compounds by EI-MS

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Today, all state-of-the-art mass spectrometric methods require at least one sample preparation step to be conducted under atmospheric conditions. This makes the analysis of volatile and oxidation sensitive compounds by mass spectrometry, e.g. metal organyles or phosphanes, virtually impossible. The construction of an inlet system similar to the AGHIS[1] (all-glass heated inlet system), which is easy to replace and does not hinder the routinely employed sample introduction using a push rod, overcomes this limitation. Using the cold inlet system, electron impact mass spectra of volatile compounds exhibiting high sensitivity towards moisture and/or air are obtained in a straight forward fashion.

Experimenteller Teil

The cold inlet system consists of an interface made from stainless steel with a needle valve for controlled leaking of the sample into the source of the mass spectrometer and a flange for the attachment of custom made lockable test tubes, containing the sample. The interface is placed in the same position as the usually employed push rod. An internal interface made from glass is used to guide the sample to the ionisation chamber of the mass spectrometer. The internal interface is held in place by a stainless steel mounting plate with an opening for the push rod. EI Mass spectra are recorded with an Autospec X (VG, now Waters). Data analysis is performed with Opus 3.7.

Ergebnisse und Diskussion

The cold inlet system was tested using a stannane compound. The first measurements yielded signals with an isotopic pattern typical for ions containing tin, however, no molecular ion was detected. Intensity of the detected peaks was also quite low, with no signals present above *m/z* 300. To improve the sensitivity when using the cold inlet system, an internal interface was constructed. This internal interface presents no obstacle for the operation of the mass spectrometer with the direct inlet system using a push rod. The internal interface consists of a mounting plate with a hole for the push rod, holding a small funnel made from fused silica in place, which guides the gaseous sample directly into the ionisation chamber of the ion source. This modification enhanced the sensitivity of the inlet system significantly. However, molecular ions are rarely detected for most compounds as they fragment fast. Only in the presence of stabilizing ligands molecular ions can be detected.

As the internal interface remains inside the ion source all the time, switching between direct inlet system using a push rod and the cold inlet system is facilitated within seconds. Cross-talk between different samples is prevented by pumping out the inlet system thoroughly. The cold inlet system presents an opportunity to obtain mass spectra of compounds which decompose rapidly when standard sample preparation procedures under ambient conditions are employed. EI mass spectra of compounds, which were so far not possible to analyse using mass spectrometry, have been obtained in this manner. Accurate mass measurements have been performed also, enabling the verification of the elemental composition.

Referenzen

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Neue Aspekte

Cold Inlet system enabling MS analysis of volatile compounds exhibiting high sensitivity towards moisture and/or air.

PIE 20

UV Photodissociation of Conformer-Selected Protein Ions

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Direct top-down sequencing of larger proteins *via* mass spectrometry is often challenging when established slow heating techniques such as collision-induced dissociation (CID) are used. Due to an internal redistribution of the energy during activation, cleavages occur non-statistically at the weakest peptide bonds, which typically results in limited sequence coverage. A promising alternative is ultraviolet photodissociation (UVPD) were extensive and rather statistical fragmentation along the backbone leads to a considerably increased informational content.[1] So far, however, it is essentially unknown in how far the structure of the protein, which – for one and the same charge state - can range from very compact, native-like conformations to extended and unfolded structures, affects the UVPD fragmentation behaviour.

Experimenteller Teil

A technique that is sensitive to the higher order structure of protein ions is ion mobility mass spectrometry (IM-MS) were the molecules are separated in the gas phase according to their angular averaged collision cross section (CCS). In order to evaluate the impact of the protein structure on the UVPD fragmentation pattern, we here used IM-MS to pre-select distinct gas-phase conformers of ubiquitin 11+ prior to photodissociation. To do so, ions were generated in a nanoelectrospray source and separated according to their CCS in a home build drift tube IM-MS cell. Subsequently, the isolated population was mass/charge filtered using a quadrupole and irradiated with light of an excimer laser at 193nm. Finally, the resulting fragments were analyzed *via* time-of-flight MS.

Ergebnisse und Diskussion

Basis for our investigation was the 8.5kDa protein ubiquitin, which has been studied extensively in the gas phase. At a charge state of 11+ ubiquitin adopts two distinct conformations, a more compact one with a CCS of 1800Å^2 and an extended one with a CCS of 1870Å^2. UVPD fragmentation of both pre-selected conformers yielded complex spectra, with many similarities but also very obvious differences. Assignment of the peaks revealed that the sequence coverage is generally very high, which is in good agreement with previous studies.[2] Surprisingly, however, the majority of differences in the UVPD spectra of both conformers was found to arise from cleavages at one and the same position around Pro19. In solution, the *cis-trans* isomerization of this particular residue was found to be crucial for the global folding of ubiquitin. Molecular dynamics (MD) simulations revealed that such a *cis-trans* isomerization of Pro19 is also a major determinant for the structure of ubiquitin in the gas phase. The theoretical CCS of *cis*-Pro19 ubiquitin agrees very well with the experimental value of the more compact conformation; the theoretical CCS of *trans*-Pro19-ubiquitin on the other hand agrees well with the CCS of the extended conformer. Therefore, we conclude that *cis-trans* isomerization of Pro19 is key for both the structure as well as the UVPD fragmentation pattern of gas-phase ubiquitin. In a more general context these results imply, that conformer selected UVPD has the potential to be used as a tool to monitor subtle structural differences in gas-phase proteins.

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Neue Aspekte

First ultraviolet photodissociation experiment of conformer-selected protein ions reveals crucial impact of proline *cis-trans* isomerization on gas phase structure.

PIE 21

Challenges in Microscope Mode Imaging Mass Spectrometry

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Imaging mass spectrometry (IMS) in microscope mode offers the possibility to analyse a large area on tissue sections and on sample arrays simultaneously. Linear microscope IMS experiments are limited in their application by a number of factors: The low resolving power of the employed ion optics; The time resolution afforded by the scintillator screen detector (P47, 70 ns emission life time); The multi-hit capability, per pixel, of the employed imaging sensor. To overcome these limitations, we introduce an ion velocity corrected post extraction differential acceleration (PEDA) method to achieve higher ToF resolutions [1,2], a newly developed scintillator with shorter emission lifetime [3], and couple the experiment to the PImMS sensor with multi-mass imaging and time stamping capabilities [4,5].

Experimenteller Teil

Based on ion-trajectory simulations we have developed a five electrode ion optic using the PEDA technique optimised for mass tuneable imaging of samples up to 4 mm in diameter. The setup was tested with PEG and dye samples to measure its spatial and ToF resolution. The new scintillator used, was grown in a vacuum sublimation process and mounted behind a 40 mm MCP detector. The performance of the resulting imaging detector was characterised by a number of key parameters, including: Fluorescence decay time, brightness, stability and spatial resolution. Furthermore, the CMOS based PImMS sensor was applied to the system to demonstrate its “multi-hit” detection capabilities.

Ergebnisse und Diskussion

To improve the achievable ToF resolution of a linear microscope mode IMS experiment the post extraction differential acceleration method was employed. After initial extraction of ions from the source region, the extractor lens is pulsed to a higher voltage to differentially accelerate ions that have passed this electrode. This pulse compensates the initial velocity spread of the ions desorbed from the sample surface and allows time focusing of a narrow mass range of ions (±20%) onto the imaging detector. The experimental ToF resolution was determined ($m/\Delta m \approx 2000$) while the obtained spatial resolution was ~25 µm. To tune the optimised mass range to any mass of interest, the extraction field was adjusted such that the particle trajectories match those of the previously optimised mass range. Four different mass ranges, peaking around 400 Da, 500 Da, 600 Da and 700 Da were investigated. In each case we demonstrate that the spatial and ToF resolution of the spectrometer is preserved.

The characterisation measurements for the new scintillator screens developed for imaging and ToF applications showed an emission life time below 4 ns while the brightness exceeded that of a P47 screen by a factor 2x. No signal degradation was observed over an extended period. The spatial resolution of the new screens is comparable to commercial imaging detectors, and they are therefore fully applicable to any ion imaging application requiring a high time resolution. Further tests employing Single Photon Avalanche Diodes indicate that such scintillators can be used for charged particle detection without the need for MCPs.

First results are presented using the PImMS sensor as an imaging device for IMS. The camera can operate with a time precision of 12.5 ns at a resolution of 72x72 pixel and is capable of detecting up to 4 events per pixel within each ToF cycle.

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Neue Aspekte

Construction of a Microscope Mode Imaging Mass Spectrometer; Development of a novel scintillation detector and application of the PImMS sensor

PIE 22

Performance Results of a Mobile High-Resolution MR-TOF Mass Spectrometer with MS/MS Capability for in-situ Analytical Mass Spectrometry

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A mobile multiple-reflection time-of-flight mass spectrometer (MR-TOF-MS) has been developed. In contrast to conventional time-of-flight mass spectrometers, the mobile MR-TOF-MS provides ultra-high mass resolving power and sub-ppm accuracy in a compact and portable format [1]. Thus it allows resolving isobars and enables accurate determination of the composition and structure of biomolecules for in-situ measurements. Furthermore the device offers high mass resolving MS/MS capability via selective ion re-trapping and collisional-induced dissociation (CID) in the same trap from which they are injected into the time-of-flight analyzer.

Experimenteller Teil

The spectrometer consists of an atmospheric pressure interface (API) for various atmospheric ion sources, a dedicated RFQ beam preparation system (ion guide, mass filter, ion cooler and ion trap) and a compact time-of-flight analyzer (~ 0.5 m) with MCP detector. Inside the analyzer the ions are reflected by electrostatic mirrors, so the analyzer can be traversed up to a thousand times by the ions. This entails a flight path of up to 1 km length and therefore ultra-high resolution in a compact system can be achieved [2].

All electronics, pumps and the control system are mounted with the spectrometer in one single frame of only 0.8 m^3 volume.

Ergebnisse und Diskussion

The MR-TOF-MS has been successfully commissioned and measurements with a Cs ion source as well as with different atmospheric ion sources have been carried out. A mass resolving power exceeding 300,000 (FWHM) and a repetition rate of over 1 kHz have been measured [3]. In addition ion re-trapping has been performed, which is a requirement for the high resolution MS/MS capability of the system. With the option of re-trapping ions in the RFQ ion trap, the device is also able to perform multiple consecutive trap and measurement cycles (MS^N) with high resolution (> 10,000) in every stage in the same analyzer. In order to optimize the device, ion-optical simulations with SIMION are performed supplementary to the measurements.

With the device many applications like waste water monitoring at hot spots, mass-based classification of biomolecules and breath analysis are possible. While the current system makes use of standard components, the device is readily scalable to achieve even further reduction in size and weight to fit specialized applications in the future.

Referenzen

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Neue Aspekte

The device offers mobile ultra-high-resolution MR-TOF-MS with MS^N with high resolution in every stage.

PIE 23

Microarrays for mass spectrometry: from single-cell analysis to routine quantification

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Recently, *Urban et al.*[1] introduced a new microarray platform for high-throughput analysis combined with label free readout using MALDI-MS. The microarray platform consists of an "omniphobic surface" with hydrophilic spots in the micrometer size[1]. The microarray especially turned out to be beneficial, where small and difficult to handle solutions had to be analyzed as demonstrated for single cell analysis in a metabolomics study of single yeast cells[2].

Through the past years, microarrays for mass spectrometry found its way to several other applications from a combined platform with microfluidics and liquid chromatography as well as to routine quantitative applications in clinical studies.

Experimenteller Teil

Microarray targets (Microarrys for Mass Spectrometry, MAMS) were produced as described in *Urban et al. 2010*[1], *Pabst et al. 2013*[3]or *Küster et al. 2014*[4]. For AB Sciex MALDI-MS instruments, slides were mounted onto a standard AB Sciex sample target carrier using the mask from the "LaserBio Labs™ Mass Spectrometry Imaging Starter Kit" or the MTP Slide Adapter II for Bruker MALDI instruments. Analysis was performed using an AB Sciex TOF/TOF 5800 or a 4800 Plus mass spectrometer (Darmstadt, Germany), as well as a Bruker solariX FTMS (Bremen, Germany). MALDI matrices, standards and solvents were obtained from Sigma- Aldrich (Fluka, Buchs, Switzerland).

Ergebnisse und Diskussion

Microarrays for mass spectrometry offer specific features: i) Self-aliqouting properties allow an automatic aliqouting as well as focusing of solutions to hydrophilic spots ii) Nanoliter volume reaction cavities generate a platform for microfluidic applications. iii) Depending on microarray substrate, spots can be selectively functionalized for an on-chip affinity assay.

Overall, the microarray is designed as a high-throughput platform for a label free or combined readout using MALDI-MS as well as fluorescence and optical microscopy[1]. We provide an overview of current applications, where we show further developments of the single-cell analysis technique as well as demonstrate a microarray platform for advanced identification of post translational protein modifications[1,2,5]. The later one is realized by integrating the microarray into the workflow of nLC-MS with the assistance of microfluidics[5].

Since quantitative analysis is always an issue in biological as well as clinical applications, we designed also a chip for quantitative MALDI-MS applications. Here we demonstrate the usefulness of our microarray platform for drug analysis as well as for biotechnological applications like monoclonal antibody and metabolite level monitoring of cell batch processes.

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Neue Aspekte

We present a newly developed microarray plate, which brings new perspectives to MALDI-MS applications

PIE 24

A hydrodynamically optimized nano-electrospray ionization source and vacuum interface

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Ions generated in atmospheric pressure ionization (API) sources like electrospray ionization (ESI) have to be transferred into vacuum in order to be analyzed by mass spectrometry (MS), ion mobility spectrometry (IMS) or to be deposited on a substrate (soft landing[1,2]). The coupling of an API source to a vacuum chamber is made via an interface, typically a transfer capillary or a pinhole of small diameter, as pumping speed is limited. Present interfaces are very inefficient, as they only transfer a small fraction of around 1% of the generated ions into vacuum. Nano-ESI sources are typically better, reaching transmissions around 20%.

Experimenteller Teil

To improve the performance of the vacuum transfer, we optimized the geometry of the transfer capillary, taking all influences on the ion motion into account: electrostatic forces from applied voltages and space charge forces as well as hydrodynamic drag forces.[3]

Ergebnisse und Diskussion

Our new interface shows transmission into vacuum of up to 100% of the ions generated at a nanoelectrospray emitter for analyte concentrations below 10^{-3} M. For high concentrations and high electrospray emitter voltages a threshold current in the range of 2-40 nA is reached beyond which the transmission decreases. The performance of the source was tested with current measurements, mass spectrometry, and ion beam deposition experiments[1,2]. It was shown that the ion beam generated by the source is well defined and free of contamination.

Based on computational fluid dynamics calculations of the gas flow within the capillary and ion motion simulations, we show that hydrodynamic forces of an optimized gas flow collimate the ion cloud, which effectively counteracts the space charge forces that are the main reason for ion losses.

This ion source represents a major improvement over present nanospray sources in terms of transmission. In particular measurements requiring high sensitivity or high ion flux will profit from this development. Further, the approach of hydrodynamic optimization will allow improving other atmospheric pressure ion sources.

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Neue Aspekte

In-situ scanning tunneling microscopy allow for unprecedented resolution of individual molecules, only possible because of ultrahigh-vacuum sample-preparation by ion soft-landing.

PIM: Imaging

PIM 1

Investigation of different hierarchal clustering approaches for protein identification directly from tissue section in a MALDI imaging experiment

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Mass spectrometry imaging (MSI) allows for the correlation of spatial localization and chemical information directly from biological surfaces. A typical data set can contain thousands ion signals with varying degrees of co-localisation. We can also utilise an additional dimension called high efficiency ion mobility separation (IMS) based on travelling wave (T-wave) technology incorporated into the mass spectrometer to add specificity to the MSI experiment. This leads to highly complex data sets of several gigabytes in size which necessitates the need for advanced automated computerized processing to help maximise the data potential. Here we investigate the use of different Hierarchal Clustering Analysis (HCA) methods to aid the analysis of tryptically digested tissue section by clustering ion images based on their correlation.

Experimenteller Teil

Four peptide digests; Bovine Serum Albumin, Phosphorylase B, Alcohol dehydrogenase and Enolase) were spotted at 100 fmol forming a 6x6 array comprising four 4x4 overlapping squares.

Sample tissue sections (a mouse fibrosarcoma model) were washed, then on-tissue tryptic digestion was carried using a SunCollect sample preparation device. After an overnight incubation, alpha-cyano-4-hydroxycinnamic acid matrix was applied evenly to the sample in several coats also using the SunCollect.

Data were acquired using a MALDI SYNAPT G1 and G2-S mass spectrometer in MS mode with tri-wave ion guide optics to separate ions according to their ionic mobility in the gas phase. The mass range of acquisition was 700-3,000 Da where matrix, lipid and tryptic peptides can be detected.

Ergebnisse und Diskussion

The information rich data sets were subsequently processed and visualised using High Definition Imaging (HDI) 1.2 MALDI software. The first step in data reduction of the large dataset was achieved by peak picking the data using the multidimensional Apex3D detection algorithm in the m/z and drift time dimension. The second step of the routine was to generate ion distributions comprising X,Y coordinates (i.e. ion images). The third step was to correlate all processed ion distributions using the Pearson product-moment algorithm, which generates a peak correlation matrix. The degree of data correlation is expressed using a correlation coefficient that is a measurement of the linear dependence between two variables. The result of the calculation is R value that ranges from -1 to +1. Negative numbers illustrate a negative correlation and positive a positive one.

In order to help understand the timing, the efficiency and the quality of the correlation, the array of tryptic peptides data was used. The different HCA methods were assessed in terms of their ability to cluster peaks from the tryptically digested imaging data set into groups of related peptides, which can then be used to yield protein identifications via PMF analysis. The methods were also evaluated in terms of the time required to complete the clustering analysis and the number of hierarchal levels created.

The optimum HCA method (top-down K-medoid) was then applied to the mouse fibrosarcoma model tissue data. It successfully clustered tryptic peptides with multiple protein identifications from a complex tryptically digested tissue section.

Neue Aspekte

Study of different HCA approaches based on the whole ion images to identify proteins via PMF directly from tissue section.

PIM 2

Spatial correlation combined with hierarchal clustering analysis for reducing complex multi-dimensional MALDI imaging dataset

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Mass spectrometry imaging (MSI) allows for the correlation of spatial localization and chemical information directly from biological surfaces. Recently, it has allowed simultaneous visualisation of thousands of lipid species in an entire tissue section, leading to the generation of several gigabytes of complex and high dimensional data from a single tissue experiment, especially if ion mobility separation (IMS) was used to add specificity to the experiment. This has result in the growing need for automated computerized processing.

Here we are presenting a novel MALDI imaging workflow that includes acquisition of the MALDI imaging data using ion mobility, processing using High Definition Imaging software and a novel data spatial correlation approach using Pearson product-moment correlation statistical tool.

Experimenteller Teil

Data were acquired using a MALDI SYNAPT G2-S mass spectrometer in MS mode with tri-wave ion guide optics to separate ions according to their ionic mobility in the gas phase. The mass range of acquisition was 100-1,000 Da where lipid species can be detected, as well as matrix species and small endogenous metabolites.

The information rich data sets were subsequently processed and visualised using High Definition Imaging (HDI) 1.2 MALDI software. This approach is based of grouping the processed ion images using a Pearson product-moment algorithm, which correlates the images based on their ion distribution throughout the tissue, rather than comparing MS spectra at different locations.

Ergebnisse und Diskussion

Proof-of-principle experiments have been carried out using parts of a thin section of a rat brain section, produced using a cryotome and deposited on tape and mounted on a standard MALDI target. Alpha-Cyano-4-hydroxycinnamic acid matrix was applied evenly to the sample in several coats using a TM-Sprayer sample preparation device.

The first step to reduce the large dataset was to process it by peak picking the data using the Apex3D algorithm in the m/z dimension and drift time dimension.

The second step of the routine was to generate ion distributions containing X,Y information (i.e. ion images).

The third step was to carry out the Hierarchal Clustering analysis by comparing the ions images between themselves and clustering by similarity. All processed ion distributions were correlated using the Pearson product-moment algorithm, which generates a peak correlation matrix. The degree of data correlation is expressed using a correlation coefficient that is a measurement of the linear dependence between two variables. The result of the calculation is an R value between -1 and +1, representing a measure of the degree of correlation. Negative numbers show a negative correlation and positive a positive one. Hierarchal clustering analysis (HCA) was performed on the correlation matrix w K-Means top down divisive clustering where, with K equaling 2 for the division of each cluster. HCA by K-means allowed for useful visualization of the correlation matrix. This, subsequently, enables easy determination of groups of peaks with increasingly correlated distributions, within the large complex dataset.

Neue Aspekte

New method to simplify MALDI imaging complex data analysis combining Pearson product-moment correlation statistical tool with K-Means top down HCA.

PIM 3

Imaging MALDI mass spectrometry in microscope mode with infrared lasers – breaking the diffraction limit on biological samples

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Infrared MALDI mass spectrometry (IR-MALDI-MS) at wavelengths around 3 µm can be utilized to exploit the intrinsic water content of biological samples as a MALDI matrix. Especially for imaging of small structures, were proper application of an external matrix is challenging, this could be beneficial. The spatial resolution of conventional MALDI imaging MS in the microprobe mode however is ultimately limited by diffraction, preventing the analysis of small structures with IR-MALDI-MSI. In microscope mode MSI, the spatial distribution is defined only by the ion optical system and the ion detector [1]. Therefore the combination of IR-MALDI and microscope mode MSI with a TimePix detector allows for IR-MALDI-MSI below the diffraction limit utilizing water as an intrinsic matrix.

Experimenteller Teil

A mass microscope based on a triple focusing time of flight (TRIFT) mass spectrometer (Physical Electronics Inc., Eden Prairie, MN, USA) equipped with a Timepix detection system [2] is used for microscope mode MSI. A Nd:YAG pumped OPO laser system operating between 2.7 and 3.1 µm was used for desorption. The laser is coupled to the instrument via a hollow waveguide. Standard matrix preparations covered by a grid for well-defined spatial features as well as frozen cryo-sections of fish tissue are employed as samples. Efficient cooling of the sample is necessary to stabilize the water used as a matrix in the second case under high vacuum conditions. A LN2 based cooling stage is employed for that purpose.

Ergebnisse und Diskussion

In order to use the existing parts for laser beam delivery on the TRIFT instrument with only minor modifications, the laser beam was coupled into a hollow wave guide (500 µm core diameter, 2 m long) connected to the instrument. Telescope optics are used to image the exit aperture of the waveguide onto the sample 3:1. Mode-mixing within the waveguide results in an inhomogeneous beam profile on the sample comprising of 4-6 “hot spot” areas observed in the direct ion image. This was prevented by utilizing artificially increased mode mixing prior to the hollow wave guide in order to produce a flat top beam profile on the sample.

First measurements were carried out on standard dried droplet sample preparations of different matrix-analytes combinations covered with a grid (pitch 36.3 µm) providing a well-defined spatial structure. Such benchmark samples were useful to tune the instrument and to determine both mass and spatial resolution. A lateral resolution of 9.8 µm was achieved on these samples, thereby breaking the (IR) optical limit of diffraction of 14 µm for the given system.

In a second step, untreated frozen tissue sections of a bait fish were measured, utilizing their intrinsic water content as a matrix. In combination with principal component analysis, the method allowed for the differentiation of tissue types like stomach and liver within one laser spot. To our knowledge this constitutes the first example of IR-MALDI imaging in microscope mode on untreated frozen tissue.

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Neue Aspekte

IR-MALDI-MSI in microscope mode with a timepix detector, IR-MALDI-MSI with water as an intrinsic matrix

PIM 4

Investigation of fungal metabolites using LESA-, DESI- and UHPLC-OrbitrapXL mass spectrometry

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The majority of global occurring, wood-decaying fungi live in habitats with overlap in niches of different fungal species. In consequence of the close vicinity fungi compete for territory and resources affecting decay rates in woodland ecosystems. The competition includes antagonistic actions by released metabolites causing changes in mycelial morphology, metabolic processes, secondary metabolites and extracellular enzyme patterns. However, antagonistic metabolites are still largely unknown.

Fungal interactions are difficult to investigate, because most methods are limited and not suitable. In former studies, we demonstrated LESA-OrbitrapXL-MS as powerful method to analyze metabolites of microorganisms directly from surfaces in agar plates [1]. Here we present that a combinatory approach of LESA-, DESI- and UHPLC-OrbitrapXL-MS is also very efficient to study fungal interactions.

Experimenteller Teil

Wood decaying fungi were cultivated in confrontation assays on Petri dishes for 28 days at different temperatures. Co-cultivation induced metabolites were either directly extracted from the surface of the agar plates and analyzed using LESA- and DESI- or extracted and investigated with UHPLC-OrbitrapXL mass spectrometry. Raw data files were interpreted and further converted to mzXML file format. Differentially analysis and PCA were performed in R using the packages xcms [2, 3] and pcaMethods [4].

Ergebnisse und Diskussion

To understand the fungal distribution in woodland ecosystems there is a need to comprehensively study fungal interactions. Fungi show a wide range of different morphological growth behavior while interacting with other fungi. In addition, some fungi release pigments due to co-cultivation. Using UHPLC-OrbitrapXL-MS and software ‘xcms’, we will demonstrate that metabolic profiles qualitatively and quantitatively differ between mono- and co-cultivated fungal strains. Furthermore, we visualized several metabolites (e.g. indigo) in different zones on an agar plate using LESA- and DESI-MS and will show distribution of respective metabolites.

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Neue Aspekte

Metabolic profiling and visualization of metabolites of co-cultivated fungi using UHPLC-, LESA- and DESI-OrbitrapXL mass spectrometry will be presented.

PIM 5

Mass spectrometry imaging of surface lipids on intact Drosophila melanogaster flies

Filip Kaftan[1,2,3], Vladimír Vrkoslav[3], Phillip Kynast[1,4], Purva Kulkarni[4], Sebastian Böcker[4], Josef Cvačka[3], Markus Knaden[1], Aleš Svatoš[1,3]

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Mass spectrometry imaging (MSI) providing information about the spatial distribution of molecules occurring on the sample surface and was recently adapted to insects[1]. Analytical technique MALDI MSI could be used for studying surface lipids (e.g. pheromones) on the whole intact specimen of insect for instance *Drosophila melanogaster* fly. Because of the 3-D character of analyzed insect the mass shift is observed during the 2-D MALDI MSI experiments. The rate of the mass shift is possible to reduce by gilding. However the mass shift could be completely corrected only by software applied to MALDI MSI datasets.

Experimenteller Teil

D. melanogaster flies were fixed in the dedicated MALDI targets with integrated complex profiled males and females cells[2]. The MSI experiments of surface lipids of virgin and copulated 6 days old flies were performed in positive reflectrone mode by LDI-TOF and MALDI-TOF technique. In MALDI-TOF experiments the lithium 2,5-dihydroxybenzoate (LiDHB)[3] matrix was sprayed on the samples by airbrush. The MALDI targets with samples were coated with a layer of gold in various thicknesses using sputter coater. The samples were imaged using step size of 100 µm or 50 µm. Raw MSI data were processed with custom-made software into spatially differentiated data and then converted to the 2-D maps of ions intensity maps by BioMap software and in-house developed software.

Ergebnisse und Diskussion

Using dedicated MALDI targets with profiled cells adapted to the dimensions of males and females imagoes of *D. melanogaster*, the reproducible height level and correct spatial orientation of flies on the target was achieved[2]. Based on the LDI/MALDI MSI experiments performed on MALDI Micro MX (Waters, UK) the visualization of males anti-attractants 11-*cis*-vaccenyl acetate (cVA)[4] and 3-*O*-acetyl-1,3-dihydroxy-octacosa-11,19-diene (CH5*O*3)[4] distribution was achieved. The strongest signals of these compounds were located on the tip of the male abdomen. In case of copulated pairs of flies the signals of cVA and CH5*O*3 were observed also from the upper part of female abdomen what indicate that the male marking the female with this mixture of anti-attractants during the copulation. On the other hand the distribution of the female pheromones 7*Z*,11*Z*-heptacosadiene[4] and 7*Z*,11*Z*-nonacosadiene[4] was different. MSI results were supported by GC-MS analysis of hexane extracts of relevant parts of flies. However all the acquired MSI datasets were affected by mass shift (predominantly between ca ± 0.2 Da to 0.3 Da) caused by 3-D nature of analyzed biological samples of *D. melanogaster* flies. For the mass shift correction the in-house developed software was used. The comparison study between BioMap software and in our laboratory developed method was demonstrated on values for matrix ($[DHB+K]^+$) ion at *m/z* 193.1 (MALDI MSI), tricosadiene ($[M+Li]^+$) ion at *m/z* 327.5 (MALDI MSI) and cVA ($[M+K]^+$) ion at *m/z* 349.4 (LDI MSI) and in addition on TGs ion at *m/z* 815.8 in different selected mass windows. Based on the output from in-house developed software was possible to eliminate the mass deviation effects as well as help with the detection of low-intensity mass signals.

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Neue Aspekte

Using in-house developed software the mass shift observing during LDI/MALDI MSI experiments of 3-D biological object (*D. melanogaster*) was corrected.

PIM 6

Detection of Individual Cells in Tissue using MALDI-TOF Imaging at 10 µm Pixel Size

Alice Ly[3], Michael Becker[1], Eckhard Belau[1], Jane-Marie Kowalski[2], Janine Rattke[1], Soeren-Oliver Deininger[1], Detlev Suckau[1], Axel Walch[3], Marius Ueffing[3]

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As MALDI Imaging instrumental parameters approach the low µm range, sample preparation becomes the resolution limiting experimental factor. Here, we combine sample preparation by matrix sublimation with optimized instrument settings to explore the limits of MALDI Image resolution. We have used biological model systems that have features on the 10 µm scale to evaluate these limits (rat cerebellum and porcine retina).

Experimenteller Teil

Ten µm sections of fresh-frozen tissue were mounted on conductive glass slides. After tissue desiccation, DHB matrix was sublimated using a home-built device following P. Chaurands protocol. MALDI images were acquired on a MALDI-TOF MS in reflector mode, at a laser focus diameter of 5µm. At 1 kHz laser frequency 100 shots were accumulated per pixel with a 10µm raster width. Post-acquisition H&E staining followed standard protocols; the resulting microscopic images were co-registered with the MALDI images.

Ergebnisse und Diskussion

In rat cerebellum are several prominent anatomical features such as the granular layer and molecular layer. Located between the granular and molecular layer are the bodies of purkinje neurons, these appear as individual cell bodies with a ~30 µm diameter. These cells were clearly identified in our datasets, e.g., by a lipid peak at m/z 878.9. Individual cells with 30 µm diameter were represented by 3x3 pixels, indicating that the true pixel size was indeed 10 µm.

Porcine retina displays several highly organized layers of cells at ~ 10 µm thickness. The absence of unique m/z signals prevented obtaining high resolution images based on single peaks. However, multivariate statistical analysis such as Hierarchical Clustering allowed resolving the layered structure of the retina based on peak populations rather than individual peaks.

Neue Aspekte

We explore the limits of MALDI Image resolution

PIM 7

MALDI Tissue Image Analysis of Rat Testis at 10 µm Pixel Size and 200 k mass

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Spatial and mass resolutions are both important parameters in MALDI imaging. We have used the sublimation method to obtain 10µm spatial resolution from tat testis sections providing a rich tissue morphology at mass resolution >200k using MALDI-FTICR.

Experimenteller Teil

Frozen rat testis sections were cut at 10 µm thickness using a cryo-microtome, transferred to ITO-coated glass slides and dried under vacuum. DHB matrix was sublimated in a home built device as described by Chaurand et al. 2011 MCP. MALDI images were acquired on a 7T-FTICR instrument with a 1 kHz smartbeam II laser and 10µm flat top focus. The analyzed mass range: m/z 500-900 comprised lipids, which were identified based on accurate mass by matching against Lipidmaps.org database.

Ergebnisse und Diskussion

Even anatomic features such as sub-structures inside the seminiferous tubules as small as 10µm were clearly resolved in rat testis sections. Notably, ion images at m/z 758.545 and m/z 788.607 show clear differentiation of the interstitial space from the smooth muscle layer lining the basal lamina of the tubules. The muscle layer (<10µm) and the interstitial space (<40µm) were clearly resolved, indicating that a real image resolution of 10 µm pixel size was achieved, without oversampling conditions. The mass resolution in the lipid mass range was 200k, providing for the unambiguous assignment, e.g., for a pair of ions with a mass distance of only 3mDa. The lipid at m/z 808.5819 was found in the interstitial space, m/z 808.5849 in the lumen of the seminiferous tubules. The high mass accuracy allowed a tentative assignment of the molecular formulas of the two substances and their identities as $[C44H84NNaO8P]^+$ as phospholipid PC36:2 and $[C46H83NO8P]^+$ PC38:5.

Neue Aspekte

MALDI imaging with high spatial and high mass resolution by MALDI FTICR

PIM 8

Development of a DESI MS-based method for pesticide screening

Stefanie Gerbig[1], Sabine Guenther[1], Gerold Stern[2], Rolf-Alexander Düring[1], Hubertus Brunn[2], Bernhard Spengler[1]

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Pesticides are widely applied all over the world to protect plants against insects, mold or fungi. Some of these pesticides are banned or restricted in industrialized countries, but are still extensively used in some non-european countries. The toxic and cancerogenic hazards of these pesticides require systematic control of the residues on food items, especially at the points of entry of crops, for example at large airports. These controls are carried out routinely in targeted and random sampling, but routine analysis methods like GC and HPLC-MS are time consuming. This problem has been addressed by utilizing DESI-MS which offers sensitive *in-situ* analysis with little sample preparation.

Experimenteller Teil

Analysis of twenty different pesticide standards as well as different fruit homogenates and extracts has been carried out using a custom-built DESI ion source that was mounted onto a motorized 2D stage. Motorized stage was controlled by Servo Design Kit hardware and software. Sample homogenates and extracts were prepared according to the QuEchERS protocol[1] and analyzed with no further sample treatment. Mass spectra were acquired using an Orbitrap Exactive mass spectrometer (Thermo Fisher Scientific Inc., Bremen, Germany). DESI Imaging experiments were carried out with a spatial resolution of 70 µm per pixel, data analysis of imaging experiments was performed with home-built Mirion software V2_441.

Ergebnisse und Diskussion

In collaboration with Landesbetrieb Hessisches Landeslabor (LHL) in Giessen, Germany, the development of a system for *on-site* detection of pesticide residues on imported food items of plant origin from developing countries is planned. To guarantee the feasibility of the project, a selection of pesticide standards were analyzed using DESI mass spectrometry and limits of detection (LOD) were determined. The LODs that were previously reported in the literature[2] were readily achieved. To test the setup under realistic conditions, authentic samples from Frankfurt airport were provided by the LHL. This sample set included the extracts of fruit and vegetable goods generated by QuEchERS sample preparation protocol. Furthermore, homogenates of the respective samples were provided and DESI-MS analyses were carried out. The results of these experiments showed that the presence of sample matrices has a strong influence on the ionization efficiency of the pesticide compounds, but we were nevertheless able to detect the majority of the substances detected by LHL by routine methods. Additionally, DESI imaging experiments on spiked eggplant samples were performed to determine whether or not pesticides that were applied to the surface intrude into the fruit. For this purpose, the pesticide was applied to the surface of the intact fruit and fresh frozen sections of 100 µm thickness were prepared. Preliminary data suggests that invasion does not take place, at least not in a limited amount of time. Parallel to the MS analysis using high resolution instruments, the detection of pesticide standards was also tested on a portable Mini 11 mass spectrometer purchased from Aston Labs of Purdue University.[3] Pesticide standards were easily detected with the portable instrument, enabling the planned on-site analysis of pesticides. Future experiments include the development of a semi-quantitative method for the surface analysis of fruit and vegetable items.

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Neue Aspekte

Collaboration with national institution to develop a screening method for pesticides in imported goods; imaging of pesticides in plant sections

PIM 9

Complementary MALDI MSI by inkjet printing

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Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) is a powerful tool in the rapidly expanding field of "-omics" providing information of molecular abundances and distributions on tissue sections.[1] As the applied matrix determines the nature of analytes detected, we present a method to correlate different fields of "-omics", *i.e*. lipidomics and proteomics, on a single tissue section.

Inkjet printing allows the precise deposition of picoliter volumes in defined patterns.[2,3] Therewith, different matrices are deposited on a tissue section in a pixelated, alternating way to result in a raster of dots for each matrix. MALDI MSI can measure each matrix grid separately and with the individually required measurement conditions to generate complementary imaging data from one single tissue section.

Experimenteller Teil

Tissue sections (12 µm) of different organs (liver, brain, kidney) of a C57BL/6J mouse or a rabbit were sliced in a microcryotome (Leica CM 1850). The application of various matrices (α-CHCA, sinapinic acid, DHB or ferrulic acid) was performed by inkjet-printing (microdrop Technologies GmbH). The complete tissue section was covered with matrix in an automated way by a predetermined offset dot grit. For the acquisition of the mass spectra either an Ultraflex III MALDI-TOF/TOF or an UltrafleXtreme TOF/TOF instrument (Bruker Daltonics) was used. The teaching of each matrix raster grid prior to the measurement was carried out using the spot microarray tool in FlexImaging (Bruker Daltonics).

Ergebnisse und Diskussion

The first example presents the combination of two complementary matrices DHB and ferulic acid on a rabbit kidney in a print distance of 800 µm for the analysis of proteins. The matrices were deposited in offset grids and measured under the required conditions including individual laser intensities, adapted laser focus and laser random walk settings. The overlay of protein ion images from the two matrices shows complementary information, which would not be obtained using only one matrix. The second example shows a mouse liver tissue, where α-CHCA was used for the analysis of lipids and sinapinic acid for the analysis of proteins in a print distance of 500 µm. As expected, the distribution of lipids as well as proteins was more or less homogeneously. To increase the print distance further and hence the spatial resolution an optimization of the print head parameters in the inkjet-printer was performed to obtain droplets as small as possible. The droplet ejection process performed with the novel triple pulse reduction allowed a decrease of the drop size in comparison to the single pulse of about 77% to a 35 pL volume droplet.[4] First experiments show that this method enables a printing distance of 150 µm.

The ability to print different matrices on a single tissue sections allows an extensive and tissue-saving analysis of rare and limited medical biopsies or pathological tissues by receiving multiple data from a unique section. No further biopsy-consumptive parallel sections are required that can show differences in morphology because of increasing cutting-depth. Furthermore, loss of signal intensity by matrix removal, washing steps or degradation among multiple consecutive preparations can be prevented.

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Neue Aspekte

Alternated matrix application by inkjet printing for the simultaneous analysis of MALDI MSI in different "omics" fields

PIM 10

Mass Spectrometric Imaging in Malaria Research

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After a first demonstration in 1994 (1), MALDI mass spectrometry imaging (MSI) became a valuable histological tool providing topological information on phospholipids (2), peptides (3), and drug compounds with high reliability, validity, and performance. Lipids are not only essential building blocks of cells but also play an important role in signal transduction, e.g. in malaria parasites, their mosquito vectors, and human hosts. The spatial distribution of lipids was imaged with high resolution from *Plasmodium berghei*-infected and non-infected mouse liver focusing on differential expression of lipids in control and infected animals in a rodent malaria study. Infection-specific lipid signals also have been imaged from pathogen-infected *Anopheles stephensi* mosquito sections in several technical replicates, being relevant also for rodent malaria marker studies

Experimenteller Teil

Normal and infected mouse liver sections of 10-20 µm thickness were prepared with a microcryotome. Mosquito samples were cut in 20 µm thick longitudinal sections, after carboxymethyl cellulose embedding with a microcryotome. A high-resolution atmospheric-pressure MALDI ion source (AP-SMALDI10, TransMIT GmbH, Giessen) was used for imaging (2). For positive ion measurements 2,5 dihydroxybenzoic acid (DHB) was homogeneously deposited by means of a dedicated high-resolution pneumatic sprayer. The source was coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific GmbH, Bremen), set to a mass resolving power between 50,000 and 140,000 at m/z = 200. Internal calibration was performed using a matrix ion signal as a lock mass, resulting in a mass accuracy of typically better than 3 ppm.

Ergebnisse und Diskussion

The software package 'MIRION' was used to generate mass images from raw files generated by the mass spectrometer. Matrix cluster peaks were used for internal calibration and ions formed by 30 laser pulses per spot were accumulated prior to detection. Mass spectra from 10-25 µm pixels were obtained with a mass accuracy of ≤ 3 ppm RMS. After measurement the matrix was removed from sections with 70% ethanol, and sections were H&E-stained to correlate the structure with m/z images both from mouse liver and *Anopheles* sections. Optical images of samples also were taken before the measurement with a light microscope.

Anatomical structures and localizations of different classes of phospholipid compounds were determined with high mass resolution and mass accuracy, classified as glycerophospholipids, glycerolipids, sphingolipids and sterols within the mass range of m/z 400-1000 in positive ion mode. Heme was detected in blood vessel regions of mouse liver with high mass accuracy (m/z = 616.16). MS imaging of heme in liver has a potential for new therapeutic interventions during liver stage malaria, as parasite development is highly dependent on heme biosynthesis (4). All Images were generated with a bin width of $\Delta m/z$ = 0.01. The identity of lipid molecules was confirmed by MS/MS analysis directly from tissue.

Differential distribution of phosphatidylcholines including PC(34:3), LPC(16:0), LPC(18:0) and LPC(20:4) has been observed around the infected hepatic portal triad surface of mouse liver. Lysophosphatidycholine activity has the ability to change the surface properties during infection, inflammation and injury (5). Also an unknown metabolite distribution was observed only in infected liver by MSI and validated by comparing to control mouse liver.From pathogen-infected *Anopheles stephensi* tissue sections, distribution and localization of PC(28:1), ceramide(34:1) and PC-P(38:3) were found specifically in the gut and head region of infected Anopheles from different measurements

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Neue Aspekte

High resolution MALDI imaging was employed to investgate infection mechanisms of malaria in mosquitos and mouse liver

PIM 11

Dithranol is an Efficient Matrix for MALDI-MS Imaging of Glyco- and Phospholipids with High Lateral Resolution

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Mass spectrometry imaging (MALDI-MSI) of bioactive lipids directly from tissue slices has recently found an enormous interest. Here, we demonstrate that dithranol constitutes a matrix that offers a particular high potential for the MSI analysis of phospho- and acidic glycolipids (including sulfatides and gangliosides). The matrix produces uniform micro-crystalline sample preparations with average crystal sizes in the low µm-range and high analyte ion yields in the negative ion mode. MS experiments characterizing the performance of the matrix for the detection of gangliosides and SEM images characterizing the tissue coverage are presented. Examples demonstrating the MALDI-MSI analysis of various tissue types, in particular mouse brain slices (C57BL/6), with lateral resolutions down to ~10 µm are moreover shown.

Experimenteller Teil

Dithranol [1] as well as GM1-containing ganglioside preperations (mouse brain, CBA/J) were dissolved in $CHCl_3$:MeOH (2:1 v/v). In particular, this solvent system ensures an efficient extraction of (glyco-) lipids from sprayed tissue (prepared to 10-20 µm thickness, C57BL/6). A semi-automated pneumatic spray system [2] was used to produce uniform matrix layers with crystal sizes of a few µm. A Synapt G2-S HDMS mass spectrometer (Waters) was employed for the MS experiments. The MALDI ion source of this instrument was modified for operation at elevated pressure of 0.7 mbar and for achieving an improved lateral resolution close to 10 µm (see contribution of Kettling et al. at this conference for details). All MS experiments were performed in the negative ion mode.

Ergebnisse und Diskussion

SEM and optical microscope images revealed that exceedingly uniform sample preparations with average crystal sizes in the low µm-range were obtained by spraying the dithranol matrix onto mouse brain tissue slices. Typically 10 bouts with 3 s lengths followed by 15 s drying intervals were employed at a sprayer-sample distance of 10 cm. Optimal ion yields were obtained at elevated ion source pressures (N_2) of 0.7 mbar, about a factor of 2-3 above the default settings of the G2-S. At this settings, the maximum signal intensities for the detection of GM1 gangliosides, as achieved from standard MALDI sample preparations at optimum laser fluence settings, surpass those obtained with the standard MALDI matrix 2,5-dihydroxybenzoic acid (DHB) threefold. The high extraction efficiency for plasma membrane lipids provided by the $CHCl_3$:MeOH solvent system, the formation of uniform microcrystalline matrix layers, and the high ion yields obtained upon desorption/ionization render the dithranol matrix particularly suitable for high-resolving MALDI-MSI of endogenous lipids in tissue slices. We demonstrate this feature with selected examples of molecular images that were obtained from different tissue types. A minimum lateral resolution of about 10 µm was achieved in the MSI-analysis of mouse brain tissue.

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Neue Aspekte

First report on using a dithranol matrix for the sensitive analysis of plasma membrane lipids with close-to-cellular lateral resolution.

PIM 12

Comparison of MALDI-TOF imaging and MALDI-FT-ICR imaging of formalin-fixed, paraffin embedded tissue samples

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MALDI-TOF and MALDI-FT-ICR instruments are readily available for mass spectrometric imaging application. While TOF analyzers have regularly been used for imaging of proteins and peptides, FT-ICR is often the primary choice of instrument for studies focusing on the analysis of lipids and metabolites.

FT-ICR instruments deliver superior mass resolution and mass accuracy, as well as a potentially higher sensitivity by accumulating ions from multiple laser shots in the ICR cell [1]. While FT-ICR mass analyzers technically outperform TOF analyzers in many aspects, the impact of the selected mass analyzer on the quality of imaging data remains to be examined. In this study, we compared two imaging datasets acquired from biological replicates either on an FT-ICR instrument, or a TOF instrument.

Experimenteller Teil

Two sections of a formalin-fixed, paraffin-embedded bladder cancer tissue microarray were prepared, containing a total of 851 samples. Antigen retrieval was performed and the tissue samples were tryptically digested and coated with CHCA matrix. Subsequently, one section was analyzed by MALDI-TOF imaging on a Bruker autoflex speed, the other one by MALDI-FT-ICR imaging on a Bruker solariX 9.4T, both equipped with a smartbeam MALDI laser. The number of distinct signals in all spectra, as well as the number of occurrences of each signal were used to compare the imaging data acquired on the TOF and the FT-ICR instrument.

Ergebnisse und Diskussion

In the FT spectra, we observed more than twice as high a density of signals in the peptide-relevant m/z range compared to TOF spectra. While this is not surprising due to the enhanced mass resolution, our data suggest that the selection of signals also differs between TOF and FT-ICR mass spectrometers, as about 25% of the signals detected by the TOF analyzer were not found in FT-ICR the dataset.

In mass spectrometric imaging, the m/z value is the only measure to distinguish analytes in the highly complex tissue sample. It therefore benefits from the superior mass resolution and mass accuracy of the FT-ICR mass analyzer. Besides the apparent advantages of FT-ICR, we found that both FT-ICR and TOF mass analyzers may reveal signals exclusively to either, and conclude that both methods can be used complementarily to get a deeper insight into the molecular constitution of tissue samples.

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Neue Aspekte

Comparison of TOF and FT-ICR imaging datasets on formalin-fixed, paraffin-embedded biological samples.

PIM 13

A spatially-aware highly-sensitive peak picking method for MALDI-imaging

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MALDI-imaging is a spatially-resolved mass spectrometric technique which can obtain spatial distribution of hundreds of molecules in a thin tissue section. Manual analysis is time-consuming since it requires going over all m/z-values, plotting their m/z-images, and examining them visually.

If there is prior knowledge available about the spatial structure of the sample, then a certain region of interest can be defined and correlated with the intensity patterns of the m/z-images [1, 2]. However, often there is no prior knowledge given. In this poster we introduce a novel method that automatically detects structured m/z-values without specifying a region of interest a priori and without manual visual examination [3]. We demonstrate it on 2D and 3D MALDI-imaging data sets.

Experimenteller Teil

The new approach automatically selects spatially structured m/z-images by ranking all m/z-images by their level of spatial structure. The ranking is based on the original measure of spatial chaos.

The calculation of the measure of chaos performs mainly in three steps. At the first step we use an edge detection filter to detect edges inside an image. This step results in a binary edge mask. At the second step, we build a one-nearest neighbor graph connecting all edge-pixels. Additionally, for each graph edge the distance between pixels connected is calculated. Finally, the mean length of the graph edges represents the measure of chaos. We show that unstructured m/z-images have a significantly higher mean edge lengths than structured m/z-images.

Ergebnisse und Diskussion

For evaluation of the proposed method we applied it to a MALDI-imaging data set of a 2D rat brain coronal section [1,3], and data sets of 3D mouse kidneys made of consecutive serial sections [4,5].

For the 2D rat brain data set we statistically evaluated the proposed measure of spatial chaos. First, we selected test sets of unstructured and structured m/z-images, defined by us after visual examination of all m/z-images. Then, for each m/z-image we calculated its value of the measure of spatial chaos. Based only on the measure of spatial chaos, for these test sets we classified structured versus unstructured images and determined the accuracy, sensitivity, and specificity of the classification. We were able to automatically reproduce the expert judges (structured versus unstructured).

The state-of-the-art protocol for 3D MALDI-imaging includes serial sectioning of a sample, measuring each section using 2D MALDI-imaging, and merging individual 2D data sets corresponding to serial sections into one 3D data set. We applied the proposed measure of spatial chaos to 3D data sets of mouse kidney specimens. For these data sets the proposed algorithm produces a three-dimensional neighbor graph. We show that the algorithm can be extended to 3D and automatically discovers m/z-values corresponding to structured images if the quality of the consecutive 2D MALDI-imaging datasets is reproducibly good.

Our motivation to develop this approach was to be able to detect unknown molecules. Selecting structured m/z-images corresponding to molecules after visual examination of m/z-images is the well-accepted approach of manual analysis and is a part of everyday work. Our parameter-free and unsupervised method supports the imaging mass spectrometrist at this task. The method also complements spectrum-wise peak picking increasing its sensitivity, as it does not depend on peak intensity, but only on the measure of spatial chaos of the corresponding m/z-image.

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Neue Aspekte

The proposed measure of spatial chaos automatically and unsupervisedly detects structured m/z-images and thus complements spectrum-wise peak picking.

PIM 14

MALDI MS von Polymeren unter Verwendung der Dried-Droplet Probenpräparation und ionischer Matrices

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Bei der Dried-Droplet Probenpräparation werden der Analyt und die Matrix in einem geeigneten Lösemittel gelöst und einige Hundert nL der Lösung auf das Probentarget aufgetropft. Nach Verdampfen des Lösemittels ist anstatt einer homogenen Fläche oft eine Ringbildung beobachtbar. Diese hat einen großen Einfluss auf die Qualität und Reproduzierbarkeit der Spektren. Ungeachtet des Nachteiles der lateralen Reproduzierbarkeit ist die Dried-Droplet Probenpräparation für MALDI-MS auf Grund der einfachen Handhabung weit verbreitet. Durch die Verwendung einer ionischen Matrix kann eine Verbesserung der Oberflächenhomogenität bei nahezu identischer Probenpräparation erreicht werden.

Experimenteller Teil

Zur Synthese der ionischen Matrizen werden klassische Matrixsubstanzen (z.B.: Dihydroxybenzoesäure, DHB; Sinapinsäure, SA) verwendet und mit einer equimolaren Menge einer organischen Base (z.B.: n-Butylamin) in einem Lösemittel (z.B.: Methanol) gemischt [1]. Die Bildung der ionischen Flüssigkeiten wurde mit ^{1}H-NMR Spektroskopie überprüft.

Die Dried-Droplet Probenpräparation erfolgte durch Mischen der ionischen Matrices mit den gelösten Polymeren (z.B.: PEG, PMMA) und auftragen eines Tropfens dieser Lösung. Alternativ wurde auch die sogenannte Sandwich Methode angewandt. Dabei wird ein Tropfen der Polymerlösung auf das Target aufgetragen. Nach Verdampfen des Lösemittels erfolgt die Auftragung der (ionischen) Matrix auf den eingetrockneten Spot. Die Homogenität der Probenspots wurde MALDI-MS Imaging (MSI) analysiert.

Ergebnisse und Diskussion

Bei Verwendung einer ionischen Matrix zeigen die Messergebnisse eine erhebliche Verbesserung der Homogenität des Probenspots. Der typische laterale Intensitätsabfall, welcher bei einer Ringbildung auftritt, konnte stark vermindert bzw. verhindert werden. Die Probenspots konnten auch mehrfach gemessen werden, ohne eine sichtbare Änderung der Massenspektren feststellen zu können.

Neben der einfachen Synthese zeichnen sich diese Verbindungen durch eine hohe Vakuumstabilität aus. Selbst nach 24h Aufenthalt in der Quelle des MALDI MS wurden reproduzierbare Spektren gemessen. Die Intensität der Spektren ist dabei deutlich höher als bei der Verwendung konventioneller Matrices. Auf Grund der niedrigeren Laserenergie die für eine Messung benötigt wird, nimmt auch die Polymerfragmentierung ab. Ein weiterer Vorteil der ionischen Matrices besteht in ihrer Unempfindlichkeit gegenüber der verwendeten Scanmethode (z.B.: „Zeile-für-Zeile“). Diese zeigte keinen Einfluss auf die aufgenommenen Spektren bzw. das Image. Speziell die Methode bei der im ersten Durchgang nur jeder zweite Messpunkt in jeder zweiten Zeile gemessen wird und die verbleibenden Spots in einem zweiten Durchgang gemessen werden, ist für eine klassische Matrix ungeeignet. Durch den erhöhten Einfluss des Lasers auf die Messspots des zweiten Durchgangs weisen diese eine erheblich niedrigere Intensität auf. Da ionische Matrices diesen Effekt nicht aufweisen, kann man auf eine Regeneration der Probenspots, trotz der hohen Viskosität, schließen. [2]

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Neue Aspekte

Durch die Verbesserung der Reproduzierbarkeit bei gleicher Probenpräparation sind ionische Matrices eine ausgezeichnete Alternative für die Analyse von Polymeren.

PIM 15

Elemental Bioimaging of Nanosilver-Coated Prostheses in Bone Tissue by Laser Ablation-Inductively Coupled Plasma-Mass Spectrometry (LA-ICP-MS)

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Silver is known to exhibit bactericidal properties. Therefore, elemental silver is integrated into surgical prostheses. While the release of ions is assumed to reduce infections, the behavior and fate of silver in living organisms is not fully understood. To find out about possible long-term effects of the respective prostheses the development of analytical methods for silver is required. Elemental bioimaging by means of laser ablation coupled to inductively coupled plasma mass spectrometry (LA-ICP-MS) is a capable method for the investigation of metals in biological samples. Its high sensitivity and good detection limits permit the determination of elements in low concentration ranges.

Experimenteller Teil

Nanosilver-coated prostheses from a medical study involving dogs were investigated. Bone sections were investigated using a laser ablation system coupled to an inductively coupled plasma quadrupole mass spectrometer. The ablation system was equipped with a pulsed Nd:YAG laser operating at 213 nm. The laser was focused on the sample via a CCD camera viewing system. Optimization procedure for the laser ablation method was performed regarding spot size, laser energy, scanning speed as well as carrier gas flow, until the best balance between spatial resolution, amount of ablated material, signal intensity and washout behavior was achieved. The thin section layers were ablated in line scans (0 µm gap), which were directed from the hard cortical bone to the implant material.

Ergebnisse und Diskussion

Explanted tissue sections containing the medical device as well as surrounding tissue were analyzed using LA-ICP-MS. Method development was challenging considering the inhomogeneity of the examined samples including metal, hard and soft tissue or embedding media with their different physical properties. With this approach, visualization of the surface was achieved and the distribution of different metals was investigated.

Using LA-ICP-MS, the required sensitivity is available for simultaneous imaging of trace elements such as silver or zirconium in excellent resolution and without interferences. The visualization of all major implant constituents was shown and metal distribution from the implant surface into the soft tissue was observed for silver, zirconium, titanium, vanadium and aluminum. Furthermore, the detection of zirconium revealed remaining blasting abrasive particles from preoperative surface treatment. As a complementary analytical technique, µ-X-Ray fluorescence spectroscopy (µ-XRF) was employed for validation of the data. Phosphorous and sulfur were determined as well. It was demonstrated that combining the benefits of two powerful elemental imaging techniques allows to obtain complementary information, which is useful for the investigation of biological samples consisting of a challenging matrix. Non-destructive µ-XRF provided highly resolved images for Ca, P and S in fast analysis times, whereas the correlation of phosphorus and calcium was attributed to crystallized bone.

Neue Aspekte

LA-ICP-MS proves the distribution of metals from bone implant materials into surrounding tissues.

PIM 16

Studying Nephrogenic Systemic Fibrosis by Laser Ablation-Inductively Coupled Plasma-Mass Spectrometry

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The disease *nephrogenic systemic fibrosis* (NSF) was first described in 2000 [1] and is only diagnosed in patients with impaired renal function. The development was linked to the administration of gadolinium based contrast agents (GBCA) during magnetic resonance imaging (MRI) six years later. [2] Because free Gd^{3+} is toxic, it is chelated by polyaminocarboxylic acids and therefore hindered to be released during MRI. Nevertheless, release of Gd^{3+} induced by transmetallation, especially by free iron, is possible. A subsequent precipitation as insoluble deposits like $GdPO_4$ is likely at that point. Only little information on the fate of GBCA and potential risk of free Gd^{3+} in NSF patients is available until now, so that further investigations are required.

Experimenteller Teil

In the first step, the total gadolinium concentration in a tissue section of a NSF patient was determined by means of inductively coupled plasma-mass spectrometry (ICP-MS) after microwave digestion. Subsequently, the distribution of gadolinium in 5 µm thin tissue sections was carried out utilizing laser ablation (LA) coupled to ICP-MS. The use of LA with ICP-MS combines spatial resolution with a sensitive and selective detection method. Besides gadolinium, calcium and phosphorous were detected simultaneously in order to visualize the distribution in the tissue. Quantification of gadolinium by LA-ICP-MS was performed using of matrix matched standards for calibration.

Ergebnisse und Diskussion

Tissue biopsy specimen of a NSF patient was first analyzed by means of ICP-MS in order to determine the total gadolinium concentration. After a microwave digestion, concentrations in the lower ppm range of gadolinium were recognized. To gain further information on the fate of gadolinium in the skin of the patient, 5 µm thin sections were analyzed using LA coupled to ICP-MS. Phosphorous and calcium were monitored simultaneously with gadolinium in order to visualize the structure of the tissue. Additionally, the presumed correlation of these two elements with the gadolinium distribution was proven. Received data was processed by a home-made software resulting in elemental distribution maps of all three elements in the ablated area, which correlate with microscopic images taken prior analysis.

Furthermore, quantification of gadolinium in the skin sections was performed. Matrix matched standards spiked with gadolinium in concentration range of 0.01 µg g^{-1} to 200 µg g^{-1} were made of gelantin and ablated directly prior the analysis of the deparaffinized tissue section utilizing equal laser ablation and mass spectrometric parameters. The received calibration function exhibited a good linearity in the chosen concentration range, whereas the RSD of each standard ranged from 3.8% to 10.8%. Furthermore, the limit of detection (LOD) was determined with a value of 1.7 µg kg^{-1} and the limit of quantification (LOQ) was calculated with 5.8 µg kg^{-1} using the 3σ and 10σ criterion. Based on these findings, gadolinium concentrations in the investigated samples ranging from 55 ppm to 400 ppm were determined.

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Neue Aspekte

LA-ICP-MS was applied for determination of gadolinium distribution and quantification in a tissue section of a NSF patient.

PIM 17

AP-SMALDI mass spectrometry imaging of crop plants, natural products and insect metabolites

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Chemical imaging by mass spectrometry has gained significant attention due to its ability to map complex distributions of molecules in biological systems. The technique so far mainly focused on clinical investigations. Herein, we present recent advances in sample preparation, high resolution in mass and space (HR^2), high mass accuracy (< 3 ppm mass error) and tandem Mass Spectrometry Imaging (MS^2I) for different types of tissue sections from fungus, plant and insects. The visualization of metabolites with high spatial resolution helped to understand complex biological processes.

Experimenteller Teil

Tissue sections from plants and insects were obtained using a cryotome after optimizing various preparation techniques, such as carboxymethyl cellulose (CMC) and tragacanth gum embedding. 2,5-dihydroxybenzoic acid (DHB), 30 mg/mL in acetone:water (50:50), 0.1 % trifluoroacetic acid, was applied as a matrix with a high-resolution matrix-preparation robot (SMALDIPrep, TransMIT GmbH, Giessen, Germany). An atmospheric-pressure high-resolution MALDI imaging source (AP-SMALDI10, TransMIT GmbH, Giessen, Germany) was used for ion imaging. The SMALDI source was operated with a nitrogen laser at 337 nm wavelength with 60 Hz repetition rate. The source was coupled to an orbital trapping mass spectrometer (Exactive or Q Exactive Orbitrap, Thermo Fisher Scientific GmbH, Bremen) set to a mass resolving power of 50,000 to 140,000 at m/z = 200.

Ergebnisse und Diskussion

Images were generated using the software package 'Mirion'. All measurements including mass spectra from 5 µm pixels were acquired with a mass accuracy of better than 3 ppm (root mean square of mass error). Molecular images were generated with a bin width of $\Delta m/z$ = ±5 ppm. Images from seeds (oilseed rape and wheat), roots (rice and *Paeonia*), rhizome of licorice and insects (*Paederus* and *Harmonia*) were generated at 5 to 25 µm spatial resolution. Various metabolites like amino acids, carbohydrates, glucosinolates, phonolic compounds, flavonoids, lipids and glycosides were identified and visualized at 10 µm pixel size from germinating rapeseed in a single experiment. Highly resolved MS images revealed different anatomical structures of the germinating oilseed rape like the seed coat, endosperm and embryo. An experiment with 5 µm pixel size was performed on wheat seed. Different regions of the wheat seed were specific for certain compounds, like a polyphenol glycoside located in the radicle, coleoptile and plumule of germ; phosphatidylcholine in the scutellum; and a phosphatidylglycerol specific for the pericarp. In the case of licorice, the free flavonoids like glabrene were specifically located in the cork layer of the rhizome and the flavonoid glycosides like isoviolanthin were detected with high abundances in the internal part such as medulla, xylem and phloem rays, next to the cork layer of the rhizome. Whole insect imaging of *Paederus riparius* was performed at 20 µm spatial resolution and the defensive compound pederin was observed with high mass accuracy, as well as its metabolite pseudopederin. These molecules were prominent in the fourth abdominal segment, where the reservoir of the defensive gland is located. Additionally mass signals specific for different organs like brain, nerve cord, egg, ovary, gut etc. were identified and visualized.

Neue Aspekte

Sample preparation for plant and insect tissue sections. High resolution in mass and space method for understanding metabolite distributions.

PIM 18

Controlling the Structure of Peptides and Proteins on Solid Surfaces with Electrospray Ion Beam Deposition in Ultrahigh Vacuum

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Biological systems generate functional nanoscale structure through hierarchical self-assembly, for which protein folding is the most prominent example. Mimicking this complex yet robust process outside of biological systems may allow observing and influencing the folding process in great detail. This would be highly beneficial for the fundamental understanding of the structure formation through folding and could lead to a novel approach to the bottom-up fabrication of nanostructures.

Experimenteller Teil

We developed an electrospray ion beam deposition (ES-IBD) system capable of depositing nonvolatile molecules including proteins and polypeptides on surfaces in ultrahigh vacuum (UHV).[1] In this environment we study their structure and electronic properties with resolution at the atomic level using in-situ scanning tunneling microscopy (STM) [2], while chemical information is provided by a secondary mass-spectrometry measurement [3].

Ergebnisse und Diskussion

We show that the structure formation of polypeptides adsorbed on a surface exhibits similarities to protein folding in solution and can, moreover, be controlled on several length scales, using features unique to ES-IBD. Bradykinin, a short peptide of 9 amino acids (AA), folds into monomer and dimer equilibrium structures that, on the next level of hierarchy, arrange themselves into ribbons.

Long polypeptide chains, like the unfolded protein cytochrome-c (104 AA, 12 kDa), do not reach thermal equilibrium, but instated fold randomly, perfectly following polymer statistics in two dimensions (2d).[4] In this case, ES-IBD allows us to select the mechanical properties of the gas phase ion before deposition and steer the structure on the surface between extended chains for high charge states and refolded 2d-random coils for low charge states.

Our results show that ES-IBD combined with high resolution UHV-STM, opens the door to conformational control of 2d polypeptides and its application to rationally folded functional nanostructures at surfaces.

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Neue Aspekte

We quantitatively measure the ion transmission and identify the mechanisms with help of a fluid dynamics and ion motion simulations.

PG: Grundlagen der MS

PG 1

Efficient gas phase dehydrogenation of MALDI-derived peptide ions by nitroarene matrices or -additives

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Previously, we showed [1] that the cinnamic acid derivative α-cyano-3-hydroxy-4-nitrocinnamic acid when used as MALDI matrix has a high efficiency to dehydrogenate, fragment and/or oxidize analyte molecules from numerous substance classes such as (phospho)lipids, peptides, polymers and carbohydrates in the gas phase. We tentatively assumed that intermediate matrix radicals are involved which abstract analyte hydrogen radicals. In this contribution, the analyte dehydrogenation reaction is elucidated using high level Hartree-Fock quantum calculations of involved matrix and analyte intermediates and by a series of MALDI-MS and -MS/MS experiments. Further nitroarenes are introduced that partially show extremely high efficiencies for dehydrogenation of analytes in the gas phase. Possible application fields for use of these new MALDI compounds will be discussed.

Experimenteller Teil

The nitrated α-cyanocinnamic acid (CCA) derivatives 4-NO_2CCA, 3-OH-4-NO_2CCA, 3-OH-5-NO_2CCA, 2,4-DiNO_2CCA as well as the 4-NO_2CCA methyl and ethyl esters were synthesized by a Knoevenagel condensation reaction according to [2].

Picric acid and trinitrobenzene solutions as well as substance P and several dipeptides were purchased from Sigma-Aldrich.

Ab-initio calculations were performed with the software Gaussian 09 after initial structure building using Chem 3D Ultra 10.0. Geometry optimization with subsequent frequency, zero-point and vibrational energy, entropy and spin density calculations were performed using the Møller-Plesset perturbation theory at the MP2(Full)/6-311+G(2d,2p) level of theory. MALDI MS and -MS^2 experiments with selected analyte compounds were performed with a hybrid oTOF mass spectrometer (QStar Pulsar i, AB SCIEX) equipped with a pulsed N_2-laser (337nm).

Ergebnisse und Diskussion

The dehydrogenation efficiency of the investigated nitrated CCA derivatives was studied using several peptides as analytes. Moreover, the effect of adding multiply nitrated additives such as picric acid or trinitrobenzene to conventional MALDI matrices was investigated. Together, these results demonstrate that the abstraction efficiency of hydrogen atoms from X-H bonds of analytes depends only on the number of matrix/additive NO_2-groups. In contrast to initial assumptions, the presence of additional ortho-substituted hydrogen-donors is not necessary [1]. As reactive species that is initiating the dehydrogenation reaction the radical cationic nitro compounds were identified. Calculation of the energy levels of the involved matrix and analyte species as well as the assumed intermediates clearly supports the assumed underlying reaction pathway. This comprises a strongly exergonic analyte hydrogen abstraction to one of the two nitro-oxygens. Subsequently, a second hydrogen transfer of only low exergonicity with generation of oxidized analyte species and doubly hydrogenated nitro-radicals is assumed to occur. To some extent this species can undergo a subsequent loss of water upon intramolecular rearrangement.

Analyte species whose two X-H bonds to be dehydrogenated are connected by a vinyl group exhibit strongly increased dehydrogenation efficiencies compared to those comprising directly neighbored X-H bonds. This preference is probably caused by an optimized distance between analyte hydrogens and matrix/analyte nitro oxygens.

As possible areas of applications for in-situ analyte dehydrogenation, MS^2 differentiation between Leu/Ile by means of different ammonia loss efficiencies of the corresponding Leu/Ile immonium ions will be presented.

In addition, we demonstrate the generation of v-ions at low energy CID conditions. This reaction becomes possible by intramolecular proton shifts upon amino acid side chain dehydrogenation and is specific for certain amino acids. Particular high dehydrogenation efficiencies are found for tryptophan which can potentially allow a rapid identification of the presence of Trp within the AA chain.

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Neue Aspekte

Nitrated MALDI matrices and -sample additives result in efficient gas phase peptide dehydrogenation: pathways are elucidated and possible applications discussed.

PG 2

1,8-Di(piperdinyl)-naphthalene as a novel matrice for low-mass MALDI-MS

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Requiring relatively little sample preparation and being able to analyzed lot of samples in a single run, matrix assisted laser desorption/ionisation mass spectrometry (MALDI-MS) has proven to be a powerful analyzing technique. However the conventional matrices used for MALDI-MS generate multiple interfering signals below 600 *m/z* and thus restricting this technique predominantly to the analysis of larger molecules like peptides, proteins, oligo-and poly saccharides as well as other natural or synthetic polymers. To extend the scope of possible analytes to compounds in the low mass region, new MALDI-matrices are required which do not show signals below 1000 *m/z*.

Experimenteller Teil

New superbasic MALDI-matrices were developed and synthesized in our lab based on the structure of 1,8-bis(dimethylamino)-naphthalen (DMAN) [1] an excellent matrix for neg. ion mode MALDI-MS. These were then screened for their potential as MALDI-matrices with compounds and metabolites of varying degree of acidity e.g. fatty acids, citric acid and trifluoroacetic acid as well as complex mixtures like fruit juice.

Ergebnisse und Diskussion

Because of the superbasic nature of DMAN, the ionization of the analytes takes place during crystal formation on the MALDI-plate prior to desorption. This occurs via proton exchange from the analytes to the superbasic matrix as a result of a simple acid/base reaction between analyte and matrix. Therefore an optimal intensity of analyte signals can only be achieved when an approximate 1:1 molar ratio of analyte and DMAN is used.[2]

Switching to the superbasic 1,8-di(piperdinyl)-naphthalene as a matrix in neg. ion mode MALDI not only results in an improved sensitivity, but in contrast to DMAN the matrix can be used in excess, an equimolar ratio of analyte and matrix is not required anymore to obtain the best signal. This allows for complex mixtures of analytes in a wide range of concentrations to be detected on one spot, without sacrificing signal intensity. The range of detectable analytes was extended and now includes weaker acidic compounds like amino acids and nucleotides.

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Neue Aspekte

New matrix for negative ion mode MALDI-MS suitable for the detection of small molecules with the potential for metabolomic studies.

PG 3

UV-MALDI-MS Analysis of Non-Covalent Peptide-Peptide Complexes with a 6-Aza-2-thiothymine-matrix: Effect of Wavelength and Fluence on the Detection of the Complex

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The analysis of non-covalent complexes by MALDI-MS poses a challenge. Factors that all can result in their unintended dissociation are the acidic character of most MALDI matrices, the requirement of analyte-matrix co-crystallization, and the thermal load during the overall MALDI-MS process. Best results are frequently obtained with the "pH-neutral" 6-aza-2-thiothymine (ATT) matrix [1,2]. Interestingly, this compound exhibits a peak absorption close to 260 nm, far off the standard MALDI wavelengths of 337 and 355 nm. Here, we investigated the influence of the laser wavelength in the range of 220-400 nm and that of the laser fluence on the ion generation. The data reveal distinct regions in which maximum intensities of the peptide-peptide complexes are obtained.

Experimenteller Teil

Human gastrin I (Pyr-GPWLEEEEEAYGW) and either kemptide (LRRASLG) or a protein kinase C substrate (VRKRTLRRL) were used as receptor-ligand systems [1]. Samples were prepared from aqueous solutions using the dried droplet method to produce molar analyte-to-matrix ratios of ~1/350. MS experiments were performed with an oTOF-mass spectrometer [3,4] that is equipped with an optical parametric oscillator laser (OPO; versaScan, GWU-Lasertechnik) providing tunable laser light with 5 ns pulse duration. The elliptical laser spot size on the target was ~300x600 µm². To record each mass spectrum 600 laser shots were applied. Ion signals were recorded for excitation wavelengths between 220-400 nm (step size: 10 nm) and by varying the laser fluence between ion detection threshold and approximately twice this value.

Ergebnisse und Diskussion

In agreement with our previous studies [3,5], the wavelength course of the threshold fluences for the total ion generation (including analyte- and matrix-derived molecular and fragment ions) follows the inverse of the optical absorption curve. Consequently, the lowest threshold fluences and presumably highest overall ion yields [5] were found close to the optical peak absorption. A different picture is obtained for the specific Gastrin I-Kemptid and Gastrin I-PKCS complexes. For these aggregates, optimal ion yields (relative to the TIC or the monomers) are obtained in narrow wavelength-fluence bands that are found along the declining slope of the absorption curves of the ATT matrix tailing towards longer wavelengths. The major reason for this finding is probably the reduced thermal energy content in the MALDI plume at these red-shifted (relative to the absorption peak) excitation conditions [5]. In addition, it is conceivable that the ejection of a larger matrix volume at wavelengths of reduced absorptivity could also support the survival of the weakly-bound aggregates. In total, the data provide important hints on how MALDI mass spectrometry of non-covalent complexes can be optimized.

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Neue Aspekte

First study investigating the wavelength-fluence dependence of MALDI-MS with an ATT matrix for detection of specific non-covalent peptide-peptide complexes

PG 4

Formation of multiply charged ions in MALDI mass spectrometry

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For more than 25 years MALDI was the method best known for simple spectra of singly charged analyte ions, bringing along advantages especially for complex samples and imaging mass spectrometry of biological tissue. Surely, drawbacks like less attractive fragmentation conditions or the need of mass analyzers with wide mass ranges appeared, but were compensated mostly by spectral simplicity. Recent work of several groups, however, focuses on enhancement of multiple charging (e.g. laser spray ionization or advantageous sample preparation with liquid components)[1],[2], with the goal of analyzing larger proteins with mass spectrometers of limited mass range. Our attempt was to reproduce these successful experiments and enhance and confirm parametric influences by improved sample preparation and statistical clearness.

Experimenteller Teil

For our experiments, a sample preparation as used for high resolution mass spectrometry imaging was employed, forming small crystal sizes and uniform coverage. This should improve sample surface homogeneity, and uniform coverage and small crystal sizes would result in a high shot-to-shot-reproducibility, allowing for data analysis with statistical methods.

For mass spectrometry a high-resolution MALDI imaging ion source (TransMIT GmbH, Giessen) was used on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific GmbH, Bremen).

Investigated parameters were laser fluence, voltage and temperature, as derived from publications on this field.[3] For each parameter combination a line of 100 spectra was measured. Different matrices were used to compare results under various conditions.

Ergebnisse und Diskussion

As described before,[3] higher temperatures of the transfer capillary were found to be beneficial for higher abundances of multiply charged ions, but only in combination with voltage shutdown (so-called laserspray ionization, LSI). This parameter combination, however, is problematic for certain instrumental situations. As shown by Cramer et al., multiply charged ions are indeed detectable when voltage is applied, if a liquid matrix is used for sample preparation. Our experiments with liquid matrices showed that voltage shutdown is even disadvantageous for signal intensity of multiply charged ions. Acceleration voltage thus is not an essential parameter for obtaining higher charge states.

Furthermore, we investigated the influence of capillary temperature on adduct formation. We found that singly charged protonated ions are not affected strongly by temperature increase (as published by Cramer et al. for liquid matrices), while the abundance of sodium adduct ions increases with temperature. We assume the cationization mechanisms being affected in a similar way as in the case of high laser irradiances.[4]

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Neue Aspekte

We provide a statistical analysis on multiple charging in MALDI. Adduct formation may be a result of cluster formation.

PG 5

Comparison of Peptide Fragmentation Pathways under ToF-SIMS, ME-SIMS, and ESI Low-energy CID Tandem MS Excitation Conditions

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ToF-SIMS and MALDI-MS are increasingly applied in parallel for molecular imaging of biological tissue. ToF-SIMS provides a sub-µm resolution but is generally accompanied by a more extensive fragmentation of larger biomolecules. In contrast, predominantly intact molecular ions are generated by MALDI-MS, however at a lower lateral resolution (typically >10 µm). In order to efficiently combine these two complementary MS imaging techniques a better understanding of the involved fragmentation pathways is required. Here, we characterized the type of peptide fragments that are generated with ToF-SIMS, matrix-enhanced (ME-)SIMS, and MALDI-MS. Comparison of the dissociation products to those generated by ESI low-energy CID MS/MS allowed differentiation of the occurrence of "thermal" versus rapid, high-energetic dissociation mechanisms to some extent.

Experimenteller Teil

Bradykinin (MW 1060.21 g/mol) was employed as peptide standard and 2,5-dihydroxybenzoic acid (DHB) and a-cyano-4-hydroxycinnamic acid (HCCA) as matrices for MALDI-MS and ME-SIMS. For ToF-SIMS, aqueous analyte samples were spin-coated on silicon wafer targets, for ME-SIMS analyte-matrix samples (molar ratio: 10^{-4}) were prepared by pneumatically spraying the solutions. For MALDI-MS samples were prepared by the dried droplet method. SIMS experiments were performed with a modified TOF-SIMS Instrument using 30 keV Bi^+, Bi_3^+, Bi_5^+, and Bi_7^7 as primary ions. Two QStar–type o-TOF mass spectrometers (AB Sciex) were employed for MALDI-MS and ESI tandem MS experiments, respectively. Ar was used as collision gas and CID energies (E_{lab}) between 20-40 eV were applied. Theoretical bradykinin fragments were calculated using ProteinProspecor website (http://prospector.ucsf.edu/prospector/mshome.htm).

Ergebnisse und Diskussion

Exclusively „thermal" peptide fragments are produced under ESI low-energy CID conditions. Therefore, the ESI tandem MS spectra of bradykinin reflect the well-known series of y-, a-, and b-type ions and this ion series can serve as a reference for a thermally-induced (equilibrium) dissociation process. Mostly, thermal peptide fragment ions are also generated within the MALDI-process [1]; these ions are often referred to as "post-source decay (PSD)" products. In addition, a smaller fraction of the fragment species, resulting from fast chemically-induced "in-source decay (ISD)" processes, is detected in the MALDI-MSI spectra acquired with ISD-active DHB matrix [1].

In contrast, next to the molecular $[M+H]^+$ ion a mixture of thermal and higher-energetic fragment ions of bradykinin (e.g., c-,x-,v-,w-species) is generated by both ToF-SIMS and ME-SIMS. In addition, a series of internal fragments, that are containing a variable number of amino acid (AA) residues, is found in the mass spectra. Similar peptide fragment series are produced by ToF-SIMS and ME-SIMS and also using either of the two matrices (DHB/HCCA). As expected, the softest desorption/ionization conditions are obtained for sputtering with the largest Bi_5^+- and Bi_7^+-cluster ions [2], as indicated by an increased detection of larger fragment species and increased yield for the molecular ion.

Because of the absence of any matrix-derived background ions, fragment identification is facilitated for the ToF-SIMS analysis from neat peptide. However, all mass spectra readily allow complete identification of the peptide sequence. In contrast to the ESI tandem MS analysis, based on the detected side chain losses and cleavages giving rise to v-, and w-type fragments, respectively, even the extended annotation of several side chains of the AAs becomes possible for the SIMS-, and to a lower extent for the MALDI-MS analyses. This feature can allow the identification of isobaric Leu/Ile residues [3].

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Neue Aspekte

First study in which the peptide fragmentation pathways in ToF-SIMS/ME-SIMS and in MALDI-MS/ESI-CID-MS/MS are compared

PG 6

Discovery of multiple sites of intra-molecular protonation and different fragmentation patterns within the fluoroquinolones using ion mobility mass spectrometry

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In this study high definition mass spectrometry (HDMS) is explored as a tool for method development to support the unequivocal identification of fluoroquinolones (FQ) residues in complex matrices. HDMS uses a combination of high resolution MS and high efficiency ion mobility based measurements and separations. Compounds can be differentiated based on size, shape and charge. In addition both precursor ion and fragment ion information can be acquired in a single injection in an HDMS experiment ($HDMS^E$). Ion mobility differentiates molecules as they tumble through a buffer gas and their progress is related to the average rotational collision cross section (CCS). Ion mobility drift time can be used to determine the CCS of a molecule.

Experimenteller Teil

The extracts of blank and fortified porcine muscle tissue were kindly provided by RnAssays. The mass spectra were acquired on a Waters Synapt® G2-S HMDS mass spectrometer in ESI+ ionization mode after separation with a linear gradient of water/acetonitrile containing 0.1% formic acid on an ACQUITY BEH C18, 1.7 µm, 2.1 x 50 mm column at 45 °C using a Waters ACQUITY UPLC® system. Data were acquired using MassLynx and processed using UNIFI v 1.6.5 Research Edition software.

Ergebnisse und Diskussion

By using the described gradient 25 antibiotics could be separated in a 10 minutes UPLC run. The 9 FQ compounds were eluting between 2.0 and 2.6 min. If the data is reviewed using the component drift plot summary 18 mobility resolved FQ species are observed. Each FQ compound was found to be comprised a minimum of two protomers, i.e. protonation occurring at either the acidic or basic region of the molecule. For ciprofloxacin protomers estimated CCS values of 108.7 Å² and 119.1 Å² were determined. The two mobility resolved protomers are showing different fragmentation behavior. The fragments at m/z 288 and 245 are believed to arise from a species were ionization occurs on the basic group. Protonation occurring on the acid group results in the formation of fragment ions at m/z 314 and 231.

A series of porcine extracts have been screened for the presence of fluoroquinolones with and without the application of ion mobility spectral clean-up. Two protomers of danofloxacin could be identified. In this example, ion mobility has been used to resolve the matrix derived interferences from the identified component of interest, danofloxacin. For both protomers the following performance was observed: mass accuracy < 1ppm and %CCS is within 2% of the expected values. The data confirms that further consideration should be given to method development since the ratio and formation of the protomers was seen to vary with eluent flow rate, capillary voltage, cone voltage and extract composition. These $HDMS^E$ observations have the potential to explain the differences sometimes observed in inter-laboratory studies where participants report results obtained from monitoring specific MRM transitions.

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Neue Aspekte

With ion mobility the separation of protomers forming different fragmentation patterns could be achieved.

PG 7

Protein Microsolvation: Identification of Crown Ether Binding Sites on Ubiquitin Mutants

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There is ongoing debate about the extent to which protein structures are retained after electrospray ionization and transfer into the gas phase. In this context, it is widely accepted that the repulsion between equal charges is one of the major determinants: low charge states typically adopt compact, native-like conformations, while high charge states tend to unfold.[1] Recently, we showed that not only Coulomb repulsion but also the much more subtle coordination of charged side chains onto the protein backbone can severely influence the conformation of gas-phase proteins.[2] Here, we are investigating these effects further by comparing the impact of solvent-exposed residues with those involved in intramolecular salt bridges.

Experimenteller Teil

In order to study the influence of side chain-backbone coordination on the structure of a gas-phase protein, we non-covalently attached different amounts of crown ether (CE, 18-crown-6) to the charged lysine side chains of a series of ubiquitin mutants (Sigma Aldrich). The resulting protein-CE complexes were analyzed via ion mobility spectrometry (IMS) using a commercially available traveling wave IMS apparatus (Waters, Synapt G2-S). For comparison between the charge states we estimated the CCS using an established calibration protocol.[3]

Ergebnisse und Diskussion

Basis of our investigation are ubiquitin mutants, which instead of the 7 lysine residues of the wild type (*wt*) contained no lysine (*noK*), or exclusively one lysine residue at a particular site (*K6*, *K27*). All remaining lysines are replaced by arginine, which due to its size is expected to bind much weaker to CE, if at all. In the *K6* mutant, the lysine side chain is assumed to be exposed to the exterior and easily accessible for CE complexation. In *K27* on the other hand, the lysine is involved in an intramolecular salt bridge.

Independent of the charge state, complexes with up to five CEs were observed for *wt* ubiquitin as well as all mutants at gentle ionization conditions. However, a considerably reduced signal intensity is observed for the protein-CE complexes of the *K6*, *K27*, and *noK* mutants, which points to a rather non-specific attachment.

Ubiquitin ions of high (unfolded) and low charge states (compact) essentially retained their shape upon CE complexation. This was expected and has been observed previously for other proteins.[2] Intermediate charge states, which in general show a multitude of coexisting conformations, are more sensitive to CE microsolvation. Here, *wt* ubiquitin undergoes an unusually distinct compaction with increasing number of attached CEs. Once the CE is bound, the charged side chains cannot coordinate to the backbone any longer and unfolding is prevented. In contrast, no significant structural transition was observed for the *noK* mutant, which can be explained by the non-specific nature of the CE binding. Similar results were also observed for the salt-bridged lysine in *K27*. Surprisingly, however, a clear compaction upon CE binding was found for *K6* (solvent-exposed lysine). Taken together, this indicates, that the microsolvation induced compaction of gas-phase proteins results from binding of the CE to few specific, non salt-bridged lysine side chains.

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Neue Aspekte

Identifying crown ether binding sites with ion mobility-mass spectrometry using different ubiquitin mutants

PG 8

Cleavage efficiency of endoproteinase GluC nearby to phosphorylation sites

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The determination of the phosphorylation degree of proteins is important for understanding the regulation of biological processes. This can be done by comparing the phosphorylated and the corresponding non-phosphorylated peptides after the digest[1]. For this reason the applied protease should not be influenced by phosphorylated amino acids nearby the cleavage site. However, it is known that trypsin is negatively influenced by phosphate groups which are nearby to the tryptic cleavage site[2]. That leads to different cleaved peptides, when comparing the phosphorylated with the non-phosphorylated protein.

Here we evaluated the influence of phosphorylation sites on endoproteinase GluC which cleaves at the C-terminus of glutamate and aspartate[3].

Experimenteller Teil

We used a set of custom-made synthetic peptide pairs (JPT, Berlin) with and without phosphorylated serine. The distance between the phosphorylation and the cleavage site was varied between 0 and 3 amino acids. A reference peptide was added to each peptide pair for data normalization. The digests were done with an endoproteinase from *Staphylococcus aureus* for 2-6 hours, dissolved in 50 mM HEPES (pH 7.4). Enzyme activity of each sample was stopped by adding a solution with 5% phosphoric acid (PA). Samples were desalted with C-18 tips. The eluate was then mixed with 2,5-dihydroxybenzoic acid + 2% PA in equal volumes[4]. Samples were analyzed with a MALDI-TOF/TOF (Bruker Daltonics) on a MTB ground steel plate.

Ergebnisse und Diskussion

After testing several matrices, it turned out, that using 2,5-dihydroxybenzoic acid + 2% PA resulted in reasonable results for all used peptides. Furthermore the digest conditions were varied depending on the amino acid sequence of the different peptides.

First we used the peptide YTFE-pS-LPAK with the phosphorylation directly C-terminal to the cleavage site. After 30 minutes 99% of the control peptide YTFESLPAK and 0% of peptide YTFE-pS-LPAK were digested. Peptide YTEF-pS-LPAK with one amino acid between the phosphorylated serine and the glutamate was 25% cleaved after 24 hours. The digest of the control peptide YTEFSLPAK was 100% complete after 24h. The peptide YETF-pS-LPAK, with two amino acids between the phosphate group and the cleavage site showed also a reduced cleavage because of the phosphate group, but the effect was weakened. After 24h the digest was complete for the control peptide and phosphorylated peptide.

The same procedure was done with synthetic peptides with phosphate groups at the N-terminal side of the cleavage site. The results for these peptides showed the same negative effect of the phosphate group on the cleavage efficiency of GluC. In summary, this indicates a strong influence of phosphate groups on both sides of the cleavage site, which decreases with the distance between the phosphorylated amino acid and the cleavage site.

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Neue Aspekte

The cleavage of an endoproteinase GluC is negatively influenced by phosphorylated amino acids next to glutamate.

PG 9

Analytical and functional assessment of chemical modifications sites in therapeutic proteins

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Chemical modifications like asparagine deamidation, aspartate isomerization, and methionine oxidation are typical degradations for therapeutic proteins. Recombinant monoclonal antibodies (mAbs) are exposed to bio-process and storage conditions that might influence the rate and extent of these modifications. Previous studies have shown that the described degradation can affect *in vitro* stability and *in vivo* biological functions.

Experimenteller Teil

For the identification and functional evaluation of antibody critical quality attributes (CQAs) derived from chemical modifications of amino acids, an approach employing specific stress conditions, like elevated temperatures, pH and oxidizing agents, was applied. Stressed samples were analyzed by proteolytic peptide mapping, quantitative liquid chromatography mass spectrometry, ion exchange chromatography, size exclusion chromatography, and surface plasmon resonance analysis.

Ergebnisse und Diskussion

The described approach was adequate to identify and quantify deamidation, isomerization and oxidation sites in susceptible complementary-determining regions and the conserved regions of recombinant IgG1 antibodies. Surface plasmon resonance analysis of stressed mAbs demonstrated a loss of target binding activity of specific degradation variants. However, none of the assessed degradation products led to a complete loss of functionality. In summary, this approach facilitated the identification and *in vitro* assessment of potential critical quality attributes (CQA) in recombinant mAbs.

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PG 10

Sequencing of Copolymers using Mass Spectrometry

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The sequencing of polymers has started to elucidate the detailed structure of polymers, which was initially performed with proteins and peptides. Firstly, this kind of sequencing approach has been applied to homopolymers by the determination of the chain length and fragmentation series. In 2007, Thalassinos *et al.* presented a software for the determination of end groups in homopolymer MS/MS spectra. Furthermore in 2011, Baumgärtel *et al.* reported first results of the application of a software for *de novo* identification of fragmentation mechanisms of homopolymers. Copolymers have an even more complex spectra in comparison to homopolymers. In 2012, Weidner *et al.* developed the software MassChrom2D, a fraction dependent method using liquid chromatography hyphenated offline to MALDI enabling 2D copolymer compositions plots.

Experimenteller Teil

MALDI-ToF MS measurements were performed with an Ultraflex III ToF/ToF (Bruker Daltonics, Bremen, Germany) mass spectrometer equipped with Nd:YAG and a collision cell. All spectra were measured in the positive ion reflectron mode. For the MS/MS mode, argon was used as a collision gas at a pressure of 2×10^{-6} mbar. The instrument was calibrated prior to each measurement with an external standard PMMA standard $H(CH_2CCH_3COOCH_3)_nH + Na^+$, m/z = 425 or 2526, measured with sodium iodide) from PSS Polymer Standards Services (Mainz, Germany) in the required measurement range. MS and MS/MS data were processed using software FlexAnalysis (Bruker Daltonics), Data Explorer 4.0 (Applied Biosystems) and an isotope pattern calculator from Bruker Daltonics.

Ergebnisse und Diskussion

A new method is established to provide the analysis of linear copolymers, which is independent of their composition and polymer class. A statistical model was proposed for the polymerization process and to reduce the model fitting to high-dimensional numerical optimization and NP-hard combinatorial problems. A simple model for the polymerization as well as a complex method for estimating the model parameters from MS and MS/MS spectra is proposed. Quantification of the abundancences of every linear copolymer chain after optimization of certain parameters has been performed. Currently, the synthesis of copolymer libraries and additional experiments are conducted to demonstrate the plausibility of the computational results. The libraries will also help to explore the feasibility limits of the method. Furthermore, chemical questions will be correlated to a statistical model and supported by interpretable visualizations.

PG 11

Zeitaufgelöste Untersuchung der Ionenbildung durch eine niederfrequente Tieftemperatur-Plasmaquelle

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Dielectric-Barrier-Discharge (DBD) Quellen eignen sich für eine direkte Desorption und Ionisation von Molekülen von Oberflächen ohne vorherige Probenpräparation. Diese einfachen und flexiblen Quellen zeichnen sich durch eine geringe Leistungsaufnahme sowie Gasverbrauch aus.

Obwohl die Verwendung solcher Ionenquellen immer weiteren Einzug in die Massenspektrometrie erhält, sind die grundlegenden Mechanismen weiterhin nicht vollständig geklärt. Die meisten Studien konzentrieren sich auf die Charakterisierungen des Plasmas sowie der gebildeten angeregten und ionischen Spezies durch optische Spektroskopie.

Experimenteller Teil

Um ein tieferes Verständnis der zugrundeliegenden Ionenbildung zu erlangen wurde eine DBD mit verschiedenen Anregungswellenformen betrieben und die gebildeten Ionen in Echtzeit detektiert. Die Verwendung eines Time-of-Flight Instruments ermöglichte die Aufnahme kompletter Spektren bei 400 Hz. Die verwendete DBD bestand aus einer Quarz-kapillare mit einer ringförmigen Außenelektrode und einer innen liegenden Draht-Masseelektrode und wurde mit Helium betrieben. Der über den Verlauf der verschiedenen Spannungsverläufe gemessene Strom gibt zusätzliche Aufschlüsse über verschiedene Entladungsmechanismen.

Ergebnisse und Diskussion

Die Stromverläufe der verschiedenen Spannungs-Wellenformen zeigen deutliche Unterschiede in den Endladungscharakteristiken. So zeigen sowohl eine Sinus- als auch eine Dreiecks-Anregungsfunktion eine Streamer-Entladung. Dies ist an vielen zufällig verteilten Mikroentladungen mit einer Dauer < 40 µs zu erkennen. Die Sägezahn Wellenform zeigt über den Verlauf der flachen Flanke ebenfalls Streamer-Entladungen, bei der schnellen Polaritätsumkehr kommt es schlagartig zu einer Townsend-Entladung. Die Stromprofile einer Rechteckfunktion lassen ausschließlich diese Townsend-Entladungen erkennen.

Obwohl die angeregten und ionischen Spezies, die sowohl währen der Streamer-Entladung als auch der Townsend-Entladung gebildet werden, zuerst sowohl zeitlich als auch räumlich höchst konzentriert sind, zeigen die korrespondierenden Ionensignale eine deutliche zeitliche Verbreiterung. Dieser Effekt wird durch Stöße und turbulente Durchmischung unter Atmosphärendruck, bevor die Ionen in den Niederdruckbereich des Massenspektrometers extrahiert werden, hervorgerufen. Ebenfalls kommt es durch die Verbreiterung der durch Streamer-Entladung gebildeten Ionen zu einer Überlagerung, so dass diese Ionen im zeitlichen Verlauf eher breite Signale zeigen.

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Neue Aspekte

Es wurde die zeitliche Bildung von Ionen in einer DBD-Quelle in Abhängigkeit der angelegten Spannungsform mittels schneller transienter MS untersucht.

PG 12

1c2p-REMPI von Anisol-Komplexen

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Für diesen Beitrag wurden 1c2p-REMPI-Spektren von nicht-kovalenten Anisol-Komplexe aufgenommen. Diese Ergebnisse sind für das Verständnis und die Erklärung von supramolekularer Organisation von Bedeutung. Werden aus Anisol und einem anderen kleinen Aromaten Dimere gebildet, ergeben sich elektronische Übergänge, die grundsätzlich anders als die der Monomere sind [1]. Es kommt zu Verschiebungen von Banden im Vergleich zum Monomer. Anders als Phenol-Dimere, bei denen das eine Phenol als Protonendonor, das andere als Protonenakzeptor wirkt und somit zu zwei 0-0-Übergängen führt, gibt es bei den Anisol-Komplexen nur einen [1]. Die Komplexpartner des Anisols werden variiert und ihre Auswirkungen auf die Änderung der elektronischen Struktur der Komplexe untersucht.

Experimenteller Teil

Es wurde ein Molekularstrahlexperiment an einem modifizierten ReTOF (*Bruker*) durchgeführt. Die verwendeten Substanzen wurden ohne weitere Aufreinigung in einem Mischungsverhältnis von 1:1 (V:V) verwendet. Der Molekularstrahl wurde mit Argon (Hintergrunddruck 4 bar) erzeugt. Zur Ionisierung der Komplexe wurde ein von 265 nm bis 277 nm durchstimmbarer Farbstofflaser (Coumarin 153, Scanmate, *Lambda Physics*) verwendet, der von einem Nd:YAG-Laser mit 355 nm (SpitLight, *InnoLas*) gepumpt wird. Die Messungen wurden von einer im Arbeitskreis programmierten Software über ein Oszilloskop (LSA1000Series, *LeCroy*) und eine MCP aufgenommen [2].

Ergebnisse und Diskussion

Im Experiment können homologe Komplexe des Toluols mit bis zu drei Molekülen und homologe Komplexe des Anisols mit bis zu drei Molekülen nachgewiesen werden. Heterogene Komplexe werden in der Zusammensetzung Toluol: Anisol 1:1 gefunden. Ein nicht-kovalenter Anisol-Phenol-Komplex hat nur einen 0-0-Übergang, der im Vergleich zu beiden Reinstoffen rotverschoben ist [1]. Auch Anisol-Toluol-Komplexe zeigen dieses Verhalten. Änderungen am Substitutionsmuster des komplexierten Aromaten werden auf ihre Auswirkungen auf die Wellenlängenverschiebung des 0-0-Überganges bezüglich der Reinsubstanzen untersucht. Die Spektren zeigen bei Frequenzen oberhalb des 0-0-Übergangs des Komplex ein komplexes Bandenmuster. Anhand von Rechnungen wird die Zuordnung der Banden vorgenommen.

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Neue Aspekte

1c2p-REMPI-Spektren von Anisol-Komplexen.

PG 13

Fragmentierung von Alkylsubstituierten Coumarinen mittels CID und Vis-mPD

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Coumarine sind sowohl als Laserfarbstoffe als auch als Label für Fotofragmentierungen bekannt. Im Rahmen dieser Arbeit wurde ein Coumarinlabel mit unterschiedlich langen Alkylketten substituiert und die Fragmentierung mittels CID und Vism-PD untersucht. Für die Vism-PD Messungen wurde ein neues Experiment aufgebaut, sodass es möglich ist mittels Nanosekundenpulsen bei einer Wellenlänge von 532nm Untersuchungen in der ICR Zelle durchzuführen. Hierbei zeigte sich ein deutlicher Einfluss der Kettenlänge auf das Fragmentierungsverhalten der Seitenkette als auch auf das Labelmolekül an sich. Dieses zeigt ab einer bestimmten Kettenlänge ebenfalls Fragmente.

Experimenteller Teil

Alle Messungen wurden mit einem 7.05T FT-ICR Massenspektrometer der Firma Bruker Daltonik (Bremen) durchgeführt. Die unterschiedlichen Proben wurden mittels Elektrospray Ionisation in die Gasphase überführt und anschließend durch CID (*Colliosion Induced Dissiociation*) mit Argon als Stoßgas sowie Vism-PD fragmentiert. Hierzu wurde ein Nd:YAG Laser von Continuum zur Fragmentierung mit Nanosekundenpulsen bei einer Wellenlänge von 532nm verwendet. Alle verwendeten Substanzen wurden aus Grundchemikalien von Sigma Aldrich synthetisiert.

Ergebnisse und Diskussion

Bei den durchgeführten Fragmentierungsreaktionen mittels CID und Vis-mPD an den substituierten Coumarinmolekülen wurden Fragmente sowohl aus der Alkylkette als auch vom Coumarinlabel direkt beobachtet. Neben den Abspaltungen an beiden Seiten der bei der Synthese gebildeten Iminbindung konnten weitere Fragmente wie Propen oder Buten aus dem Bereich der Alkylketten nachgewiesen werden. Zusätzlich zu diesen Fragmenten kommt es bei verkürzten Alkanketten unerwarteteter Weise auch zur Bildung von Fragmenten des Labelmoleküls im Bereich der Diethylaminoseitengruppe. Bei kurzen Alkylketten als Substituent an der Iminbindung konnten neben der Propanabspaltung aus der Diethylaminoseitengruppe auch kleinere Fragmente wie Methan oder Ethan neben verschiedenen Radikalabspaltungen beobachtet werden. Insgesamt konnte gezeigt werden, dass die Alkylsubstituierten Coumarine bei Fragmentierungsuntersuchungen mittels CID und Vis-mPD viele Fragmente bilden und das unter bestimmten Bedingungen auch das Labelmolekül an sich fragmentiert. Ebenso wie bei den Xanthenfarbstoffen [1] zeigt sich auch bei den substituirten Coumarinen eine Verletzung der *Even Electron Rule*. Es konnte außerdem gezeigt werden, dass Farbstoffmoleküle mittels eines Nd:YAG Lasers in der ICR Zelle fragmentiert werden können.

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Neue Aspekte

Fragmentierung von Coumarinlabeln mittels CID und Vis-mPD

PG 14

Kinetic Energy Release bei der Fragmentierung substituierter Amine

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Der Verlust von Propan aus der Diethylaminoseitengruppe wurde am Beispiel von Rhodamin B gezeigt [1]. Zum besseren Verständnis dieser Ergebnisse wurden Alkylaminogruppen in kleinen Molekülen unter anderen Reaktionsbedingungen untersucht. Dazu wurden mono- und dialkylsubstituierte Aniline und di- und trialkylsubstituierte Amine mit unterschiedlichen Alkylkettenlängen verwendet, um Abhängigkeiten der auftretenden Fragmente von der Länge der Alkylketten und dem aromatischen System zu untersuchen.

Experimenteller Teil

Alle Messungen wurden mit einem Micromass ZAB-2F der Firma Vacuum Generators durchgeführt. Die Proben wurden in die Gasphase überführt und mittels Elektronenionisation ionisiert. Mass-Analysed-Ion-Kinetic-Energy-(MIKE)-Spektrometrie der gebildeten Ionen lieferte Werte für den Kinetic Energy Release (KER).

Ergebnisse und Diskussion

Die untersuchten alkylsubstituierten Aniline und Amine zeigten das gleiche Fragmentierungsmuster und lieferten vergleichbare kinetischen Energien beim Verlust analoger Neutralteilchen. Im Gegensatz zum Rhodamin B trat jedoch kein Propanverlust aus diethylsubstituierten Anilinen und Aminen auf. Stattdessen ergaben die Untersuchungen mittels MIKES bei allen alkylsubstituierten Anilinen und Aminen als vorherrschenden Prozess aus dem Molekül-Radikalkation den Verlust eines Alkylradikals mit einer um ein Kohlenstoffatom kürzeren Kette als die Kettenlänge der substituierten Alkylgruppen. Dieses Verhalten trat unabhängig vom Substitutionsgrad des Stickstoffatoms und vom aromatischen System auf. Die Fragmente der di- und trisubstituierte Verbindungen, die durch den Radikalverlust entstanden, zeigten Alkenabspaltungen. Dabei ließen sich für Alkenverluste, bei denen die Kettenlänge der Länge der substituierten Alkylketten entsprach, geringe Werte für die freigesetzte kinetische Energie bestimmen. Alkenabspaltungen, bei denen die Alkenkette im Vergleich zum Substituenten um ein Kohlenstoffatom verkürzt war, zeigten sowohl bei den Anilinen als auch bei den Aminen einen deutlich erhöhten KER. Auch hier zeigte sich keine Abhängigkeit vom aromatischen System.

Referenzen

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Neue Aspekte

Es wurden Werte für den Kinetic Energy Release von Alkylfragmenten bei der Fragmentierung alkylsubstituierter Amine bestimmt.

PG 15

A new 2D quantum mechanical method based on self-consistent partial charge calculations and atomic energies

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The prediction of preferred bond cleavages after protonation of a compound based on theoretical methods represents still a challenge. These predictions require at least two steps in the calculations: 1st the determination of the preferred protonation sites and subsequently 2nd the prediction of the weakest bond to be cleaved. It could be shown that high quality quantum mechanical calculations are principally able to predict these bonds correctly [1, 2]. However, these calculations are not applicable for evaluating large compound databases concerning their fragmentation because of long calculation times.

Experimenteller Teil

We developed a very fast method based on 2D-structural formulas of compounds to predict both the preferred protonation sites and the bonds with lowest bond order. Simultaneously, the reaction energies for each possible fragmentation pathway can be determined. The results of our calculations in comparison with experimental gas phase proton affinities for a set of amines are presented in this context. Furthermore, the method is applied to the fragmentation of nicotine and quercetine as model compounds and compared with experimental data.

Ergebnisse und Diskussion

The energies of all calculated fragments show a good correlation with the electronic energies obtained by semiempirical AM1 energies. In high advantage, in comparison to the semiempirical methods our 2D-based calculations are more than one thousand times faster and do not require a 3D-structure energy optimization.

Altogether, these preliminary results based on the application of our new method offer an optimistic outlook with respect to the prediction of fragmentation patterns of parent molecules from databases with thousands of structures by calculations in a reasonable time. The combination of the energy calculations with *in-silico* methods such as MetFrag (http://msbi.ipb-halle.de/MetFrag/) can help to identify compounds with tandem MS among candidate structures obtained from general purpose compound libraries. The integration will remarkably improve the possibility for identification of metabolites from MS/MS data.

The project was support by the Deutsche Forschungsgemeinschaft (Br 1329/14-1 and NE 1396/5-1).

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PG 16

Wavelength and Fluence Dependence of UV-MALDI-MS with 3-Hydroxypicolinic Acid and Dithranol Matrices for the Analysis of Oligonucleotides and Acidic Glycolipids

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Using a laser wavelength that corresponds to a high optical absorption of the utilized matrix is a key element for sensitive UV-MALDI mass spectrometry. However, even many classical MALDI matrices exhibit absorption profiles in the solid state that overlap only weakly with the emission lines of the two common UV-MALDI-lasers of 337 nm (N_2-laser) and 355 nm (Nd:YAG-laser, 3xf). Here, we investigated the influence of the wavelength and laser fluence for UV-MALDI-MS with two matrices: The first, 3-hydroxypicolinic acid (3-HPA), is the standard matrix for the analysis of oligonucleotides and has a peak absorption at ~310 nm. The second, dithranol, has recently been identified by us as particular powerful for MALDI-MS imaging of acid lipids such as gangliosides [1,2].

Experimenteller Teil

Dithranol exhibits a broad modulated absorption profile that spans the full near-UV range to values above 400 nm. Local absorption maxima are found at about 260 and 380 nm. Diffusive reflection spectroscopy was used to determine the solid state absorption profiles [1]. An oTOF mass spectrometer was employed for the MS experiments [3-5]. Tunable light was generated with either a frequency-doubled dye laser (for the experiments with 3-HPA) or with an optical parametric oscillator (dithranol) (the laser pulse durations are ~5 ns for both lasers). The investigated wavelength range was 290–350 nm for 3-HPA and 220–420 nm for dithranol. Fluence was varied between the ion detection thresholds and a factor of up to 5 above.

Ergebnisse und Diskussion

A mix of DNA reference compounds served as analytes for MALDI with 3-HPA while GM1 ganglioside-containing extracts from mouse brain were used in connection with the dithranol matrix.

For the DNA/3-HPA system the highest ion yield (determined by dividing the molecular ion abundances by the total ion count) is found at around the absorption maximum (i.e., between 300-320 nm). In particular, a better signal-to-noise ratio is found at these wavelengths as compared to 337 nm (where the absorption decreases by about a third), suggesting that the overall ionization efficiency increases. With respect to laser fluence, protonated molecular DNA species dominate the spectra in the optimal fluence range (2-3 times the threshold fluence), while higher abundances of sodium and potassium adducts are obtained at higher laser fluences.

Upon spraying dithranol [dissolved in $CHCl_3$:MeOH (2:1, v/v)] on mouse brain tissue slices, especially uniform microcrystalline sample coatings as well as high ion yields of acid lipids are obtained in MALDI-MS imaging applications [1,2]. First rule-of-thumb experiments suggested that better analytical sensitivities are obtained when the results obtained with 337 and 355 nm were compared, in line with the higher absorption coefficient found at 355 nm. In this presentation, detailed results from currently ongoing work will be presented that investigated this finding in more detail.

In particular, a very wide wavelength range from 220 to above 400 nm will be screened for the first time, thereby covering the local absorption maxima as well as the long-tailing red flank of the absorption band of dithranol matrix. The data will not only allow identification of optimal excitation conditions but also provide exciting insights into the MALDI mechanisms.

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Neue Aspekte

Detailed study on the effects of wavelength and laser fluence on the UV-MALDI-MS performance characteristics with 3-HPA and dithranol matrices

PLK: Lipid- und Kohlenhydrat-Analytik

PLK 1

Compositional analysis of asphaltene sample using FT-ICR Mass Spectrometry with different ionization techniques

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Asphaltenes are one of the most problematic fractions of crude oil since they are considered to have properties that can cause problems during oil refining processes and transportation. The exploration of their structure is a huge challenge for researchers because of their complexity, polycondensed aromatic construction and limited solubility. According to their definition they are insoluble in paraffinic solvents and soluble in toluene supporting their high aromaticity and polar, high heteroatom containing condensed aromatic rings and aliphatic chains.[1]

Mass spectrometry with high mass accuracy and ultrahigh resolution with different ionization methods enables to provide a thorough image on their composition on a molecular level.[2]

Experimenteller Teil

The final properties of a freshly prepared asphaltene sample from a crude oil are highly sensitive to every single parameter applied during the procedure (e.g. contact time with solvent, solvent-to-crude oil ratio, temperature, level of washing). Asphaltene samples were prepared from bitumen sample according to IP 143 method and different ionization methods were applied to provide different types of cations (protonated ions or radical cations). The results with ESI, as the most widespread ionization method for polar components, were compared with other ion sources for the analysis of heavy crude oil, like APPI (Krypton VUV lamp) and laboratory-built APLI (KrF excimer laser, 248 nm).[3] Mass analysis was performed on a 7 T LTQ FT-ICR MS.

Ergebnisse und Diskussion

Asphaltene preparation was performed under strictly controlled parameters, as it was observed that a slight change can have a big impact on the final properties (e.g. solubility, composition, degree of aromaticity, range of double bond equivalents) of the asphaltene sample. The results of the compositional analysis show that the capability to ionize and detect the highly aromatic structures, such as asphaltenes, depend strongly on the ionization method itself. The discrimination of different compound types can be observed, as each ionization method only shows a part of the components present in the complex mixture.

As it was expected, the formation of radical cations was significant compared to their corresponding protonated ions in the cases when APPI and APLI were used for ionization. Furthermore, there was a detection of radical cations generated by the ESI ionization as well under the applied circumstances (sprayed from toluene/methanol, 50/50 and 4.0 kV needle voltage).

The observed radical cations were mostly N-containing species or hydrocarbons with high DBE number (in the range of 20-40) and low H/C values (0.4-1.0). The formation of these unexpected species might be explained with chemical oxidation or electrochemical oxidation in the ES needle.[4] This observation is significant because it can extend the utility of electrospray ionization technique and the ability to analyze different type of molecular species (aromatic, polar and nonpolar compounds) with electrospray in asphaltene investigation.

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Neue Aspekte

The delicate preparation of the heavy asphaltene fraction from crude oil is investigated with different ionization methods.

PLK 2

Analysis of lecithins used in chocolate production by HPTLC-FLD, HPTLC-ESI-MS and HPTLC-MALDI-TOF/TOF MS

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Lecithin or rather phospholipids are added as emulsifier E 322 to various products in food industry [1]. Most of the lecithin used in chocolate production is obtained from soy beans requiring declaration with regard to intrinsic allergens and GMOs. Efforts have been made to use non-genetically modified sunflower lecithin instead. Phospholipids are crucial for the miscibility of the different components and stability of the chocolate mass. Of special interest were phosphatidy-choline (PC) and phosphatidyl-ethanolamine (PE). Both phospholipids influence the rheology of molten chocolate differently. While PC reduces viscosity, PE is more effective reducing the rheological *Casson* yield value than the viscosity. The lecithin samples were quantitatively analyzed by HPTLC for their phospholipid content, rheological behavior and fatty acid pattern. [2]

Experimenteller Teil

After development on HPTLC plates silica gel 60 F_{254} with chloroform – methanol – water – ammonia (25 %) (60:34:4:2) [3] and derivatization by immersion into the primuline reagent, densitometric evaluation was performed via fluorescence measurement at UV366/<400 nm.

Rheological data were measured for three different chocolate masses (dark, brown and white) with a viscosimeter at 40 °C.

For HPTLC-ESI^+-MS, zones of interest were directly eluted via the TLC-MS Interface and recorded by a single quadrupol MS (ExpressIon CMS, Advion, Ithaca, USA).

For HPTLC-MALDI-TOF, the developed aluminium sheets (5 x 7.5 cm) were homogeneously coated with DHB matrix solution, fixed in the TLC-MALDI adapter target and MALDI-TOF mass spectra were acquired with the ultrafleXtreme TOF/TOF MS (Bruker Daltonics, Bremen, Germany). [2]

Ergebnisse und Diskussion

The quantitative analysis showed that there are significant differences in soy bean and sunflower lecithin samples concerning their PC and PE content. The ratio PE/PC for soy lecithin was <1, while the ratio for the sunflower lecithin samples was >1.

The rheology of the respective chocolates using sunflower or soy lecithin showed overall comparable results. Slightly higher viscosity was measured for dark chocolate when using sunflower lecithin 1 instead of soy lecithin. When using sunflower lecithin 2 instead of soy lecithin the rheological yield value of milk chocolate was slightly increased. No significant differences in flow parameters could be detected for white chocolate. When comparing the PE and PC contents to the rheological results, no causal correlation between the PE content of the lecithin and the yield value in any of the used chocolates could be found. In addition, neither the lower PC content of sunflower lecithin 1 nor the higher amount of PC in sunflower lecithin 2 compared to soy lecithin had a divergent effect on the viscosity of dark or milk chocolate. Only for white chocolate, a negative correlation (r = -0.99) was indicated. Finally, the differences in PC and PE content in the three lecithin samples were too marginal to be the reason for the differences in the rheology of milk or dark chocolate.

Due to mass spectrometric analysis via HPTLC-ESI^+-MS, two molecular species for each PC and PE could be identified. Dilinoleoyl- and palmitoyl-linoleoyl-derivates could be identified in correspondence with literature. This result about the fatty acid profile was confirmed by HPTLC-MALDI-TOF/TOF MS.

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Neue Aspekte

Quantitation of lecithins used in chocolate production. Comparison with rheological data. Fatty acid determination by HPTLC-ESI^+-MS. Comparison with HPTLC-MALDI-TOF MS.

PLK 3

Global analysis of mycobacterial cell wall lipids by combination of high resolution mass spectrometry and fractionation of lipids during extraction

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High resolution mass spectrometry is an important tool for characterization and identification of selected *Mycobacterium tuberculosis* (*M.tb*) cell wall lipids, which comprise crucial factors of mycobacterial virulence and pathogenicity [1]. State of the art procedures for global characterization of the M.tb lipidome are based upon time-consuming lipid class separation using TLC and liquid chromatography (LC)[2]. Here we present a new strategy for profiling mycobacterial lipids by stepwise fractionation of lipids according to their hydrophobicity during extraction. Thereby, mass spectrometric acquisition time requirements can be reduced from hours to minutes. This workflow is the first step towards a diagnostic tool for M.tb isolates based upon lipid profiling.

Experimenteller Teil

Freeze dried bacterial cells (*M.tb*, H37Rv) were extracted with petroleum ether and according to Bligh and Dyer, respectively[3]. Analyses of the lipid extracts were performed in the positive and negative ion mode using an Apex Qe FTICR-MS equipped with a Triversa Nanomate nano-ESI source. Instruments' TOF time was ramped from 0.0012s to 0.0028s to cover the mass range from 200 *m/z* to 3000 *m/z* in a single experiment. Resulting mass spectra were subsequently smoothed, baseline subtracted and centroidized using a customized VBA script (Data Analysis 4.0). Lipids were identified by their monoisotopic masses using LipidXplorer[4] applying a mass accuracy of 3ppm. MFQL files were createdaccording to the entries of the MtbLipidDB database[5].

Ergebnisse und Diskussion

Bligh and Dyer lipid extracts of *M.tb* were directly infused into the mass spectrometer and analyzed in the positive and negative ion mode. Evaluation of accurate mass spectra using LipidXplorer permitted fast (~5min) identification of a number of complex lipid species, including phosphatidylinositol (PI), phosphatidylinositol mannosides (PIMs), sulfolipids (SL), and phthiocerol dimycocerosate (PDIM). Petroleum ether lipid extracts contained primarily trehalose 6,6'-dimycolate species (TDM) and detailed characterization of TDM species present in the bacterial extracts could be performed using these fractions. These examples show that stepwise extraction of lipids is a valuable tool for the analysis of complex mycobacterial lipid extracts and replaces time-consuming TLC and/or LC separation prior to MS analyses.

We are currently trying to optimize the pre-separation and implementing data-dependent MS/MS analyses into our workflow for verification of lipid assignments. Future work will focus on the use of custom made internal standards to compare lipid signatures of different mycobacterial strains. This data will then be used to correlate mycobacterial lipid signatures with virulence and drug-resistance data of different strains.

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Neue Aspekte

Improved workflow for the mass spectrometric analysis of mycobacterial lipid extracts.

PLK 4

A novel method to simultaneously measure long-chain aldehydes and fatty acids using HPLC-ESI-QTOF

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Sphingosine-1-phosphate (S1P) is a novel bioactive sphingolipid involved in the regulation of cell proliferation, differentiation and survival [1]. S1P can be degraded by a specific S1P lyase leading to the formation of (2*E*)-hexadecenal and phosphoethanolamine. Interestingly, the further metabolism of (2*E*)-hexadecenal is unknown. In general, it is proposed to re-enter the sphingolipid metabolism after oxidation to hexadecanoic (palmitic) acid. To examine the further metabolism of (2*E*)-hexadecenal, we developed a mass spectrometric method to simultaneously measure (2*E*)-hexadecenal, (2*E*)-hexadecenoic acid and hexadecanoic acid using HPLC-ESI-MS/MS. These compounds are poorly ionisable by electrospray-ionisation. Therefore we established a derivatisation method with 2-diphenylacetyl-1,3-indandione-1-hydrazone (DAIH) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) capable for the simultaneous detection of long chain fatty aldehydes and acids [2].

Experimenteller Teil

Basal (2*E*)-hexadecenal concentrations in commonly used cell lines are low, probably due to the chemical reactivity of the α,β-unsaturated aldehyde function. For metabolism studies lysed HepG2 cells were spiked with either unlabeled or deuterated (2*E*)-hexadecenal and incubated at 37 °C. Samples were taken at different time points and free fatty aldehydes and acids were extracted using the method of Dole [3]. After derivatisation (2*E*)-hexadecenal and the corresponding acids were separated using a Rapid Resolution Liquid Chromatography system equipped with a ZORBAX Eclipse XDB-C18 column. Ionisation of the analytes occurred within an electrospray source operating in the negative ion mode. Quantification of the analytes was performed by selected reaction monitoring using a G6530A quadrupole/time-of-flight mass spectrometer (all from Agilent, Waldbronn, Germany).

Ergebnisse und Diskussion

We developed a sensitive method to simultaneously detect the long-chain aldehyde (2*E*)-hexadecenal and the fatty acids (2*E*)-hexadecenoic and hexadecanoic acid using DAIH as derivatisation reagent and EDC as coupling agent. We achieved a limit of detection (LOD) of 10 fmol per injection for the DAIH-derivatives of (2*E*)-hexadecenal and the corresponding acids. The method validation revealed a good reproducibility, linearity and high recovery. Derivatives of the aldehyde and the corresponding fatty acids were baseline-separated within 12 min. The method was applied to examine the metabolism of (2*E*)-hexadecenal by incubating unlabeled or deuterated (2*E*)-hexadecenal with HepG2 cell lysates for up to one hour. For the first time, we were able to show that (2*E*)-hexadecenoic acid but not hexadecanoic acid was formed from (2*E*)-hexadecenal. Since the conversion of (2*E*)-hexadecenal to (2*E*)-hexadecenoic acid did not proceed quantitatively, further not yet identified metabolic routes remain to be studied. This finding is in contrast to the general assumption that (2*E*)-hexadecenal re-enters the sphingolipid metabolism via hexadecanoic acid and, therefore, provides a good basis for further metabolism studies. Indeed, first results show that co-treatment of HepG2 cells with (2*E*)-hexadecenal and the acyl-CoA-synthetase inhibitor triacsin C leads to an enrichment of (2*E*)-hexadecenoic acid in the cell lysates. This indicates that an activation of the unsaturated hexadecenoic acid rather than a saturation to hexadecanoic acid is involved in the metabolism of (2*E*)-hexadecenal. In summary, our method will be instrumental to clarify the metabolic fate of (2*E*)-hexadecenal, a degradation product of the bioactive sphingolipid S1P, and could be useful to study diseases known for abnormalities in long-chain fatty acid metabolism, *e.g.* the Sjögren-Larsson syndrome, more in detail.

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Neue Aspekte

New insights into the metabolism of (2*E*)-hexadecenal, a degradation product of sphingosine-1-phosphate, via HPLC-ESI-QTOF.

PLK 5

High throughput analysis of differentially induced metabolites using metaXCMS and automated fragmentation tree alignment.

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The qualitative and quantitative analysis of all metabolites represents the metabolite profile of a plant at a given instant. In addition to factors like stage and state of a plant, biotic stresses e.g. leaf herbivory or bacterial infection could significantly alter the metabolite profile of a plant. In metabolomics, characterization of such metabolites induced due to particular biotic stress is still a challenging task. High-throughput analysis of metabolites using modern LCMS techniques can be combined with XCMS[1] and automated fragmentation trees alignment programs[2,3] for rapid characterization of such metabolites.

Experimenteller Teil

Arabidopsis thaliana Col-0 plants were grown under standard growth conditions. Plants at early flowering stage (25 days old) were used for all experiments. Wild type plants serve as control. One plant set is subjected to *S. littoralis* leaf herbivory while the other set is subjected to *P. syringe* infection. Plants were cut into above and below ground parts and extracted separately using methanol:water (80:20, v:v). Extracts were analyzed using UHPLC (C-18 RP column) coupled to Orbitrap MS. Mass spectra were acquired at various fragmentation energies in CID or HCD mode. Analysis of differentially up/down regulated masses was performed using metaXCMS[1]. Extracted masses are categorized into related chemical classes using the automated fragmentation tree alignment approach[2,3].

Ergebnisse und Diskussion

LCMS data were analyzed using MetaXCMS which identified total of 6560 differentially induced mass features (fold change > 2; P value >0.05). We acquired MS/MS data using online data-dependent settings in which total of 873 differentially induced mass features were fragmented. Molecular formula and corresponding fragmentation tree (FT) was computed for each mass. We assigned hypothetical identifications to some mass features based on their presence in AraCyc or KEGG. These mass features were then clustered together using automated comparison of computed FT. To encompass all mass features, we will be employing nearline data-dependent tandem MS strategy (MetShot)[4]. The metaXCMS analysis and FT alignment results for MetShot based MS/MS acquisition will be reported shortly.

Biotic and abiotic stresses cause induction of different pathways and thus whole new ensemble of metabolites in each stress. It is highly likely that metabolites involved in same pathway depict similar up/down differential induction. They might share chemical similarity if they arise from the same precursor molecule. We propose that such clusters comprise of compound arising from same or similar pathways and/or belong to the same chemical class. This is more evident when we tried to assign hypothetical identifications for some masses based on their presence in AraCyc or KEGG. The hypothetical identification and clustering of these masses matched well the previously identified compound classes. In future, we will confirm it by true identification of mass features clustered together.

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Neue Aspekte

Application of metaXCMS and FT alignment algorithm to identify compound class and to unravel underlying metabolic networks.

PLK 6

Matching Thin-Layer Chromatography and Desorption Electrospray Ionisation Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry for Lipid Analysis

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Due to the complexity and heterogeneity oft biological samples their analysis employing mass spectrometric methods often poses a serious challenge which can be addressed by use of mass analysers providing a very high resolving power such as Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometers. Alternatively, sample complexity can be reduced by chromatographic separation, e.g. thin layer chromatography (TLC) preceding MS analysis. Coupling desorption electrospray ionisation (DESI) to a mass spectrometer represents a convenient technique to probe the chromatographic planar phase of a TLC plate under ambient conditions [1]. Here, we show preliminary data on the direct desorption and ionisation of lipids separated on TLC plates followed by their structural elucidation by use of infrared multiphoton dissociation (IRMPD).

Experimenteller Teil

Mixtures of phospholipids and glycosphingolipids were separated on silica gel-coated high-performance (HP)TLC plates. Subsequently, developed plates were submitted to DESI FT-ICR-MS analysis. Ions were generated by use of an in-house built DESI source matched to the Apollo ESI source of a Bruker Apex II FT-ICR instrument (7 T). The setup includes an automated sample holder for sample positioning and scanning of surfaces as well as an electrically heated extension of the vacuum interface capillary. Gas-assisted electrospray is generated by a modified ESI emitter that can be positioned unrestrictedly to vary the distance to the sample surface and the impact angle of the spray. Analyte ions were isolated and submitted to IRMPD MS/MS analysis employing a continuous wave infrared laser.

Ergebnisse und Diskussion

The spatial resolution of the DESI source employed was probed by imaging test patterns drawn on a glass plate with permanent markers. A pattern from two different dyes could be imaged with a spatial resolution of about 300 µm sufficient for application of the current setup to analyse two well separated analyte bands on the TLC plate. Mixtures of phospholipids and glycosphingolipids were separated on silica gel-coated HPTLC plates and submitted to DESI FT-ICR MS. Mass spectra mainly revealed the presence of intact sodiated analytes. Depending on the *m/z* range resolutions of 50000 to 100000 were achieved routinely with mass accuracies in the low ppm-range (external calibration). For structural elucidation precursor ions were isolated inside the ICR cell and activated by irradiation with IR photons. The observed fragmentation patterns are similar to those obtained by low energy collisional activation and evaluation of the MS/MS spectra allows for determination of the structure of the analyte ion species under inspection. The results presented in this study demonstrate the potential of the hyphenation of planar chromatographic separation and formation of gaseous ions directly from the surface of the TLC plate by DESI for the analysis of lipids.

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Neue Aspekte

Direct coupling of TLC with FT-ICR-MS IRMPD for lipid analysis

PLK 7

Lipid mediators in pathogen infection: LC-MS/MS approach to analyze eico- and docosanoids

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Lipid mediators are oxidized polyunsaturated fatty acids that derive from cell membrane phospholipids. By successive enzymatic actions, the released precursors arachidonic acid, eicosapentaenoic acid and docosahexaenoicacid can be converted to lipid mediators which hence are classified as eico- and docosanoids. These small bioactive lipids play a prominent role in cell signaling during inflammatory processes, e.g. pathogen infections (Serhan et al., 2008). Briefly, acute and chronic inflammations are described by increased levels of pro-inflammatory eicosanoids, e.g. prostaglandins, whereas pro-resolving eico- and docosanoids, e.g. lipoxins and resolvins, indicate that an inflammation is ceasing (Norris and Dennis, 2013, Tam, 2013). To gain insight into the regulation of macrophage activity a LC-MS/MS method was developed for eico- and docosanoid profiling.

Experimenteller Teil

To monitor alterations in the mediator compositions during pathogen infection, we are establishing an analytical platform for mice infection models of *Mycobacterium tuberculosis* (M.tb). The supernatant of (un-) infected peritoneal and bone marrow-derived macrophage cells is analyzed for segregated lipid mediators using solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Sample preparation are performed using the Strata™-X series (Phenomenex®) and LC analysis using an Agilent 1100 LC-system with a Luna C18(2) column (150 x 0.3 mm, 5 µm, Phenomenex®). The mobile phase is comprised of a water/acetonitrile/formic acid gradient at a flow rate of 10 µl/min. For the acquisition of negative ion mode MS^2 (m/z 100 to 400) a QTof Ultima™ is utilized using electrospray ionization.

Ergebnisse und Diskussion

Preliminary data show, that we can unequivocally identify isomeric lipid mediators using the quadrupole time-of-flight mass analyzer. The eicosanoid members 5-HETE and 5,6-EET (precursor ions: m/z 319) as well as Lipoxin A4 (LXA4) and prostaglandin E2 (PGE2) (precursor ions: m/z 351) show specific fragment ions after collision induced dissociations. PGE2 is the most prominent member of pro-inflammatory eicosanoids and is increased upon M.tb-infection in bone marrow-derived macrophages from mice (Paudyal, unpublished). In addition, the docosanoid members Resolvin D1 (RvD1) and Resolvin D2 (RvD2) (precursor ions: m/z 375) showed specific fragmentation patterns. Based on these first datasets, we can demonstrate that the chosen fragment ions of LXA4, RvD1, (un-) labelled RvD2 are quantifiable in the range from 1-1000 fmol/µl ($r^2 > 0.997$). Extraction efficiencies for LXA4 and RvD1 were determined to 102 ± 10% and 100 ± 17% using weak anion exchange SPE. We further can show that these three chemical similar lipid mediators could be quantified using the chosen conditions for the LC-MS^2. Future work will focus on the optimization of sample preparation procedures for eico- and docosanoid profiling during pathogen infection using different biological matrices.

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Neue Aspekte

We have established a method for lipid mediator profiling using SPE and LC-MS^2.

PLK 8

Dual extraction protocol of bioactive lipids and RNA for lipidomic and transcriptomic profiling in the same tissue

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Lipids are molecular components which play essential roles in many physiological processes and pathological conditions. In neurobiology, the lipids serve not only a constitutive role, as previously believed, but also as the source for signaling molecules, such as endocannabinoids or eicosannoids that underscore essential neurobiological functions [1],[2],[3]. To gain a better understanding of their specific functions and to define the signaling networks, especially under pathological conditions, accurate identification and quantification of lipids in biological samples, as well as profiling of related genes is essential.

Experimenteller Teil

We used an advanced targeted mass spectrometry method, based on liquid chromatography (LC) and multiple reaction monitoring (MRM) to identify and quantify the lipid signals, such as endocannabinoids and their phospholipid precursors and a quantitative polymerase chain reaction (RT-qPCR) for the analysis of the corresponding RNA. The extraction of the RNA was done by using the RNeasy Mini kit from Qiagen, combined with a liquid-liquid extraction method for the isolation of lipids from the same sample.

Ergebnisse und Diskussion

To date, lipidomic and transcriptomic profiling is typically carried out in distinct experiments (animals/tissue regions). Here we report the development of a method which allows the dual extraction of lipids and RNA from the same mouse brain tissue sample. By combining the isolation of lipids and RNA in one experiment it is possible to not only reduce the number of animal models, but also the time and costs necessary to carry out lipidomic and transcriptomic profiling. Even more, the analysis of signaling pathways is more reliable by obtaining the molecular components from the same tissue instead from two distinct samples, circumventing thereby the pitfalls originating from tissue heterogeneity. The approach aims at enabling a better understanding of the mechanisms and metabolical pathways of a biological system, thereby saving tissue, time and money.

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Neue Aspekte

Combination of lipid and RNA extraction in one experiment to reduce tissue amount and increase time and cost effectiveness.

PLK 9

Coupling of TLC immunodetection and ESI MS for identification and structural characterization of hydroxylated globo-series glycosphingolipids in mouse kidney

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Glycosphingolipids (GSLs) play important roles in a wide variety of cellular processes like signal transduction and cell-cell interaction. The hitherto investigated globo-series GSLs globotriaosylceramide (Gb3Cer), globotetraosylceramide (Gb4Cer) and Forssman GSL are well known specific receptors of Shiga toxins [1], a group of bacterial toxins, which may cause severe kidney complications upon infection. The structural variety within globo-series GSLs is mainly related to the ceramide portion due to the substitution with fatty acids of different chain length.

Here, we report on the identification of Gb3Cer and Gb4Cer variants with hydroxylated ceramide moieties derived from mouse kidney and their structural characterization by combining the thin-layer chromatography (TLC) overlay assay and ESI MS [2].

Experimenteller Teil

GSLs were isolated from the kidneys of female CBA/J and male NMRI mice. Extraction of GSLs was performed with different mixtures of chloroform/methanol followed by saponification [3]. Samples were adjusted in chloroform/methanol (2/1, v/v) to a defined concentration and analyzed by TLC overlay assays using polyclonal anti-Gb3Cer and anti-Gb4Cer antibodies. The silica gel of the antibody-positive bands was scraped off the TLC plate and GSLs were extracted with pure methanol. The extracts were dried and dissolved in methanol, 1 % formic acid for structural characterization by ESI mass spectrometry with a Q-TOF instrument equipped with a nanoelectrospray manipulator. MS/MS experiments by collision-induced dissociation (CID) were performed with argon as collision gas [2, 4].

Ergebnisse und Diskussion

Both lipid extracts derived from mouse kidneys were separated by TLC and stained with orcinol resulting in detection and visualization of GSLs. For comparison and to preliminarily assign different GSLs in mouse kidney well characterized reference mixtures of neutral GSLs were co-separated. The orcinol stain suggested the presence of Gb3Cer and Gb4Cer with Cer (d18:1, C22–C24) and Cer (d18:1, C16) in both kidney GSL preparations. Interestingly, a triplet of bands was detected for Gb3Cer indicating the presence of more polar variants with decreased TLC mobility (lower triplet band) in addition to the species carrying unmodified ceramides with long C22–C24 (upper triplet band)- and short-chain C16 (middle triplet band) fatty acids. This modification was also visible but less pronounced for Gb4Cer. The orcinol-based preliminary assignments were further verified by TLC overlay assays with anti-Gb3Cer and anti-Gb4Cer antibodies.

The following ESI MS analysis of the silica gel extracts derived from the antibody-positive bands confirmed the presence of a huge variety of Gb3Cer and Gb4Cer lipoforms with an exceptional high degree of ceramide hydroxylation with up to 3 additional hydroxy groups. The structures were further elucidated by low-energy CID whereupon the detection of diagnostic ions enabled the assignment of the hydroxylation to the sphingosine base (d18:0 and d18:1) and/or the fatty acid with C16 – C24 chain length.

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Neue Aspekte

Identification of a high variety of hydroxylated Gb3Cer- and Gb4Cer-lipoforms in mouse kidney by TLC overlay assay and ESI MS

PLK 10

Analysis of free fatty acids and other lipids by different MS methods

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Free fatty acids [FFAs] are involved in many physiological processes. For instance, FFAs are key players in the fat metabolism of the liver and their oxidation products (normally released from oxidized phospholipids under catalysis of the enzyme phospholipase A_2) are involved in many inflammatory reactions.

Established methods of (quantitative) analysis of FFAs are gas chromatography [GC] - normally coupled with mass spectrometry [MS] - and soft ionization MS techniques such as MALDI MS or ESI MS. All these methods have their particular advantages but also drawbacks . The aim of this study was the comparison of different MS methods (including the recently developed matrix-free UV-("fly-assisted")-LDI MS using Drosophila wings as sample substrates [1]) for the analysis of (oxidized) FFAs.

Experimenteller Teil

Selected FFAs and all chemicals for oxidation reactions as well as the phospholipase A_2 were purchased from Sigma and used as supplied. Selected phosphatidylcholines [PCs] with different double bond contents were from Avanti Polar Lipids. Oxidations were performed by using the reagent NaOCl or Fenton conditions. Lipids (from selected biological tissues) were extracted according to Folch for comparative purposes. ESI and MALDI mass spectra were recorded on a Bruker Amazon SL or a Bruker Autoflex, respectively. UV-LDI mass spectra (using wings from sacrificed *Drosophila melanogaster*)were recorded on a "QSTAR-type" orthogonal-TOF MS with a fine vacuum of ca. 2 mbar in the oMALDI2 ion source, providing "collisional cooling" [1].

Ergebnisse und Diskussion

The MS analysis by the FALDI approach of FFA mixtures prepared in a dilution series revealed a linear response over almost 3 orders of magnitude in the presence of an internal standard. Highest sensitivities (ca. 1-10 pmol) were obtained in the positive ion mode by analyzing the $[M+K]^+$ adducts of FFAs. However, other alkali ion adducts (for improved MS/MS) can be easily generated by rinsing the wings with alkali base solutions (for instance, LiOH). Importantly, the FFA signals are not suppressed by the presence of abundant other lipids typically found in crude tissue extract, such as PCs or TAGs. Moreover, because the analysis is carried out under moderate vacuum, no compositional changes of FFA mixtures occur due to evaporation of the unsaturated species. Therefore, crude tissue (e. g. liver) extracts can be rapidly screened regarding their FFA composition without previous sample purification. This is an advantage in comparison to the so far established MS and particularly MALDI-based methods.

Another aim was to investigate whether this approach is also suitable to study oxidized fatty acids, which are generated under physiologically-relevant conditions by the action of the enzyme phospholipase A_2 (PLA_2) from oxidized phospholipids. Phospholipids were treated with oxidizing agents such as HOCl or the complete Fenton system (Fe^{2+}/H_2O_2) and afterwards digested with PLA_2. It could be shown that the chlorohydrins as well as fatty acid peroxides are easily detectable. The extent of unwanted fragmentation reactions (for instance, HCl loss from the chlorohydrins or H_20 loss from hydroperoxides) could be successfully minimized. All data were additionally compared with common MALDI spectra (recorded in the presence of 2,5-dihydroxybenzoic acid or 9-aminoacridine) as well as ion trap ESI MS measurements.

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Neue Aspekte

Oxidized fatty acids can be successfully released by PLA_2 digestion from the corresponding phospholipids and characterized by MS.

PLK 11

Computational Tool for Visualizing and Comparing Lipidomes

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Advances in mass spectrometric identification of lipids has led to an increased number of published lipidomes [1-3]. However, methods for analyzing lipidomes are limited as compared to proteomes or genomes. Specifically, existing methods focus entirely on lipid abundances for determining correlation-based similarities between lipidomes. Such approaches cannot account for lipids that are unique for a lipidome, hence limit the ability to compare lipidomes across species or tissues comprehensively.

We present a novel software framework for visualizing and comparing lipidomes. Our approach employs structural similarity between lipids to compute a scalable chemical space of a lipidome. Further, we developed a scoring system to measure the extent of structural overlap between lipids analogous to sequence comparison analysis.

Experimenteller Teil

The following workflow describes key issues addressed in the design of computational tool. First, we investigated ways to process large ensembles of lipid structures in a memory-efficient manner. Second, we developed and tested methods for determining structural similarity between lipids. Third, we applied Principal Component Analysis (PCA) to convert structural similarity values into a three dimensional chemical space. We tested PCA routine using pairwise similarities of 30150 lipids of LIPIDMAPS structure database. Fourth, we examined methods to determine the extent of overlap between lipidomes by comparing the spatial distribution of lipids in the chemical space. Finally, as a proof of principle we tested our computational workflow for comparing four yeast strains that were grown at 24°C and 37°C respectively [1].

Ergebnisse und Diskussion

SMILES (Simplified Molecular Input Line Entry Specification) is a linear representation of three-dimensional structure of a molecule, routinely used for storing and retrieving chemical structures from databases [4]. In our computational workflow, we employed SMILES representation of lipid structures for processing lipidome data.
First, we tested three algorithms (CACTVS Canonical SMILES, Open Babel's Canonical and Non Canonical SMILES) for converting three-dimensional lipid structures into SMILES and adjudged Open Babel's Non Canonical SMILES algorithm to be the suitable choice for conversion.
Second, we examined six algorithms for determining structural similarity between lipids. Word frequency based methods, Fingerprint and LINGO failed to differentiate positional isomers while alignment based algorithms, Bioisosteric, Smith-Waterman, SMILIGN and Levenshtein distance did. Levenshtein distance provided most consistent similarity scores.
Third, we show that PCA produces unique coordinates for all 30150 lipids of the LIPIDMAPS structure database and cluster lipids according to the defined nomenclature. Plotting lipids in orthogonal principal component planes provided an intuitive visualization system for navigating within the yeast lipidome comprising 248 unique lipids.
Fourth, we developed and tested a scoring system to measure the extent of overlap between a pair of lipidomes by calculating the average distance between unique lipids. Lipidome-distance is a numerical measure of the combined structural differences between a pair of lipidomes.
Finally, as a testing system we applied Lipidome-distance to compare four yeast strains [1]. Lipidome-distance based clustering brought the wild-type BY4741 and mutant-Elo1 strains together, indicating that the metabolic alteration caused by the elongase mutation was smaller then the temperature effect. For the mutant strains Elo2 and Elo3 we noticed stronger influence of the elongase mutation, separating them from the wild-type BY4741 and mutant-Elo1 strains.
In summary, we successfully employed structural relationship between 248 lipids to quantitatively estimate the influence of growth temperature and metabolic pathway alteration in yeast lipidome.

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Neue Aspekte

For the first time, a scoring system was developed for global comparison of lipidomes.

PLK 12

Shotgun lipidomics of glycosphingolipids

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Glycosphingolipids are found in the cell membranes of organisms from bacteria to men and are the major glycans of the vertebrate brain. Nearly all glycolipids in vertebrates are glycosphingolipids whose ceramide backbones vary in length, saturation, and hydroxylation. This structural diversity has an impact on the presentation of the attached glycan at membrane surfaces. In this study we present the method of extraction of glycosphingolipids from different cell lines and their analysis by means of shotgun lipidomics supported by thin layer chromatography. We demonstrate that this approach enables high throughput and broad coverage of glycosphingolipid species and classes.

Experimenteller Teil

Glycoshingolipids were extracted by chloroform:methanol:water (4:8:3 v/v/v) [1] followed by a two-phase partitioning in di-isopropyl ether: 1-butanol (6:4, v/v) performed as described [2] with modifications. The aqueous phase collected after the two-phase partitioning was then cleaned-up by solid phase extraction [3]. Thin layer chromatography was performed on Silica gel 60 F254 plates in chloroform:methanol:CaCl2 (65:35:8) and glycolipids were visualized by staining with 20% H_2SO_4. Shotgun lipidomics analyses were performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) equipped with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences). Lipids were identified by LipidXplorer software [4] developed in MPI-CBG. The analysis encompassed GA2, GA1/Gb4, Gb3, GD1, GM3, GM2, GM1, GT1, GD3, GD2, GT3, GT2, GQ1, GP1, sulfatides, Forssman antigens.

Ergebnisse und Diskussion

In this study, top-down shotgun lipidomics analyses and thin layer chromatography were applied to characterize the glycosphingolipid profile of mammalian cell lines. Porcine brain total ganglioside extract (Avanti Polar Lipids) served as a reference. The glycosphingolipids were extracted by a chloroform:methanol:water mixture, followed by a two-phase partitioning and further solid phase extraction. Total extracts were directly infused into a Q Exactive mass spectrometer and species were identified by LipidXplorer software by accurately determined masses. In less than 4 min acquisition time the analysis encompassed 16 major glycosphingolipid classes: GA2, GA1/Gb4, Gb3, GD1, GM3, GM2, GM1, GT1, GD3, GD2 , GT3, GT2, GQ1, GP1, sulfatides and Forssman antigen with no carry-over of lipid material between the samples. Thin layer chromatography further confirmed the lipid class assignments made by shotgun lipidomics. The method is robust, sensitive (~200 ng to 1 µg of lipid per analysis) and is integrated into the generic shotgun pipeline that covers glycerolipids, glycerophospholipids, ceramides, sphingomyelins and sterols.

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Neue Aspekte

Rapid shotgun lipidomics profiling of glycosphingolipids.

PLK 13

TOP DOWN APPROACH FOR GLYCAN ANALYSIS OF THERAPEUTIC ANTIBODIES BY HPLC-MS

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Monoclonal antibodies are one of the most rapid growing classes of therapeutic agents as they can be applied in a various number of diseases like different caner variants, arthritis and multiple sclerosis. A perfect knowledge about structure and structure related function is therefore essential for development and quality control of these biopharmaceutics. Since antibodies are glycoproteins, there glycosylation patterns play an important role in biological function and efficiency. For instance it was found that antibodies show a higher immune response in the absence of fucose sugars.

Experimenteller Teil

Here we show a top down approach for structural analysis of glycosylated antibodies by LC-ESI-q/TOF mass spectrometry by comparing two different preparations of the therapeutic antibody Trastuzumab (Herceptin®). Compared to commonly used bottom up analysis, a top down approach doesn't involve further chemical treatment, enzymatic digest or sample enrichment leading to a smaller consumption of sample and preparation time. Top down analysis was performed of the native antibody as well as of the reduced antibody using only an amount of few picomole. The multiple charged spectra were deconvoluted using a maximum entropy deconvolution and highly resolved spectra were obtained, showing different glycopatterns of the two preparations.

Ergebnisse und Diskussion

Analysis of the light chain led to isotopically resolved ion patterns, showing more posttranslational modifications such as phosphorylation and glycation with a single hexose. Additional information about glycan composition and *N*-terminal amino acid sequence could be obtained by in source fragmentation of the native and the reduced antibody. Furthermore this technique allows determining not only qualitative but also quantitative information about the amount of modifications without any labeling. In summary this method provides a fast and accurate determination of differences in preparations, especially posttranslational modifications, as well as additional information about glycan composition and amino acid sequence by in source fragmentation.

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Neue Aspekte

In source fragmentation of the intact and redurced antibody led to structural information about amino acid sequence and glycan composition.

PLK 14

HPTLC-ESI-MS-Kopplung zur Charakterisierung von Saponinen in unterschiedlichen pflanzlichen Matrices

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Saponine (lat. *sapo* = Seife) sind im höheren Pflanzenreich weit verbreitete Glykoside. Strukturell basieren sie auf einem lipophilen Aglykon (Sapogenin), an dem diverse hydrophile Kohlenhydrate gebunden sind. Insbesondere Hülsenfrüchte (z.B. Erbsen), der Seifenrindenbaum *Quillaja saponaria*, Spargel oder auch Kartoffeltriebe aus keimenden Knollen („Kartoffelaugen“) sind saponinreiche Quellen. Saponine verfügen über keine ausgeprägten Chromophore, was die Analyse über HPLC-DAD erschwert. Praktisch keine der derzeit existierenden Chromatographieverfahren bietet eine allumfassende Charakterisierung und Quantifizierung, die für die Qualitätssicherung von Nöten ist [1]. Eine Möglichkeit ist die HPTLC, da sie u.a. eine Bandbreite an Detektionen zulässt. Erst in den letzten Jahren vermehrt verwendet, ist die *off-line*-Kopplung der Dünnschichtchromatographie (*thin-layer chromatography*, TLC) mit der Massenspektrometrie (MS) über ein TLC-MS-Interface.

Experimenteller Teil

Gegenstand dieser Untersuchungen war es Saponine aus verschiedenen Matrices, u.a. aus Trockenerbsen (Schale, geschält), Spargel und Kartoffeltrieben, zu isolieren und durch Kopplung der HPTLC mit der ESI-MS über ein TLC-MS-Interface zu identifizieren. Nach methanolischer Extraktion und Aufreinigung mittels Festphasenextraktion (SPE) wurden die saponinreichen Extrakte auf HPTLC-Kieselgelplatten unter Verwendung des Laufmittelgemisches Chloroform/Methanol/Wasser (6/4/0,9; v/v/v) getrennt. Die postchromatographische Visualisierung erfolgte mit *p*-Anisaldehyd-Schwefelsäure Reagenz unter UV- bzw. Weißlicht. Zur spezifischeren Differenzierung zwischen Spiro- und Furostanolen im Spargel wurde eine Färbung mit Ehrlich’s Reagenz vorgenommen. Relevante Substanzen wurden auf underivatisierten Platten markiert, von der Oberfläche semi-automatisiert extrahiert und über eine flüssige Phase in ein ESI-Massenspektrometer (amaZon ETD, Bruker Daltonik GmbH, Bremen) überführt [2]. Die Messung erfolgte im positiven Ionenmodus bei m/z 500-1500.

Ergebnisse und Diskussion

Mithilfe der elutionsbasierten Interface-Technik wurden in Erbsen zwei charakteristische triterpenoide Saponine, das DDMP-Saponin (Sojasaponin βg, m/z 1091,5 $[M+Na]^+$) sowie das Saponin B (Sojasaponin I, m/z 965,5 $[M+Na]^+$) nachgewiesen. Spargelproben enthielten vorzugsweise die Furostanole Asparasaponin II (m/z 925,4 $[M+Na]^+$) und Protodioscin (m/z 1071,5 $[M+Na]^+$). In Kartoffeltrieben wurden als steroidale Glykoalkaloide das α-Chaconin (m/z 852,5 $[M]^+$) und das α-Solanin (m/z 868,5 $[M]^+$) detektiert [3, 4, 5].

Die Kopplung an das ESI-Massenspektrometer ist einfach und schnell zu handhaben. Ausschließlich Substanzzonen von Interesse werden analysiert, weshalb die HPTLC-MS zielgerichtet anwendbar ist. Durch nahezu vollständige Elution der Banden in das ESI-MS werden hinreichend sensitive und annähernd kontaminationsfreie massenspektrometrische Signale innerhalb weniger Minuten erhalten.

Referenzen

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Neue Aspekte

HPTLC-MS-Kopplung zur Analyse von Saponinen

PLK 15

Massenspektrometrische Charakterisierung von Enzym-Lactose-Derivaten

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Enzymatische Arzneistoffe werden im Rahmen der Pharmakotherapie häufig zur Substitution körpereigener Proteine eingesetzt. So wird ein Mangel an körpereigenen Verdauungsenzymen im Rahmen der Pankreasinsuffizienz durch Verabreichung von pankreatischen Enzymen (wie z.B. α-Amylase und Lipase) therapiert [1, 2].

Eines der Hauptanliegen der Enzym-verarbeitenden pharmazeutischen Industrie besteht in der Bewahrung der nativen Proteinstruktur während der Formulierungsentwicklung und Produktion. Interaktionen zwischen enzymatischen Arzneistoffen und pharmazeutischen Hilfsstoffen wie z.B. Lactose, einem häufig eingesetzten Füllstoff in der Tablettierung, können zur Modifizierung der Proteine des Arzneistoffs und somit zu sinkender biologischer Aktivität und immunologischen Reaktionen führen. Die Charakterisierung und Identifizierung der dabei entstehenden Produkte bilden die Grundlage zum Verständnis des resultierenden Einflusses auf die Eigenschaften des Präparats bzw. seiner Risiko-Nutzen-Bewertung.

Experimenteller Teil

In der vorliegenden Studie wurden tablettierte Präparate auf der Basis von α-Amylase und Lactose charakterisiert. Die Untersuchungen der Präparate erfolgten nach proteolytischem Verdau mit Trypsin und der Messung der tryptischen Peptide mittels MALDI-TOF(/TOF)-MS (ultrafleXtreme, Bruker Daltonik GmbH, Bremen)[3]. Zuvor erfolgte eine Auftrennung der Peptide mittels HPTLC (*High performance thin layer chromatography*) unter Verwendung von Cellulose als stationäre Phase und eines Laufmittelgemischs aus 2-Butanol, Pyridin, Ammoniak und Wasser [4]. Nach der chromatographischen Trennung erfolgte schließlich die massenspektrometrische Charakterisierung durch direkte HPTLC-MALDI-MS-Kopplung. Für ergänzende Informationen wurden die Peptide außerdem mittels UV-Detektion sowie verschiedener Derivatisierungsreagenzien (Fluorescamin-, Ninhydrin- oder Diphenylamin-*p*-Anisidin-Reagenz) visualisiert (TLC Visualizer, CAMAG Chemie-Erzeugnisse & Adsorptionstechnik AG & Co. GmbH, Berlin).

Ergebnisse und Diskussion

Neben der Anwendung der bislang beschriebenen MALDI-MS-Analyse von Glycopeptiden [3] erfolgte im Rahmen dieser Arbeit zusätzlich eine Charakterisierung der tryptisch erzeugten Peptide mittels HPTLC-MALDI-MS. Durch die vorangehende dünnschichtchromatographische Auftrennung sollte eine gezielte Detektion und verbesserte Zuordnung möglicher Modifikationen bewirkt werden. Die simultane Auftrennung mehrerer Proben bietet dabei die Möglichkeit des direkten Vergleichs zwischen behandelter und nicht-behandelter α-Amylase. Ebenso konnte eine direkte Gegenüberstellung des Einflusses verschiedener Parameter bei der Tablettierung und Lagerung erreicht werden. Ferner wurden durch verschiedene Derivatisierungen der Analyten ergänzende Informationen über die entstehenden Produkte erhalten. So konnten bei den Enzym-Lactose-Derivaten mit Hilfe der UV-Detektion sowie des zuckerspezifischen Diphenylamin-*p*-Anisidin-Reagenzes Spots detektiert werden, welche im Fall der nicht umgesetzten α-Amylase-Kontroll-Probe fehlten. Mittels direkter HPTLC-MALDI-MS-Kopplung konnten diesen Spots in Bezug auf die Kontrollprobe abweichende m/z-Werte zugeordnet werden.

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Neue Aspekte

HPTLC-MS-Kopplung zur Peptid-Analyse von Enzym-Lactose-Derivaten

PLK 16

Glycan and glycopeptide profiling of biopharmaceuticals

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Glycosylation is among the most complex post-translational modifications (PTM) undergone by proteins. Because glycans are assembled from various, partly isomeric monosaccharides linked in multiple regio- and stereospecific ways, their analysis is extremely challenging. However, many research endeavours and industrial applications rely on rapid and efficient profiling of PTMs. MS features high selectivity and broad applicability. Thus, in combination with efficient sample preparation and separation techniques, MS enables increasingly detailed glycosylation profiling.

Glycosylation profiling is important in the development and production of biopharmaceuticals, such as therapeutic antibodies and growth hormones. Specific differences in glycosylation can have a significant effect on pharmaceutical properties, such as plasma half-life and efficacy. Therefore, detailed MS based glycan profiling is gaining importance for the development and quality control of functional glycosylation.

Experimenteller Teil

At the CPM, we are currently using mainly three MS based methods for glycosylation analysis: For immunoglobulin G (IgG) F_C glycosylation, 1) an LC–ESI-MS method [1] and 2) a MALDI-MS method [2], and 3) a MALDI-MS method for PNGase F released N-glycan profiling. The MALDI methods include a solid phase extraction in a pipet tip format for glycan or glycopeptide enrichment. Both hydrophilic interaction (2, 3) and reversed-phase stationary phases (3) are employed. Released glycans are also modified by ethyl esterification of sialic acid groups for stabilization and resolution of sialic acid linkage (α2-3 vs α2-6). Using Herceptin, an IgG1 therapeutic monoclonal antibody, these methods were compared to multiple chromatographic and spectrometric methods.

Ergebnisse und Diskussion

Overall, the glycosylation profiles obtained for Herceptin with the different methods were well comparable. For example, the value of the relative abundance of the main glycoform varied with a relative standard deviation below 5% and a maximum deviation of 8% between the 12 methods. Consequently, the biases within each method seem to be small. One important source of bias and a major distinctive factor between the methods is sensitivity. Of the three in-house methods, the released glycan method was the most sensitive. While we generally handled a 0.2% cut-off value (relative abundance), this method hinted at bisecting species at a level just below 0.1%. Though all three in-house methods require little hands-on time, the MALDI methods are more laborious, due to the SPE purification and sample spotting steps. However, the LC–MS method requires significantly more instrument time (13 min vs. 10 s) which gives the MALDI methods a cost advantage, especially for larger sample numbers.

The derivatization by ethyl esterification presents an efficient way to stabilize sialic acids and thereby avoid the fragmentation of sialic acid containing glycans which is otherwise observed in MALDI-TOF-MS with reflectron mass analysis. Consequently, sialic acid containing glycans are no longer underestimated by the signal loss resulting from the formation of metastable ions. In addition, the ethyl esterification is selective for α2-6 linked sialic acids. α2-3 linked sialic acids are also stabilized, but in the form of lactones. The resulting mass difference of 46.04 Da allows easy distinction without the need for fragmentation.

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Neue Aspekte

We present the results of an extensive method comparison for glycosylation analysis and a novel derivatization method for sialic acids.

PLK 17

Qualitative and Quantitative Investigation of Glyco-proteoforms from Prostate-specific antigen (PSA) in Healthy and Cancer Samples

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Qualitative and quantitative changes in glycosylation play a crucial role in medical and in biopharma applications. In the current approach, N-glycosylation changes of PSA, an important cancer biomarker with a high variety of glycan structures on a single *N*-glycosylation site, were investigated by mass spectrometry. This was part of a study organized by the ABRF gPRG group in 2013. Glycan identification was performed on the glycopeptide level, whereas the intact proteoforms were used for relative quantitation. This combined bottom-up and top-down intact protein approach provides a powerful tool for analyzing the glycosylation profile - the first for in-depth glycan structure elucidation and the second for elimination of digestion artifacts and reduction of glycan charge derived artifacts.

Experimenteller Teil

For the bottom-up approach, reduced, alkylated and tryptically digested PSA was separated by LC-MS/MS using ESI ion trap MS. Glycopeptide spectra were extracted from the LC-MS/MS dataset using a classifier algorithm and masses of the peptide moiety were automatically determined. Glycan database searches using the GlycoQuest search engine were performed. Glycan quantitation was carried out on intact PSA using an Ultrahigh Resolution (UHR)-ESI QTOF. Monoisotopic masses were derived from maximum entropy deconvoluted spectra.

Ergebnisse und Diskussion

More than 50 glycans were identified. Quantitation performed on intact PSA showed significant changes toward sialylated glycans in the cancer sample. For the proper quantitation, the mass areas for all previously identified glycans were compared: about 30% up-regulation in the cancer sample, and about 30% were down-regulation resulted. This result was in coincidence with quantitation results on released glycans of the identical PSA samples.

Neue Aspekte

Quantitation performed on intact PSA showed significant changes toward sialylated glycans in the cancer sample

PLK 18

Characterisation of a lectin from Trichosanthes dioica with high specificity towards glycoconjugates harbouring α2-6-linked sialic acid

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Lectins are a class of carbohydrate-binding proteins or glycoproteins that are ubiquitously found in nature. Among the plant lectins, leguminous lectins are characterised best but information on lectins from other families are rare. Thus, a seed lectin from *Trichosanthes dioica* (TDSL) belonging to the *Cucurbitaceae* family was studied comprehensively both with respect to structure and carbohydrate binding specificity. Amino acid sequence and posttranslational modifications such as glycosylation and formation of disulfide bridges of TDSL were determined by use of quadrupole time-of-flight mass spectrometry (Q-ToF-MS) and low energy collision-induced dissociation (CID). Binding characteristics of TDSL towards carbohydrates was probed by a combined approach using glycosphingolipids (GSLs) and glycoproteins as interaction partners.

Experimenteller Teil

For structural characterisation on the protein as well as on the glycan level, TDSL was enzymatically digested in-gel and in-solution by means of proteases with varying specificity and derived (glyco-)peptides were analysed by nanoESI Q-ToF-MS and low-energy CID experiments as described recently [1]. Binding to GSLs was investigated by thin-layer chromatography (TLC) overlay assays with GSLs from different sources. Glycoproteins comprising various types of *N*-glycans were separated by SDS-PAGE under reducing conditions and binding specificity of TDSL was analysed in Western blots using an antibody directed against xylose present in pauci-mannose type *N*-glycans [2].

Ergebnisse und Diskussion

Previous studies suggest that TDSL is a glycosylated lectin of 55 kDa comprised of 2 subunits of 24 and 37 kDa covalently linked by one or more disulfide bonds [3]. *De-novo* sequencing of peptides obtained by proteolytic cleavage of TDSL indicate high amino acid sequence homology with a galactose-specific lectin from the seeds of snake gourd (*Trichosanthes anguina*, SGSL) [1]. As in type II ribosome-inactivating proteins the molecule consists of a lectin chain and a catalytic chain which are joined through a disulfide linkage. Preliminary results obtained by mass spectrometric experiments suggest that the catalytic chain is comprised of two subunits which form a non-covalent complex similar to recent findings reported for SGSL [1]. Inspection of *N*-glycopeptide ions by collisional activation revealed the presence of pauci-mannose type *N*-glycans in both the lectin and the catalytic chain of the analysed protein. Previous data indicated a specific binding of TDSL towards galactose and/or *N*-acetylgalactosamine [3]. Here, we show that careful investigation of the carbohydrate-binding specificity of TDSL exhibits strong binding to gangliosides and glycoproteins comprising α2-6-linked sialic acid as well as weaker interaction with Galβ1-4GlcNAc present in neutral GSLs. Thus, TDSL has to be considered as a lectin with preferential binding specificity towards oligosaccharides harbouring terminally α2-6-linked sialic acid rather than a galactose/*N*-acetylgalactosamine specific lectin.

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Neue Aspekte

Structural characterisation and investigation of the carbohydrate-binding specificity of a lectin from the seeds of *Trichosanthes dioica*.

PLK 19

Gas-phase ion chemistry of oligosaccharide silver cation adducts

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Typically, electrospray ionization (ESI) of oligosaccharides yields protonated and sodiated analytes. Low-energy collision induced dissociation (CID) of these analyte species predominantly gives rise to B- and Y-type ions formed by cleavage of the glycosidic bonds. From these fragment ions information on the glycan sequence can be derived. However, especially if branched oligosaccharides are considered, B- and Y-type fragment ions are of little diagnostic value with respect to linkage sites. In the presence of silver ions CID gives rise to specific cross-ring cleavages due to stabilization of the glycosidic bonds, thus providing enhanced information on the molecular structure, i.e., branching sites. Here, we present the use of silver adducts as a means to elaborate the structure of lichen-derived oligosaccharides by nanoESI-CID.

Experimenteller Teil

Oligosaccharide samples were obtained by hydrolysis of lichen-derived polysaccharides and sample solutions were supplemented with silver ions if formation of argentized analyte molecules was desired. NanoESI-MS experiments were carried out using a quadrupole time-of-flight (QToF) mass spectrometer equipped with a Z-spray atmospheric pressure ion source (Micromass, Manchester, UK) in positive ion mode. A capillary voltage of 1100 V and a cone voltage of 40 V were applied. For CID experiments precursor ions were selected in the quadrupole analyzer and fragmented in the collision cell using Ar as a collision gas (2,5 x 10^{-5} mbar) and collision energies were adjusted to obtain sufficient fragmentation.

Ergebnisse und Diskussion

The mass spectra (MS1) obtained from silver-spiked analyte solutions differed significantly from those obtained from acidified samples. Intact ionized molecules were detected exclusively as the corresponding Ag^+ adducts, clearly recognizable by the isotopic pattern of silver ($^{107}Ag/^{109}Ag$, ~1:1).

Collisional activation of the precursor ions harboring silver ions yielded B- and C-type ions resulting from cleavage of the glycosidic bond. More interestingly, peaks of lower intensity were detected that clearly indicate the presence of A-type fragment ions which result from cross-ring cleavages. When protonated and sodiated analytes obtained from acidified sample solutions were submitted to CID fragmentation patterns significantly distinct from the previous were observed. Fragmentation of protonated precursors exclusively led to cleavages of the glycosidic bonds, whereas precursor ions comprising sodium yielded also ions resulting from cross-ring cleavages, though to a much lesser extent than the argentized precursors.

Therefore, we conclude that silver as a dopant enhances the mass spectrometric analysis of oligosaccharides, since Ag^+ obviously stabilizes the glycosidic bonds, thus allowing processes leading to cross-ring cleavages to compete with the simple rupture of glycosidic bonds.

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Neue Aspekte

Collisional activation of oligosaccharide silver adducts gives rise to cross-ring cleavages.

PLK 20

Improved enzymatic degradation of oversulfated glycosaminoglycans

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Glycosaminoglycans (GAGs) are natural polysaccharides which occur in many biological tissues [1]. There is also increasing evidence that oversulfated GAG derivatives are useful as implant coatings [2]. The detailed characterization of these polysaccharides is challenging: although mass spectrometry (MS) is a powerful tool to elucidate their structures, MS is not applicable to high mass polysaccharides, but characteristic oligosaccharides are needed. These oligosaccharides are normally generated by enzymatic degradation. Unfortunately, chemically modified (particularly sulfated) GAGs are refractive to digestion.

This study focuses on the digestibility of glycosaminoglycans with different degrees of sulfation by hyaluronidase. It will be shown by MALDI MS and ESI MS that all investigated GAG derivatives can be basically digested if the reaction conditions are carefully adjusted.

Experimenteller Teil

Solutions of native hyaluronan and three oversulfated hyaluronan derivatives with degrees of sulfation of 1.2, 1.8 or 3.0 [3,4] were digested with bovine testicular hyaluronidase. For the optimization of the digestion conditions, the sodium chloride concentrations and the pH values were varied.

The relative amounts of digestion products indicated by the Reissig signal (absorbance at 585 nm) were monitored by a colorimetric assay according to Muckenschnabel et al. [5] modified by Asteriou et al. 2001 and further adapted.

All MALDI-TOF mass spectra were acquired on an Autoflex™ mass spectrometer (Bruker Daltonics, Bremen, Germany) in the linear detection mode with saturated 9-aminoacridine in methanol as matrix. Negative ion ESI spectra were recorded on a Bruker Amazon SL device.

Ergebnisse und Diskussion

The hyaluronidase activity under standard conditions (37° C, pH 5.7, no additional NaCl added) depends significantly on the substrate and, thus, the degree of sulfation of the GAG: the digestion of the native (non-sulfated) HA results in the highest increase of the Reissig signal, which is a measure of the concentration of the reducing endgroups. The sulfated HA derivatives with ds_s = 1.2 and 1.8 are digested to comparable extents; however, only about 20 % of the effect that is detectable in the case of the native HA could be achieved. Considering the accuracy of the applied method, the very low absorbance detected in the case of the highly sulfated derivative (ds_s = 3) suggests that no significant degradation has taken place.

The generated (native and sulfated) hyaluronan oligosaccharides could be detected by MALDI-TOF MS. The linear mode in combination with negative ion detection (using 9-aminoacridine as matrix) gave optimum results. In the case of the sulfated HAs with ds_s = 1.2 and 1.8, however, only sulfated oligosaccharides (by MALDI and ESI MS) are detectable, whereby the tetra-, hexa- and octasaccharides with one to a maximum of three sulfate residues could be unequivocally identified.

The digestion of the HA with the highest extent of sulfation (ds_s = 3) was most challenging. Nevertheless, careful optimization of reaction conditions (particularly the sodium chloride concentration and the pH value) led to a fourfold increase of the Reissig signal and, thus, to a significant increase of the oligosaccharide yields. Under these optimized conditions, even the corresponding HA tetrasaccharide with up to six sulfate residues was successfully detectable by MS. Hopefully, these optimized conditions will enable the generation of larger amounts of GAG oligosaccharides with defined sulfation patterns because such compounds are not yet commercially available.

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Neue Aspekte

Oversulfated hyaluronan polysaccharides can be successfully digested by hyaluronidase and the released oligosaccharides characterized by MALDI and ESI MS.

PLK 21

Characterization of pronase E preparations for biopharmaceutical glycosylation analysis

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The digestion of glycoproteins with the enzyme preparation pronase E is an established method for the generation of N- and O-glycopeptides.[1] The analysis of pronase-generated glycopeptides by LC-ESI-MS and MALDI MS workflows has been described numerous times [2,3,4]. However, very little attention has been paid so far to the nature and ratio of the ingredients of pronase mixtures. As pronase is an enzyme mixture isolated from the extracellular fluid of *Streptomyces griseus*, its composition may underlie natural variation. Here, we present the analysis of intact proteins from different vendors' pronase preparations via mass-spectrometry based methods. Furthermore, the reproducibility of proteolytic digestion using pronase mixtures is examined.

Experimenteller Teil

Pronase preparations were purchased from Roche and Sigma Aldrich. Furthermore, two different batches of pronase from Roche were compared. Intact protein analysis was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (ABSciex) with sDHB as matrix or employing a microTOF-QII ESI-QqTOF mass spectrometer (Bruker Daltonics). For ESI MS analysis, proteins were first separated and desalted on a micro/nano HPLC system (SunChrom GmbH) equipped with a 250 µm x 200 mm RP-C4 column packed in-house. Pronase-generated N- and O-glycopeptides from different biopharmaceuticals were analyzed in positive and negative ion mode using a MALDI LTQ Orbitrap XL mass spectrometer (Thermo Scientific) and DHB as matrix.

Ergebnisse und Diskussion

Several enzymes known to be ingredients of pronase mixtures could be confirmed by intact LC-ESI-MS as well as MALDI MS analysis. The examined pronase preparations were very similar in composition and in results of glycoprotein digestion. Although SDS-PAGE analysis of incompletely digested glycoproteins revealed some differences in digestion efficiency, glycopeptide patterns were comparable. Pronase digestion also showed to be very reproducible and insensitive against small changes in digestion conditions such as digestion temperature or enzyme-to-substrate ratio. To sum up, the composition of pronase preparations seems to be consistent between different batches and vendors. Therefore, pronase digestion can be employed for a reproducible analysis of biopharmaceutical glycosylation.

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Neue Aspekte

Investigation of pronase enzyme mixture composition and digestion reproducibility

PLK 22

Acetonitrile boosted nanoLCMS of N-glycans and its applications in biopharmaceutical development

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N-glycosylation is an important post-translational modification influencing the function, efficacy, safety and structure of therapeutic proteins, especially of monoclonal antibodies[1]. For the comprehensive analysis of these complex and heterogenic structures sensitive and robust methods are necessary. NanoLCMS has proven its use for N-glycan analysis in several investigations especially after derivatization of N-glycans with frequently used fluorescence labels like 2-AA or 2-AB [2]. Especially during early biopharmaceutical development when the sample amount is rather limited N-glycan characterization is difficult. We have therefore developed a high-throughput sample preparation for the purification of monoclonal antibodies from cell culture supernatant and subsequent N-glycan processing and reversed phase NanoLCMS analysis. The use of acetonitrile saturated nebulizer gas thereby increases the sensitivity drastically.

Experimenteller Teil

96-Well Protein G based affinity purification of mAbs from cell culture/ harvest broth. On-column deglycosylation of immobilized mAb.

Purification and 2-AA labeling with use of ultrafiltration and reductive amination.

96-well based gel-filtration of 2-AA glycans to remove excess label

NanoLCMS using reversed phase chromatography and ion-trap mass spectrometry using a recently marketed nano spray. nanoESI spray is source is flushed with acetonitrile saturated nitrogen to enhance ionization.

Ergebnisse und Diskussion

The sample preparation is highly reproducible and efficient enabling the purification of less than 1 µg mAb in cell culture supernatant or harvest broth. On-column deglycosylation using PNGaseF resulted in high N-glycan yields.

The analytical method encompassing RP nanoLC and on-line ion-trap mass spectrometry was qualified in terms of linearity, sensitivity, robustness and reproducibility. MS sensitivity was in the attomolar range for a single N-glycan on column and the method is linear over five orders of magnitude.

In an exemplary study N-glycans from different clones of an IgG Fc part fusion protein were analyzed with the developed approach and compared against the glycan maps of purified drug substance demonstrating the suitability in early development.

It is further shown that the use of acetonitrile saturated nitrogen which is flushed into the nano spray chamber increases the MS intensity and signal to noise of N-glycans drastically.

The results demonstrate the use of the developed method for high sensitivity N-glycan characterization in early biopharmaceutical development. However, the method is not limited to this application, it can also be used for other analytical questions where sample amount is very limited but high sensitivity demanded (e.g. glycan biomarker discovery).

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Neue Aspekte

High-throughput sample preparation (on-column PNGaseF digest, small scale gel filtration). RP NanoLCMS of 2-AA N-glycans. ACN boosted nanoESI increases sensitivity.

PLK 23

Adding chemistry as a tool for selective analysis to analyze heavy crude oil sample

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The demand for affordable energy drives people to continue to focus on fossil-based materials including crude oil from unconventional resources. [1] Although only a small percentage of non-hydrocarbon compounds (such as N-, S-, and O- compounds) are present in crude oil, they are among the most deleterious to refining catalysts and confer adverse stability properties. Crude oil is the most complex natural mixture available which overall characterization represents a challenge to any analytical system. [2] The combination adding selective organic reactions to ultrahigh resolution and high accuracy mass spectrometry (Fourier transform ion cyclotron mass spectrometry, FT-ICR MS) is a successful approach to determine the distribution of these classes and provide selectivity for the analysis of crude oil.

Experimenteller Teil

A heavy Bitumen was dissolved in dry 1, 2-dichloroethane (DCE) and was reacted with a series of different amount of derivatization reagents in the presence and absence of silver tetrafluoroborate ($AgBF_4$) for 24 h. The precipitate was removed by centrifugation and washed with dichloromethane.

Mass spectra were recorded using a 7 T FT-ICR MS (Thermo Fisher Scientific, Bremen) equipped with an ESI source. External mass calibration was performed using a tune mix solution.

Ergebnisse und Diskussion

As known, electrospray ionization (ESI) allow analyzing polar compounds; nevertheless, most of components of crude oil are non- or less-polar. Here, we introduced the use of selective chemical reactions to crude oil analysis by studying the derivatization of crudes. This approach allows to analyze the non- and less-polar components (such as hydrocarbon-, Oxygen-, and sulfur-species) under positive ESI conditions, but also to selectively tag the polar compounds (such as nitrogen-species) by the derivatization agent.

Different solvents were screened and dry DCE proved to be the best choice of solvent for the reaction. Before derivatization, bitumen separately dissolved in wet and dry DCE were introduced into FT-ICR MS under ESI positive mode. Polar N- and NS-species were observed only in wet DCE where nitrogen compounds were protonated, but no signals were detected when using dry DCE.

Different derivatization reagents were tested and different levels of selectivity were obtained according to the reaction parameters. In the absence of $AgBF_4$, as the increase of chemical reactivity of ethylated reagents, nitrogen-species which are not dominant decrease until they disappear. For example, with ethyl bromide as derivatization reagent, S-, S_2-, N-, NS-, OS_2- and CxHy-species were observed. With ethyl iodide, S-, S_2-, N-, NS- and OS_2- were observed; but only S-, S_2- and OS_2- showed up with ethyl triflate. While in allylation process of bitumen, for example, nitrogen compounds are dominant compounds with allyl chloride as derivatization.

At the presence of $AgBF_4$, nitrogen compounds totally disappear but oxygen compounds are observed, and the high selectivity of sulfur- and CxHy-species was realized.

These results show that it is possible to selectively characterize the classes of compounds in crude oil by adding a chemical reaction to the analysis scheme. The FT-ICR MS allow following the details of these complex reactions.

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Neue Aspekte

Combining chemical reactions with FT-ICR MS analysis is used to selectively analyzing crude oil.

PLK 24

Ion mobility mass spectrometry (IM-MS) separation and detection of oxidized lipid-peptide adducts

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Lipid peroxidation derived carbonyl compounds, (oxoLPPs) are strong electrophiles and thus highly reactive towards nucleophilic residues in proteins. The resulting Michael and Schiff base adducts formed between α,β-unsaturated aldehydes (e.g. 4-hydroxy-2-nonenal) and proteins were intensively studied during last decades. On the contrary, only few studies described the modification of proteins and peptides with phospholipid-bound carbonyls (PL-oxoLPPs). Such analyses are challenging for conventional LC-MS/MS due to the mixed chemical nature of the compounds, their low abundance in biological samples and presence of multiple isobaric adducts. Here we report for the first time the successful combination ion mobility (IM) and MS for gas phase separation and identification of PL-oxoLPP-peptide adducts.

Experimenteller Teil

1-Palmitoyl-2-(9-oxo-nonanoyl)-*sn*-glycerophosphatidylcholine (PNPC, 1 mmol/L) was incubated in aqueous ammonium bicarbonate (1.5 mmol/L, pH 7.4) with peptide Ac-FSHVE**K_6**LGNNPVS**K_{14}**-OH (1 mmol/L) for 2 h at 37°C. Samples were stored at -20°C and thawed shortly before infused in static nanoESI on a Synapt G2-S^{TM} (Waters, Manchester). The Synapt combines an orthogonal quadrupol-ToF analyzer with a lossless travelling wave ion mobility separator (TWIMS). Spectra were acquired with a ramped TWIMS wave velocity of 1200-300 m/sec and nitrogen pressure of 3 mbar in the ion mobility cell, and over a mass range of 50-2000 *m/z* in the ToF mass analyzer. Data mining was done using DriftScope^{TM} software (Waters, Manchester).

Ergebnisse und Diskussion

The reaction mixturesof PNPC and peptides were separated by IM-MS on SynaptG2-S into several major "trend-lines" representing unreacted peptide, PNPC and PNPC-peptide adducts. Thus ions corresponding to peptide-lipid adducts were easily isolated and analyzed by MS and MS/MS. The primary amine of PNPC allows the formation of Schiff-bases due to the absence of a double bond in α-position. When incubated with a peptide containing two lysine residues, the mixture of singly and doubly PNPC-modified peptides was observed. IMS separated the isomeric mono-PNPC-adducts in three signals allowing to identify the lipid modification site at Lys_6 and Lys_{14}. Interestingly, the Schiff-base product of PNPC-K_6 was detected as two separated signals reflecting most probably different orientations of the hydrophobic lipid along the peptide backbone (N- or C-terminal orientation). This observation was further confirmed by detecting two different IM signals of the doubly-modified PNPC-adducts. Thus, IM-MS allowed separation of different classes of compounds (peptide vs lipid vs peptide-lipid) and various structural conformers within a single peptide-lipid adduct, driven most probably by the orientation of the hydrophobic lipid along the peptide backbone.

Neue Aspekte

IM-MS was for the first time successfully applied to separate and identify oxidized lipid-peptide adducts.

PLK 25

Mass spectrometry and fluorescence detection of carbonylated lipid peroxidation products after TLC separation

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Lipid peroxidation products (LPP) generated during oxidative stress *in vivo* are recognized as important biomarkers of several human disorders, such as metabolic syndrome, obesity and diabetes. The different physicochemical properties of low and high molecular LPP as well as their low contents in complex biological matrices challenge their detection and quantification. Recently, we introduced a new technique to simultaneously profile both low (volatile) and high molecular weight carbonylated products obtained by phospholipid oxidation using derivatization with 7-(diethylamino)coumarin-3-carbohydrazide (CHH)[1]. For complex biological samples, however, it is necessary to separate the mixtures before MS analysis. Here we show that thin layer chromatography(TLC) can separate CHH-derivatized LPP allowing their fluorescence-based quantification on the plate followed by MS identification of the extracted compounds.

Experimenteller Teil

Different phospholipids (PL), low molecular weight aldehydes (e.g. hydroxy-alkenals and alkanals) and lipid extracts from Cu/ascorbic acid oxidized human plasma samples were analyzed by TLC before and after CHH derivatization. Lipid extracts were loaded on HPTLC silica gel 60 plate and separated with a mixture of chloroform, methanol, water and triethylamine (eluent A; 28/33/6/33, v/v). CHH-derivatized lipids and low molecular weight aldehydes were chromatographed in dichloromethane/acetonitrile (9/1, v/v). CHH-derivatized compounds were detected first by their fluorescence before all lipids were visualized with primuline (0.5% in acetone/water, 8/2, v/v). Selected bands were scratched off, lipids were extracted (0.9% NaCl/methanol/chloroform, 1/1/1, v/v/v) and identified using data dependent acquisition on ESI-LTQ-Orbitrap.

Ergebnisse und Diskussion

Eluent A allowed the separation, identification and relative quantification of phospholipids, cholesterol and cholesteryl-esters in human plasma samples before and after oxidation (Cu/ascorbic acid). CHH-derivatization prior to lipid extraction allowed the simultaneous analysis of low and high molecular weight lipid-bound carbonylated LPPs. The overlay of the CHH-specific fluorescence (carbonylated LPP detection) and primuline stain (PL and cholesterol detection) of a single TLC-plate allowed the relative quantification of all compounds of a given product class. Furthermore, high resolution Orbitrap MS and tandem MS/MS in the linear ion trap allowed the identification of PL-bound carbonylated compounds (e.g. 1-hexadecanoyl-2-(hydroxy-keto-octadecadienoyl)-sn-glycero-3-phosphoethanolamine), hydroxy-alkenals (e.g. hydroxy-nonenal, andhydroxy-hexanal) and different alkanals (e.g. hexanal, nonenal, decanal). Additionally, carbonylated oxysterols and cholesteryl-esters were detected. All CHH-derivatized carbonylated LPP were confirmed by the specific reporter ion at *m/z* 244 detected after CID fragmentation. Thus fluorescence and MS allows monitoring plasma lipid oxidation. The combination of NP-HPTLC and ESI-LTQ-Orbitrap-MS provides a powerful technique to separate and identify lipid oxidation products with high accuracy in biological samples.

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Neue Aspekte

Carbonyl specific derivatization, TLC separation, fluorescence quantification and MS identification allows profiling oxidized lipidomes in complex biological samples.

PLK 26

Quantification of sugar-related intermediates of in vitro glycation systems by GC-MS

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Thermal processing of food induces the Maillard reaction in which carbohydrates react with free amino groups of peptides and proteins. The resulting early glycation products (Amadori compounds) can undergo further oxidative and non-oxidative degradation accompanied with formation of various carbohydrate and dicarbonyl intermediates yielding advanced glycation end products (AGEs), which may represent biomarkers of diabetes, Alzheimer's disease and ageing. Though the complex steps of glycation reactions and sugar degradation were intensively studied in recent decades, the pathways and mechanisms of particular AGE formation are still not completely characterized. As glucose degradation products are important intermediates in the Maillard reaction, their identification and quantification could provide more details about the reaction mechanisms.

Experimenteller Teil

Cooking conditions were simulated by incubating mixtures of *D*-glucose (25 mmol/L) and synthetic peptide (Ac-AFGSARASGA-NH_2, 0.5 mmol/L) at 95 °C for up to 2 h in 100 mmol/L phosphate buffer containing 18 µmol/L $FeSO_4$. As a control, *D*-glucose was incubated in absence of peptide. Quantification of carbohydrates was achieved by GC-EI-sector field MS. Phosphate was removed by reversed-phase/anion exchange solid-phase extraction (RP-AX-SPE), carbohydrates were derivatized with methoxyamine hydrochloride (MOA)/*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), separated by GC and quantified by the corresponding extracted ion chromatograms (XICs). The external calibration relied on carbohydrate mixtures spiked to incubation solution (100 mmol/L phosphate buffer containing 18 µmol/L $FeSO_4$ and 0.5 mmol/L peptide).

Ergebnisse und Diskussion

GC-MS allowed simultaneous quantification of twenty carbohydrates in glycation mixtures, which together with efficient two-step derivatization procedure (conversion into oximes followed by silylation) provided satisfactory sensitivity and linearity. Thereby, the presence of peptide in the analyzed samples did not influence the sensitivity. In contrast, phosphate present in the incubation mixtures resulted in considerable disturbance of the analyses. To overcome this, RP-AX-SPE was applied to remove the phosphate that was achieved with > 99% efficiency and high precision (RSD < 10 %) for quantification of carbohydrates in the reaction mixtures. After removing phosphate by RP-AX-SPE from the samples and subsequent derivatization, at least 7 sugars (prospective glycation agents and further Maillard reaction intermediates) were identified and quantified by integration of corresponding XICs. Thus, our approach allows the parallel analysis of carbohydrates and peptide Maillard reaction products in the presence of phosphate buffers and iron (II) salts. Its application to the analysis of carbohydrates formed during *in vitro* glycation could provide better insights into the mechanistic aspects of the Maillard reaction and the pathways of AGE formation.

Neue Aspekte

GC-MS-based method for simultaneous quantification of twenty carbohydrates in glycation and glycoxidation systems.

PO: Poster Organische MS

PO 1

Comparison between stable-isotope (deuterium) labeling and radiolabeling (14C) for the elucidation of the in vitro metabolic pattern

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The elucidation of the *in vitro* metabolism is an essential and safety relevant investigation during the development of a pharmaceutical drug. The analytical procedure consists of two steps: (i) finding the metabolites in the complex matrix and (ii) elucidating the metabolite's molecular structure. The standard technique to spot metabolites is the use of a radiolabeled drug. Due to the inherent drawbacks of techniques using radioisotopes, as an alternative stable-isotope (deuterium) labeling and LC-MS with recognition of the specific isotopic pattern was applied. To demonstrate the equivalence of both approaches, a set of *in vitro* drug metabolism studies were performed once with the classical radio-approach and once with stable-isotope labeling for a detailed comparison.

Experimenteller Teil

A set of commercially available pharmaceutical compounds was selected and their non-, stable- (deuterium) and radio- (^{14}C) labeled form was purchased.

For each of these compounds incubations were performed with

- Rat microsomes, 1µg/mL and 10µg/mL up to 90 min
- Rat hepatocytes, 1µg/mL and 10µg/mL up to 20 hours

For the two approaches, the isotope mixtures were:

- Radioisotope approach: non-labeled / ^{14}C labeled drug ~1:0.3
- Stable-isotope approach: non-labeled / deuterated drug 1:1.

For both incubation types several control incubations, control samples and interim time-points were also investigated.

Ergebnisse und Diskussion

For each of the compounds, the *in vitro* metabolism was elucidated in detail first using the stable-isotope approach with structure elucidation of all spotted metabolites. In a second step the *in vitro* metabolic pattern was elucidated using the radioisotope-approach including LC-offline-radiodetection and MS. Finally, both results were compared and the found metabolic patterns were aligned between the two approaches. As a consequence, for all individual studies (all compounds incubated with either rat microsomes or rat hepatocytes) the equivalent conclusions could be extracted:

All metabolites which were found with the radioisotope-approach could also be found with the stable-isotope approach. Additionally, selected metabolites could only be seen with the stable-isotope approach. Overall, both approaches show individual advantages and disadvantages. The radioisotope approach offers quantitative data and indicates non-covered metabolites eluting during the chromatographic break-through, the stable-isotope approach offers direct access to the m/z value of the metabolites, which is often challenging with the radio-approach.

Based on the results discussed here and a large set of proprietary in-house studies, it can be concluded that the stable-isotope approach is a highly valuable tool for the elucidation of the drug metabolism during early *in vitro* metabolism studies.

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Neue Aspekte

Stable isotope labeling offers a valuable alternative to radiolabeling for early *in vitro* metabolism investigations

PO 2

Comprehensive toxicology screening by ion trap MSn using the Toxtyper® workflow

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Screening solutions in clinical and forensic toxicology require automated, robust and specific technologies overcoming some of the well known issues of current GC-MS, LC-UV and immunoassay solutions. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) can reveal more valuable information than LC-UV detection while covering a broader and complementary range of analytes when compared to GC-MS. In addition LC-MS/MS is far more specific compared to immunoassays. The use of comprehensive library searching tools combined with LC-MS/MS is regarded as the future benchmark for toxicology screening solutions. This study describes the development and challenging of a robust and easy-to-use ion trap based LC-MS^n solution (Toxtyper) for the detection and identification of common drugs, drugs of abuse and their metabolites in the shortest time possible.

Experimenteller Teil

Sample preparation for serum or urine extraction was performed according to a liquid-liquid extraction (LLE) protocol with the goal to remove matrix compounds. For chromatographic separation of compounds a fast reversed phase LC separation (11 min full duty cycle) was used. Based on a scheduled precursor list approach an amaZon speed ion trap MS system was used to generate MS, MS^2 and MS^3 data. Acquired data were matched against a spectral library currently containing around 850 toxicology relevant compounds taking retention time and MS^n into account. Data evaluation and reporting was carried out by an automated spectral library search algorithm.

Ergebnisse und Diskussion

In order to challenge the new Toxtyper MS^n based screening solution several blank serum samples were spiked with a set of common drugs (e.g. antidepressants, benzodiazepines and hypnotics). The blinded samples were automatically processed and analyzed by 7 independent Toxtyper amaZon speed systems at 5 different locations. The automated screening approach revealed a high level of reproducibility and correct identifications. The overall transferability of this workflow between different laboratories has been proved successfully. In particular, Bruker's SmartFrag technology ramps the fragmentation voltage ensuring an ultimate level of reproducibility during compound fragmentation of different amaZon LC-MSn systems. In conclusion the presented screening method offers a fast and reliable routine identification tool for clinical and forensic analysis.

Neue Aspekte

Automated and fast LC-MS screening solution for toxicological compounds based on MS^n library approach.

PO 3

Quantification of Immunosuppressants Drugs by LC-MS/MS ion trap analysis with a new smart MRM mode

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LC-MS technologies are playing an increasing role in Therapeutic Drug Monitoring (TDM) due to the high demand for fast method development and high throughput in the clinical environment. Immunosuppressive drugs reduce or inhibit the activity of the immune system and are used to prevent the rejection of transplanted organs and tissues or to treat autoimmune diseases. As most of these drugs are non-selective they cause a general decrease in the immune defence which means a higher risk of infections and spread of malignant cells. Because of the narrow therapeutic range, e.g. for Tacrolimus (4-15 µg/L) in blood, therapeutic drug monitoring (TDM) is essential in immunosuppressive therapy to maintain the efficacy of the immunosuppressant drugs while minimizing their toxicity.

Experimenteller Teil

After protein precipitation (Methanol, $ZnSO_4$) from whole blood a fast 3.5 min UHPLC gradient on a Hypersil Gold C8 column (1.9 µm, 2.1 x 150 mm) was used for the separation of four different immunosuppressants (Tacrolimus, Sirolimus, Everolimus, Cyclosporin A) plus three internal standards (Ascomycin, Cyclosporin A and D). For the MS/MS analysis an amaZon speed ion trap MS system was used. Quantification was carried out by the new smart Multiple Reaction Mode (sMRM) using the most abundant fragment ions. Qualification was performed by library search of the full scan MS/MS spectra against a homemade library.

Ergebnisse und Diskussion

This work describes the use of the amaZon speed equipped with a new sMRM mode for quantification of immunosuppressants. The sMRM mode allows an easy and quick method set-up for multiple MS/MS events. It considers retention time, MS/MS parameters and allows for positive/negative switching even on a UHPLC time scale. The LOQs that are required for quantification of the four drugs (down to 1 ng/ml) were reached easily with good signal-to-noise values. Calibration curves reveal excellent correlation coefficients ($R > 0.999$) for all compounds and cover the required therapeutic concentration range. For the quality control samples that were prepared using the same methods as for real patient samples accuracies of more than 85% were reached. In addition to the pure quantitative data the identity of the targets was confirmed via library search using full scan MS/MS spectra. The ion trap specific features allow for the generation of quantitative and qualitative results in one run.

Neue Aspekte

Novel smart MRM mode for quantitative ion trap applications in combination with library search for verification of compound identity.

PO 4

MALDI-MS for quantification of Doxorubicin in plasma – reduced contamination risk with cytostatic drugs

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Cytostatic drugs are highly effective substances used in cancer therapy. People are working with these compounds in research and analytic have to keep in mind that these drugs are harmful because they interfere with the metabolic processes in human organism. The cytostatic Doxorubicin is an anthracycline derivative that intercalates into DNA and inhibits the progression of the enzyme topoisomerase-II which relaxes supercoils in DNA for transcription.

Here we evaluated a MALDI-MS method for rapid quantification of Doxorubicin under reduced risks. The method can be used in different analytical areas, such as: dissolution testing in pharmaceutical technology, but also in all areas of medical and environmental diagnostics. The method entirely can keep up with published ESI methods with respect to the quantification limit.

Experimenteller Teil

Method evaluation is performed in samples from calf plasma as matrix, using the internal standard method. Internal standard is Daunorubicin, which carries one hydroxyl group fewer than Doxorubicin. The plasma was precipitated with pure acetonitrile and then centrifuged. The supernatant was taken and mixed with matrix solution - alpha-cyano-4-hydroxycinnamic acid (CHCA; 2 mg/mL). The MALDI measurements were carried out on an Orbitrap LTQ (Thermo Scientific, Bremen) in the negative ion mode. 50 spectra were acquired for each spot. A comparison of an existing HPLC method with fluorescence detection and the new MALDI-MS method for a timeline of dissolution tests was performed.

Ergebnisse und Diskussion

The method was validated following the criteria set out by the FDA. LLOQ, linearity, recovery, inter-day, intraday, precision and all stability tests are under the acceptance criteria. All measurements comply with the guidelines of the FDA. The lower limit of quantification (LLOQ) is 10 ng/mL, which is competitive to any published results for Doxorubicin. Compared to the HPLC method with a fluorescence detector, the MALDI method is more sensitive and even faster in a daily routine.

Our method has two advantages: First of all, it is faster than any existing method because the measurement takes only several minutes. Secondly, compared to ESI-MS the contamination risk is severely reduced. Immediately after generation of the harmful sample it is embedded in the matrix, it can be analyzed by mass spectrometry without spraying at atmospheric pressure. It can be transported and stored for further measurements without risk.

Neue Aspekte

MALDI-MS quantify cytostatics from plasma and to determine the concentration out of dissolution tests.

PO 5

Analysis of Isoprenoid Pathway Metabolites by LC-MS

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Isoprenoid pathway metabolites such as the isoprenoidphosphates and isoprenoid-pyrophosphates are central metabolites leading to sterols, dolichols, ubiquinones, prenylated natural products and proteins. The development of new methodologies for the preparation as well as the separation and quantitation of pure phosphorylated isoprenoid metabolites is key for detailed investigations of these pathways on a molecular level. It is therefore of much interest to develop new high-performance separation methods which are able to determine the whole range of isoprenoid-(pyro)phosphates.

Experimenteller Teil

The analysis of a whole range of synthetic metabolites of isoprenoid pathways by LC-MS methods will be presented.

Ergebnisse und Diskussion

New HLPC-MS methods for the simultaneous analysis of the stereoisomeric dimethylallyl- and isopentenyl-(pyro)phosphates respectively have been established using the cyclo-dextrine-based stationary phase Supelco Cyclobond 2000 and a buffer/acetonitrile eluent in HILIC mode. Whereas the separation of these polar single unit isoprenoid-(pyro)-phosphates is based on selective ionic interactions on a cyclodextrin-based stationary phase, the separation of the larger isoprenoid-(pyro)phosphates has been achieved with IPC-UHPLC-MS using ion-pair chromatography on a Supelco Ascentis Express C8 column and dihexylamine acetate. The resolution of this IPC has been improved significantly with Fused Core particles.

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Neue Aspekte

This method turned out to be very versatile and applicable for a large range of other phosphorylated and polar metabolites.

PO 6

25-Hydroxyvitamin D Serumkonzentrationen gesunder Erwachsener bei KarMeN, einer Metabolomics-Studie mit Focus Ernährung

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Bei der KarMeN-Studie (Karlsruhe Metabolomics and Nutrition) wurde der Einfluss von Ernährung und körperlicher Aktivität auf das Metabolom untersucht. Die 312 gesunden Probanden wurden ausführlich charakterisiert (Anamnese, Körperzusammensetzung, klinische Parameter, Ernährung, körperliche Aktivität, Fitness). Die Metabolom-Analytik in Plasma und Urin erfolgte über NMR, GCxGC sowie targeted LC-MS. Lebensmittelverzehr und Nährstoffaufnahme wurden mit zwei 24-h-Recalls im Abstand mehrerer Wochen geschätzt. Für Vitamin D ist damit aber keine Beurteilung der Versorgung möglich, da der größte Teil in der Haut durch UV-Licht gebildet wird und nur ein kleiner Teil durch die Nahrung aufgenommen wird. Aufgrund der zentralen Bedeutung im Stoffwechsel (Knochen, Immunabwehr) ist es wesentlich den Versorgungsstatus von Vitamin D im Rahmen von KarMeN durch eine direkte, quantitative Bestimmung im Blut zu erfassen.

Experimenteller Teil

Vitamin D_3 wird im Körper gebildet oder stammt aus tierischen Quellen, *Vitamin D_2* stammt aus Pflanzen. Anerkannter Parameter für den Versorgungsstatus ist die Speicherform *25-Hydroxyvitamin D* (*25OH-D_2/D_3*) im Serum. Die Bedeutung isomerer Formen ist unklar, es gibt aber Hinweise, dass die *3-Epimere* teilweise physiologisch wirksam sind. Im Rahmen der KarMeN-Studie wurde daher eine Methode zur Bestimmung von *25OH-D_2/D_3* sowie *epi-25OH-D_3* im Serum mit LC-MS etabliert.

Methode: UPLC-MS/MS (Acquity H-Class + TQD, Waters), PFP-Säule 150x2.1mm (Acquity HSS PFP, Waters), Methanol-Gradient, ESI positiv, MRM 2 Übergänge je Analyt

Probenvorbereitung: Proteinfällung (Methanol/ZnSO4) plus Flüssig-Extraktion (Hexan)

Kalibration und Kontrollen: matrixadaptiert, gespiktes Humanplasma (Chromsystems), deuterierter interner Standard (*d_6-25OH-D_3*)

Ergebnisse und Diskussion

Das hormonell wirkende Vitamin D ist ein essentieller Mikronährstoff, der in den letzten Jahren zunehmend in den Focus gerückt ist und bei dem eine Unterversorgung in der Bevölkerung diskutiert wird. Ein Mangel beeinflusst den Calcium-Stoffwechsel und ist mit gesundheitlichen Risiken assoziiert, z.B. Störungen des Knochenmineralisierung (Osteomalazie) und erhöhte Infektanfälligkeit. Für einen Zusammenhang mit systemischen Erkrankungen (Krebs, Diabetes Typ II, Herz-Kreislauf) gibt es Hinweise, aber bisher keine ausreichende Evidenz.

Mit der etablierten Methode können die einzelnen Epimere von *25OH-D* ab 5 nmol/L sicher erfasst werden, der lineare Bereich geht bis 72 nmol/L, für *25OH-D_3* bis 166 nmol/L. Die Präzision der Kontrollen liegt bei 5-7% (Serie, Tag-zu-Tag), der Bias bei <3% (Richtigkeit) und die analytische Wiederfindung inklusive Probenvorbereitung bei 72-81%.

Die Bestimmung von *25-OH-D* ermöglichte die direkte Überprüfung des Versorgungstatus der Probanden und erlaubt im Rahmen der Studie wertvolle Rückschlüsse auf mögliche Zusammenhänge zwischen Vitamin-D-Serumkonzentrationen, metabolischem Status, Ernährung sowie körperlicher Aktivität. Bei *25OH-D_3* liegt der Median mit 51.1 nmol/L knapp über dem DGE-Referenzwert von 50 nmol/L, 47% der Werte liegen unterhalb. Es zeigte sich keine signifikante Korrelation mit Alter oder Geschlecht. Für *25OH-D_2* wurden nur in 21 Proben (7%) Werte oberhalb der Bestimmungsgrenze gefunden, für *epi-25OH-D_3* in 47 Proben (15%). In diesen 47 Proben korreliert *epi-25OH-D_3* mit *25OH-D_3* ($p < 0.0001$). Der erwartete saisonale Einfluss (Sonneneinstrahlung) zeigt bei *25OH-D_3* einen deutlichen Sinus-Verlauf und kann durch eine rechnerische Kompensation eliminiert werden. Dies ist wichtig um mögliche Einflüsse von Vitamin D auf andere Stoffwechsel-Parameter erkennen zu können, die ansonsten von der jahreszeitlichen Schwankung verdeckt werden könnten. Mittelwert und Median sind nach der jahreszeitlichen Kompensation nahezu unverändert, die Streuung der Daten wird etwas geringer. Im Rahmen weiterer Auswertungen ist geplant mögliche Zusammenhänge mit anderen KarMeN-Parametern aus den Bereichen Ernährung, körperliche Aktivität, Gesundheit und Metaboliten-Profilen zu untersuchen, jeweils mit und ohne Korrektur des jahreszeitlichen Verlaufs.

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Neue Aspekte

Erfassung der Vitamin-D-Versorgung eines gesunden Studienkollektivs in Abhängigkeit von Ernährungsstatus, körperlicher Aktivität und Metaboliten-Profil.

PO 7

Rapid mining of the metabolic diversity of antibiotic compounds produced by symbiotic Streptomyces bacteria using Compound Discoverer software

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The role of antibiotic compounds produced by bacteria in the natural environment is largely unknown. This includes the purpose of production, the timing, the diversity of compounds, and the evolution of resistance against antibiotics [1]. A valuable model system to study the role of antibiotics in nature is the symbiosis of *Streptomyces* bacteria with ground-nesting digger wasps of the genus *Philanthus* [2]. Symbiont-produced antimicrobial compounds protect the larva and pupa of the wasp from fungal infestation [3]. Here, we extracted the antibiotic compounds from the natural environment, i.e. the wasp cocoons and subjected it to LC-HR/AM MS/MS analysis. We aimed to fully characterize the diversity of the antimicrobial compounds present in the natural environment using the novel Compound Discoverer software.

Experimenteller Teil

Wasp cocoons were taken from a laboratory population and extracted with methanol. Extracts were subjected to LC-MS analysis and HR/AM data were acquired on a Q Exactive mass spectrometer using a TopN method (full scan and ten data-dependent HCD scans triggered on the most intense ions). Several approaches were undertaken to identify derivatives of the two main antibiotic components (streptochlorin and piericidin A1): derivatives of the chlorine containing streptchlorin were screened for using the characteristic chlorine isotope pattern from the fullscan. Derivatives of piericidin were identified using predicted modifications and therefore mass shifts in the full scan. In addition, piericidin derivatives were identified in the HCD scans using predicted MS2 fragments (Fragment Ion Search FISh).

Ergebnisse und Diskussion

Several search strategies within the Compound Discoverer software were employed to mine the data acquired on the Q Exactive mass spectrometer for derivatives of the two antibiotics known from the symbiotic *Streptomyces* bacteria. First, the full scan data were screened for the characteristic chlorine isotope pattern of streptochlorin (“Isotope Ratio Tracer”). This feature not only revealed the streptochlorin peak, but also a peak representing the pentenylstreptochlorin, a derivative not known so far from this symbiosis. Derivatives of the piericidin were identified employing several complementary search strategies. First, piericidin analogues were screened using predicted modifications and, therefore, predicted mass shifts in the full scan (“Metabolite Generator”). This search revealed about 15 compounds resulting from one or several methylation, oxidation, desaturation or glucoside conjugation steps. Very much congruent to the results from the “Metabolite Generator”, piericidin analogues were identified using predicted MS2 fragments (“FISh Tracer”). In this search mode, the parent structure is subjected to artificial fragmentation. This search assumes that derivatives of the same parent structure share characteristic MS2 fragments and marks the calculated fragments in all HCD scans. This search mode confirmed the piericidin analogues identified with the “Metabolite Generator” and therefore, makes the results more reliable.

The presented workflow will allow for a rapid screening of antibiotic compounds produced in different symbiont species. This could help to understand the role of antibiotic compounds in nature and may reveal useful insights for the current antibiotic crisis.

This work represents a proof of principle study to demonstrate the customizable workflow of the Compound Discoverer software and its value to identify compounds originating from a known parent structure. The Q Exactive mass spectrometer and Compound Discoverer software are a well suited platform to screen for derivatives of known precursors like the identification of metabolites, degradation products or synthesis byproducts.

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Neue Aspekte

This study presents new software to identify substances stemming from a parent compound from LC-MS data using various search strategies.

PO 8

HPTLC-ESI-MS zur Bestimmung von Anthocyanen in Futtermitteln und Lebensmitteln

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Anthocyane werden als Farbstoff E 163 verschiedensten Lebensmitteln zugesetzt. Außerdem werden Trester anthocyanhaltiger Früchte als Futtermittelzusatz verwendet. Für die Anthocyan-Analytik werden heutzutage überwiegend RP-HPLC-Methoden mit UV/Vis/DAD/MS-Detektion eingesetzt. Diese Methoden sind meist zeitaufwändig und erfordern eine aufwändige Probenaufarbeitung. Daher sollte eine schnelle und matrix-robuste HPTLC-Methode entwickelt werden, die einen hohen Probendurchsatz erlaubt.

Die neu entwickelte und validierte Trennmethode für 11 Anthoycyane eignete sich gut zur Quantifizierung der wichtigsten Anthocyane in Futter- und Lebensmitteln [1]. Saft- und Weinproben dergleichen Pflanze zeigten ein vergleichbares Anthocyanin-Muster, wohingegen sich dieses zwischen Pflanzenspezies charakteristisch unterschied. Die Bioaktivität (*Aliivibrio fischeri*) und Radikalfänger-Eigenschaften (DPPH•) der einzelnen Anthocyane wurden simultan bestimmt. Unbekannte Anthocyan-Zonen wurden nach Elution mittels TLC-MS-Interface durch Aufnahme von Massenspektren (HPTLC-ESI$^+$-MS) weitergehend identifiziert.

Experimenteller Teil

Anthocyanine in 21 Proben wurden auf HPTLC-Platten Kieselgel 60 mit Ethylacetat – 2-Butanon – Ameisensäure – Wasser (7:3:1.2:0.8) in 20 min getrennt. Waren im oberen hR_F-Bereich Anthocyanidine erkennbar, wurde nach Abschneiden der unteren Plattenhälfte der obere Plattenteil mit Ethylacetat – Toluen – Ameisensäure – Wasser (19:3:1.2:0.8) 13 min entwickelt. Die Absorptionsmessung (Mehrwellenlängenscan) erfolgte bei 505, 520, 530 und 555 nm.

Radikalfänger-Eigenschaften wurden durch Tauchen der entwickelten Platte in methanolische DPPH•-Lösung bestimmt. Antioxidative Zonen waren als helle Zonen auf violettem Hintergrund zu erkennen.

Die Bioaktivität wurde mittels *Aliivibrio fischeri* Leuchtbakterien-Bioassay bestimmt. Entwickelte Platten wurden in die Bakterien-Suspension getaucht [2] und die Lumineszenz-Differenz in der jeweiligen Zone gegenüber dem Plattenhintergrund bestimmt.

Unbekannte Zonen wurden mittels TLC-MS-Interface von der Platte eluiert und direkt mittels ESI$^+$-MS analysiert.

Ergebnisse und Diskussion

Die erhaltenen Chromatogramme wurden dokumentiert und mittels Mehrwellenlängenscan nach Bestimmung der jeweiligen substanzspezifischen Absorptionsmaxima quantitativ ausgewertet. Dabei zeigte die Methode gute Validierungskenndaten mit Korrelationskoeffizienten ≥ 0.9993, Vergleichspräzision von 1.4 % bzw. 1.8 %, Laborpräzision von ≤ 6.7 % und Robustheit von ≤ 5.5 %, sowie niedrige Bestimmungsgrenzen (≤ 100 ng/Zone).

Die Detektionsgrenze (LOD) für die Bestimmung der Radikalfänger-Eigenschaften der einzelnen Anthocyane mittels DPPH•-Reagenz lag zwischen 10 und 80 ng/Zone.

Die Bestimmung der Bioaktivität mittels marinen *Aliivibrio fischeri* – erkennbar durch Lumineszenzminderung/-erhöhung im Vergleich zum Plattenhintergrund – zeigte für Anthocyanidine vergleichbare Detektierbarkeiten zum DPPH•-Reagenz. Für einige Anthocyanine hingegen waren Werte im unteren µg/Zone-Bereich nötig.

Nach quantitativer Auswertung der Anthocyane wurden Aglykone von unbekannten Zonen einfach und schnell mittels HPTLC-ESI$^+$-MS ermittelt. In einem Tollkirschenextakt konnten auf diese Weise zwei unbekannte Zonen als Cyanidin- und Petunidin-Derivate identifiziert werden. [1]

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Neue Aspekte

Quantifizierung von 11 Anthocyanen in Futter-/Lebensmitteln. *In situ* Bestimmung der Bioaktivität und Radikalfänger-Eigenschaften der Anthocyane. Identifizierung unbekannter Anthocyan-Zonen durch HPTLC-ESI$^+$-MS.

PO 9

Fast Quantitative Determination of Methylphenidate in Rat Plasma and Brain ex vivo by MALDI MS/MS

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Methylphenidate (MPH) is known to block the pre-synaptic reuptake of dopamine and noradrenalin in order to reduce symptoms of attention deficit hyperactivity disorder (ADHD), which affects 4-10 % of children world-wide.[1] The therapeutic mechanism of the drug is not yet fully understood. It is not known how the therapeutically relevant blood plasma levels of MPH in rats translates to brain levels of the drug. This study presents a simple and sensitive high-throughput method for *ex vivo* quantification of MPH in rat plasma and brain based on matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI MS/MS).

Experimenteller Teil

All MALDI MS and MS/MS analysis were performed with a 4800 MALDI TOF/TOF analyzer (Applied Biosystems, CA, USA), equipped with a 200 Hz ND:YAG laser (355 nm). Spectra were automatically acquired using positive reflector mode with fixed laser intensity. At least 1250 shots per spectrum were accumulated. The MALDI spotting is based on an optimized dried droplet preparation protocol using the common MALDI matrix alpha-cyano-4-hydroxycinnamic acid (CHCA).

Method development was done on plasma and brain samples spiked with MPH in different concentrations. The internal standard methylphenidate-d_9 hydrochloride was used for quantification. The method was validated according to FDA guidelines and afterwards plasma and brain of rats pre-treated with MPH were analyzed.[2]

Ergebnisse und Diskussion

The complex question relating to MPH distribution in plasma and brain places enormous demands on instrumental analysis. A highly sensitive and robust analytical method is needed for the analysis of MPH in these differing environments. Over the last decade, a broad range of methods has been developed, with mainly LC-ESI setups as analytical tool.[3] MALDI poses an alternative to these common LC-ESI methods. Due to the high salt and buffer tolerance of MALDI, an LC separation step can be omitted. In addition, very little sample is needed with only a small amount being ablated during measurements. Because of the simple sample preparation based on liquid-liquid extraction (LLE) and MALDI measurements requiring approximately 10 s per sample, analysis time can be significantly reduced. Interferences by matrix background, which often hamper the analysis of small molecules, are circumvented by using MS/MS. By this means, MPH was quantified within a concentration range of 0.1-40 ng/mL in plasma and 0.4-40 ng/mL in brain homogenate with an excellent linearity ($R2 \geq 0.9997$) and good precision. Method validation was carried out following the criteria set out by the FDA, and included the following parameters: selectivity, recovery, linearity, lower limit of quantification (LLOQ), intra-day accuracy, inter-day accuracy, precision (% RSD), short term stability and freeze/thaw stability. The intra- and inter-day accuracies fulfilled the FDA's ± 15/20 criteria. The recovery of MPH ranged from 93.8-98.5% and 87.2-99.8% in plasma and homogenate, respectively. We show that MPH is successfully quantified in spiked plasma and brain and also in plasma and brain of pre-treated rats.

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Neue Aspekte

Rapid quantification of Methylphenidate in plasma and brain of rats by MALDI MS/MS is shown.

PO 10

High-Throughput screening and sensitive quantitation of carbendazim down to the low ppb range in wine and orange juice by UHPLC-QqQ

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Carbendazim is a broad-spectrum fungicide commonly employed in Brazil to combat black spot mold, yet illegally in the United States. However, it was detected in imported Brazilian orange juice products in the US in 2011. In 2012, the same residue was found in wine from Chinese origin. This has raised the effort of regulatory agencies to check food supplies for carbendazim Multiple-reaction monitoring (MRM) using LC-triple quadrupole mass spectrometry has been recognized as an easy and sensitive assay for pesticide residue analysis in complex matrix. In the current work, a MRM assay developed on a novel UHPLLC-MS/MS system is used for rapid screening and quantitation of carbendazim in orange juice and wine samples.

Experimenteller Teil

Carbendazim and carbendazim-d3 (IS) standards were dissolved in MeOH to prepare two 100 ppm stock solutions. 1 mL organic orange juice and wine samples were diluted 50x by water and filtered respectively to serve as blank matrices. The carbendazim stocks were diluted in the matrix to prepare calibration solutions in a range between 0.02 ppb and 100 ppb. The final IS concentration in each carbendazim matrix solution was 1.5 ppb. The UHPLC (Bruker Advance) run time was 6 min and the MRM transitions (Bruker EVOQ triple quad) for carbendazim and IS have been optimized for most sensitive quantitation as follow: carbendazim 192 > 160 (18eV), 192 > 132 (30eV); carbendazim-d3 195 > 160 (18eV). Carbendazim eluted at ca. 3 min.

Ergebnisse und Diskussion

First, an evaluation study was carried out for the dilution effect on the matrix interference. At the 50-time dilution, the matrix effect is minimized and does not have any effect on the sensitivity anymore. The lowest sensitivity level of 0.02 ppb can be easily achieved. Software was applied for auto-MRM transition development. Under direct sample infusion, the software picked the specified target masses or automatically scanned the available most intense ions in the mass spectrum for product ion scans. The collision energy (CE) was automatically ramped in Q2 to find the optimized value for each MRM transition. The optimized MRM parameters including transitions and CE values were automatically saved for later use. At the matrix dilution factor of 50, carbendazim was quantified within a dynamic range of 1 ppb to 5 ppm (> 3 orders of magnitude) in orange juice and wine , sufficient for real-life routine food testing. More method validation such as reproducibility test will be presented.

Neue Aspekte

A high-throughput, sensitive MRM assay with simple sample preparation for carbendazim quantitation in juice and wine

PO 11

Rapid Wine Profiling: Exploring an Ambient Ionization MS Metabolomics Approach coupled with Extraction Techniques

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Fingerprinting and profiling of wines using mass spectrometric (MS) approaches are fundamental for authentication and characterization of wines. A robust analytical approach combining ambient ionization with tandem high-resolution mass spectrometry was developed to rapidly identify marker compounds and classify varietal species. Wine fingerprinting can be assessed with this method by directly measuring the wines without any sample pre-treatment to distinguish between red and white wines.

Experimenteller Teil

Selective wine profiling using phenolic characterization was carried out by a targeted liquid-liquid extraction employing ethyl acetate to further determine the varietal grape species used in the production of red and white wines. The liquid extracts were subjected to both high resolution mass spectrometry (HRMS) LC-MS (Information Dependent Acquisition (IDA)) and Direct Analysis in Real Time (DART) MS analysis generating individual profiles for over 150 wine samples.

Ergebnisse und Diskussion

Comparing the DART-MS approach with the traditional LC-MS method, increased sample throughput was achieved and alternatively direct coupling of DART with an automated solvent-free extraction employing solid phase micro-extraction (SPME) sorbents, including polystyrene-divinylbenzene-polyacrylonitrile (PS-DVB-PAN), C18-polyacrylonitrile (C-18-PAN), phenylboronic acid-polyacrylonitrile (PBA-PAN) and a mixed-mode phase (C18+ benzenesulfonic acid cation exchanger) a direct wine profiling approach was developed [1]. The SPME ambient MS workflow demonstrates a novel approach for wine analysis with rapid all-in-one analyte extraction and desorption yielding complementary selective profiles as compared to liquid-liquid extraction. Data interpretation was handled using principal component analysis (PCA), generating scores plots clearly differentiating several red and white wine varieties.

Acknowledgements: The Bundesinstitut für Risikobewertung (BfR) is kindly acknowledged for providing wine samples and PAS Technology Deutschland GmbH for the loan of the Concept 96 SPME automation system.

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Neue Aspekte

Rapid MS sample analysis employing ambient ionization SPME coupled with statistical data processing for a metabolomics approach to wine characterization.

PO 12

Better sensitivity in LC-MS for sample from biological fluids by superior sample preparation with zirconia-coated silica

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Protein precipitation is a widely accepted and applied sample preparation method for biological samples such as plasma or serum due to its simplicity and gross level removal of proteins. Phospholipids are also present in high concentrations in biological fluids. Phospholipids are known to be a major cause of ion-suppression in LC-MS leading to irreproducible results and less sensitivity.

Experimenteller Teil

In the experimental section we describe new procedures for the analysis of strong acids, strong bases and hydrophobic compounds using zirconia-coated silica.

Ergebnisse und Diskussion

HybridSPE-Phospholipid technology merges the simplicity of protein precipitation and selectivity of SPE leading to higher sensitivity. MeOH/salt is an alternative protein crashing method (if ACN/FA does not work; works in 80 %) leading to improved recovery for difficult compounds.

Referenzen

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Neue Aspekte

New methods for strongly acidic, strongly basic or very hydrophobic compounds using zirconia-coated silica.

PO 13

Anion exchange solid-phase extraction towards selective removal of phosphate in presence of organic acids for GCMS analysis

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In biological matrices, most of the intermediary metabolites are present in very low concentrations causing major problems in their quantitative analysis. Deleterious effects on signal response due to matrix interferences affect the sensitivity, reproducibility and accuracy of the assay. It has been reported earlier that highly abundant compounds in biological extracts such as phosphate in human cancer cells cause severe problems in quantitation using GC-MS [1]. We anticipated to selectively remove phosphate and focused in particular the separation from other organic acids. For this, anion exchange sorbent materials for solid phase extraction (SPE) were screened to selectively retain phosphate based on differential (de-)protonation dissociation constants of selected target compounds in aqueous solution

Experimenteller Teil

The sample was prepared as a mix of the 13 different analytes at a concentration of 50 µM in 1.5 mM phosphate buffer. The SPE protocol was optimized depending on the pH modifications (2.4 and 3.8) and Lewis base strength with sodium fluoride. After SPE, the eluates were dried and treated with methoxyamine hydrochloride/ pyridine and MSTFA before GCMS analysis. Silica-based and polymer-based SPE columns were tested for their abilities of selective phosphate removal. Metal precipitation was compared as a traditional approach. Experimental parameters like solvent activity, temperature, pH, and change of elution volumes were also investigated to achieve optimal conditions for selective phosphate removal.

Ergebnisse und Diskussion

Polymer-based columns appeared the best compromise between efficient phosphate removal and quantitative recoveries of organic acids and sugars compared to the silica-based columns. While silica-based columns effectively removed the phosphate, they did not give satisfactory recoveries of the target analytes mainly due to their highly polar nature of scaffold as well as presence of a positively charged tertiary amine functional group tethered to its surface. We suggest the higher retention of the target analytes, the organic acids and phosphate, through their interactions with the functional groups present on the surface of the column silica scaffold.

Among the protocols tested, polar organic solvents such as methanol proved to be the best choice. Organic acids and sugars show relatively high miscibility in organic solvents like methanol. Further, high elutropic strength and low viscosity of methanol might benefit the quantitative recoveries of organic acids. In addition, this observation suggests the involvement of reversed-phase interactions in the SPE process.

Metal salts not only precipitated phosphate to the extent of biological acceptability, but they also resulted in poor quantitative recoveries of organic acids and sugars, most likely due to precipitation upon interaction with metal salts. Poor quantitative recoveries after treating the samples with metal salts could also be the result of poor derivatisation of the analytes post precipitation. In either cases, unacceptable losses of analytes were encountered which impacted the absolute quantitative outcomes.

Among various other parameters, lower temperature, pH and lower elution volumes were found to be the optimum conditions suitable to achieve best recoveries for organic acids and sugars. In conclusion, using solid phase extraction we developed an optimized protocol achieving the selective depletion of phosphate with acceptable quantitative recoveries of the target analytes.

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Neue Aspekte

Selective removal of phosphate as a frequent, often highly abundant compound in biological matrices and bioassays

PO 14

Improving LC-MS detection limits and sensitivity by dedicated solvents and additives

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In recent years both high efficiency HPLC columns (such as Fused-Core®) and new UHPLC-MS systems enabled chromatographic separations with decreased limits of detection. This advantage may also become a disadvantage when even the smallest amounts of impurities in the solvent or additives cause background noise and unwanted peaks.

Experimenteller Teil

A comparison of LC-MS Ultra with other gradient grade solvents is shown.

Ergebnisse und Diskussion

New LC-MS Ultra CHROMASOLV solvents are especially tested for UHPLC-MS providing lowest drift, noise and background in UV and MS detection in both ESI(+) and ESI(-) mode. This ensures lowest limits of detection and reliable results, even when applying steep gradients.

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Neue Aspekte

A new generation of high-purity solvents for enhanced sensitivity in LC-MS is presented.

PO 15

Rapid and Simple Approaches to Multi-residue Pesticide Analysis by both GC-MS/MS and LC-MS/MS

Qingyu Sun[2], Stephan Kühne[1], Zicheng Yang[2], Kefei Wang[2]

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Tandem mass spectrometry coupled to chromatography, such as GC-MS/MS and LC-MS/MS, operated in MRM mode, is the method of choice for targeted screening of multi-residues analysis in complex food matrix samples. However, improving sample preparation for multi-class multi-pesticide analysis still presents a challenge, and MRM method development, including setting up the MRM acquisition table and data processing, is complicated and time consuming.

This study has two main goals: first, a new sample preparation method is evaluated as an alternative to the conventional QuEChER) for extracting pesticides from vegetable and fruit matrix; second, a new software approach of Compound Based Screening (CBS) was introduced to simplify MRM method development on both GC-MS/MS and LC-MS/MS instruments.

Experimenteller Teil

The vegetable and fruit samples were extracted following the procedure recently developed at US FDA Irvine. 10 g of finely chopped sample was mixed with 10 mL of acetone, and poured into a tube containing a mixture of C18, PSA, MgSO4, Fructose and NaCl. After the mix, 1 mL of petroleum either was added. After centrifuge, the top organic layer (acetone with petroleum either) was taken for GC-MS/MS analysis directly or reconstituted in initial mobile phase for LC-MS/MS analysis. The GC-MS/MS was carried out on Scion 436 GC coupled to a TQ model triple quadrupole mass spectrometer, the LC-MS/MS on an EVOQ Elite model triple quadrupole mass spectrometer equipped with an Advance model UHPLC.

Ergebnisse und Diskussion

The sample extraction method was applied for more than analyzing more than 200 pesticides for LC-MS/MS analysis. The sample preparation was very simple and less expensive compared with the traditional QuEChER methods. The final solution in acetone makes it suitable for direct analysis by GC-MS/MS and easy for evaporation to be reconstituted in LC mobile phase.

The method development for both LC-MS/MS and GC-MS/MS analysis employed a unique compound based screening (CBS) approach involving using a MRM library (different for GCMS and LCMS) and automatic optimization of scan (dwell) time for each pesticide residue. For GC-MS/MS analysis, retention index of pesticides from the MRM library can be used to predicate retention time ranges of the target residues, shortening the method development time. The acquisition method is dynamically linked to the processing method, simplifying the data processing. Pesticide analysis in real vegetable/fruit matrix showed the limit of quantitation (LOQ) down to low and sub ppb range, and the results are reproducible, demonstrating sensitive and robust GC-MS/MS and LC-MS/MS systems.

Neue Aspekte

Unified and simpler extraction method for sample preparation, compound based screening (CBS) simplifies multi-residue MRM method development.

PO 16

Studies of 1,2-unsaturated derivatives of (1-4) linked disaccharides and their 2-C-functionalized analogs by positive ESI-MSMS

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The synthesis of highly functionalized carbohydrate *C*-analogs is of current interest for glycochemistry.[1)] The 1,2- unsaturated derivatives of β(1-4) linked disaccharides are versatile building blocks for the synthesis of biologically active compounds.[2)]

We investigated the use of electrospray ionization (ESI) combined with tandem MSMS in positive mode for the differentiation of some hexa-acetyl / hexa-benzyl-D-lactals,- and - maltals and for their corresponding 2*C*-branched malonates.[3)]

The influence of the malonate substituents at the *gluco* ring on the fragmentation under ESI-CID was investigated. The disaccharides derivatives consisted of four pairs of stereo isomers. The ESI- MSMS spectra of these stereo isomers were analyzed to make stereo chemical conclusions.

Experimenteller Teil

The mass spectra were obtained in positive ion mode using an ESI-Q-TOF mass spectrometer (Micromass Manchester, UK). All samples were injected (15µL/min) with a Harvard syringe pump. The capillary voltage was set to 3.2 kV. The desolvation temperature was 180°C. For MS/MS argon was used as collision gas.

The adduct ions with lithium were selected and activated in CID mode by increasing the collision energies (5-75eV) up to the energy where the relative abundance of 50% for the selected ions were found. The elemental compositions of principal fragment ions were confirmed by accurate mass measurements with standard deviations less than 3 ppm.

Ergebnisse und Diskussion

All the MSMS spectra of the lithium $[M+Li]^+$ adducts of the disaccharides showed the typical cross - ring cleavages and the glycosidic bond cleavages.[4)] The unsaturated carbohydrates **1-4** exhibit B,- and C-ions as the preferred products from the glycosidic bond cleavages. The corresponding disaccharides with a methoxy group in position C_1 and malonate group in position C_2 show additionally the Y,- and Z-ions. The peracetylated lactals **1** and **5** give these ions with higher intensities than for the maltals (**2** and **6**). In contrast the benzylated compounds show the opposite behaviour; even ions with higher intensities for the maltal derivatives **4** and **8** than for the lactals **3** and **7** were detected. As expected for the acetylated compounds **1**, **2**, **5** and **6** peaks are due to the loss of the substituents $[M-AcOH]^+$ and $[M-2xAcOH]^+$ and $[M-OBn]^+$ for the benzylated compounds **3** and **4** were found. The major fragmentation observed in the CID spectra of **3**, **4**, **7** and **8** seems to be the concerted two bond cross ring cleavage to give the ions m/z 261 as the base peaks. The fragments for the typically loss of the methoxy group neighbouring to the ring oxygen were not detected for compound **5** and **6** and for **7** and **8** only with very low intensities RA < 5%. Furthermore the mass spectra of compounds **3**, **4** and **7**, **8** show abundant *retro*-Diels-Alder (RDA) reaction products. Finally it can be concluded, that the ESI CID spectra of the $[M+Li]^+$ ions of the glycal disaccharide analogs provide information about their fragmentational behaviour and may allow to differentiate these (1-4) linked unsaturated disaccharides and 2-*C* branched analogs.

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Neue Aspekte

Characterization of the fragmentation behaviour and differentiation of 1,2-unsaturated and functionalized carbohydrate *C*-analogs with ESI- MSMS of their $[M+Li]^+$ ions

PO 17

Negativ-Ionen DART-FT-ICR-MS hoch fluorierter Massenstandards und deren Einsatzmöglichkeiten zur Kalibrierung über einen weiten m/z-Bereich

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Direct Analysis in Real Time (DART) [1] hat inzwischen vielfältige Anwendungen, die durchweg zuverlässiger und leicht durchführbarer Massenkalibrierung bedürfen; ganz besonders, wenn Summenformeln mittels exakter Masse bestimmt werden sollen [2, 3]. Hier ist das für negativ-Ionen DART an einem Fourier-Transform Ionencyclotronresonanz (FT-ICR) Massenspektrometer realisiert. Es wird eine Methode beschrieben, die auf einer kommerziellen Tuning-Lösung (Agilent ES Tunemix) beruht und eine negativ-Ionen Kalibrierung über einen sehr weiten *m/z*-Bereich ermöglicht. Der Tunemix besteht aus Ammoniumtrifluoracetat, Betain, 2,4,6-Tris(heptafluorpropyl)-1,3,5-triazin und symmetrischen Hexakis-(fluoralkoxy)-phosphazenen [4]. Mit 2% (v/v) Trifluoressigsäure liefert er negative Ionen von *m/z* 69 bis *m/z* 2834. Basierend auf dieser Kalibrierung werden weitere hoch fluorierte Massenstandards mit DART-MS untersucht; nicht zuletzt zur Validierung der Kalibriermethode. Außerdem werden die experimentellen Bedingungen und eine Referenzmasssenliste angeführt.

Experimenteller Teil

Die DART-Spektren wurden an einem Bruker ApexQe FT-ICR-Massenspektrometer (Bruker Daltonik, Bremen) mit ESI-MALDI-Kombiquelle aufgenommen. Daran wurde eine DART-SVP-Quelle (IonSense, Saugus) mit OpenSource verwendet, die über eine zusätzliche Pumpstufe (VapurInterface) am ESI-Interface montiert wurde. Je 2–5 µl der Proben wurden in Lösung bei 0.1–1 mg ml^{-1} auf die Drahtgitter der Open Source-Karten aufgetragen. Helium wurde als DART-Gas bei 150–300°C eingesetzt. Die Ionen wurden im RF-only Hexapol (h2) für 0.2–1.5 s akkumuliert und dann in der ICR-Zelle analysiert. Es wurden 4–12 Transienten für ein Breitband-Spektrum mit 512 kB oder 1 MB Datensätzen akkumuliert. Die externe Massenkalibrierung wurde mit Agilent ES Tunemix erstellt. Die Gerätesteuerung erfolgte mit Bruker ApexControl, die Auswertung mit Data Analysis.

Ergebnisse und Diskussion

Das negativ-Ionen DART-Spektrum von Agilent ES Tunemix zeigt neben Trifluoracetat, $[C_2F_3O_2]^-$ (TFA^-), nominell *m/z* 113, und $[TFA_2–H]^-$ Cluster-Ionen bei *m/z* 227, nur noch Signale von $[M+TFA]^-$ Ionen der Hexakis-(fluoralkoxy)-phosphazene bei *m/z* 734, 1034, 1634, 2234 und 2834. Signale von 2,4,6-Tris(heptafluorpropyl)-1,3,5-triazin oder Betain werden nicht gefunden. Setzt man jedoch 2,4,6-Tris(heptafluorpropyl)-1,3,5-triazin als einzelne Substanz ein, wird ein DART-Spektrum mit Molekül-Radikalanion und Adduktionen erhalten, allerdings immer von relativ geringer Intensität. Ebenso wenig führt das in der ESI-MS [5] etablierte Ultramark 1621 oder etwa Ultramark 2500F (Fomblin) zu intensiven Spektren. Ultramark 1621 lässt sich aber mit HTFA zur Bildung von $[M+TFA]^-$ Ionen bringen. Anders verhält es sich mit Perfluortributylamin (FC43 oder auch PFTBA) und Perfluorkerosin (PFK), die sich beide als für die Kalibrierung in negativ-Ionen DART-MS ungeeignet erweisen. Prinzipiell gut eignet sich dagegen Perfluornonansäure; jedoch gibt es aktuell keine käufliche Mischung dieser Verbindungsklasse.

Daher wurde eine Massenkalibrierung mit den relativ weit auseinanderliegenden Peaks des ES Tunemix entwickelt, die sich in unserem Labor bereits bewährt hat. Die Referenzmassenliste ist auch elektronisch erhältlich.

Die DART-Spektren der basierend auf ES Tunemix-Kalibrierung untersuchten hoch fluorierten Standards belegen aber die Tauglichkeit der vorgestellten Methode, auch wenn sie sich selbst nicht für einer derartige Anwendung eignen.

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Neue Aspekte

Weitbereichskalibrierung und Massenrekord von *m/z* 2834 für negativ-DART-FT-ICR-MS.

PO 18

Abspaltung von atomarem Stickstoff aus geradelektronischen Ionen? Eine Untersuchung an Benzodiazepinen

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Das Auftreten von Fragmentierungen mit Δm = 14 gilt gemeinhin als unmöglich und ist bislang nur in seltenen Fällen schlüssig dokumentiert [1,2]; hierbei handelt es sich um Fragmentierungen ungeradelektronischer Ionen. Bei der Untersuchung einiger Benzodiazepine mit ESI-MS/MS wurde ein Fragmention mit Δm =14 aus dem protonierten Molekülion beobachtet; HRMS-Messungen ergaben, dass dies dem Verlust von atomarem Stickstoff entspricht. Es wurde vermutet, dass hier unter Ringverengung eine Extrusion des N-Atoms aus dem Diazepin-Ring stattfindet (Ringverengungen unter Abspaltung von Neutralteilchen aus Heterozyklen unter ESI-MS/MS-Bedingungen sind in der Literatur bekannt [3,4]). In dieser Untersuchung sollten die vorliegenden Ergebnisse verifiziert und erweitert werden.

Experimenteller Teil

Alle Messungen wurden an einem Bruker Esquire-LC Ionenfallen-Massenspektrometer durchgeführt. Die Proben wurden in Methanol gelöst ($c \approx 10^{-5}$ mol/l) und mit einer Spritzenpumpe (Cole-Parmer Mod. 74900) bei einer Flussrate von 3 µl/min zugeführt. Die Ionenquellen-Parameter waren: *capillary voltage* 4 kV, *nebulizer gas* 5 psi, *dry gas* 5 ml/min, *dry gas temp.* 300°C. Als *nebulizer/dry gas* wurde entweder Stickstoff oder Druckluft verwendet. MS/MS-Experimente wurden mit He als Stoßgas durchgeführt.

Ergebnisse und Diskussion

ESI-MSn-Untersuchungen von verschiedenen Benzodiazepinen mit einem Ionenfallen-MS zeigten, dass das $[M+H-14]^+$-Ion nur bei NO_2-substituierten Benzodiazepinen auftrat. Hauptfragment in den MS/MS-Spektren dieser Verbindungen ist $[M+H-NO_2]^+$. Isolierung dieses Ions aus dem MS^2-Spektrum führte zum Auftreten eines um 32 u schwereren Adduktions, dessen Masse also nominal dem $[M+H-14]^+$ entspricht. Die Vermutung, dass es sich hierbei um die Anlagerung von O_2 im Analysator handelt, wird gestützt durch ein Experiment, bei dem der O_2-Partialdruck im Restgas in der Ionenfalle erhöht wurde (durch Verwendung von Druckluft statt Stickstoff als *nebulizer/dry gas*); dies führte zu einer deutlichen Erhöhung des $[M+H-NO_2+32]^+$-Signals. Die Fragmentierung dieses Ions führte darüber hinaus zu demselben Fragmentspektrum wie die direkte Fragmentierung des $[M+H-14]^+$.

Dies legt den Schluss nahe, dass das $[M+H-14]^+$ nicht durch die Abspaltung von atomarem Stickstoff unter Kontraktion des Diazepinrings entsteht, sondern es sich in Wirklichkeit um ein $[M+H-NO_2+O_2]^+$ handelt. Weitere Untersuchungen müssen zeigen, ob dieses Verhalten auch bei anderen NO_2-substituierten Verbindungen beobachtet werden kann, und inwieweit der radikalische Charakter des $[M+H-NO_2]^+$ die Anlagerung des Diradikals O_2 ermöglicht bzw. begünstigt. Dementsprechend wäre zu prüfen, ob auch andere Radikalkationen in der Ionenfalle ein ähnliches Verhalten zeigen.

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Neue Aspekte

Anlagerung von O_2 an radikalische Fragmentionen unter ESI-MS/MS-Bedingungen

PO 19

Determination of chemical composition changes in crude oil after ozonation by using ultrahigh resolution mass spectrometry

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Ozonation is an effective method of petroleum physicochemical properties regulation. It can be used in many fields of petroleum industry from crude oil production to waste water treatment. Nevertheless the mechanism of ozone reaction with petroleum compounds and the reaction products are not really studied. As petroleum is a very complex mixture and ozonation is not a selective process because it can produce thousands of different products. Application of techniques such as IR-spectroscopy can only provide general information about the reaction. Using ultrahigh resolution mass spectrometry gives a chance to go deeper into chemical changes of the petroleum composition and finally to have a better understanding of the ozonation process.

Experimenteller Teil

Three different crudes (10 ml volume) were ozonated for 30 minutes under the same experimental conditions (ozone-oxygen mixture flow rate of 100 ml/min, ozone concentration 90 mg/l).

Ultrahigh resolution FT-ICR MS (LTQ-FT; Thermo Fisher Scientific) was applied to investigate chemical composition of crudes before and after ozonation. Atmospheric pressure photon ionization (APPI), electrospray ionization in both positive and negative (ESI(+), ESI(-)) modes were applied for analysis in m/z range 150-1200. Each sample was measured using a resolution power 400 000 (@*m/z* 400). The mass spectra were externally and internally calibrated. The data were then analyzed using a combination of XCalibur (Thermo Scientific) and Composer software packages (Sierra Analytics, Modesto,California, U.S.A.).

Ergebnisse und Diskussion

The results show that drastic changes occur during the ozonation of crude oil samples. While these mixtures are already extremely complex the reaction still can be followed. The data reveal that a wide range of oxygen containing species O_x (x=1-6), O_xN (x=1-6), O_xS_y (x=1-6, y=1-2) is being formed during the ozonation. The most abundant of them are O_x (x =2-5) species, which involve acids, ethers, ketones and other combined functionalities. Additionally, sulfur containing classes are easily being oxidized. O_xS classes (x =3-5) which corresponds to sulfonic acids and their derivatives are present in higher intensities.

One side effect of the reaction is that after ozonation the DBE values tend to become lower as well as the molecular mass of the products. NO2S and NOS species found in crude are seem to degrade as in two out of three crudes these comonents disappear after the ozonation process. In general, the first indication is that heteroatom containing compounds in crude oil seem to react easier to ozonation than pure hydrocarbons as it is supposed to be in theory.

Neue Aspekte

Using ultrahigh resolution mass spectrometry to get a better understanding of the petroleum ozonation mechanism and the reaction products.

PO 20

Laserspektroskopische Untersuchungen an heterosubstituierten Halogenbenzolen mittels REMPI- und MATI-Spektroskopie

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Ionenspektroskopie ist eine essentielle Methode zur Untersuchung der vibronischen Struktur von molekularen ionischen Spezies und angeregten Neutralen. Theoretische Konzepte zur Kopplung von elektronischen und Vibrationszuständen lassen sich durch spektroskopische Untersuchungen vibronischer Zustände validieren. Die vibronische Struktur des ersten angeregten Zustands (S_1) wurde mit Hilfe der Resonance Enhanced Multi Photon Ionization (REMPI) Spektroskopie untersucht, der ionische Grundzustand (D_0) mit Hilfe der Mass-Analyzed-Threshold Ionization Spektroskopie. Bei der Analyse der REMPI-Spektren richtet sich ein besonderer Fokus auf starke Aktivitäten formal symmetrieverbotener Moden und möglicher Schwingungsprogressionen und Kombinationsbanden. In den MATI-Spektren wird besonders auf Abweichungen von der $\Delta v=0$ Vorzugsregel [1] geachtet. Das Ziel der aktuellen Untersuchungen ist es, den Einfluss der Fluorsubstitution in Halogenbenzolen zu analysieren. Die experimentellen Ergebnisse werden durch quantenchemische Rechnungen gestützt.

Experimenteller Teil

Das Experiment besteht aus einem mit einstufiger Ionenquelle und Reflektron ausgerüstetem Time of Flight (ToF)-Massenspektrometer und zwei separat (Nd:YAG) gepumpten, durchstimmbaren Farbstofflasern. Die Dye-Wellenlänge beider Laser wird in einem BBO-I frequenzverdoppelt. Der Aufbau lässt somit Zwei-Farben-Experimente zu. Ein Überschall Molekularstrahl aus Probenmolekülen und Seed-Gas (Argon) wird durch ein gepulstes Jet-Ventil in die Ionenquelle expandiert. Im REMPI-Modus werden die gebildeten Photoionen direkt in das ToF beschleunigt. Im MATI-Modus hingegen wird im Anschluss an die Laserionisation ein schwaches elektrisches Feld angelegt, das die prompt gebildeten Ionen von den Rydberg-Neutralen diskriminiert. Schließlich wird ein Hochspannungspuls angelegt, der die Rydberg-Neutralen ionisiert. Die nach ihrem m/z-Verhältnis separierten Ionen werden durch einen MCP-Detektor erfasst. Das Ionensignal wird von einem digitalen Oszilloskop ausgelesen.

Ergebnisse und Diskussion

Die oben genannten Untersuchungen lieferten sehr genaue Werte für die elektronische Anregungsenergie (AE) und Ionisierungsenergie (IE) von 1,2-Dichlor-4-fluorbenzol, 1,3-Dichlor-2-fluorbenzol und 1,2-Dichlor-4-fluorbenzol. Desweiteren konnte erstmals eine detailierte Analyse der Schwingungsstruktur durchgeführt werden. Das REMPI-Spektrum von 1,3-Dichlor-2-fluorbenzol zeigt eine ungewöhnlich hohe Aktivität einer niederfrequenten out-of-plane-mode ($17b^2$). MATI-Spektren uber verschiedene Moden zeigen eine Verletzung der $\Delta v=0$ Vorzugsregel [2].
Außerdem lässt sich im MATI-Spektrum über die 17b-Mode eine kurze, dreigliedrige Progression der Mode 17b mit einem Shift des Frank-Condon-Maximums zu niedrigeren Energien beobachten. Während die Rechnungen für den elektronischen und ionischen Grundzustand eine planare Struktur zeigen, legen die Rechnungen für den angregten Zustand eine verzerrte, nicht planare Struktur nahe.

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Neue Aspekte

Geometrieänderung bei Anregung und Ionisation

PO 21

The transformation pathway of fluoxetine under iron- and sulfate-reducing conditions

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Fluoxetine is a crucial antidepressant. In Germany, the amount of prescribed fluoxetine was 1.1 tons in 2012. [1] After administering, fluoxetine and its human metabolites are excreted via urine and reach wastewater treatment plants (WWTPs). Fluoxetine is known to pass municipal WWTPs and hence, is discharged into rivers and streams. Obviously, current biological aerobic/anoxic treatment processes of WWTPs are inappropriate for its total removal [2]. Although its acute toxicity is low, sub-lethal effects on aquatic organisms have been reported at concentration levels in the ng/L-range [3]. Therefore, alternative biological systems have been studied to remove fluoxetine. For this, strict anaerobic batch has been used in order to biodegrade fluoxetine.

Experimenteller Teil

Anaerobic lab-scale experiments with fluoxetine were conducted using activated sludge as inoculum which was diluted 1:4 with WWTP effluents. To establish iron- or sulfate-reducing conditions, an iron(III)-hydroxide suspension or a sodium sulfate solution was spiked to the batch experiments. A search for transformation products (TP) was conducted using an LTQ-Orbitrap Velos mass spectrometer in negative and positive ionization mode. A screening on new masses in the mass range of fluoxetine exhibited a chromatographic peak that increased during the lab-scale experiment and hence was nominated as a potential TP. The quasi-molecular ion of this TP was then fragmented by MS^n experiments on the Orbitrap mass spectrometer. Based on their fragmentation behaviour a tentative molecular structure was assigned for the TP.

Ergebnisse und Diskussion

The Fluoxetine-TP294 was unstable under sulfate-reducing conditions, whereas iron-reducing conditions showed enrichment over time. Fluoxetine-TP294 which was only detected in the negative ionization mode showed a well-defined fragmentation pattern during MS^2 experiments. To elucidate further the chemical structure of TP294, the fragment (m/z 203.0327) which was formed by in-source fragmentation was further fragmented by MS^2 experiments yielding m/z 159.0442. This fragment was identified as a toluyl moiety containing a trifluoromethyl group. This trifluoromethyl group was then cleaved during the MS^3 experiment to yield the isolated toluyl group with m/z 91.0552.

A tentative transformation pathway of fluoxetine was deduced from the structural differences between TP294 and fluoxetine. The pathway comprises the methylation of an aromatic ring in ortho- or meta-position relative to the trifluoromethyl group, the β-cleavage of the amino group and a rearrangement of the resulting aldehyde to an ester. NMR experiments of the isolated TP294 will be used for a final confirmation of the proposed structure. Further experiments will also elucidate the importance of fluoxetine transformation at pilot scale reactors which are maintained under anaerobic redox conditions.

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Neue Aspekte

This is the first description of an anaerobic fluoxetine-TP. Fluoxetine-TP294 exhibits an unusual fragmentation pattern enabling structure elucidation.

PO 22

Aryl-phenyl scrambling in intermediate organopalladium complexes. A gas-phase study of the Mizoroki-Heck reaction.

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Phosphine ligands are commonly used to control the regio- and stereochemistry in important transition metal mediated C-C bond forming reactions such as Mizoroki-Heck, Suzuki or Stille couplings. However, exchange reactions between phosphorus- and palladium-bound aryl moieties lead to unwanted product mixtures.[1-2] This aryl-aryl scrambling process was studied in solution by means of NMR[1] or by examining the collision induced dissociation (CID) behavior of palladium phosphine aryl complex ions using tandem MS.[2] We performed ion/molecule reactions (IMRs) between $[Pd(phosphine)(aryl)]^+$ complex ions and a model olefin (2,3-dimethylbutadiene) in the gas phase and were able to examine the scrambling reaction mechanism of the aryl/phenyl interchange during the coupling reaction in the absence of solvent effects, counter-ions and aggregation processes.

Experimenteller Teil

Ion/molecule reactions (IMRs) were performed in the LTQ-part of a *Thermo Fisher* LTQ Obitrap XL (Bremen, Germany) hybrid instrument which was modified as described elsewhere.[3-5] In brief, the helium buffer-gas supply of the LTQ was upgraded with an extra septum inlet which allowed the injection of dimethylbutadiene (DMB) using a pump-driven syringe. This way, a gaseous mixture of helium and DMB was introduced into the LTQ resulting in partial pressures of ca. 2.5 mTorr (helium) and ca. $2.5 \cdot 10^{-3}$ mTorr (olefin) inside the ion trap. The yield of the aryl/phenyl exchange reaction was determined by means of the ratio of scrambled and unscrambled hydride complex ions formed during CID of the carbopalladation product ions.

Ergebnisse und Diskussion

Different *para*-substituted aryl iodides were reacted with bis(dibenzylideneacetone)palladium ($Pd(dba)_2$) and 1,3-bis(diphenylphosphino)propane (dppp) in acetonitrile at room temperature. (+)ESI-MS analysis of the reaction mixture yielded the complex ions $[Pd(dppp)(Ar)]^+$, which were characterized by exact mass measurement, their characteristic isotopic distributions and the indicative fragmentation behavior obtained from MS^2 and MS^3 product ion experiments.

To probe the gas-phase Mizoroki-Heck coupling, complex ions $[Pd(dppp)(Ar)]^+$ were mass selected in the ion trap and reacted with dimethylbutadiene (DMB) introduced into the trap *via* the gas flow of the helium background gas.[3-5] The IMRs of all complex ions $[Pd(dppp)(Ar)]^+$ with DMB led exclusively to the formation of product ions with a characteristic mass shift of 82 Da (C_6H_{10}, DMB), representing olefin insertion product ions. CID of these species yielded exclusively two hydride complex ions, $[Pd(dppp\text{-}Ph)(H)]^+$ and $[Pd(dppp\text{-}Ar)(H)]^+$, which are rationalized assuming an interchange between the palladium-bound aryl moiety and a phosphorus-bound phenyl group during isolation of $[Pd(dppp)(Ar)]^+$ in the ion trap. Unambiguously proven by NMR, the aryl/phenyl exchange does not take place in solution *prior* to ESI-MS and thus, it is an exclusive gas-phase phenomenon. Interestingly, we find elevated aryl/phenyl interchange reactivity in the gas phase compared to analogous scrambling reactions in solution, where $[Pd(dppp)(Ar)]^+$ complex ions with electron-poor aryl moieties do not show scrambling at all.

The ratio of the abundance of scrambled and unscrambled hydride complex ion pairs were logarithmized and plotted *vs.* the respective Hammett σ_P parameter of the corresponding substituted aryl moieties. The slope of the linear fit (ρ = -0.90) indicates that the scrambling reaction is favoured for aryl ligands with electron-donating *para*-substituents. This result is consistent with the proposed mechanism for the scrambling reaction proceeding *via* the formation of an intermediate phosphonium ion.[1-2]

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Neue Aspekte

Complete gas-phase Mizoroki-Heck reactions are performed and linear free-energy correlations are applied to elucidate the aryl/phenyl scrambling mechanism.

PO 23

Selective-Reagent-Ionization Mass Spectrometry Studies on 10 New Psychoactive Substances (Designer Drugs)

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New psychoactive substances (NPS), which are sometimes also called designer drugs or legal highs, are a hot topic in our society [1]. NPS are chemical compounds with intoxicating effects similar to illegal drugs. However, due to differences in their chemical structures and compositions, NPS are not affected by common drug legislation and thus readily available via vendors on the Internet. Recently we undertook detailed studies on a broad variety of NPS, ranging from stimulants and entactogens to psychedelics utilizing Selective-Reagent-Ionization Mass Spectrometry (SRI-MS), which is an advanced form of Proton-Transfer-Reaction Mass Spectrometry (PTR-MS). Here we report on these studies, which include data on detectability of the active ingredients and purity of the compounds supplied by online vendors.

Experimenteller Teil

The term "SRI-MS" was introduced by Karl et al. in 2012 [2] describing an advanced PTR-MS instrument which is capable of switching the reagent ions, most notably between H_3O^+, NO^+, O_2^+ (and Kr^+). One major advantage of SRI-MS is increased selectivity (compared to PTR-MS) which has been demonstrated e.g. for explosives in [3]. Similar to PTR-MS, SRI-MS instruments consist of an ion source for the generation of reagent ions at high purity levels and a drift tube where chemical ionization between the reagent ions and the analytes take place. Subsequently, the resulting product ions are analyzed and detected in a mass spectrometer, which was a Time-Of-Flight (TOF) mass analyzer in the present studies.

Ergebnisse und Diskussion

We analyzed 10 readily available (at the time of purchase) new psychoactive substances, namely 4-fluoroamphetamine, methiopropamine, ethcathinone, 4-methylethcathinone (4-MEC), N-ethylbuphedrone (NEB), ethylphenidate, 5-MeO-Dalt, dimethocaine, 5-(2-aminopropyl)benzofuran and two forms of nitracaine (powder and crystal) utilizing H_3O^+, NO^+, O_2^+ and Kr^+ as reagent ions in an SRI-MS instrument [4]. The samples had been purchased from different online vendors located in different European countries. With the exception of nitracaine in powder form, which did not contain the active ingredient (most probably due to improper synthesis, as compounds used for nitracaine synthesis could be detected in the powder) all samples contained the advertised ingredients with a low level of impurities present. This is noteworthy as in a previous study on illegal drugs by us, a sample of "street heroin" contained only 40% pure heroin [5].

Two of the analyzed NPS are isomers, namely 4-MEC and NEB. Utilizing H_3O^+ as reagent ions for the detection of these compounds only the protonated molecules were observed at m/z 192 ($C_{12}H_{18}NO^+$); i.e. the two isomers could not be distinguished. However, by switching to any of the alternative reagent ions available in SRI-MS immediately solved this problem as characteristic fragment ions were detected with $C_4H_{10}N^+$ (m/z 72) being the most abundant product ion for 4-MEC and $C_5H_{12}N^+$ (m/z 86) for NEB. This example serves to illustrate how SRI-MS can be used to definitively improve selectivity (compared to PTR-MS) by enabling the identification of isomers.

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Neue Aspekte

A series of new psychoactive substances (designer drugs) is analyzed with SRI-MS. Switching reagent ions results in increased selectivity.

PO 24

Untersuchung der Transformation von iodierten Röntgenkontrastmitteln unter anaeroben Milieubedingungen mittels komplementärer Massenspektrometrie

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Die weit verbreiteten intravasal eingesetzten iodierten Röntgenkontrastmittel (IRKM) werden in Kläranlagen nicht oder nur unvollständig abgebaut. Ein im gereinigten Abwasser häufig nachgewiesenes IRKM ist Diatrizoat, das in der Regel in Konzentrationen von über 1µgL^{-1} vorliegt [1].

Durch Verregnung des gereinigten Abwassers auf Rieselfeldern gelangen IRKM in Böden und Sedimente; dort kann eine biotische sowie abiotische Transformation und/oder eine Verlagerung ins Grundwasser stattfinden [2]. Im Hinblick auf anaerobe Transformationsprozesse sind entstehende Spezies des Diatrizoats weitgehend unaufgeklärt - zur Beurteilung der ökotoxikologischen Relevanz ist eine Identifizierung sowie Quantifizierung der gebildeten Transformationsprodukte unerlässlich. Oftmals fehlen zur validen Quantifizierung neuartiger Transformationsprodukte mittels LC/ESI-MS geeignete Standards - als innovativer, komplementärer Quantifizierungsansatz ohne den Einsatz spezies-spezifischer Standards wurde daher die Kopplung der LC/ICP-MS genutzt.

Experimenteller Teil

Zur Untersuchung des anaeroben Abbaus von Diatrizoat wurden Laborexperimente durchgeführt. 100g Boden wurden mit der 4-5-fachen Menge an Grundwasser versetzt und nach Austreiben des gelösten Sauerstoffs unter Argonschutzatmosphäre mit einer definierten Menge an Diatrizoat dotiert. Die Probengefäße wurden verschlossen, dunkel gelagert und unter ständigem leichten Schütteln bei Raumtemperatur inkubiert. In regelmäßigen Abständen (bis maximal 200 Tage nach Inkubationsstart) wurden ca. 3mL Probe unter Argonschutzatmosphäre entnommen, über einen 0,45µm Filter filtriert und dunkel und kühl bis zur Analyse gelagert. Die Identifizierung der Transformationsprodukte wurde mittels LC/ESI-tandem MS durchgeführt; die Quantifizierung der vollständig deiodierten Transformationsprodukte wurde über kommerziell erworbene Standards erreicht. Für die iodierten Spezies waren keine Standards verfügbar; deren Quantifizierung wurde mit einer neu entwickelten komplementären HPLC/ICP-MS-Methode nach Retentionszeitenvergleich erreicht.

Ergebnisse und Diskussion

In anaeroben Laborexperimenten waren nach einer „Lagphase“ von 50-80 Tagen eine Reihe an Transformationsprodukten des Diatrizoats nachweisbar. Auf Basis der Ergebnisse konnte ein (potentieller) Abbauweg von Diatrizoat vorgeschlagen werden. Unter aeroben Bedingungen ist bekannt, dass der Abbau des Diatrizoats hauptsächlich unter Deacetylierungsreaktionen stattfindet [3, 4]. In der vorliegenden Studie konnte jedoch erstmals gezeigt werden, dass unter anaeroben Bedingungen der Abbau zuerst unter Iodidabspaltung verläuft; erst im Anschluss hieran werden die im Molekül vorhandenen Acetylgruppen abgespalten - als Endprodukt wird ein vollständig deiodiertes und deacetyliertes Molekül erhalten. Zur weiteren Untersuchung des Transformationspfades sowie zur weiteren Beurteilung der ökotoxikologischen Relevanz der entstandenen Spezies ist eine valide Quantifizierung unerlässlich. Durch den komplementären, innovativen Einsatz der HPLC/ICP-MS Kopplung konnten auch Transformationsprodukte quantifiziert werden, für die keine Standards vorlagen. Hierdurch war es möglich die Massenbilanz des Abbaus über alle während des zeitlichen Abbaus entstehenden Transformationsprodukte durch den Einsatz beider Techniken zu schließen. Dies zeigt erstmals ein vollständiges, zeitaufgelöstes Bild des biologischen Abbaus von Diatrizoat unter anaeroben Bedingungen auf.

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Neue Aspekte

Komplementärer Einsatz von LC/ESI-MS und LC/ICP-MS zur speziesanalytischen Untersuchung der Transformation von Diatrizoat unter anaeroben Bedingungen.

PO 25

Drugs enable MALDI-TOF MS of their drug delivery system

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Drug delivery systems (DDSs) have been established to overcome the insolubility in aqueous media of many hydrophobic drug molecules.[1] The hydrophobic character of the drug molecules often arises from a π-system, either of aromatic or conjugated character. These π-systems result in a bathochromic shift of the absorption wavelength into the UV region, which is a basic prerequisite for the matrix in the matrix-assisted laser desorption/ionization (MALDI) process.[2,3] Assuming that a π-system is in principle sufficient to provide matrix qualities, several drug molecules might account as assistance in the MALDI process. This approach, to use the encapsulated drug molecules as matrix, could allow the detection of the drugs as well as the DDSs in *ex vivo* and *in vivo* applications.

Experimenteller Teil

Five different π-system containing drug molecules were chosen for a comparative study as matrix, namely madurahydroxylactone (MHL), tetrakis(4-hydroxyphenyl)-porphyrine (THP), chartreusin (Chart), amphotericin B (AmB) and retinoic acid (RA). Their ability to act as matrix was investigated for a selection of polymers, such as PEG, PMMA, PS, PEtOx and PEG-*b*-PCL. The DDSs were prepared by adding water and an acetone solution of PEG-*b*-PCL to a defined amount of drug. The acetone was allowed to evaporate and the micelles characterized.

For the MALDI measurement the dried-droplet sample preparation method was applied. An array of three to fourteen spots was prepared for the fourteen different laser energies investigated. MALDI-TOF spectra were recorded on an Ultraflex III TOF/TOF of Bruker Daltonics (Bremen, Germany).

Ergebnisse und Diskussion

In a first MS approach, the selected drug molecules were submitted to LDI measurements to study their behavior under laser irradiation in the positive mode as the majority of polymers is ionized and detected as cation. The base peak was provided for, *i.e.*, Chart, RA and THP by a quasi-molecular ion, either a protonated species (RA and THP) or a sodiated molecule (Chart). Thus, it can be concluded that the drug molecules are suitable for the LDI process.

To assess their suitability as matrix four different spectra characteristics were evaluated in dependence of the investigated polymer, the matrix, and the laser energy. Firstly, the m/z value of the peak with the highest intensity, the maximum peak, of each obtained distribution was determined. Furthermore, its intensity, signal-to-noise (S/N) ratio, and resolution were used for the analysis. In summary, RA as established matrix for MALDI delivered the best quality profile for all homopolymers. Nevertheless, surprisingly good volatilization enhancing effects have been detected for the four other drug molecules making them also suitable as matrix. For the block copolymer, RA and AmB produced the best results.

Micellar solutions of MHL, THP and RA encapsulated by PEG-b-PCL with the optimum inclusion capacities were selected for further MS investigations. However, only for the micellar solution of THP the drug as well as the block copolymer were detected in MALDI-TOF MS proving this system to be suitable for detection of a drug as well as its carrier. The imaging of these DDSs will be part of a future study as further optimization is required for the (MA)LDI-TOF MSI detection of this small entities.[4]

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Neue Aspekte

Drugs and their DDSs are detected without matrix. Both will be analyzed in biological media and imaged on cellular level.

PO 26

Untersuchung des Kinetic Energy Release des Verlust von 44 Da aus Xanthenfarbstoffen

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Die im Arbeitskreis Grotemeyer untersuchte Abspaltung von 44 Da aus Rhodamin B zeigte im 9,4 T-FT-ICR eine Abspaltung von C_3H_8 anstelle des oftmals publizierten CO_2 [1]. Diese Abspaltung findet mutmaßlich an den Diethylaminoseitenketten des Rhodamin B statt. Um genauere Aussagen über das Verhalten von Rhodamin B zu machen, wurde dieses und andere Xanthenfarbstoffe in einem Sektorfeldgerät untersucht. Hierbei wurde besonderes Augenmerk auf die metastabilen Zerfallsmechanismen gelegt. Zudem wurden für die 44er Abspaltungen der *kinetic energy release* (KER) bestimmt.

Experimenteller Teil

Verwendet wurde ein ZAB-2F der Firma Vacuum Generators mit inverser Nier-Johnson Geometrie. Die mittels Elektronenionisation bei 70 eV generierten Ionen wurden mit *mass analysed ion kinetic energy* (MIKE)-Spektrometrie untersucht. Hierbei wurde das Magnetfeld so eingestellt, dass nur ein bestimmter Vorläufer passieren kann, die Fragmente, die im zweiten feldfreien Raum durch metastabilen Zerfall entstehen, wurden mit dem elektrischen Feld analysiert.

Ergebnisse und Diskussion

Verglichen wurden drei beobachtete Verluste von 44 Da aus Rhodamin B. Die erste Abspaltung wurde aus dem Vorläufer mit m/z 442 beobachtet, welcher dem $[M\text{-}H]^{+\cdot}$ entspricht. Diese zeigt einen ungefähr doppelt so hohen KER wie die darauf folgenden Abspaltungen, was darauf hin deutet, dass im metastabilen Prozess zuerst das CO_2 und dann zwei C_3H_8-Moleküle abgespalten werden. Um diese Annahme zu verifizieren, wurde das Rhodamin 110 untersucht, welches an den Stickstoffatomen keine organischen Reste aufweist. Hierbei wurde eine Abspaltung von 44 Da beobachtet welche einen KER besitzt der ungefähr so hoch ist, wie der KER des ersten 44 da Verlustes aus Rhodamin B. Wie erwartet traten keine weiteren Abspaltungen von 44 Da auf.

Durch diese Vergleichsmessung konnte gezeigt werden, dass im metastabilen Zerfallsmechanismus aus Rhodamin B, wider Erwarten zuerst CO_2 abgespalten wird und anschließend zwei C_3H_8-Moleküle. Zudem deutet das Fehlen von weiteren 44 Da Abspaltungen aus dem Rhodamin 110 darauf hin, dass diese beim Rhodamin B aus den Diethylaminoseitenketten stammen.

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Neue Aspekte

Untersucht wurden verschiedenen Verluste von 44 Da aus Xanthenfarbstoffen, der KER dieser Abspaltungen wurde bestimmt.

PO 27

Fragmentierung von unmarkierten und markierten Azofarbstoffen

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Es ist bekannt, dass Xanthenfarbstoffe wie Rhodamin B unerwartete Fragmentierungsmuster aus der Dialkylaminogruppe beim Zerfall im Massenspektrometer zeigen [1-3]. Um den Mechanismus dieser Fragmentierungsreaktion zu verstehen wurden weitere Moleküle untersucht, die andere Strukturmerkmale besitzen. Eine Gruppe bildeten die Azofarbstoffe, welche eine große Verbreitung und vielfältige Anwendungen besitzen. Dennoch wurde diese Gruppe der synthetischen Farbstoffe nur wenig massenspektrometrisch charakterisiert. Dies führte dazu, dass das Fragmentierungsverhalten einiger Vertreter dieser Stoffklasse am hochauflösenden FT-ICR Massenspektrometer untersucht wurde.

Experimenteller Teil

Die untersuchten Farbstoffe wurden durch Azokupplung der Anilinderivate oder durch Alkylierung von 2-Aminoazotoluol synthetisiert. 2-Aminoazotoluol wurde von *abcr*, alle weiteren Chemikalien von *Sigma Aldrich* bezogen.

Massenspektren wurden mit einem Bruker APEX III FT-ICR Massenspektrometer mit einem 7.05 T Magneten (Bruker Daltonik, Bremen) aufgenommen. Die Proben wurden in MeOH/H_2O gelöst und mittels der ESI-Quelle in die Gasphase gebracht.

Die Fragmentierung erfolgte durch Stoß- oder Laseraktivierung in der ICR-Zelle. Hierfür wurden die entsprechenden Ionen in der Zelle isoliert und gespeichert. Die Stoßaktivierung erfolgte mit Argon durch SORI-CID Anregung. Für die Laseraktivierung wurde ein verdoppelter Nd:YAG Laser (*Continuum Inlite II*) mit einer Wellenlänge von 532 nm verwendet.

Ergebnisse und Diskussion

Es wurde untersucht, wie sich die Fragmentierung der Alkylaminogruppe mit verschiedenen Alkylgruppen verändert. Weiterhin zeigen die untersuchten Azofarbstoffe auch Fragmentierungen des konjugierten Systems. Hierbei zeigt sich eine große Übereinstimmung bei fast allen untersuchten Molekülen.

Sehr überraschend ist das Verhalten der monosubstituierten Aminoazotoluole. Diese zeigen den Verlust von Ammoniak (NH_3), welcher nicht aus den Strukturen der Moleküle erklärbar ist. In der protonierten Form ist ein quartäres Amin (Alkyl-NH_2^+-Azotoluol) vorhanden. Dies lässt den Schluss zu, dass es zu einer Umlagerung kommt, in deren Folge das neutrale Ammoniak abgespalten wird. Das dritte Wasserstoffatom kann hierbei nicht aus den als Alkylgruppen verwendeten Methyl- oder Ethylsubstituenten stammen. Die Markierung dieser Gruppen mit Deuterium führt ebenfalls zu dem Verlust von unmarkiertem Ammoniak. Die Beteiligung der in ortho Position befindlichen Methylgruppe an der Umlagerung ist somit sehr wahrscheinlich.

Die disubstituierten Varianten dieser Moleküle verhalten sich anders. Ein Verlust von Ammoniak wird nicht beobachtet. Die auftretenden Fragmente können neutralen und radikalischen Abspaltungen aus der Dialkylaminogruppe zugeordnet werden.

Mehrere unerwartete Fragmentierungen konnten bei der Untersuchung der Azofarbstoffe beobachtet werden. Mit Hilfe von Deuterierungsexperimenten war es möglich Schlüsse auf den vorliegenden Fragmentierungsmechanismus zu ziehen.

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Neue Aspekte

Neue Abspaltungen aus aminhaltigen Azofarbstoffen.

PP: Proteomics

PP 1

Travelling Wave Ion Mobility Assisted Duty Cycle Enhancements for Targeted and Non-Targeted Proteomics Experiments

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The average duty cycle of Time of Flight analysers is dependant on the m/z data acquisition range but the integration of travelling wave ion mobility devices in QToF geometries can afford significant duty cycle improvements. In this High Duty Cycle (HDC) mode, the pusher number is synchronised with the pusher delay time and this delay time related to the drift time of species through the TWIMS. The system can be programmed to optimise this parameter for a specific charge state over the entire mass range. Results show that the HDC mode of operation can give at least a five times increase in sensitivity over the entire mass range for chosen charge states in both targeted and non-targeted experiments.

Experimenteller Teil

Protein digests and synthetic peptide mixtures were analysed by either direct infusion, nanoscale LC-MS or nanoscale LC-IM-MS. A direct flow nanoscale LC system was configured with a trapping and an analytical column. A 300nL/min reversed phase gradient changed the organic composition from 1 to 40% in 30 or 90 minutes, depending on sample complexity.

The mass spectrometer was operated in either a non-targeted data directed manner, whereby species eluting from the column are identified for CID fragmentation automatically or in a targeted MS/MS mode, enabling quantitation. The HDC mode was tuned to enhance the signal of either multiply charged precursor ions or singly charged fragment ions.

Ergebnisse und Diskussion

The Time of Flight analyser was programmed to enhance the charge state of interest by selection from an m/z *vs.* drift time distribution the region populated by these ions. The output of this selection produced a calibration file for use in different Ion Mobility enabled mass spectrometry methods.

We show that a signal improvement with HDC mode enabled for both multiply charged precursors and singly charged fragment ions of approximately five to seven fold is achieved during the infusion of Glu-Fibrinopeptide B.

Injection of an *E. Coli* tryptic digest onto the LC system were performed with the instrument operating in Ion Mobility enabled DDA mode. The enhancement in fragment ion signal with HDC mode employed lead to more confident identifications when the data was interrogated through post processing software.

For targeted MS/MS analyses of a four protein digest spiked into the *E. Coli* digest, the limit of detection can be extended by at least an order of magnitude when the exact mass traces for several fragment ion masses are combined. We show from the dilution series of the four protein mixture that the limit of quantitation is also extended with HDC on compared to HDC off.

Neue Aspekte

Utilising Ion Mobility and Time of Flight analyser characteristics to enhance duty cycle and mass spectral signal intensity.

PP 2

Specific lysosomal diagnostic for MPS II and MPS VI patients by fluorimetry and mass spectrometry

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Lysosomal storage diseases (LSDs) are a group of diseases that lead to accumulation of glycosaminoglycans (GAGs) in different organs and tissues. People with one of this disease do not produce enough one of the specific lysosomal enzyme required to break down this long sugar chains in small pieces. Accumulation of GAGs within lysosomes can produce progressive cellular damage leading to skeletal malformations, pulmonary deficits, short stature, retarded growth, hepatic and cardiac abnormalities and sometimes neurological abnormalities.

In this report we describe a specific and sensitive diagnostics on dry blod spots (DBS) for single and multiplex assay of LSDs by multiple reactions monitoring mass spectrometry (MRM-MS) and by fluorimetry.

Experimenteller Teil

We optimized enzyme reaction condition and procedure for the assay, including concentration of substrates and internal standard, composition of extract buffer and mass spectrometer parameters. We used specific substrate for specific diseases and internal standard to directly quantify the enzymatic activities, and tandem mass spectrometry for enzymatic product detection. The internal standard (umbelliferyl standards) were synthesized by Pechmann condensation and used for development of new single and multiplex assays. Rehydrated dried blood spots were incubated with an extraction buffer and then with the enzyme substrates. Clinical diagnostics assays were developed by MS-MRM determinations with an Esquire 3000 ion trap mass spectrometer (Brucker, Germany).

Ergebnisse und Diskussion

The quantification of accumulated byproduct in DBS by tandem mass spectrometry can be used to detect more than 40 genetic disorders in newborn-screening programs. We have developed a high-throughput single and multiplexing assay for MPS II and MPS VI in DBS. Mass spectrometric methods have been developed for the detection of accumulated metabolites resuting from different lysosomal storage disease such as Fabry disease, Gaucher disease and Krabbe disease. These methods are sensitive and specific for quantification of metabolites. The data obtained suggest that the method is reliable for newborn screening for these two lysosomal enzyme deficiencies.

The method described here, in which tandem mass spectrometry is used to directly quantify enzymes in DBS and should be applicable to almost any enzyme that retains activity in a rehydrated DBS.

We analyzed dried blood spots for single and multiplexing assay from MPS II and MPS VI patients and in all cases the enzyme activities were below the minimum activities measured in patients in comparison with healthy controls. This method is easy, fast and reliable and can be successfully used in clinical trials.

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Neue Aspekte

Optimized method for lysosomal storage disease diagnostics using MRM-MS and fluorimetry determination.

PP 3

Quantification of endogenous peptides in biological fluids by immunoprecipitation coupled to MALDI-MS

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Reliable quantification of endogenous peptides in body fluids is a challenging task in analytical sciences. In recent years peptide immunoprecipitation in combination with MALDI (also known as iMALDI) has evolved as a reliable and high-throughput technique to solve this analytical task. Firstly, endogenous peptides are isolated by peptide-specific antibodies out of a biological matrix like plasma or CSF (CerebroSpinal Fluid). Secondly, enriched peptides are analysed by MALDI mass spectrometry to reliably detect the peptides of interest using their exact mass. Further, relative as well as absolute quantification is obtainable by adding stable isotope labelled standard peptides. We have applied this technique for the quantification of amyloid beta peptide species in CSF of patient with AD (Alzheimer's Disease) and controls.

Experimenteller Teil

Initially, we spike CSF with heavy N15-isotopically labelled Abeta fragment 1-40 as an internal standard. Then endogenous and spiked Abeta fragments are enriched from the CSF through IP with an antibody recognizing a variety of different Abeta peptides. The enriched Abeta peptides are analysed by MALDI-TOF MS (ABSciex MALDI TOF/TOF 4800), where the endogenous Abeta peptides are detected in parallel to the spiked internal standard reference peptide. This method enables relative quantification of endogenous Abeta peptides detected across different CSF samples. Here, we have applied this method on a cohort of 62 CSF samples. 31 AD (Alzheimer's disease) and 31 control samples have been comparatively assessed by statistical analysis.

Ergebnisse und Diskussion

The concentration of amyloid beta peptides in CSF is in the subnanomolar to lower nanomolar range. To enable a mass spectrometric detection of the Abeta peptides an enrichment step is needed. Due to the physicochemical properties of abeta peptides liquid chromatography is difficult for a larger set of abeta peptides. Therefore MALDI is the preferred mass spectrometric platform. This has further the advantage that the throughput is drastically increased because time consuming LC gradients are not necessary. On the other side immunoassays rely only on the discriminative power of antibodies. By this co-influence of peptides, different only in one or a few amino acids, is highly likely. Using the IP-MALDI method a second separation step is possible by using the different masses of abeta species.

We have downscaled the Abeta IP-MALDI method from 1 ml input volume of CSF to 0.5 ml and down to 0.2 ml. We could reproducibly detect up to 13 Abeta peptides and have used 9 of them for relative quantification. The ratio of ratios (endogenous peptide / spiked standard 1-40 heavy) for 9 of the measured peptides have a mean analytical CV of $\leq$26%, and for 7 of $\leq$ 11%, proving reproducibility of the IP-MALDI method for the relative quantification of the peptides Aß 1-17, 1-19, 1-33, 1-34, 1-37, 1-38, 1-39, 1-40 and 1-42. The method is able to distinguish CSF samples based on different concentration of Abeta peptides simultaneously and thus provides an efficient tool to compare sets of human CSF samples in the context of Alzheimer's disease (disease progression, treatment, diagnosis etc.). In the tested study cohort (31 AD vs. 31 controls) the quantitative pattern of a subset (1-37 to 1-40) is very similar and shows higher values in AD samples (factor of ~2).

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Neue Aspekte

Methodical improvement, evaluation and application of endogenous peptide quantification in body fluids by immunoprecipitation coupled to MALDI mass spectrometry

PP 4

Protein C Inhibitor Proteotypic Peptide Quantitation by LC-Free

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Biomarker validation remains one of the most important constraints to development of new clinical assays. To address this challenge, we recently reported on the development of a SISCAPA-MALDI assay, which allows high throughput mass spectrometric quantitation of protein analytes in large sample sets. Here we describe the application of this assay to measure a surrogate peptide from protein C inhibitor (PCI) in sera from 51 prostate cancer patients to verify the use of the surrogate peptide as a biomarker for monitoring prostate cancer.

A 2-plex SISCAPA-MALDI assay was developed for quantitation of surrogate peptides from PCI and soluble transferrin receptor (sTR) and was applied to 159 trypsin-digested sera collected from 51 prostate cancer patients.

Experimenteller Teil

Corresponding Stable Isotope Standard peptides were produced with a label resulting in a mass shift of +10 Da, allowing quantitation using mass spectrometry. Patients who experienced biochemical recurrence of prostate cancer after treatment with androgenic hormones/radiation showed decreased levels of the PCI analyte within 18 months of treatment. Levels remained high in the plasma of patients who did not experience cancer recurrence. Prostate specific antigen (PSA) levels had no predictive value in the same time-period.

Ergebnisse und Diskussion

Results from the study show that the high-throughput, LC-free SISCAPA-MALDI assay is capable of analyzing peptide analytes in clinical samples with accuracy and in a short period of time. The lower limit of detection for PCI in the assay was found to be 1 fmol, which is 150 times lower than the endogenous level of PCI in 10 µL of plasma. The CV at the endogenous concentration was 3.8%. Adoption of this assay will allow systematic, rapid validation of putative protein biomarkers using large sample sets. An even much larger cohort (perhaps more than 1000 patients) will be required to unequivocally validate the PCI analyte for clinical use.

Neue Aspekte

Adoption of this assay will allow systematic, rapid validation of putative protein biomarkers using large sample sets.

PP 5

Characterization of the Breast Cancer Marker Candidate LAG3 Protein in Human Plasma by Direct SPRi-MALDI-MS Analysis from Antibody Arrays

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Proteomics plays an important role in biomarker discovery for clinical applications. In this study, we coupled Surface Plasmon Resonance imaging (SPRi) with MALDI-TOF mass spectrometry to permit the multiplexed quantification of binding by SPRi and the molecular characterization of interacting partners by subsequent MS analysis. This adds a dimension of specificity as MS permits the differentiation of molecules that are difficult to tell apart by use of antibodies, such as truncation variants or protein isoforms.

Experimenteller Teil

The LAG3 protein was spiked in human plasma at ~1µg/mL in a proof of concept study to detect, identify and characterize it as potential breast cancer marker. LAG3 was bound to □-LAG3 antibodies that were covalently attached to the chip surface. SPRi binding kinetics were obtained in real time, followed by tryptic digestion, matrix deposition and MALDI MS and MS/MS spectra acquisition. LAG3 was identified through Mascot interrogation.

Ergebnisse und Diskussion

The density of bound antibody on the surface (~7fmol/mm²) was compatible with both, quantitative determination of binding parameters and the identification thru bottom-up MS/MS analysis for the SPRi-MALDI workflow. MALDI image analysis of the chips confirmed the co-localization of LAG3 peptides with the array spots whereas serum albumin – used to block the reactive chip surface after □-LAG3 deposition – was only detected between the array spots. This indicated that the matrix coating and trypsin application processes employed (piezoelectric nebulisation) did not significantly cause any delocalisation of the array content.

The rapid, multiplexed and automated on-chip MALDI-MS analysis shows robustness at the femtomole level and opens numerous applications in the proteomic field such as ligand screening and lead optimization.

Neue Aspekte

The rapid, multiplexed and automated on-chip MALDI-MS analysis shows robustness at the femtomole level and opens numerous applications in proteomics

PP 6

Urinary bladder cancer proteome profiling by nanoLC-ESI-IMS-MSE analysis – proof-of-principle

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Prognostic diagnosis of bladder cancer is still a major limitation in the treatment of urinary bladder cancer. Bladder cancer is known as 4th most common cancer in males and accounts for 4.1 % of total cancer deaths in Europe [1]. Due to the highly heterogeneous properties of the cancerous tissue, efficient methods need to be developed in order to obtain individual proteome signatures that may become useful for patient stratification. In this project we aim at generating quantitative proteome signatures for bladder cancer using solid tumor samples. Also, detailed protein structure information, particularly phosphorylation differences, shall be analyzed to further our understanding of molecular processes associated with cancer development and/or metastasis potential [2-4].

Experimenteller Teil

Tumor and control tissue sample pairs (ca. 400 mg each) from 15 patients with different grading and stages mainly G3N2, G3N0 and G2N0 have been collected by radical cystectomy and transurethral resection, respectively. Corresponding protein extracts from these three groups have been generated according to in-house SOPs. For one sample pair *in-solution* tryptic digestion was performed. Further, 1µl of samples (peptide conc. 0.89 µg/µl) have been subjected to nanoLC-ESI-IMS-MS^E (Synapt G2, WATERS) analysis for quantitative proteome profiling [5].

Ergebnisse und Diskussion

Using our work-flow, nanoLC-ESI-IM-MS^E revealed a total of 32,000 ion signals (including precursor and fragment ions) using a 120 min LC gradient. From these approx. 4800 peptides were identified with a minimum of 3 fragment ions per peptide. Finally, ca. 800 proteins were identified in one run. A protein was considered positively identified when 3 matching peptides were determined. Identification numbers were confirmed by triplicate analyses of the tumor material. Proteins were quantified by spiking a known amount of yeast enolase into the samples as a standard. Comparison of nanoLC-ESI-IM-MS^E results of the control tissue with those of the tumor enables differential expression analysis.

All steps starting from tissue harvesting during surgery, protein extract generation, *in-solution* digestion, mass spectrometric peptide analysis, and protein identification have proven successful. Each of the mentioned steps has been optimized to yield in high quality data sets. Differential protein expression analysis will be performed in order to determine a proteome signature of urinary bladder cancer.

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Neue Aspekte

Proteome profiling of Urinary bladder cancer using solid tumor tissue

PP 7

Frit or Filter? Application of Porous Silica Frits as Inert Protective In-Line Filters for Nanoflow - HPLC-MS/MS in Quantitative Proteomics

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Quality of the separation, reproducible and reliable retention times of peptides across series of nanoflow HPLC-MS/MS analyses are prerequisites for label-free quantitative proteomics. Micrometer- and submicrometer particles and debris originating from samples, solvents and equipment lead to column compression and clogging compromising separation and reproducibility of proteomics experiments. We have developed in-capillary filters of porous silica that do not increase void volumes, do not interfere with peptide retention or recovery of phosphorylated peptides and are easy to make and use. The application of porous silica filters improves stability and reproducibility of nanoflow HPLC separations.

Experimenteller Teil

Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were flushed with 50% methanol/water and dried in a stream of helium using a pressure cell. Capillaries were cut to desired length (20-30 cm), quickly dipped into a droplet of Kasil solution (Kasil 1: Kasil1624: formamid; 3:1:1, NextAdavance, USA) and filled to the length of ca. 1 – 2 mm by capillary forces. Filled capillaries were left for 3h at room temperature and the solution was cured into porous silica frits at 100°C overnight. Finally the frits were cut to desired length of less than 1 mm with a ceramic cleavage tool.

Ergebnisse und Diskussion

Quantitative proteomic experiments by HPLC-MS/MS rely on the quality of peptide separation. The peak shape influences peak picking and integration of ion signals for quantification and high reproducibility of retention times is required to match peptide signals between analyses.

The quality of the nanoflow separation is often comprised by micrometer- and submicrometer-sized debris that accumulate on both trap columns and separation columns and lead to their clogging and increased wearing of valve seals and rotors. We found that common sources of these particles are C-18 desalting cartridges, gel material from direct injection of protein in-gel digests, microscopic precipitates and debris of valve seals.

Metal microfilter discs with pore sizes down to 1 µm were inefficient and also substantially increased the void volume of nanoflow LC setup. Therefore, we developed zero-dead volume inert porous silica frits and evaluated their application as filters.

Porous silica frits were excellent filters due to their porous structure that also excluded sub-micrometer sized debris. These frits did not affect peptide recovery and retention of unmodified peptides as well as phosphorylated peptides. Cured inside capillaries, the filters added no void volume and did not impact the resolution or symmetry of chromatographic peaks. These filters could be embedded within fused silica capillaries of various inner diameters from 20 µm to 200 µm and, because of the very low flow resistance, placed at any position of the HPLC system at the flow rates of 70-1000 nl/min. We used to place porous silica frits in the connecting line between autosampler and HPLC, at inlets and outlets of valves, at the vented columns. Altogether, they significantly enhanced the stability and reproducibility of nanoflow separations and improved the quality of label-free quantitative proteomics analyses.

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Neue Aspekte

Porous silica filters for improved quality, stability and reliability of nanoflow HPLC for quantitative proteomics

PP 8

Quantitative and specific derivatization of arginine residues by an improved protocol using malondialdehyde

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The dominance of arginine-containing peptides in MALDI-MS spectra of tryptic digests has been reported in previous studies[1]. Unfortunately, arginine-containing peptides require higher energy for a sufficient fragmentation than lysine-containing peptides[2]. The explanation for both phenomena is based on the high gas-phase proton affinity of arginine compared to those of other amino acids[3]. Thus, a reduction of basicity by derivatization of arginine could yield better peptide identification results. Several methods to modify arginine residues have been investigated. Apparently, the reaction with the highest quantitative output is the reaction with the acetal form of malondialdehyde (MDA) in a strongly acidic environment[4]. However, undesired side reactions occur. The work presented here aims at reducing these side reactions through an additional purification step.

Experimenteller Teil

TMP (1,1,3,3-Tetramethoxypropane)(5 ml) was dissolved in 180 ml of isopropanol and added to 5,5g of Amberlite-resin. The mixture was shaken for 2 hours. Afterwards the liquid was decanted and the contained solvents were removed by rotary evaporation. After two additional exchange steps (re-dissolving in 180 ml isopropanol, shaking, evaporating) a pale yellow solution was obtained[4].

The obtained solution was purified by column chromatography using hexane/ethyl acetate to yield TiPP as colorless fluid.

50 µg of peptides were dissolved in 50 µl of hydrochloric acid (12 M), and 1,5 µl of TiPP was added. After 1 hour of incubation at room temperature the mixture was diluted 1 : 100 with water and purified by solid phase extraction using C18-spin columns.

Ergebnisse und Diskussion

Educt for derivatization of arginine residues is the acetal form of MDA. Acetal forms of MDA may cause esterification (methylation in case of TMP) of carboxyl groups during the reaction through released alcohol. The tendency towards esterification is much lower for large and branched alcohols compared to isopropanol. As the isopropyl acetal form is not commercially available, TiPP was synthesized by transacetylation of TMP. The reaction yields a mixture of 80% tetraisopropyl form and 20 % triisopropyl-monomethyl form. The additional purification step of the reagent shows several improvements:

- the amount of derivatization side products is reduced (no methylation through absence of the monomethyl form)
- Possible polymers were removed (probable reason for yellow color)
- Storable for a longer time at 4°C in the dark is possible.

All in all, the procedure is improved by the additional purification step.

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Neue Aspekte

Less side products during modification of arginine residues by an improved purification protocol.

PP 9

Novel ImageID Workflow Combining MALDI Imaging and LC-MALDI for Obtaining Identification and Spatial Localization of Proteins from Eye Lens Tissu

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MALDI MS imaging of proteins is a well-established technique that compliments traditional histological methods in studying various tissue pathologies on the molecular level, comparing different tissue states and finding potential biomarkers. While information about spatial localization of intact proteins is obtained relatively fast it might be challenging to identify these proteins. Identification steps for the proteins of interest often require significantly more time than MALDI imaging experiments, particularly, if they have to be isolated from tissue first and in particular if FFPE tissue is being analyzed. This presentation is focused on the novel ImageID workflow that combines LC-MALDI with MALDI imaging thus correlating protein identification results in a bottom-up proteomics approach with protein spatial localization on tissue.

Experimenteller Teil

In this workflow 2 consecutive equatorial tissue sections of bovine eye lens were subjected to on-tissue proteolytic digestion by nebulizing trypsin solution followed by incubation at 37˚C for 2 hours. One of the two tissue slices was covered with 0.1% TFA to extract tryptic peptides for LC-MALDI-TOF/TOF analysis. The other tissue slice was sprayed with 2, 5-dihydroxybenzoic acid matrix for MALDI MS imaging at 100 µm spatial resolution. The list of identified peptides grouped by protein from LC-MALDI dataset was correlated to the peak list from MALDI MS imaging dataset using a software utility. The correlated peptides grouped by protein were visualized in the imaging software.

Ergebnisse und Diskussion

More than 100 proteins from bovine eye lens were identified and spatially localized using the combined MALDI imaging and LC-MALDI workflow. On average 12 of these proteins belong to the crystallin family which comprises up to 90% of protein content in the eye lens. The crystallin family is the most studied by MALDI imaging and best understood group of eye lens proteins. This workflow allows us to gain insight into spatial distribution of less abundant proteins. For example, tryptic peptides from retinal dehydrogenase co-localize in the nucleus of the lens in contrast to peptides from filensin that co-localize in the peripheral cortical region.

This combined workflow is vastly superior to direct MALDI-TOF/TOF sequencing of peptides from tissue in terms of number and search scores of identified proteins. The LC-MALDI component of the workflow allows identification and visualization of hundreds of peptide signals from imaging data set that otherwise would fail to provide peptide identities. The advantage over imaging followed by tissue homogenization and identification by LC-ESI-MS/MS is that the correlation between identification and imaging results is significantly better due to the fact that MALDI ionization was used for both parts of the workflow.

Neue Aspekte

Applying a combined MALDI Imaging and LC-MALDI workflow to determine spatial localization and protein identity to the eye lens

PP 10

Proteomic analysis of the presynaptic active zone derived from different murine brain structures

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Progress in the analysis of membrane proteins by mass spectrometry within the last decades facilitated an improved characterization of the presynaptic active zone (reviewed in [1]). Studies on this compartment from *Rattus norvegicus* were already performed [2] and protocols could be modified to isolate the presynaptic active zone from whole brain lysate of *Mus musculus*.

In this work, we are presenting results from the analysis of the presynaptic active zone derived from different brain regions, more precisely from hippocampus, cortex and olfactory bulb.

Experimenteller Teil

Synaptosomes from *Mus musculus* were freshly prepared in triplicate for each brain region similar to a previously described protocol [3]. Protein concentrations were determined by Pierce BCA Protein Assay (Thermo Fisher Scientific) and the use of detergents after immunoprecipitation (e.g. NP-40 and SDS) was omitted. Proteins were subjected to a tryptic in-gel digestion protocol.

Mass spectrometric measurements were carried out on an Easy nLC II (Thermo Fisher Scientific, Bremen) coupled to a micrOTOF-Q II instrument (Bruker Daltonics, Bremen). Peptides were identified using Mascot 2.4 (Matrixscience, London).

Ergebnisse und Diskussion

For each sample around 35 µg of protein could be isolated which appears to be consistent to the amount we determined for the whole murine brain lysate. By subsequent LC-ESI-MS/MS analysis after tryptic digestion around 150 proteins for each brain region could be identified in at least two replicates.

Even if no quantification method has been applied yet, these results can give useful insights into the differing composition of the presynaptic active zone purified from three different murine brain structures.

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Neue Aspekte

The murine presynaptic active zone of three different brain regions could be analyzed employing shotgun proteomics.

PP 11

Development a method for identification of protein in formalin-fixed paraffin embedded tissues

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Formalin-fixed paraffin embedded (FFPE) tissue is preferred in pathology for guaranteeing long-term stability of tissues (2). Formalin fixation chemically modifies proteins thus making the identification of proteins from FFPE tissue difficult (1,3). In this study, we developed a method for identification of proteins from FFPE tissues. We applied this method for assigning the identity of peptides underlying m/z signals in spectra yielded by mass spectrometry imaging (MSI) experiments.

Experimenteller Teil

20 consecutive tissue sections from FFPE tissue were sliced (6µm thickness), transferred to glass slides and deparaffinized, Tissue areas of interest were separated and transferred from the slides to defined vials (e.g. tumor tissue and non-tumor tissue), the tissue slices in the individual vials washed with antigen retrieval buffer and finally incubated with trypsin. The resulting tryptic peptides were analyzed by LC-Q-TOF, and LC-TOF-TOF. Protein identification was performed by processing the mass spectrometric data by OpenMS and by comparing the data with a protein database (SwissProt) by a search engine (MASCOT).

Ergebnisse und Diskussion

By the protocol developed in this study we were able to identify approximately 1000 proteins from only 20 sections of FFPE tissue. The identified proteins served as a database for assigning the identity of peptides observed in previous MSI studies of FFPE tissue micro arrays.

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Neue Aspekte

Development a method for identification of proteins in formalin-fixed paraffin embedded tissue.

PP 12

Achieve Low Flow Sensitivities with Micro Flow Chromatography on the QTRAP® 6500 System for Targeted Quantitative Proteomics

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There has been a significant amount of research focused on discovering proteins/peptides that are differentially expressed in specific cell and disease conditions. To confirm or refute their ultimate utility, many more samples must be analyzed with increased throughput and robustness, which means faster chromatography and/or higher flow rates. Even though best sensitivities are typically achieved using nL/min flow rates, there has been increased interest in working in the microflow regime (3-50 µL/min) to obtain a good balance between throughput, robustness and sensitivity. This study analyses the lower limit of quantitation (LLOQ) achievable for a range of peptide standards in both simple and complex matrixes while run at nanoflow on a QTRAP® 5500 system and microflow on a QTRAP® 6500 System.

Experimenteller Teil

Beta-Galactosidase digest and 6 Peptide mixture was obtained from AB SCIEX. The Six Protein Digest was obtained from Michrom BioResources. Crashed plasma matrix was prepared by mixing equal volumes of plasma and acetonitrile, followed by centrifugation. NanoLC experiments were performed on the QTRAP® 5500 System with NanoSpray® Source using a Eksigent ekspert™ nanoLC 425 System with Eksigent cHiPLC® System. MicroLC experiments were performed on the QTRAP® 6500 System with IonDrive™ Turbo V Source with 25 µm ID hybrid electrodes using an Eksigent ekspert™ nanoLC 425 System and the 1-10 µL/min flow module.

Ergebnisse und Diskussion

This comparative experiment analyzed the signal intensities for a standard beta-galactosidase digest at 10 fmol on column. The signal intensities were quite comparable between the nanoflow experiment on the QTRAP 5500 system and the microflow experiment on the QTRAP 6500 System. The total run time was reduced by 2 fold in the microflow experiment but separation resolution was preserved. The LLOQs were determined using both LCMS systems and the results across the peptides were compared. While some variation is observed across the peptide group, on average a 2x lower LLOQ was observed when using microflow configuration on the QTRAP 6500 system. The final comparison experiment was performed using the Bradykinin peptide in protein precipitated plasma. Standard concentration curves were run on both LC/MS platforms and LLOQs determined. The results for the microflow experiment on the QTRAP 6500 system provided an LLOQ of about 6 amol on column. On the QTRAP 5500 system at nanoflow, the LLOQ was found to be 12.5 amol.

Microflow chromatography on the QTRAP® 6500 system provides an easy to use, high-throughput workflow for performing targeted peptide quantitation. Assays currently performed by nanoflow LC on the QTRAP 5500 system could be easily translated to the microflow QTRAP 6500 system for accelerated sample analysis with similar sensitivities.

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Neue Aspekte

Microflow chromatography for fast and sensitive quantitation of peptides as an alternative for nano-LC separations.

PP 13

LC-MSE based quantification of heterologous expressed small cationic (antimicrobial) peptides from the leaf apoplast of Nicotiana attenuata

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The over-expression of antimicrobial peptides (AMPs) in plants is a promising approach for crop disease resistance engineering, but the small size and extreme pI range of these small cationic AMPs restricts the use of classical gel-based methods. Apart from the achieving high transgene expression, the peptide abundance in the apoplast needs to be assessed for each AMP individually to confirm their stability in *planta*. Despite recent advances in proteomics, high throughput methods for the analysis of AMPs from plant tissues remain lacking. We developed a protocol combining rapid apoplastic peptide extraction with nanoUPLC-MS^E analysis capable for high throughput plant screening to confirm stable expression of a variety of different AMPs and their quantitative comparison between different transgenic lines.

Experimenteller Teil

The Intercellular Fluid (ICF) was extracted from 35 – 45 days old *N. attenuata* plants using a modified vacuum infiltration method (Dani et al., 2005). The peptide fractions of the ICF samples were desalted by reversed phase solid phase extraction. For nanoUPLC-MS^E analysis 5 µL of desalted ICF samples (1 % of the final eluted fraction) were spiked with 1 pmol bovine serum albumin (BSA), used as internal standard for quantification of AMPs, digested with trypsin and analyzed by nanoUPLC-MS^E. The quantity of AMPs was calculated based on the relation between the intensity of the internal standard (BSA) to the peptides of interest (Silva et al., 2006, Ullmann-Zeunert et al. 2012).

Ergebnisse und Diskussion

We adapted a vacuum infiltration method for *N. attenuata* and tested different desalting procedures to optimize identification and quantitation of AMPs in ICF using nanoUPLC-MS^E analysis. Overall 7 out of 10 transgenic *N. attenua*ta lines including NaDefensin1 (DEF1), NaDefensin2 (DEF2), VrD1 (VRD), Fabatin-1 (FAB), Mc-AMP1 (ICE), Pn-AMP2 (PNA), and LJAMP2 (LEA) were confirmed for AMP expression using nanoUPLC-MS^E analysis. The defined amount of BSA, spiked into the samples, allowed the calculation of the molar concentration of each AMP per mL ICF or per g fresh mass (FM). The quantitative comparison among all plant lines indicated relatively low peptide amounts expressed within the PNA, FAB, DEF1 and VRD lines with only 0.2 – 11 pmoL g-1 FM. In contrast, three other plant lines (DEF2, ICE and LEA) indicated very high peptide amounts with 92 – 254 pmoL g-1 FM. As the DEF1 and DEF2 peptides were endogenous defensins of *N. attenuata*, peptide levels could be directly compared to native levels within an untransformed plant. The DEF1 peptide could indeed be detected in the ICF of WT plants and in most of the other transgenic lines as well. The DEF1 over-expression line showed the highest peptide amounts, which was about 16-fold higher than the average found in all other lines. This correlated with the expectations from gene expression data, which showed on average a 16-fold increase in transcript level compared to WT. The DEF2 plants showed much higher transcript levels, which were on average 450-fold higher compared to WT. This was as well consistent with the calculated peptide amounts, which were 348-fold elevated compared to the basal amount found in some transgenic lines.

Referenzen

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Neue Aspekte

Universal high-throughput screening protocol for confirming stable expression of antimicrobial peptides and their quantitative comparison between different transgenic plant lines

PP 14

Development of methods for Information-driven MS/MS (ID-MS/MS) for increased identification rates in bottom-up proteomics of human blood serum

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Shotgun bottom-up proteomics is widely used for biomarker discovery or therapeutic target analysis. The approach is still limited for biofluid samples with a wide range of protein concentrations like blood serum, preventing a comprehensive overview within a single analysis. To increase analytical depth and coverage, an iterative process of excluding previously identified peptides was applied. A set of subsequent LC-MS/MS runs with exclusion of already detected peptides allowed identification of proteins of lower abundance and decreased redundancy.

Experimenteller Teil

Sigma male AB serum was depleted of Serum Albumin and IgGs before tryptic digestion. Digested serum was used as a test sample to evaluate the efficiency of peptide exclusion in subsequent LC-MS/MS runs on a UHR-Q-TOF instrument. The effects of column load, analysis repetition with exclusion of already identified peptides and different acquisition strategies were assessed. All data were transferred to a data analysis software (ProteinScape, Bruker Daltonics) which allowed pooling of analyses and, following the database search (Matrix Science MASCOT MSMS ion search in Swiss Prot DB at 1.5% False Discovery Rate FDR), generation of peptide exclusion lists and the compilation of a non-redundant protein list.

Ergebnisse und Diskussion

The identified peptides from the iterative runs were added to an increasingly longer “Scheduled Precursor Exclusion List” (SPExL) which was extracted automatically from MASCOT search results during data analysis and applied to the MS-control software to re-acquire data from the same sample. Using a 90 min gradient with an 800 ng column load, the first run lead to 199 identified proteins. The first round SPExL run led to the identification of 179 new proteins for a total of 369, the 2nd round SPExL to additional 68 proteins, and a 3rd round SPExL increased the list to further 62 proteins. The increase of the total number from 199 to 467 unique proteins was obtained by only three additional injections.

Neue Aspekte

Stepwise use of automatically generated SPExL for subsequent LC –MS2 that enable better identification gain at each step than replicates.

PP 15

HPLC of peptides: Increasing speed and resolution with fused-core technology and decreasing detection limits in LC-MS by elimination of TFA

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The theory of fused-core technology and its application for LC-MS separation of peptides and small proteins will be presented. Additonally, an approach on how to successfully apply formic acid as additive in mobile phases for improved LC-MS sensitivity will be shown and discussed.

Experimenteller Teil

A complex mixture of several tryptic digests was used to investigate the peak capacity of HPLC columns with fully porous and fused-core particles.

In a second experiment trifluoroacetic acid was replaced by formic acid and peak size, peak shape and peak area were evaluated.

Ergebnisse und Diskussion

Fused-core particle technology provides higher efficiency, peak capacity and sensitivity for protein and peptide analysis. The use of formic acid as additive shows higher sensitivity.

Adjusting the pH of formic acid to 3.5 shows higher sensitivity and improved peak shape.

Referenzen

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Neue Aspekte

At higher pH, in particular at pH 3.5, experimental conditions are optimized for chromatography and mass spectrometry of peptides.

PP 16

Venomic profiling of the Caucasus viper by high resolution mass spectrometry

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Snake venoms are complex mixtures of bioactive peptides and proteins. A deep understanding of the composition of venoms is of high importance not only for exploring their enormous potential as sources of pharmacological novelty, but also to fight the dire consequences of snakebite envenoming [1]. In the last decade several bottom-up proteomic strategies, to explore venom proteomes have been developed [2]. Drawbacks of these approaches are the co-elution (HPLC), low mass resolution (SDS-PAGE), or impaired quantification ability (2DE) of venom components. Further, the digestion often results in difficulties assigning all proteoforms [3].To overcome these problems, we applied a fast LC-MS approach for the rapid venom characterization of the Caucasus viper (*Vipera kaznakovi).*

Experimenteller Teil

For our experiments we used freeze dried venom of *Vipera kaznakovi*, from Turkey. The LC-ESI-HR-MS experiments were carried out on an Orbitrap XL mass spectrometer (Column: Suppelco Discovery 300Å C18 column 2 x 15 mm, 3µm). MS/MS spectra were obtained in IDA mode using CID or HCD. In gel digestion of the isolated proteins was performed after LC separation and SDS-PAGE with trypsin. The resulting peptides were submitted to LC-MS/MS (Column: Grace Vydac 218MSC18, 2.1 x 15 mm, 5µm). MS/MS spectra were interpreted manually and resulting sequences were searched against a viperid protein database using blast.

Ergebnisse und Diskussion

Individuals of Vipera kaznakovi, which can only be found in the Caucasus region, were collected in north eastern Turkey and venom was „milked“ by the „parafilm method”. To profile the venome toxins we mapped the intact molecules by LC-MS and identified the proteins or peptides regarding there masses directly by IDA MS/MS or after SDS-PAGE and in gel digestion followed by LC-MS/MS. Relative protein quantification was achieved by integrating UV peak areas and XIC peak areas of co-eluting compounds.
The venom consists of bradykinin-potentiating peptide (~1%), nerve growth factors (~1%), C-type lectins (~3%), Serine proteases (~5%), cysteine-rich secretory protein (CRISP, ~7%), SVMP inhibiting tripeptides (~12%), phospholipases A2 (PLA_2, ~23%), snake venom metalloproteases (SVMP, ~44%) and non-assigned components (~4%). As isoforms we observed mass shifts of 18 and 16 Da for 2 PLA_2s and several mass differences of 146 Da for various SVMPs shown in figure 4. These findings indicate varying sugar moieties, most likely fucosylation of the metalloproteases.

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Neue Aspekte

We performed a comprehensive LC-MS/MS based venomic profiling of *Vipera kaznakovi,* which sets the frame for our further bioactivity screening.

PP 17

Identification of a novel dimeric product of the Drosophila adipokinetic hormone gene by mass spectrometry

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Drosophila melanogaster has recently become state of the art model organism for analyzing neurobiological and endocrine systems, due to its complete genome sequence and established genetic tools. Neuropeptides represent the most diversified group of endocrine transmitters found in animals, with functions ranging from circulating hormones to neurotransmitters. Different algorithm-based genome-wide searches identified 45 GPCRs and about 42 genes encoding multiple neuropeptides [1]. Even though mass spectrometry has become the most elegant method for analyzing neuropeptide sequences, posttranslational processing and spatial distribution, a number of predicted neuropeptides could not be identified to date. In this study we describe a protocol for identification of a hitherto unknown dimeric product from adipokinetic hormone (*akh*) gene of *Drosophila* using MALDI-TOF-MS from single fly preparations.

Experimenteller Teil

Retrocerebral complexes of single adult flies were dissected with forceps in cold NH_4Cl-buffered saline. Isolated tissues were transferred on a MALDI sample plate for direct tissue profiling or homogenized in acidified methanol.

MALDI-TOF-MS was performed on an UltrafleXtreme (Bruker Daltonik, Bremen, Germany) using 1,5-diaminonaphthalene (DAN) and 2,5-dihydroxybenzoic acid (DHB) as matrix substances. DAN was dissolved in 0.1% TFA containing 50% acetonitrile at a final concentration of 10 mg/ml. 10mg/ml DHB was dissolved in 1% formic acid containing 20% acetonitrile.

Since DAN preparations were well suited for identification and characterization of monomeric subunits but insufficient for complete sequence confirmation, further experiments were performed with tissue extracts that were reduced and alkylated with iodoacetamide and dithiothreitol following standard protocols.

Ergebnisse und Diskussion

Detetection of dimeric Drm-APRP

Direct profiling of single retrocerebral complexes of *D. melanogaster* (DHB) yielded mass matches with double and single charged dimers ([M + 2H] 5146.4625, [M+H] 10291.918) of the AKH precursor related peptide (APRP) from the *akh* gene. This peptide was not identified from *D. melanogaster* so far, although an ion signal mass identical with the potential single charged monomer was recently reported [2]. To confirm the sequence of the monomer and validate the proposed dimeric organization, different disulfid bridge-reducing methods were applied (DAN, alkylation).

Confirmation of dimerization and protein sequence

Preparations of single retrocerebral complexes were analyzed with DAN resulting in the detection of the putative single charged monomer of APRP. MALDI-TOF-MS/MS experiments using these DAN-samples, however, did not reveal sufficient fragments for sequence confirmation. Therefore, analysis of reduced and alkylated extracts of retrocerebral complexes was subsequently performed using the same instrument settings and confirmed the sequence of APRP as SVGGAGPGTFFETQQGNCKTSNEMLLEIFRFVQSQAQLFLDCKHRE-OH.

While the physiological function of AKH is known - it represents the insect analog of vertebrate glucagon, a function of APRPs has so far not been elucidated [3]. However, complex posttranslational processing of the APRP as reported for locust and cockroaches, a simultaneous release with AKH in Locust [4] as well as a highly conserved peptide structure in insects [5] support the theory of a possible physiological role of APRP in Drosophila and other insects. The specific function can now be studied using a vector with an altered *akh* gene construct.

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Neue Aspekte

The confirmation of the dimeric structure of *Drosophila*-APRP enables the design of studies regarding the function of this conserved peptide.

PP 18

Enhanced Protein/Peptide Characterization Using Electrochemically Assisted Disulfide Bond Reduction

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In this poster, we present electrochemical (EC) reduction of biologically active peptides and proteins containing disulfide bonds followed by on-line mass spectrometric detection. The method does not use any chemical agents and is purely instrumental.

Experimenteller Teil

In infusion mode experiments, typically 0.9 - 5 µM solutions of the target compound in 1% formic acid /acetonitrile (90/10, v/v) were pumped into the electrochemical (EC) cell at a flow rate of 50 µL/min and the outlet of the cell was directly connected to the ESI-MS.

In flow injection experiments, the sample was introduced in 0.1% formic acid and 5% acetonitrile using 5µL injection loop. The mobile phase consisted of 0.1% formic acid and 25% acetonitrile. 1% formic acid was mixed with the mobile phase prior the electrochemical cell. The total flow rate was 100µL/min. The cell was operating in pulse mode to reduce the compounds of interest.

Ergebnisse und Diskussion

To demonstrate the performance of the EC reactor cell online with electrospray mass spectrometry, insulin (a small protein of 5733 Da containing 3 disulfide bridges) and somatostatin (one disulfide bond (1638 Da)) were used as model compounds. Efficient reduction is achieved in continuous infusion mode using an EC reactor cell with a specially developed titanium based working electrode and a square wave potential pulse. Under optimized conditions, the presented method shows almost complete reduction of insulin and somatostatin. The method does not require any special sample preparation, and the EC reactor cell makes it suitable for automation.

Online EC reduction followed by collision induced dissociation fragmentation of somatostatin showed more backbone cleavages and improved sequence coverage.

By adjusting the settings, the EC reaction efficiency was gradually changed from partial to full disulfide bonds reduction in α-lactalbumin (four bonds (14 178 Da)), and the expected shift in charge state distribution has been demonstrated. The possibility of online and gradual disulfide bond reduction adds a unique dimension to characterization of disulfide bonds in mid and top-down proteomics application.

The effects of different experimental parameters such as potential, flow rate, mobile phase composition, were tested and resulted in an optimized protocol for the electrochemical reduction of disulfide bonds [1]. Furthermore, we show data on long term stability and repeatability based on flow injection analysis.

A new instrumental set-up based on electrochemical reduction of the disulfide bonds after the HPLC separation will be shown, i.e., on-line LC/EC/MS, to develop a fully automated S-S bond assignment system. Examples for somatostatin and Insulin will be presented.

The use of electrochemical reduction of disulfide bonds facilitates the determination of disulfide bond arrangements and further supports efficient peptide and protein identification using top- and mid- down strategies.

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Neue Aspekte

Efficient reduction of disulfide bonds using a novel electrochemical approach based on a new electrode type and square wave pulses.

PP 19

Purification and digestion of low amounts of protein on PVDF membrane

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The interfering effects of ionic detergents on MALDI-MS are well-known [1]. However, in many fields of research they are essential, since they serve as a solubilizing agent for hydrophobic (membrane) proteins [2] or in SDS-PAGE protein separation. If preparative electrophoresis is chosen, the detergent often has to be removed for further sample processing [3].

The existing removal methods are as various as their disadvantages: They are elaborate (dialysis, gel filtration), expensive (RP spin columns, RapiGest) or reduce sample concentration (dilution) [4].

This work intends to develop an alternative method for detergent removal which doesn't require any extra effort or expense. Furthermore, a reduction/alkylation protocol for proteins blotted on PVDF membranes was developed and sample preparation for MS measurement was optimized.

Experimenteller Teil

Round pieces (diameter = 3 mm) of PVDF membrane (pore size = 0,2µm) were used to adsorb the protein from the initial solution and were transferred to a new tube containing 20 µl of 0,5% Octylglycoside/10% Acetonitrile in 25 mM ammonium bicarbonate buffer. The subsequent digestion was performed with trypsin with an enzyme/substrate ratio of 1:1, but at least 100 ng of trypsin.

A serial dilution of BSA was prepared to elucidate this method's LOD, considering sequence coverage and spectrum quality. MS-Measurements were performed with α-cyano-4-hydroxy-cinnamic acid as matrix in an MALDI LTQ Orbitrap XL from Thermo Scientific. PMF spectra were searched against a standard protein database using Mascot V.2.4 .

Ergebnisse und Diskussion

Purification worked down to an overall protein amount of 1,5 pmol (100 ng BSA), but sequence coverage and quantity of peptides decreased compared to somewhat higher amounts of substance, e.g. 7,5 pmol.

The development of an on-membrane reduction/alkylation method revealed that no special buffer (like required for on membrane digestion) was necessary. It can easily be performed in ammonium bicarbonate buffer. Excess iodoacetamide has to be removed by a second reduction using dithiothreitol. Removing this excess by washing the membrane showed inferior results.

Furthermore, MS measurements of the diluted digestion buffer turned out to be more advisable than extracting the peptides from the membrane with 70 % Acetonitrile in 0,1 % TFA in a second step.

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Neue Aspekte

An inexpensive method for protein purification and digestion on membrane was developed.

PP 20

Cleavage-Site Profiling of the Proteases ADAM-10 and ADAM-17

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The transmembrane proteases ADAM-10 and ADAM-17 belong to the family of the ADAM (A Disintegrin And Metalloproteinase) proteases involved in ectodomain shedding of transmembrane proteins. Disregulation of proteolytic activity of these proteases was observed to be involved in many diseases such as inflammation, cancer or neurodegenerative diseases [1].

The goal of this study was to investigate the cleavage-site specificity of recombinantly expressed ADAM-10 and ADAM-17 by using the Q-PICS approach [2] based on the PICS method established by Schilling and Overall [3]. This study compares the outcome of the experiment by using two different technologies of mass spectrometry: LC MALDI-TOF/TOF MS and LC ESI-Orbitrap MS.

Experimenteller Teil

Two different peptide libraries were generated based on the cytosolic fraction of a yeast proteome. After reduction and alkylation, the proteome was digested with either Lys-C or Glu-C as 'work-proteases'. Primary amines were blocked by reductive dimethylation and the peptide libraries were purified via SPE. Six technical replicates of the two peptide libraries were incubated with either ADAM-10 or ADAM-17.. Newly formed N-Termini, resulting from the digestion with the 'target-proteases, were labeled with TMT-Sixplex and the six internal technical replicates were combined in a ratio of 1:1:1:1:1:1. Samples were separated by IP-RP nano-HPLC coupled either offline to MALDI-TOF/TOF MS, or online to ESI-Orbitrap MS.

Ergebnisse und Diskussion

We optimized Q-PICS (Quantitative Proteomics for the Identification of Cleavage Sites) to elucidate the cleavage site specificity of recombinant murine ADAM10 and ADAM17. Two different yeast proteome-derived peptide libraries were used and analyzed by both LC-MALDI and LC-ESI MS/MS in parallel. We show that the largest difference in the cleavage site specificities of ADAM10 and ADAM17 is at the P1' site: While both enzymes cleave N-terminal of leucine, only ADAM10 shows additional preference towards aromatic amino acids, whereas ADAM17 exhibits highest preference for valine. Together with further amino acid preferences more adjacent to the scissile bond our data is in good agreement with ADAM10/17 cleavage sites previously identified in native substrates [4]. Overall, the precise identification of ADAM10 and ADAM17 cleavage specificity provides the basis for better substrate identification *in vivo* and the generation of specific inhibitors or activity based probes.

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Neue Aspekte

Elucidation of cleavage site specificities by means of complementary LC-MS platforms using proteome derived libraries and isobaric labeling.

PP 21

Mass spectrometric analysis of protein profiles in RGB-labelled tumor cells

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The method of red-green-blue (RGB) marking by using lentiviral vectors allows labelling of individual cells with highly specific colour codes. Thereby, it enables clonal cell tracking after transplantation of RGB-labelled cells into mice e.g. to investigate tumour formation [1].

We transduced the human neuroendocrine tumour cell line BON1 and focused on differential protein expression patterns of tumour cells which have been expanded ex vivo relative to defined colours before and after injecting of tumour cells into *scid*-mice. The identification of up- and down-regulated proteins will provide new insights into mechanisms related to tumorigenesis.

Experimenteller Teil

RGB-labelled cultured BON1 cells were transplanted into spleens of *scid*-mice to engraft in the liver. After 5-6 weeks mice were sacrificed and livers were dissected [2].Distinct tumours were selected and expanded in vitro.

For protein pattern analysis, tumour cells of three tumours characterized by their colour (yellow, purple and white) were subjected to 2-D gel electrophoresis. Untransduced BON1 cells were used as a control.

Protein spots, which showed an increase or a decrease of intensity in silver staining approaches were selected and subjected to tryptic digestion. Analysis of the tryptic peptides was performed on an LC-ESI-MS/MS system applying an ion-trap as a mass-analyser. Protein identification was performed via the search engine Mascot using the UniProtKB protein database.

Ergebnisse und Diskussion

The 2-D gels of proteins of the yellow, purple and white *ex vivo* expanded RGB-marked BON1 tumour cells obtained from mouse were very similar. Only few differences were detected relating to the intensities of the spots. Three spots in the gel were presented in the yellow tumour cells, which were not visible in the two other gels (purple and white tumour cells). Furthermore one spot in the gel was detected in the purple tumour, the same spot with a lower intensity was detected in the yellow tumour but no spot was detected in the white tumour. However, the differences were limited.

The spots of the 2-D gels of the different coloured *ex vivo* expanded RGB-marked BON1 tumour cells were also compared with the spots of the 2-D gel of the control BON1 tumour cells. Here, the differences in the spots were more pronounced. There were nine spots in the gel visible in the control BON1 tumour cells which did not exist in the gels of the three *ex vivo* expanded RGB-marked BON1 tumour cells. Contrary, more than twelve spots were detectable in the gels of the three *ex-vivo* expanded RGB-marked BON1 tumour cells, which were not detected in the control BON1 cancer cells.

In total 60 spots were analysed and the underlying proteins were identified. It can be assumed that the identified proteins are associated with the properties, which have to be developed by the BON1 tumour cells to survive and grow in the environment in the mouse tissue and thus may reflect mechanisms that occur during tumorigenesis. In the future by orthogonal methods we will validate the results concerning the changes in the concentration of the identified proteins and will investigate the impact of these proteins.

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Neue Aspekte

Combination of RGB marking of cancer cells with proteomic analysis.

PP 22

Characterization of Proteomics Performance of a Novel Collision Cell for Ultrahigh Resolution Time of Flight Mass Spectrometers (UHR-TOF)

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For characterizing complex samples in proteomics applications require a highly sensitive but also fast generation of high quality fragment spectra. Typical samples can consist of hundreds of thousands of different peptide species covering a concentration range of several orders of magnitude. For quadrupole time of flight instruments the collision cell is a critical part for efficiently generating and collecting fragment ions and for sampling them into the mass analyzer.

Experimenteller Teil

A novel collision cell design with an elongated axial confinement has been developed and evaluated on a UHR TOF (Bruker maxis 4G). It allows the reduction of the collision gas pressure while maintaining the fragmentation efficiency. Using this novel design, ions penetrate deeper into the collision cell until they are kinetically cooled down. To maximize width of mass transfer, dielectric strength (including new materials) and general high voltage RF capabilities have been improved. As model system for complex proteomics samples tryptic digests of whole cell lysates (1 µg human cancer cell lines HeLa and MOLT4) were separated using nano RP HPLC (90 min gradient) with captive spray ionization at a flow rate of 300 nl/min.

Ergebnisse und Diskussion

When applying this novel design to HeLa samples, reliably 2500 proteins were identified. The false discovery rate was less than 1 %. Signal intensities and identification rate remained constant for 200 consecutive replicates of this LCMSMS experiment. The improved peptide identification compared to the previous design could be attributed to a more efficient sampling of fragment ions but also to a more robust AutoMSMS fragmentation.

Neue Aspekte

The false discovery rate was less than 1 %

PP 23

Accelerated proteolytic digestion in the microwave – fiction or reality?

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The conventionally used enzymatic digestion time of 18h appears to be a major bottleneck in the accelerating world of proteomics. Therefore, many efforts were made to shorten the digestion time with different techniques and varying success[1]. Numerous protocols for ultrafast organic reactions and enzymatic digestions of proteins have been published [2]. The use of a microwave oven was reported to be a good choice to get efficient and rapid enzymatic digests of proteins when compared to the "gold standard" overnight digestion. In this study, we compare microwave digestion with a conventional digest at equal time points and temperatures.

Experimenteller Teil

In order to evaluate the digestion efficiency we used two different proteolytic enzymes, the commonly used highly specific serine protease trypsin (E:P =1:50) and the less specific serine protease elastase (E:P=1:20). Digests were performed using a CEM Discover System (CEM, Matthews, USA) with a maximumpower output of 300W and a frequency of 2.45GHz.

BSA was taken as a standardprotein in a final concentration of 0.25µg/µL. Digestion times of 5min to 2h were tested. Apart from proteolytic digestion we evaluated an accelerated reduction and alkylation protocol in the microwave.

Ergebnisse und Diskussion

Taking a look at the tryptic digests performed, the digest in the microwave after 5min indeed resulted in a better sequence coverage than the overnight digest. However, this is due to additional peptides with missed cleavages in the microwave digestion mixture at short digestion times. In contrast to that, digestions in microwave and shaker after 5 minutes at the same temperature show no difference. In a tryptic digest the sequence coverage usually decreased slightly over time due to the generation of peptides below the acquired m/z range.

For elastase, sequence coverage increased to a maximum at 30min in the thermo shaker as well as in the microwave showing no differences.

To sum up, the apparent superiority of the microwave results only from incomplete cleavage at the shorter digestion time compared with the overnight digestion.

When comparing a significantly shorter reduction and alkylation workflow in the microwave with conventionally reduced and alkylated samples no difference could be observed.Therefore, using the microwave instead of the thermo shaker for reduction and alkylation is still a good way to accelerate the overall sample preparation process.

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Neue Aspekte

Digestion in the microwave and in the thermo shaker at equivalent time points show similar results

PP 24

Mass spectrometric and bioaffinity epitope investigations of spondyloarthritides related HLA-B27

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The Human Leukocyte Antigen (HLA) class I B27 molecule is major susceptibility factor for the development of Ankylosing spondylitis (AS) and other Spondyloarthritides (*1*). In the presence of short peptides derived from viruses or bacteria and β2-microglobulin light chain the HLA heavy chain forms non-covalently associated heterotrimers which migrate from the endoplasmic reticulum to the cell surface where they are recognized by CD8+ cytotoxic T cells through their T cell receptors.

Experimenteller Teil

Two previously reported monoclonal antibodies, HD5 and HD6 (2-4), recognizing B27 free-heavy chain have been provided for this study by University of Zurich. HD5 binds specifically to HLA-B27 homodimers ($B27_2$), whereas HD6 recognizes both $B27_2$ and B27-free-heavy chains forms. To conduct this investigation, an affinity mass spectrometric approach was used. The HD5 monoclonal antibody was immobilized on a sepharose column and its affinity to the HLA-$B27_2$ was investigated by in-gel tryptic digestion. For the epitope identification an epitope-excision experiment was performed.

Ergebnisse und Diskussion

The HLA-$B27_2$ was immobilized on the antibody column and proteolytically digested with trypsin. After the wash of the supernatant, the epitope was eluted with 0.1 % TFA in milliQ and identified by mass spectrometry. The epitope proved to be discontinuous comprising of two small peptides united together by a disulphide bridge HLA-B27 [203-219]-SS-[257-273]. The two epitope peptides were synthesized via SPPS (Fmoc chemistry) and let to freely dimerize in oxidative conditions. The hetero- and homodimers were separated via RP-HPLC and their affinity was qualitatively characterized via affinity-MS experiments. It was observed that a mixture of the two epitope peptides would yield the heterodimer epitope in the presence of the antibody and this is the only species that elutes from the affinity columns. Furthermore, the precise K_D values of the epitope peptides (monomers, homo- and heterodimers) were measured by the use of a SAW Biosensor.

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Neue Aspekte

Spondyloarthritides related HLA-B27 epitope determination and characterization against HLA-B27 free heavy chain monomers and dimers antibodies.

PP 25

Improved Identification and Quantitation of Host Cell Proteins in Protein Therapeutics using 2D-LC and Ion Mobility Mass Spectrometry

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Residual host cell proteins (HCPs) are ppm-level contaminants in biotherapeutics that may elicit an unpredictable immune response and need to be monitored as part of regulatory guidelines. Data-independent analysis with 2D chromatography has been used to measure HCPs over 5 orders of magnitude in concentration (1). The inclusion of ion-mobility into the analytical method adds an orthogonal separation to chromatographic and mass spectral analysis. In this study, 2D-LC was combined with ion mobility to identify and quantify HCPs in biotherapeutic samples with increased throughput.

Experimenteller Teil

Standard proteins were spiked into a mAb sample (trastuzumab) at levels ranging from 8 to 1000 ppm to assess the ability of the method to identify and quantify proteins at typical HCP levels in a therapeutic. Multidimensional chromatographic methods were employed using high-low pH RP-RP (2) with discontinuous step-gradients. Increasing the number of LC fractions in 1st dimension from 5 to 10 improved the limit of detection from 80 to 8 ppm, while doubling the analysis time. The same benefit was achieved by incorporating ion-mobility into the analysis, with no additional instrument time. Ion mobility also yielded more reproducible and accurate quantitation of the lowest abundance HCPs.

Ergebnisse und Diskussion

A comparison was made between a traditional 2D method and a faster technique that utilized simultaneous gradients in both dimensions and multiple trapping columns in order to increase throughput. The faster technique only used 70% of the time of the typical 5-fraction method and the percentage of time savings will increase as the number of desired fractions increases. The precision and accuracy of HCP determination will be compared and contrasted with the increase in peak capacity. Measurement of the HCPs in a variety of protein therapeutics will also be presented.

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Neue Aspekte

New aspects for measurement of HCPs in a variety of protein therapeutics.

PP 26

Data Independent Analysis – A New Strategy for Discovery of Phosphopeptides in Complex Proteome Samples

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Current mass spectrometric techniques for PTM discovery in proteomic samples are often a compromise between sensitivity, speed, selectivity, robustness and comprehensive analysis. Recently, a novel data independent analysis (DIA) approach was introduced to overcome these limitations and also to allow retrospective analysis. This new innovative analytical strategy, which includes nano liquid chromatography and QqTOF mass spectrometry was developed to combine analytical sensitivity, selectivity, speed and robustness for untargeted discovery including quantitation of all detected peptides. The aim of this study was to establish a LC-data independent analysis workflow on MSMS level in combination with QqTOF system technology and T-Test/PCA analysis for the discovery of phosphopeptides a complex protein digests.

Experimenteller Teil

In this study we established a strategy for untargeted phosphorylation discovery. Therefore we split a given sample into two aliquots and dephosphorylate one of the aliquots. Only the phosphopeptides make up the difference between the samples – one carries the modification, the other doesn't. After DIA of both aliquots we generate high resolution peptide fragment XIC's with the help of a spectral library of the entire sample and integrate their areas from all samples including replicates. This information is then analyzed with T-Test and PCA on protein, peptide and individual transition level to identify those peptides/proteins which make up the difference in both samples.

Ergebnisse und Diskussion

Our preliminary results demonstrated applicability and robustness of this strategy for an untargeted PTM discovery of phosphopeptides in complex protein digests. We were able to find all peptides in replicate analysis from a mixture (Ang II: DRVpYIHPF, Cholecystokinin (10-20): IKNLQpSLDPSH, Calcitonin (15-29) DFNKFHpTFPQTAIGV) spiked into a E.coli digest as phosphorylated form in one aliquot and nonphosphorylated form the second one. In order to show the robustness of the entire workflow, including pre analysis sample treatment, we spiked an E.coli digest with α-Casein digest and followed the strategy mentioned above to identify the phoshopeptides in the complex matrix. Also here we could show that only the phosphopeptides are highlighted as a differentiator between the samples and as a consequence are clearly identified.

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Neue Aspekte

Using a data independant analysis approach for an untargeted phosphorylation analysis.

PP 27

Methylation artefacts introduced during standard proteomics sample preparation workflows and its impact on Histone PTM analysis.

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Standard procedures for detecting PTMs, which change the physicochemical properties of peptides such as phosphorylation, acetylation or glycosylation, have been developed in the last ten years. In contrast, efficient enrichment strategies for lysine or arginine methylated peptides are lacking since antibodies are mostly site-specific. In addition, the mass shift of 14.015650 amu introduced by a methyl group (or its integer multiplicity) often results in a high rate of false positive identifications by database search engines. This can be due to both misinterpretation of (i) amino acid exchanges (e.g. alanine for glycine), (ii) the false annotation of spectra originating from peptides harbouring two modifications that mimic the isobaric mono-, di- or tri-methylations, or (iii) the generation of sample preparation artefacts.

Experimenteller Teil

For unambiguous identification of arginine/lysine methylated peptides we previously introduced HeavyMethyl (HM)-SILAC. Here, we closely inspect potentially methylated peptides without HM-SILAC-labelling by (i) inspection of high quality MS2 spectra and (ii) implementation of post-labelling strategies. We interrogated all sample preparation protocol steps including fixation, staining, washing and digestion by performing the following experiments: (i) completely replacing primary alcohols with acetonitrile, (ii) selectively adding deuterated alcohols (methanol-d4 or ethanol-d6) at certain steps, (iii) post-labelling with deuterated alcohols of peptides marked with ^{18}O at their C-terminus during proteolytic digestion (trypsin, chyomotrypsin and other proteases). Taking these results about artefacts into consideration, the H2B-Nterminus with its isoforms that mimick methyl-groups, in particular PEP and PDP isoforms, was extensively studied.

Ergebnisse und Diskussion

Database search engines frequently predict methylated peptides not showing HM-SILAC peak pairs. Among these we observed many tryptic peptides supposedly harbouring a di-methyl group at the C-terminal residue even though trypsin cleavage at such sites is extremely inefficient. When spiking synthetic di-methylated peptide standards, the chromatographic retention time between “endogenous” and “synthetic” peptide was substantially different. Furthermore, Mascot results revealed many low scoring peptides predicted to carry lysine/arginine methylation, for which the y- or b-type ion series abruptly stops at Glu or Asp residues within the sequence. Therefore, we hypothesized that artifactual modifications mimicking arginine/lysine di-methylation are introduced during sample preparation.

Careful examination of our in-gel-digestion workflow on histones using methanol-d4 or ethanol-d6 revealed that Coomassie staining always results in the addition of methyl- or ethyl groups to aspartic or glutamic acid depending on the incubation time. We concluded that either completely omitting primary alcohols or replacing them with methanol-d4 can solve this problem: This strategy was extremely useful to characterize the methylation landscape of the H2B N-terminus.

Interestingly, many but not all peptides are prone to detectable incorporation of primary alcohols at the carboxyl-termini generated by trypsin. The process is driven by trypsin (not observed for other enzymes tested) and can take place during or post digestion. Since MS2 spectra cannot discriminate if a modification is placed at the carboxyl group or the side chain of the C-terminal amino acid we have set up a double labelling strategy. First the C-terminal carboxyl group is labelled with ^{18}O water resulting in the incorporation of two ^{18}O-species (4 amu mass shift). Second we measure the potential exchange of one ^{18}O (corresponds to 2 amu) for a methanol or ethanol molecule at the carboxyl terminus.

These new results enabled us to unambigously proof the existence of H2B Nterminal methylation in mamals.

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Neue Aspekte

Trypsin catalysed C-terminal esterification; methyl-group mimicking artefacts originating from in-gel digestion protocols; chemical post-labelling (deuterated alcohols); in-depth H2B N-terminus characterization

PP 28

Determination of Site Specific Acetylation Degree by All-in-One Fragmentation of Deuteroacetylation Isotopologues.

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Lysine acetylation is a reversible posttranslational modification (PTM) that plays a crucial role in many cellular processes. Acetylation is dynamically regulated by lysine acetyltransferases (KATs, aka. histone acetyltransferases HATs), and by lysine deacetylases (KDACs, aka. histone deacetylases HDACs)[1]. Localization and quantitation the acetylation degree of individual lysine residue are initial and crucial steps in deciphering the molecular mechanisms of acetylation-related biological processes.

Despite the great importance of acetylation for biological functions, its study has been hampered by the lack of suitable methods. On the global scale, exclusively relative quantitation methods (e.g. SILAC) have been applied so far. For samples of lower complexity, the acetylation stoichiometry can be determined by chemical deuteroacetylation on the protein level (Cotter et al. 2012)[2].

Experimenteller Teil

Destained/dehydrated H3 bands were resuspended in a solution containing 50µL (1:1 $C_2H_3NaO_2$: $C_2D_3NaO_2$) and 10µL (1:1 $C_4H_6O_3$: $C_4D_6O_3$) and incubated at RT for 6h. The treated gels were rinsed with ammonium-bicarbonate, dehydrated/dried in a Speed-Vac to remove remaining salts.

Proteins were digested in-gel; resultant peptides were prepared for HPLC-ESI-MS/MS analysis in an EASY-nLC1000 autosampler coupled online with Thermo Scientific LTQ-Orbitrap-Velos Pro.

Peptides of interest were chosen for a targeted all-in-one fragmentation method using a wider isolation width of ± 7 Da to contain all of their isotopologues and the dynamic exclusion was disabled to enable the collection of more MS-MS spectra over the peak.

Ergebnisse und Diskussion

In general, two main problems hamper the quantitation of PTMs degree; a) Different ionization efficiencies of modified and unmodified peptides; and b) The presence of PTMs influences the protease cleavage sites.

We developed a workflow that allows determining the site specific acetylation degree of individual proteins. We applied a method comprises in-gel acetylation of free lysine residues using deuterium-labeled acetic anhydride and saturated sodium acetate. Performing the modification reaction in such conditions results in increased specificity, because *O*-acetyl tyrosine is unstable in sodium acetate, unlike using acidic acid[1] that induces the generation of undesired side-products.

Although this method is straightforward for determining the acetylation degree of isolated acetylation sites, it is challenging when analyzing clusters of acetylation sites (typical in histones). E.g. a peptide contains 8 lysine residues generates 9 different isotopologues, each of which may consist of several isotopomers. We observed a retention time shift (deuterium-labeled species elute few seconds earlier in all analyzed samples) for isotopologues, but not for isotopomers.

For method development we generated a protein standard (recombinant H3 from *T.brucei*) with a well-defined acetylation degree of 50% on all lysine residues. The workflow has been evaluated with different proteases (Trypsin, Elastase and Thermolysin). Despite the high sequence coverage of elastase, the quantitation of these digests is difficult due to the high complexity caused by the overlap between peptides and due to the isobaric interferences of isotopic patterns. In order to reduce the complexity we had to compromise the high sequence coverage of elastase and use trypsin instead.

Targeted-MS/MS were required since Data-dependent-MS/MS methods were insufficient to gain all information needed. For peptides with many lysine residues this is very cumbersome. Therefore, we developed a targeted-workflow that applies all-in-one fragmentation of all isotopologues, which allows determining the acetylation degree of all lysine residues from a single MS/MS-spectrum.

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Neue Aspekte

Use of All-in-One targeted fragmentation method of isotopologues to determine the degree of acetylation at a lysine residue.

PP 29

Combining microfluidics with microarrays and MALDI-MS: Advanced identification and characterization of protein N-glycosylation

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Protein N-glycosylation is one of the most abundant post translational modifications and appears to be highly variable. Dozens of glycan structures on distinct glycosites are summing up to a plethora of combinations. The complex nature of glycosylation makes both, their finding and characterization, a demanding task.[1-4]

We demonstrate here a method for a reliable mining of glycopeptides as well as the subsequent characterization of the site specific glycosylation heterogeneity, by integrating a microarray platform into the workflow of nLC-MALDI-MS. Microfluidics allow further the specific treatment of each 2nd fraction with PNGAseF, to release glycans from the peptide. A pair-wise comparison between MALDI spectra of treated and untreated neighbored fractions, allows finally a reliable identification/characterization of protein N-glycosylation.

Experimenteller Teil

The nano-LC setup consisted of an Eksigent/Ekspert nanoLC400, using an Eksigent RPC18-CL-120, separation column (0,075x150mm). A flow rate of 300nL/min was maintained for all experiments. Solvent A consisted of 0.1% unbuffered formic acid in H_2O, solvent B of 95% acetonitrile in H2O and 0.1% formic acid. Fractions were collected as described in Küster et al.[5] MALDI-MS analysis was done by either using a AB Sciex TOF/TOF 5800 mass spectrometer (Darmstadt Germany) or a Bruker solariX FTMS instrument (Bruker, Bremen). Human IgM, solvents and DHB were obtained from Sigma Aldrich (Buchs Switzerland).

Ergebnisse und Diskussion

A nLC separation of proteolytic fragments of glycoproteins was fractionated onto a microarray using a microfluidic interface in a high frequency manner (1 fraction per second). One chromatographic peak is thereby spread over at least 10 individual microarray-spots.[5] Using microfluidics, each second spot is further treated with PNGAseF under a protecting oil phase.

The final MALDI-MS analysis generates two traces: I) one LC-trace with PNGAseF treated spots and II) a native trace, as directly fractionated from the nLC.

Mining for distinct glycosites was done by searching for the deglycosylated peptides in the treated trace. The identified peptides led then directly to the position of the intact glycopeptide clusters in the neighboring spots (before and after). Further, unknown glycosites can be identified by a sequential comparison of neighbored peak lists. The deglycosylated peptide could be sequenced in a simple CID MS/MS experiment. We demonstrated this approach for the identification and detailed characterization of the site-specific glycosylation heterogeneity of human IgM.

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Neue Aspekte

We report here on the integration of microarrays and microfluidics into the workflow of nLC-MALDI-MS for protein N-glycosylation analysis.

PP 30

Glycopeptide characterization by combination of CID and ETD fragmentation after charge state enhancement

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For complete glycopeptide characterization, like in biomarker discovery and therapeutic glycoprotein QC, both the localization of the glycosylation sites and determination of protein sequences are required. CID of glycopeptides mainly yields glycan fragments but produces limited information about the peptide backbone. In contrast, Electron Transfer Dissociation (ETD) dissociates the N-Cα bonds of the peptide backbone, while the glycan remains as a whole attached to the amino acid residue. Therefore, the combination of CID and ETD fragmentation is ideal for the comprehensive characterization of glycopeptides.

Experimenteller Teil

Several standards (fetuin, HCG and EPO) were reduced, carbamidomethylated and digested with trypsin, ArgC or GluC. The generated (glyco)peptides were separated on a Dionex nanoRSLC system (Acclaim PepMap C18) and analyzed with an amaZon speed ETD ion trap, equipped with a CaptiveSpray source (Bruker Daltonics). Solvent-enriched nitrogen was used as sheath gas to enhance glycopeptide ionization. CID and ETD were done in auto-MS/MS mode in enhanced resolution. Glycopeptide spectra were classified within ProteinScape 3.1 and searched against the GlycomeDB database by the GlycoQuest search engine.

Ergebnisse und Diskussion

The lower ionization efficiency and a high glycosylation micro-heterogeneity make the analysis of *N*- and *O*-glycopeptides still difficult. In particular ETD fragmentation can suffer from low charge states for high mass glycopeptides. Thus a new nano source set-up has been used to enhance glycopeptide charge states and to increase their overall signal intensities: solvent-enriched sheath gas is introduced into the CaptiveSpray source. The MS signal for glycopeptides is most enhanced for large glycans attached and/or high degrees of sialylation. Both *N*- and *O*-glycosylation are determined and similarities as well as differences are discussed in CID and ETD fragmentation.

Neue Aspekte

CID and ETD fragmentation with signal improvement and charge enhancement for characterization of glycopeptides: glycosylation site, glycan and peptide moiety

PP 31

Direct access to antibody and host cell protein levels in fed-batch cultures using microarrays and high-mass MALDI-MS

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Monoclonal antibody (mAb) production in fed-batch processes depend on several parameters, including process conditions, cell medium constitution, feeding strategies and cell density.

Inevitable, over the full span of batch cultivation, a plethora of host cell proteins (HCPs) are emitted into the culture due to secretion and cell lysis as well. The concentration of those HCPs in the cell batch supernatant is a critical quality attribute. By monitoring HCP levels, protease triggered antibody degradation could be avoided and product purification efforts could be minimized. Finally, the amount and quality of the mAb product is significantly increased.[1]

Therefore, we developed a straightforward work-flow for getting direct access to mAb and HCP levels of cell culture batches, using microarrays and high-mass MALDI-MS.

Experimenteller Teil

As a model we used a mouse hybridoma (HFN 7.1) fed-batch culture, which produces a monoclonal IgG1 antibody against fibronectin. Microarray targets were produced as described in *Pabst et al.*[3] Maltodextrin binding protein (MBP_3), used as high mass MALDI-MS internal standard and calibrant was expressed in *E. coli,* and purified as described in *Weidmann et al.*[2] For high-mass MALDI-MS analysis, we used a 4800 Plus, AB SCIEX mass spectrometer (Darmstadt, Germany) equipped with a commercially available high-mass detector (HM2tuvo, CovalX, Zurich, Switzerland). The nano-LC setup consisted of an Eksigent/Ekspert nanoLC400.

Ergebnisse und Diskussion

Our analytical approach allows a direct access to HCP profiles over the time course of a fed-batch process, which can then be implemented to fed-batch optimization and routine control. Beside this, a reliable quantitative evaluation of the intact mAb level is possible. Since MALDI-MS is usually not the method of choice for the estimation of protein concentrations we had to implement two new developments:

First of all, we employed a microarray target for having small well defined volumes with several replicates of each data point. Second, we applied an in-house developed high-mass protein standard (MBP_3, maltodextrin binding protein), which was added as an internal high mass standard for a reliable antibody quantification. This finally allows fed-batch monitoring and quality control, without prior separation techniques directly from the cell culture supernatant, in a minimum of time.

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Neue Aspekte

Evaluation of mAb and host-cell protein levels from fed-batches cultures, using high-mass MALDI-MS, microarrays and high a mass internal standard

PP32

Global proteomics and phosphoproteomics addressing differentiation of human primary fibroblast in 3D scaffolds

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Since both cell proliferation and differentiation and therefore wound healing in skin tissue is significantly influenced by the surrounding extracellular matrix (ECM) [1] use of coatings of artificial ECMs (aECM) containing collagen and glycosaminoglycans (GAGs) like hyaluron acid (HA) in different sulphation states is supposed to be a promising approach for skin graft design. Both mechanical properties and binding of cytokines (e.g. TGF-β) of ECM are known to affect fibroblasts while wound healing and induce differentiation to myofibroblasts that play a major role for restoration of tissue integrity by their contractile properties using α-smooth muscle actin (αSMA) and ECM remodeling [2].

Experimenteller Teil

For cell culture we use 3D scaffolds consisting of fibrillated collagen. Use of specific conditions while fibrillation results in scaffolds with specific properties, e.g. stiffness. Human primary fibroblasts are cultured in medium containing both either light- or heavy-labeled lysine and arginine (SILAC) and are afterwards exposed to scaffolds of different properties (e.g. stiffness, GAG-composition). After exposition cells are lysed and mixed in equal amounts. Per sample 150 µg proteins are purified and digested by FASP protocol [3] before phosphopeptides are enriched by using titan dioxide. Gained phosphopeptide samples are separated in 4 h gradient and online measured with an LTQ Velos Orbitrap mass spectrometer. MSA measurements were acquired and (phospho-)peptide identification was performed with ProteomeDiscoverer 1.4.

Ergebnisse und Diskussion

Previous studies of human primary fibroblasts in 2D cell culture comparing different aECM compositions revealed altered expression of proteins involved in matrix degradation and cytoskeletal development when exposed to highly-sulphated HA (hsHA) in comparison to non-sulphated HA [4]. Additionally a decreased differentiation of primary fibroblasts to myofibroblasts after exposition to TGF-β could be shown by decreased nuclear translocation of SMAD2/3 and subsequent decreased expression of αSMA when cultured on hsHA. These effects are attributed to be based on competitive binding of TGF-β by hsHA at receptor binding domain.

Our recent work in 3D scaffolds focus on impact of mechanical properties to fibroblast differentiation. Cell response on scaffolds of different stiffness will be studied as well as on different GAG composition. Global quantitative proteomics as well as a new implemented phosphoproteomic approach to address cellular signaling will be used for investigations.

Our pilot trails on phosphoproteomics show our ability to enrich phosphopeptides to a level of about 80%. By our mass spectrometric measurements we are able to identify about 1,000 phosphopeptides per sample by an initially sample input of only 150 µg. Among all identified proteins containing at least one phosphopeptide we found TGF-β induced proteins like SMAD2 and SMAD3 as well as nuclear interaction partners of them e.g. SNP1. However, one short-term objective is to further reduce the required sample amount and to implement a fractionation method based on use of combined C_{18}-SCX StageTips.

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Neue Aspekte

Global quantitative phosphoproteomic investigations in 3D cell cultures.

PP 33

Real-time Monitoring of Protein Phosphorylation Using High-Resolution Native MS

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Phosphorylation is the most common post-translational modification of eukaryotic proteins influencing their activity, conformation, oligomeric state or binding of interaction partners. Methods to study phosphorylation include X-ray crystallography, radioactive ^{32}P labeling or bottom-up LC/MS/MS peptide analysis. However, distribution and relative quantities of the different phospho-isoforms and dynamic structural changes co-occuring with the phosphorylation remain elusive in these approaches. Making use of a modified Orbitrap Exactive Plus [1], we designed a native MS setup to qualitatively and quantitatively investigate phosphorylation of intact biomolecular assemblies and the structural re-arrangements accompanying them. We tested our approach using cGMP-dependent protein kinase (PKG), that auto-phosphorylates upon cGMP or cAMP binding, and aurora kinase A (AurA), that phosphorylates its interaction partner protein aurora borealis (HsBora).

Experimenteller Teil

Kinase reactions were carried out under physiological pH in presence of ATP and $MgCl_2$ at 30°C with a 1000-fold molar excess of cAMP or cGMP (PKG) or at room temperature with a twofold excess of Bora (AurA). Reaction quenching was performed on ice by adding EDTA. Samples were subsequently buffer exchanged to 150–500 mM ammonium acetate pH 6.8 and diluted to 0.5–5 µM protein concentration to facilitate native MS analysis. Instrument settings were optimized to obtain high resolution and sensitivity and kept similar for all measurements. The masses of all proteoforms were determined from the *m/z* values of consecutive charge states. To obtain phosphorylation rates, the intensity weighted average number of phosphorylations was calculated and plotted against time.

Ergebnisse und Diskussion

When PKG is analyzed in presence of cGMP or cAMP but without Mg-ATP, a stable 153 kDa homodimer with 2–4 cGMP molecules or 0–2 cAMP molecules bound was detected, reflecting the protein's preference for cGMP over cAMP. In presence of Mg-ATP, all these PKG/cyclic nucleotide complexes become progressively phosphorylated over time as is evidenced by a shift of the phospho-isoform distribution to higher *m/z*. The highest number of phosphate incorporations that can be accurately assigned is eleven for cAMP- and five for cGMP-containing complexes.

To prove that this approach is also applicable to monitor heterophosphorylation we investigated the interaction of the 33 kDa kinase AurA and a 17.5 kDa N-terminal fragment of hsBora. When incubated with Mg-ATP, hsBora becomes increasingly phosphorylated with a maximum of five phosphorylations being detected. Additionally, a 1:1 complex of AurA and Bora was identified. The complex becomes more abundant with longer incubation time and shows the same trend of progressive phosphorylation as unbound hsBora.

Owing to the high resolving power of the modified Orbitrap instrument, we were able to obtain baseline resolution of multiple phospho-isoforms of PKG, hsBora and AurA as well as to monitor changes in the phospho-isoform distribution over time. Moreover, different cyclic nucleotide-bound states of PKG and the 1:1 AurA/hsBora complex could be detected, illustrating the ability to detect both covalent and non-covalent changes in a native setting with this Orbitrap mass spectrometer.

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Neue Aspekte

The described approach enables the investigation of previously poorly characterized determinants and effects of (auto)phosphorylation of (non-covalent) protein complexes.

PP 34

In-depth Mass Spectrometry Characterization of Therapeutic Antibodies for Efficient Biosimilar Development

Wolfgang Jabs[1], Romano Hebeler[1], Anja Resemann[1], Waltraud Evers[1], Laura Main[1], Carsten Baessmann[1], Detlev Suckau[1], Daniel Ayoub[2], Elsa Wagner-Rousset[2], Alain Beck[2]

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Monoclonal Antibodies (mAbs) and derived biomolecules are currently the fastest growing category of human therapeutics. More than 40 products of this class are on the market and about 30 are in late clinical trials. These therapeutic proteins are in use for a variety of indications such as inflammatory diseases and cancer. Biosimilars are copy versions of the original drug after patent expiration and it is fundamental to demonstrate the similarity between the copy and the original reference biotherapeutic molecule. Here we applied routine mass spectrometry methods for the detailed and rapid structural characterization of the therapeutic antibody cetuximab, which is an epidermal growth factor receptor inhibitor used for the treatment of colorectal cancer and head and neck cancer.

Experimenteller Teil

LC-MS runs were recorded for intact cetuximab (Erbitux, Merck) and its subunits after digestion with IdeS followed by reduction with TCEP. Ultra-high resolution (UHR)-ESI QTOF and MALDI TOF/TOF instruments were used to obtain spectra with resolved isotopes and precisely determine the monoisotopic masses of the antibody subunits. Detailed sequence information of the intact antibody and its subunits was then obtained using MALDI N- and C-terminal top-down sequencing (TDS) analyses. LC-MS/MS peptide mapping experiments on tryptic and GluC digests permitted to further localize post translational modifications and sequence variants. Using a search engine for carbohydrates structure databases, glycan profiles were automatically generated.

Ergebnisse und Diskussion

From the ESI-LC-MS mass spectra of intact cetuximab we derived the intact masses of the antibody various glycoforms. All glycoforms are 58 Da heavier than expected from public sequence data (Imunogenetic Information System). The mass shift was determined from the MS spectra of the antibody subunits generated by IdeS digestion and reduction. From this analysis the unexpected modification was assigned to the light chain.

MALDI-TDS spectra were acquired to sequence the terminal regions of the light and heavy chains of the antibody within minutes. Applied to the F_d, $F_{c/2}$ and LC subunits of cetuximab, 64% of the mAb sequence was validated with this top- and middle-down approach and the unexpected modification was localized in the light chain terminal region.

In addition, 100% sequence coverage was obtained from the trypsin and GluC peptide maps of two LC-ESI-QTOF-MS/MS datasets and a LC-MALDI-TOF/TOF datasets.The peptide map confirmed the unexpected modification revealing a sequence variant in the light chain terminal region, which turned out to be common for other biopaharmaceutical mAbs as well.

Neue Aspekte

we applied routine mass spectrometry methods for the detailed and rapid structural characterization.

PP 35

Substrate screening with MeCAT – A comparison of strategies for relative protein quantification

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Due to the high dynamics of the proteome, quantitative analysis has become increasingly more important for the understanding of processes in organisms. Nowadays, mainly methods are used, which are based on stable isotope labeling and subsequent analysis by mass spectrometry (MS). A new approach for quantification is the labeling with lanthanide chelates. By using metal coded affinity tagging (MeCAT) these complexes are covalently bound to the thiol group of cysteine residues.[1] The quantification can then be performed either by molecular or elemental MS.[2,3] Here, we applied MeCAT for screening in a complex biological sample in order to identify potential substrates of the high temperature requirement protease A1 (HtrA1). HtrA1 is of interest mainly due to its suspected tumor suppressor function.[4]

Experimenteller Teil

To meet the special requirements of a complex system, the substrate screening was performed using electrospray ionization (ESI)-MS and software-based data analysis. For the purpose of the screening, a cell lysate was divided into two aliquots. One aliquot was treated with the HtrA1 protease, whereas the other aliquot was only treated with buffer as a reference sample. Both aliquots were then labeled differentially with MeCAT and mixed. Separation of proteins was carried out by gel electrophoresis and liquid chromatography. In addition, the MeCAT based substrate screening was also performed by using inductively coupled plasma (ICP)-MS of mineralized gel bands and laser ablation (LA)/ICP-MS of two-dimensional polyacrylamide gels.

Ergebnisse und Diskussion

To achieve the identification of potential substrates, the software-based data analysis was adapted to assess MeCAT labeled peptides. Thereby, the substrate screening was implemented successfully and potential substrates were identified and relatively quantified. In addition, it was demonstrated that a MeCAT-based substrate screening can also be performed using ICP-MS. ICP-MS analyses based on the mineralization of gel bands provided an absolute quantification of the proteins. Using laser ablation (LA)/ICP-MS, the proteins were additionally quantified relatively with high spatial resolution on two-dimensional polyacrylamide gels. ICP-MS-based results were then compared with the quantification by ESI-MS. The ESI-MS results were also further compared to stable isotope labeling by amino acids in cell culture (SILAC) experiments. Consistent results were generated with both MeCAT and SILAC.

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Neue Aspekte

Application of MeCAT on a complex biological sample. Comparison of orthogonal MS-techniques for quantification.

PP 36

Novel approach for quantification of biopolymers with DOTA complexes using click chemistry

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Quantitative proteomics became pivotal recently. SILAC (stable isotope labeling by amino acids in cell culture) [1], ICAT (isotope-coded affinity tags) [2], or iTRAQ (isobaric tag for relative and absolute quantitation) [3] are examples of quantification techniques which combine the labeling with stable isotopes and molecular MS.

Recently, metal-coded affinity tag (MeCAT) [4] was introduced to track labeled species using ICP-MS (inductively coupled plasma mass spectrometry) taking advantage of its high sensitivity, multielemental capacities and structure independent response. However, some of the labeling sites in peptides and proteins are inaccessible to the bulky DOTA complexes.

This work proposes a new two-steps labeling strategy involving click chemistry [5] to reduce the steric hindrance and enable more active sites accessible.

Experimenteller Teil

Firstly, the thiol groups of cysteine in proteins or peptides were modified with a small residue containing a terminal alkyne, avoiding the introduction of bulky residues causing steric hindrance. This small group is devised as a linker and spacer that can offer more accessibility to second step labeling.

After that, without any washing step, DOTA-azide complexes harboring a lanthanide atom were introduced by click chemistry. The general features of this strategy, including the effect of loading different metals, the feasibility of peptides and protein quantification and the fragmentation behavior of the labeled species were studied.

Ergebnisse und Diskussion

A new two-step labeling strategy for peptides and proteins has been presented for the first time. It reduces the steric hindrance and allows more efficient labeling of the targeted peptides and proteins. CID fragmentation of peptides both labeled with the alkyne moiety and the DOTA-metal complex result in enough typical b- and y-series fragment ions for identification by common database search algorithms to ensure undoubted identification.

Typical reporter ion from the label was attained with the IRMPD fragmentation and HCD technology which can be used to achieved relative quantification. Successful relative quantification of labeled peptides and proteins has been demonstrated using molecular and elemental MS techniques. Accurate quantifications have been performed not only in peptide level but also in the protein level.

Furthermore, the investigation has demonstrated the multiplexing capability of the development approach for relative quantification of peptides and proteins with different proteomics workflows. High labeling efficiency and robustness have proved to be the main advantages of this approach.

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Neue Aspekte

A new approach for the quantification of proteins has been developed improving the labeling efficiency of the MeCAT reagents.

PP 37

MeCAT – New possibilities of protein analysis and quantification

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As part of proteomics, the need for reliable protein quantification methods steadily increases. Thereby, the use of stable isotopes for labeling including ICAT and iTRAQ mass spectrometry (MS) is used progressively. Labels are based on a chemical labeling reaction of the proteins or peptides with a specific reagent. Mass differences of differently labeled samples are then detected by MS to get qualitative as well as quantitative information. As a further quantification methodology, we developed MeCAT (Metal Coded Affinity Tagging) [1 - 2]. MeCAT uses chelate complexes of lanthanides for relative and absolute quantification. For the later elemental mass spectrometry is employed. Lately, we introduced the new MeCAT-IA reagent, carrying an iodoacetamide moiety that shows some distinct advantages over the previously used MeCAT-Mal with maleinimide reactivity [2].

Experimenteller Teil

Model proteins are labeled with the MeCAT-reagent, which contains a cysteine-reactive group for quantitative labeling and an elemental tag, loaded with a lanthanide ion for quantification. For separating labeled proteins from other sample components a HPLC was used online to electrospray ionization (ESI)-MS. Besides, fragmentation experiments with labeled proteins were applied, including infrared multi photon dissociation (IRMPD) and higher-energy C-trap dissociation (HCD). Relative quantification was performed with differentially labeled samples using ESI-MS, ESI-MS/MS and ICP-MS as reference [3]. Besides, first experiments of labeled peptides via MALDI-Imaging were conducted and a new cleavable linker including the MeCAT-reagent was synthesized expanding the possible applications for MeCAT as an analytical tool.

Ergebnisse und Diskussion

The investigated model proteins cover a mass range of about 14 to 67 kDa in the unlabeled state and about 20 to 92 kDa in the completely labeled state. The completeness of the labeling was examined by using substoichiometric quantities of the labeling reagent with regard to the existing thiol groups of cysteine's residues in the proteins. The obtained spectra were compared with those at which the labeling reagent was used in excess. It was shown that all four different model proteins could be labeled completely. Hence, an important requirement for reliable quantification of proteins was fulfilled and that certain labeled proteins can be used as an internal standard for the relative quantification by MeCAT-IA. In further experiments, the fragmentation behavior of labeled proteins was investigated. For that reason, the fragmentation techniques IRMPD and HCD were applied. In addition, several differentially labeled proteins were detected simultaneously in order to verify the possibility of relative quantification. Lanthanides being used for the differential labeling were europium, terbium, holmium, thulium and lutetium. All five differentially labeled proteins could be isolated and detected simultaneously for fragmentation. These results can be used for determining the concentration of proteins or peptides relatively to a labeled internal standard protein with known concentration in ESI-MS/MS-analysis. Then, this method was applied to assess the amount of HSA in a human blood serum sample. The results of the relative quantification were confirmed by ICP-MS experiments.

Increasing the analytical options with MeCAT, labeled peptides using MALDI-Imaging were investigated and a MeCAT-reagent with a cleavable part was synthesized.

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Neue Aspekte

In addition to protein quantification, MeCAT can be used as part of a cleavable reagent and in MALDI imaging.

PP 38

Proteome Analysis in Schizosaccharomyces pombe: Amino Acid Biosynthesis and Membrane Composition as Target for Improved Protein Secretion

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Protein secretion in yeast is a complex process and its efficiency depends on a variety of parameters. To investigate the influence at various steps of protein secretion and the overall proteomics response of the cell to the burden of protein production and secretion, we constructed a set of *Schizosaccharomyces pombe* (*S. pombe*) strains producing the α-glucosidase maltase in increasing amounts. The iTRAQ-based labeling strategy encompassing 2D-LC-MALDI-TOF-MS/MS analysis was applied for relative quantitative comparison of cell lysate proteins extracted from three different strains of *S. pombe*. Based on the proteome analysis results, targeted amino acid feeding and manipulation of cell membrane by addition of several surfactants were performed to increase the target protein secretion of α-glucosidase maltase in *S. pombe*.

Experimenteller Teil

Three different maltase expressing strains of *S. pombe* were constructed and cultivated in shake flask in a minimal media containing 20 g/L glucose as sole carbon source. The extracted cell lysate proteins were in-solution digested with trypsin and labeled with 4-plex iTRAQ reagents. The iTRAQ labeled peptides were first separated with analytical RP-HPLC at pH 10, followed by separation of the collected fractions by nano-IP-RP-HPLC at pH 2 coupled to MALDI target spotting. The MALDI-TOF-MS/MS analysis was carried out on an AB Sciex TOF/TOF 5800 mass spectrometer. Protein identification and quantification were performed with ProteinPilot 4.0 and in-house VBA scripts were built for the evaluation of quantification results.

Ergebnisse und Diskussion

Proteome analysis of these strains revealed complex changes in protein levels at all steps of protein secretion, from transcription and translation to protein folding and transport. We also found an unexpectedly high amount of changes in enzyme levels of the central carbon metabolism and a significant up-regulation of several amino acid biosyntheses. Analysis of the average amino acid composition of the cellular protein of *S. pombe* and comparison with our model protein showed that these very amino acids were underrepresented in the cellular protein compared to the composition of the model protein. Additional feeding of these amino acids resulted in an increase in protein secretion by factor 1.5. Ergosterol biosynthesis was identified as a second target and addition of fluconazol and Amphotericin B to the culture caused a significant decrease in ergosterol levels while protein secretion could be further increased by factor 2.1. Thus we could show that the central carbon metabolism can be one bottleneck of recombinant protein secretion in yeast and proteome analysis can be used to yield targets to efficiently increase recombinant protein secretion in yeast.

Neue Aspekte

The proteomics analysis provided a rational modification on the working strain for a more efficient protein production/secretion in *S. pombe*.

PP 39

Absolute Quantitation of Yeast Kinases by means of LC-MS/MS using QconCat and SRM Technologies

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Absolute protein quantification by mass spectrometry is an important tool in assay development and creating data for systems modeling. The large dynamic range of a proteome is the most challenging barrier to protein quantification. This abstract presents the application of label-mediated targeted mass spectrometry to quantify the kinase proteins from yeast, which span a five-order dynamic range. QConCAT technology was used to create isotopically-labeled internal standard peptides for 138 target proteins and quantification was performed by scheduled SRM triple quadrupole mass spectrometry, investigating the sensitivity of different platforms, assay specificity and quantitation dynamic range.

Experimenteller Teil

A yeast kinase QconCAT was expressed that comprises two isotopically labelled peptides for each of the targeted proteins and tryptically co-digested with a native yeast strain. Scheduled SRM experiments were conducted with a nanoAcquity system interfaced to either a Xevo TQ or Xevo TQ-S triple quadrupole mass spectrometer. A 45 min reversed phase gradient was employed using a vented trap-configuration comprising a 2 cm x 180 µm trap column and a 15 cm x 75 µm analytical column. Quadrupole resolution settings of 0.7 Da en 0.4 Da were employed, balancing the sensitivity of the two mass spectrometers, respectively.

Ergebnisse und Diskussion

Exploratory experiments with unit quadrupole mass resolution successfully quantified 70% of the target proteins but had reduced success with low abundance proteins. Of the 276 target peptides, representing 138 proteins, the QconCAT and native counterpart were observed (type A), affording direct quantitation. More than 77 peptides illustrated inferred maxima, where the QconCAT peptide was observed, but the native peptide undetected (type B). For another 55 peptides, both the QconCAT and native peptide were undetected at biologically relevant levels (type C). Reanalysis of the samples on a more advanced triple quadrupole platform, featuring an improved source for increased sensitivity and increasing Q1 and Q3 quadrupole resolution to 0.4 Da, improved the quantitation success rate to 90%, quantifying proteins ranging from one million copies per cell to fewer than 100 copies per cell. The type A, B and C peptides equaled 212, > 23, and 41, respectively. From a protein perspective, this increased the number of quantifiable proteins from 98 to 124 out of a possible 138. Both SRM assays were in good agreement with each other (Pearson's correlation 0.93 r^2 0.88) and correlate reasonably well with PAXdb (Pearson's correlation 0.96 r^2 0.92) in terms of copies per cell. Moreover, 20 proteins were quantified that have not been reported previously. Additional benefits observed were improved precision, reduced assay time and easier data processing due to less interferences.

Neue Aspekte

Increase of dynamic range and precision for absolute protein quantification is displayed.

PP 40

PEAKS LFQ – A software tool for label-free quantification with high sensitivity and high accuracy

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Label free shotgun proteomics has been used for protein identification and quantification. For system-wide application, however, maintaining high sensitivity and accuracy is challenging in label-free shotgun proteomics due to the broad dynamic range of protein abundance and stochastic identification of peptides between samples. For an intensity-based approach, the information for quantification (MS1) and identification (MS2) is measured in a single run. The deconvolution of overlapped peptide features and retention alignment between runs are the key factors for the data analysis, because the overlapped peptide feature clusters cannot be avoided, even with today's high resolution MS instruments and LC separation techniques. Here we present a software tool, PEAKS Q, for accurate label-free quantification with high sensitivity.

Experimenteller Teil

To validate our label-free quantification tool, two types of data sets were used to compare the quantitative results to a ground truth:

1. CPTAC study [1] data 6: Four datasets from Orbitrap instruments (Orbi, OrbiO, OrbiP, and OrbiW) were used. In each dataset, sample A, B, C, D, and E were yeast spiked with a mixture of 48 proteins (UPS1) at different ratios.
2. Dilution data from [2]: On the basis of the TICs, the lysate of a bacterium Streptococcus pyogenes was mixed with a lysate of human cells in a dilution series with different ratios. Each sample was measured a single time on an LTQ Orbitrap instrument.

Ergebnisse und Diskussion

The overlapped peptide isotopic clusters were commonly observed. To deconvolute overlapped peptide features, an expectation–maximization (EM) algorithm was used to auto-fit a distribution model for each peptide feature as a component in the presented isotopic clusters.

1. Detect all local maximum points on the LC-MS view.
2. Initialize a distribution model for each local maximum point and each possible charge, which represents a component in the cluster.
3. Use EM iteration to auto-fit the distribution for each component.

13.2% and 15.6% clusters were overlapped for CPTAC data and Dilution data, respectively.

To align retention time among sets of runs, a maximum weighted matching algorithm was used [3].

1. The file of a run which shares the most features with the remaining runs was chosen as the reference.
2. Given two sets of features, the retention time alignment algorithm is based on an optimization model, which works on feature matching and retention time simultaneously.

The sample sharing the most features with the remaining samples was chosen automatically as the reference sample, which was used for retention time alignment for the remaining samples. Evaluation with identified peptide feature pairs showed that the retention alignment was efficient and accurate, even with significant time shifts and distortions. After alignment, features from different runs were mapped into feature vectors/groups.

Peptides were identified with PEAKS DB and filtered at 1% FDR. Proteins were filtered at 1 minimum unique peptide. The protein abundances were estimated in each sample by correlation of the average of the feature intensities of the three most highly responding peptides per protein. The results from both datasets showed both high accuracy and high sensitivity of label-free quantification.

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Neue Aspekte

A new algorithm for label-free quantification data analysis

PP 41

Application of multi-omic and functional network analysis for paediatric patients diagnosed with idiopathic nephrotic syndrome

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Idiopathic nephrotic syndrome (INS) is the most prevalent glomerular disease in children. In spite of some progress, its pathogenesis is still unknown and the therapy options are confined to gross immune modulation. A variety of methods for diagnostic and treatment purposes are available for the patients; however, the lack of understanding regarding the pathogenic mechanisms underlying INS can lead to poor therapeutic response and adverse side-effects. Here, we describe quantitative proteomic and metabolomic approaches to reveal new molecular factors involved in pathogenesis of INS with potential diagnostic and therapeutic significance.

Experimenteller Teil

Urine samples were collected from 10 children diagnosed with INS receiving no therapy and 10 healthy children. The collected urine samples were divided into batches for proteomic and metabolomic analysis. For the proteomic studies, samples were purified and depleted of albumin using a combination of spin filters with anti-HSA resin. Proteins were reduced and alkylated prior to digestion with trypsin overnight. Label-free protein expression data were acquired with a oa-TOF using an ion mobility data independent approach. Normalized label-free quantitation results were generated using TransOmics software. In a similar fashion the diluted neat urine samples were analysed using a small molecule profiling approach. The resulting data was also analyzed using TransOmics, providing a complimentary dataset.

Ergebnisse und Diskussion

Interpretation of the data has shown a significant number of proteins to be over-expressed in the urine from INS patients, which includes a high percentage (approximately 80%) of glycosylated proteins. Metabolites of interest showing statistically significant changes include homocysteine, glutamate and uridine. Pathway analysis tools were used to review the complimentary datasets and hence provide an understanding of the underlying biology of differentially expressed proteins and metabolites. Review and validation of the suggested pathways, strongly suggests correlation with the neuronal system disorders network, specifically acute fatigue.

Neue Aspekte

We display a combined Proteomics and Metabolomics study using an ion mobility supported data independent approach.

PP 42

Efficient de novo sequencing of proteolytic peptides by use of ion mobility separation (IMS) and subsequent low energy collision-induced dissociation

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De novo sequencing of proteins is frequently performed by proteolysis, subsequent collision-induced dissociation (CID) of the resulting peptide ions, and deduction of their sequences from b- and y-type series fragment ions. However, especially in cases of larger proteins or mixtures of proteins overlapping of peptide ion signals lead to superimposed fragment ion spectra that are difficult to evaluate and bare the risk of misinterpretation. The use of HPLC-MS setups may largely avoid the overlap problems but time limitation for CID experiment to the width of a typical HPLC peak (~30 sec) often hampers the acquisition of clear cut fragment ion spectra. One way out of this dilemma is the combination of direct infusion, ion selection, and IMS prior to CID.

Experimenteller Teil

Nano electrospray ionization (ESI) MS experiments were carried out by use of a SYNAPT G2-S mass spectrometer (Waters, Manchester, UK) equipped with a Z-spray source in the positive ion sensitivity mode. Typical source parameters were: source temperature: 80 °C, capillary voltage: 0.8 kV, sampling cone voltage: 20 V, and source offset voltage: 50 V. For low energy CID experiments, the peptide precursor ions were selected in the quadrupole analyser, subjected to IMS (wave velocity 800-1200 m/s, wave height 40 V, nitrogen gas flow rate 90 ml/min, and helium gas flow rate 180 ml/min), and fragmented in the transfer cell using a collision gas (Ar) flow rate of 2.0 ml/min and collision energies up to 100 eV (E_{lab}).

Ergebnisse und Diskussion

Ions with different charge states and/or considerably distinct *m/z* values can be readily separated. This is demonstrated exemplarily for a number of ions derived from a chymotryptic digest of Jack bean α-mannosidase in the *m/z* range 672 to 674. Five peptide ion species (*m/z* 672.62, four-fold charged; *m/z* 672.71, triply charged; *m/z* 673.88 and 674.88, both doubly charged (at least partly); and *m/z* 673.32, singly charged) were separated, fragmented and their amino acid sequences were determined from a single IMS-CID experiment. Moreover, equally charged (almost) isobaric peptide ions may be as well separated by ion mobility if they are sterically distinct. As an example, two doubly charged tryptic peptide ions derived from lactotransferrin isolated from human milk at *m/z* 807.87 and 807.89 were nicely separated. Subsequent CID yielded pure fragment ion spectra allowing for a facile determination of the corresponding sequences.

The above examples show that ion mobility is a powerful tool for gas-phase separation of peptide ions. Combined with direct infusion that facilitates a broad variation of collision energy by an extended time frame for fragmentation experiments, IMS clearly improves an efficient *de novo* sequencing of proteins.

Neue Aspekte

The combination of direct infusion and IMS-CID turned out to be an excellent strategy for *de novo* sequencing of proteins.

PP 43

Molecular characterization of neuronal protein aggregates by mass spectrometry-based footprinting methods

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Aging is a major risk factor for Alzheimer's disease (AD) and Parkinson's disease (PD), and the number of people with these conditions is increasing rapidly. Formation and accumulation of fibrillar plaques of ß-amyloid peptide and α-synuclein in brain have been recognized as characteristics of AD and PD [1]. Although circular dichroism, atomic force microscopy, electron microscopy, light scattering were directed at understanding these molecules, a detailed understanding of protein aggregation remains to be achieved. Understanding how these proteins assemble will provide new targets for the development of aggregation modifiers that could potentially limit their toxicity. In our work, we are investigating two aggregating proteins, Aß and synuclein, as model systems around which we will develop MS strategies.

Experimenteller Teil

MS in combination with H/D exchange [2], and FPOP [3] (fast photochemical oxidation of proteins) were employed to study oligomerization of Aß and synuclein. A new method for determination of protein-ligand interaction was developed in our laboratory by direct coupling of SAW biosensor with MS. This method enables the determination of the binding stoichiometry and affinity of abeta peptides interactions. FPOP is a chemical footprinting method whereby exposed amino-acid residues are covalently labeled by oxidation with hydroxyl radicals produced by the photolysis of hydrogen peroxide. Modified residues can be detected by standard trypsin proteolysis followed by LC/MS/MS. The aggregation process of Abeta 1-40 during the time was also analyzed by CD spectroscopy and by simple concentration dependence measured by NanoDrop.

Ergebnisse und Diskussion

The oligomers were prepared from chemically synthesized $A\beta_{WT}$(1-40) and recombinant human alpha-synuclein (1-140) monomers by using different protocols for which we changed several factors that influence the oligomerization; examples are peptide/ protein concentration, salt nature/content, temperature, pH. By determining the global incorporation of deuteriums, we found that 29.4 ± 0.8 of the 35 backbone amide hydrogens in Aβ(1-40) exchange with deuteriums when the monomeric peptide was suspended in 95% D_2O for 60 min. Another option for measuring the incorporation of deuterium in monomeric Aβ(1-40) peptide was by analysis at the peptide level. For this, we employed pepsin, the most common protease used in H/D exchange-MS. To obtain the oligomeric conformation, Aβ(1-40) peptide was used right after dissolution in HFIP (hexafluoro-isopropanol) followed by immediate dilution with PBS to a concentration of 20 µM. The aggregation reaction was performed at room temperature under magnetic stirring. After 7 h of incubation, we observed that Abeta (1-40) still exchanges a maximum of ~ 21 hydrogens and no significant change occurred within 24 hours of incubation. Interestingly, after 48 h to 7 days, the number of deuteriums incorporated in Aβ oligomers decreased. More importantly, we observed several oligomeric states (~2.5 to 4.2 deuteriums). FPOP data gave us indication of every single amino acid modification within different times of Abeta (1-40) aggregation process. The first H/DX exchange results for synuclein aggregation are not consistent with the literature that synuclein do not aggregates itself, this process is more complex and might be influenced *in vivo* by several biological processes such as contribution of metal ions, reactive radical species or even Aβ peptides.

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Neue Aspekte

Mass spectrometry-based footprinting methods we present here are important tools to study complex processes like aggregation at amino acids level.

PP 44

Analysis of the Interaction of different length Chondroitin sulfate oligomers with Interleukin-8 by Hydrogen/Deuterium Exchange MS

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The recruitment of different chemokines and growth factors by glycosaminoglycans (GAGs) such as chondroitin sulfate or hyaloronan plays a critical key role for in wound healing processes. Thus there is a special interest in the design of artificial extracellular matrixes (aECM) with improved properties concerning their interaction with common regulating proteins. We allready showed that HDX is capable of identifying the binding surface of interleukin 8 (IL-8), a chemokine inducing chemotaxis of neutrophils, and the Chondroitin sulfate (CS) tetramer. In this work we concentrated on complexes with higher molecular CS, which NMR and X-ray failed, to contribute information about GAG binding in dimensions closer to the native state.

Experimenteller Teil

Hyaloronic acid (HA) and Chondroitin sulfate (CS) units of different length had been purchased from Iduron (Paterson Institute for Cancer Research, University of Manchester), and used as supplied. Chondroitin sulfate had been of shark, and Hyaloronic acid of Streptococcal origin. Subunits where cut by controlled endolyase scission. IL-8 was over expressed in Escherichia coli. For the HDX studies interleukin 8 and the GAGs had been exposed to deuterium isotopes in a 90 % D2O low-salt buffer for four different exchange times with several replicates. After quenching samples were immediately denatured, desalted and measured by MALDI-TOF MS (Ultraflex III, Bruker Daltonik). All analyses were performed at least in 4 replicates.

Ergebnisse und Diskussion

Binding interfaces of IL-8 and CS have been already explored by NMR (Pichert et al. 2011). Encouragingly, our results highly correlate to these data. We showed that HDX is capable of identifying binding surfaces in protein-GAG complexes with sufficient accuracy. As shown by Pichert et al. CS sulfated at position two and four bound with the helical binding area of the sequence positions 54-77 which was also observed with our analyses. Interestingly, shielding effects strengthened with higher molecular GAG's up to total shielding of the chemokine.

Neue Aspekte

We showed that binding can be observed and that HDX gives insides in complexes not resolvable by NMR and X-ray.

PP 45

Differential analysis of the mediator complex interactome

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The mediator complex regulates RNA Polymerase II-dependent transcription. To date, 25 subunits have been identified in yeast, which are grouped into four distinct modules, termed head, middle, tail and Cdk8/kinase. The modules form a stable bridge between transcriptional regulators and subunits of the RNA Polymerase II, but it is assumed, that it is also involved in chromatin remodeling and other functions[2]. It is very likely, that the mediator complex specifically interacts with other proteins to achieve its various functions. Therefore, one first aim of our studies is the identification of the yeast mediator complex interactome(s) under various conditions and the elucidation of the interactome dynamics by quantitative mass spectrometry

Experimenteller Teil

One subunit of each yeast mediator complex modules is C-terminally tagged with a triple FLAG tag [1] and immunoprecipitated with magnetic beads covalently coupled with anti-FLAG antibodies. The isolated proteins are eluted with a triple FLAG peptide solution, reduced and alkylated and digested in solution with trypsin or elastase on a 30 kDa cut-off filter[3] . For the identification of interaction partners, two immunoprecipitation were performed in parallel, one with a FLAG-tagged bait yeast strain, the other with wild-typ yeast.

Proteolytic peptides are purified over C18 stage-tips before LC-MS (Easy nLC 1000 coupled to LTQ Orbitrap Velos, Thermo) analysis on a 25 or 50 cm C18 reverse phase column and 4 hour gradients with a TOP5 HCD method.

Ergebnisse und Diskussion

Four subunits, one of each yeast mediator complex modules were C-terminal FLAG tagged: Med2 (tail), Med18 (head), Srb9 (kinase) and Med31 (middle). Co-immunoprecipitation of the FLAG tagged head module Med18 allowed identification of all 25 subunits of the yeast mediator complex. By using the unspecific protease Elastase in parallel to Trypsin, high sequence coverage for all subunits was achieved.

For a first interactome analysis, Med18-tagged and wild type yeast were cultured, lysed and immunoprecipitated completely in parallel. Using a label-free quantitation approach, true interaction partners can be distinguished from false-positive binders. Here we identified already known interaction partners like RNA-Polymerase II, Swi/SNF and telomeric proteins, but also several new potential candidates. By comparing the interactome of all four submodules we will be able to create a differential interaction map of the mediator and known and putative new interaction partners. Furthermore, dynamic analyses of the interactomes under different cell culture conditions are planned.

To estimate the relative abundance of the co-immunoprecipitated mediator complex subunits (and subsequently also of interaction partners), we calculated the intensity based absolute quantitation (iBAQ) for all identified proteins and normalized the iBAQ value to the iBAQ value of the FLAG-tagged mediator subunits (baits). It turned out, that several subunits (e.g. all subunits of the kinase module) have very low abundance compared to the bait. In the case of the kinase module it fits to the biological background: the kinase module is only bound to the mediator complex during gene repression, therefor only a subset of the isolated mediator complexes contains this module. Other low abundant subunits are maybe less strong associated. Using this way of data interpretation for further experiments we hope to get new insights in the composition and dynamic of the mediator complex.

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Neue Aspekte

We use MS as a method for monitoring dynamics of interaction partners and posttranslational modifications of the mediator complex.

PP 46

Implementing Protein/Protein Crosslinking on a State-of-the-Art QqToF Mass Spectrometer

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Chemical protein/protein crosslinking and analysis by high resolution mass spectrometry is a key tool for the structural analysis of protein complexes. By covalent coupling of spatially neighboring amino acid residues, constraints can be derived that aid in the computational modeling of complex structure. Our in-house workflow for crosslinking incorporates chemical crosslinking with BS3, reduction/alkylation/trypsinization, fractionation by size exclusion chromatography, analysis of the fractions by Orbitrap nanoLC/MS/MS, and crosslink identification using publicly available software. BS3 (Bis[sulfosuccinimidyl] suberate) is an amine-reactive, water soluble, homobifunctional crosslinker that targets lysine residues.

We have recently incorporated a fast-scanning, state of-the-art QqToF mass spectrometer into this workflow and discuss both the details of the implementation as well as preliminary performance compared to the established setup.

Experimenteller Teil

Ribonucleoprotein complexes assembled and purified from yeast whole cell or HeLa nuclear extracts were crosslinked with BS3 (Thermo Scientific) under physiological conditions. Samples were reduced with dithiothreitol, alkylated with iodacetamide and digested with trypsin. Peptide fragments were fractionated by size exclusion chromatography, and the fractions analyzed by reversed phase-C18 nanoflow chromatography (Waters TSS3 1.8 µm 250 x 0.075 mm, 75 min 5>40% ACN, 250 nl/min) coupled to a TripleToF 5600+ QqToF mass spectrometer (AB SCIEX) controlled by Analyst TF 1.6 software Build 6211. Peaklists were generated using the MS Data Converter v1.3 beta (AB SCIEX). pLink software 2013.4.28 (Chinese Academy of Sciences) [1] was used to identify peptide/peptide crosslinks against custom FASTA databases.

Ergebnisse und Diskussion

Different RNP complexes were subjected to chemical crosslinking using Bis[sulfosuccinimidyl] suberate (BS3) as the crosslinking reagent. After reduction, alkylation and trypsinization, the samples were fractionated by size exclusion chromatography. Analysis of the resulting fractions by nanoLC/MS/MS using a TripleToF 5600+ mass spectrometer allowed us to assess the suitability of the system for this application, as well as to optimize the workflow:

Size Exclusion Chromatography: Out of 7 fractions selected based on UV absorbance, two core fractions held the majority of identified crosslinks. Additional crosslinks could nevertheless be identified in the adjacent fractions.

MS parameters: Different settings were evaluated to balance between MS/MS fragment *m/z* range, accumulation time and sequencing speed. Despite lower sequencing speed, larger fragment *m/z* range (100-1750) and longer accumulation time (200 ms per spectrum) produced the best results, indicating that maintaining MS/MS spectral quality is important for the identification of low abundance crosslinks. This corresponds to a Top10 method with a cycle time of 2.2 s, resulting in a sequencing speed of 4.5 Hz.

Data conversion: the AB SCIEX MS Data Converter tool allows for export of the raw data into mgf and mzML formats. mgf was successfully used to import data into pLink.

Results: Using the improved settings we were able to identify >180 crosslink MS/MS spectra, corresponding to 140 peptide/peptide crosslinks, from a 4 MDa human RNP complex. This represents a 1.7x and 2.0x improvement over analysis of the same sample on an Orbitrap Velos mass spectrometer, respectively. Similar improvements were observed for the analysis of a 3 MDa RNP complex from yeast. A key factor in the observed improvement is the high sequencing speed of the QqToF. We propose that once optimal spectral quality is achieved, the performance of this mass spectrometric setup can compete with other state-of-the-art hybrid instruments.

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Neue Aspekte

Implementation of a fast scanning QqToF mass spectrometer for protein/protein crosslinking; testing and optimization of instrument and data processing parameters.

PP 47

Evaluating the Potential of an MS/MS-Cleavable Cross-Linker for 3D-Structure Analysis of Protein Complexes

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Chemical cross-linking combined with enzymatic cleavage of the created cross-linked protein(s) and a mass spectrometric analysis of the resulting cross-linked peptide mixture presents an alternative approach to high-resolution analysis, such as NMR spectroscopy or X-ray crystallography, for obtaining low-resolution protein structures and for gaining insight into protein interfaces. However, with increasing complexity of the investigated protein complexes, the identification of cross-linked products, i.e., the differentiation between single and cross-linked peptides, becomes increasingly challenging. Here, we investigate a new cross-linker, which allows distinguishing different cross-linking products after tandem MS experiments based on characteristic product ions and constant neutral losses. The analytical concept is exemplified for the investigation of the model proteins GCAP-2 and BSA.

Experimenteller Teil

Cross-linking reactions with recombinant guanylyl cyclase-activating protein 2 (GCAP-2) and with bovine serum albumin (BSA) were carried out in 20 mM HEPES buffer. A 100-fold molar excess of a bifunctional NHS-active ester amine-reactive TEMPO-XL reagent on the basis of the TEMPO-Bz reagent established from Han Bin Oh [1,2] over the proteins (10 µM) was used and reactions were quenched after 60 min (synthesis & proof of principle of the bifunctional TEMPO-XL reagent: pub. in preparation [3]). The cross-linking reaction mixtures were analyzed by online nano-HPLC/nano-ESI-LTQ-Orbitrap MS/MS. Potential cross-linked products were identified with the in-house software MeroX [4], fragment ion mass spectra were manually evaluated focusing on product ions caused by fragmentation of the cross-linker.

Ergebnisse und Diskussion

Nano-ESI-LTQ-Orbitrap-MS/MS analysis of cross-linked and enzymatically proteolyzed BSA and GCAP-2 allowed the unambiguous identification of various cross-linked products that are in good agreement with the known structures of the proteins. Hereby, the radical-based fragmentation of the TEMPO-linker is the main fragmentation pathway of the cross-linked products, thus allowing to automatically distinguish between unmodified peptides, intra- and interpeptide as well as “dead-end” cross-links using the MeroX software. Subsequent targeted MS^3experiments based on this information allowed the exact characterization of the cross-linked products, i.e. the unambiguous identification of the cross-linked sequences and the exact mapping of the cross-linking sites.

The characteristic fragment ion patterns of the cross-linker greatly simplify the identification of different cross-linked species, namely, modified peptides as well as intrapeptide and interpeptide cross-links, from complex mixtures and drastically reduce the potential of identifying false-positive cross-links. Ongoing experiments utilize the unique fragmentation behavior of the TEMPO-linker for selective data-dependent acquisition of MS/MS data of cross-linked products. This is expected to be highly advantageous for analyzing protein 3D-structures and protein complexes in an automated manner.

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Neue Aspekte

MS/MS cleavable cross-linker for 3D-structural analysis of protein complexes

PP 48

Characterization of Protein Kinase D Complexes at the trans-Golgi Network

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Protein kinase D (PKD) is a serine-threonine kinase comprising three isoforms, which is involved in various cellular functions, such as cell proliferation, stress and immune response, and protein transport from the Trans-Golgi network (TGN) to the plasma membrane. In particular, PKD2 mediates the fission of transport vesicles at the TGN via the interaction of its cysteine-rich subdomains C1a and C1b with diacylglycerol (DAG) and the ADP ribosylation factor 1 (ARF1) [1]. Yet, the detailed constitution of the *in vivo* PKD2-ARF1 complex and its interface regions are still a matter of debate. Also, further interaction partners of PKD2 at the TGN have to be identified. To address these issues we apply cross-linking experiments and mass spectrometry [2].

Experimenteller Teil

Chemical cross-linking is carried out with the heterobifunctional cross-linker sulfo-succinimidyl-diazirine (sulfo-SDA). Purified GST-PKD2 is incubated with sulfo-SDA to label lysine residues. After removal of non-reacted sulfo-SDA, labeled GST-PKD2 is incubated with TGN proteins and photo-reactive cross-linking occurs via exposure to UV light (365 nm). Afterwards, cross-linked complexes are isolated by affinity chromatography.

Alternatively, the photo-reactive amino acids photo-methionine and photo-leucine [3] will be incorporated into TGN proteins in HeLa or HEK-293 cells. TGN fractions will be subjected to photo-reactive cross-linking with purified GST-PKD2.

In both approaches, analysis of cross-linked products is performed by enzymatic digestion and nano-HPLC/nano-ESI-LTQ-Orbitrap mass spectrometry.

Ergebnisse und Diskussion

Full-length GST-PKD2 was purified from Sf21 insect cells using GST affinity chromatography and anion exchange chromatography. The identity of the purified product was confirmed via peptide fragment fingerprint analysis after tryptic digestion and nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS. Furthermore, purified GST-PKD2 showed considerable enzymatic activity in a luminescence-based kinase activity assay. Also, the isolation of Golgi fractions from homogenates of HeLa and HEK-293 cells by sucrose density gradient ultracentrifugation was established. Currently, initial cross-linking experiments are carried out with sulfo-SDA.

Also, a His-SUMO-tagged variant of PKD2, containing only the C1b domain is purified by metal ion affinity chromatography and size exclusion chromatography. In future cross-linking experiments, this variant may be particularly useful for elucidating the PKD2-ARF1 interaction.

Obtaining structural data of PKD2 complexes is of great importance. So far, a crucial role in the context of PKD2-ARF1 interaction has been shown for a proline residue at position 275 of PKD2 by a cell biological approach [1]. This observation will be underlined by 3D-structural data derived from cross-linking experiments. Thus, mapping the interface regions between PKD2 and ARF1 as well as the identification of additional PKD2 interaction partners will help to relate functions and mechanisms of complex formation at the TGN with structural characteristics.

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Neue Aspekte

Photochemical cross-linking to study PKD2 complexes

PP 49

Presentation of a homobifunctional azo-reagent for protein structure analysis by collision-induced dissociative chemical cross-linking (XL): Proof-of-Principle

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The combination of chemical XL and mass spectrometry has become a powerful method to study 3D-protein structures. To enable an effective analysis of XL peptides by electrospray ionization (ESI) and/or matrix assisted laser desorption ionization (MALDI) tandem MS, we and others developed novel XL-reagents, which fragment more easily than typical peptide bonds and additionally exhibit a characteristic fragmentation behavior upon collision activation, thereby allowing selective detection and sequence analysis by MS^3 [1,2]. Inspired by the results published by Beauchamp et al. [3], we synthesized a homobifunctional amine sensitive *N*-hydroxysuccinimide active ester derivative (azo-Linker) from the free radical initiator 4,4´-azobis[4-cyanopentanoic]acid and reacted it with two peptides (MRFA, RKDVY) to generate a model interpeptide XL and furthermore with Substance P to yield an intrapeptide XL [4].

Experimenteller Teil

All MS and MS^n experiments were conducted on a LTQ-Orbitrap XL instrument (Thermo Fisher, Bremen, Germany) equipped with an electrospray ion source (used in positive ion mode) and a linear quadrupole ion trap (LTQ) for ion selection and low energy CID. All exact ion masses of the ion discussed (precursor and product ions) were determined in the orbitrap analyzer and are consistent with chemical formula of the presented ion species.

All gas-phase IMRs were performed in the LTQ part of a LTQ Orbitrap XL instrument equipped with a modified helium gas inlet system for metered introduction of neutral reagents into the He buffer gas flow. The custom-made gas-handling system used is described in detail elsewhere [5].

Ergebnisse und Diskussion

Symmetric azo-compounds like the 2,2-azobis[2-methyl propionitrile] (AIBN; Vazo 64, DuPont) and also the water soluble 4,4-azobis[4-cyanopentanoic] acid (Vazo 68, DuPont) are commonly used as free radical initiators for polymerization reactions due to the effective formation of two radicals by the facilitated loss of nitrogen. The elimination of N_2 and the formation of radical species can also be triggered by CID of the azo-derivatized peptide molecular ions in the gas phase [3]. Beauchamp's results inspired us to synthesize a bis-succinimidyl active ester reagent (azoXL), which was reacted with the two peptides MRFA and RKDVY leading to a model interpeptide cross-link. The reaction products of azoXL with MRFA and RKDVY were subjected to (+)ESI-tandem-MS analysis to probe the gas-phase behavior upon CID. The product ion spectra of the singly, doubly and triply protonated molecular ions of YVDKR-azo-MRFA documented a characteristic and predominant loss of N_2 and the formation of captodative radical cations with unpaired electrons at a sterically shielded tertiary carbon. The open shell 4-cyano-4-methyl- butyricacidamide peptide cations of MRFA and RKDVY were founded with high abundance. These ions can further be collision activated in MS^3 product ion experiments for peptide sequence analysis. In doing so, CID of the captodative carbon centred peptide radical cations show the characteristic loss of 2-methylacrylonitrile (67 Da) along with extensive peptide backbone fragments, allowing an effective determination of peptide primary structure and identification of the *N*-terminal cross-link [3,4]. Characteristic mass shifted b- and a-type product ions are formed. Besides the analysis of the YVDKR-azo-MRFA interpeptide XL we reacted azoXL with Substance P (RPKPQQFFGLM-NH_2) to generate an intrapeptide XL between the *N*-terminus and the Lys residue in Position 3. To further examine the nature of captodative carbon centred peptide radical cations we conducted IMRs with allyliodide, allylbromide and dimethyl disulfide in the LTQ part of our instrument.

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Neue Aspekte

4,4-Azobis[4-cyanopentanoic] acid, a symmetrical free radical initiator, is transformed in the bis-*N*-succinimidyl-active ester (azoXL) and utilized for peptide XL.

PP 50

3D-Structural Characterization of the N- and C-terminal Regions of p53

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The tumor suppressor p53 acts as a DNA sequence specific transcription factor, which induces or represses a wide range of specific target genes that are involved in cell cycle control, senescense and apoptosis in response to genotoxic stress. Intracellular levels of p53 and its function are regulated by factors, such as post-translational modifications, degradation, and interactions with a broad palette of other proteins [1]. Briefly, p53 exerts its function as the guardian of the genome via a complex interplay of independently folded and intrinsically disordered domains. The transactivation domain (*N*-terminus) and regulatory domain (*C*-terminus) are such disordered regions. Here, we describe our efforts to obtain low-resolution structural information of p53´s termini by a combination of native MS and chemical cross-linking/MS.

Experimenteller Teil

Human p53 was overexpressed as HLT fusion protein [2] in *E. coli* BL21 (DE3) following a published protocol [3]. Purified human p53 was characterized via peptide fragment fingerprint analysis after double digestion with Glu-C and trypsin. Initial cross-linking experiments were carried out with p53 (10 µM in 50 mM HEPES, pH 7.2) using different homo- and heterobifunctional cross-linkers as well as the “zero-length” cross-linker EDC. Samples were proteolyzed with Glu-C and trypsin and the resulting peptide mixtures were analyzed by nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS (LTQ-Orbitrap XL, Thermo Fisher Scientific). Additionally, the functionality of p53 (tetramerization, DNA binding) was monitored via native MS (500 mM ammonium acetate, pH 6.8) with a modified high-mass Q-TOF II (Waters Micromass/MSVision).

Ergebnisse und Diskussion

P53 has been successfully produced using the T7-promoter-based *E.coli* expression system and purified using a strategy, which combines immobilized ion metal affinity and size exclusion chromatography steps. P53 was characterized by peptide fragment fingerprint analysis after double digestion with Glu-C and trypsin by nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS. To ensure the functionality of the purified p53, native MS measurements are carried out in the presence and absence of different p53 DNA response elements. Initial cross-linking experiments gave a hint on the functionality of purified p53 based on its ability to form homotetramers. Cross-linking experiments were performed with the zero-length cross-linker EDC and the formation of p53 dimers and tetramers was monitored via SDS-PAGE at different time points. Cross-linking experiments will be repeated in the presence of p53 response element DNA with EDC and a variety of homo- and heterobifunctional cross-linking reagents, such as BS^2G-D_0/D_4 and sulfo-SDA [4]. Analysis of the proteolytic digestion mixtures of cross-linked p53 with nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS will give insights to spatial arrangement of p53´s termini in the monomeric state and their behavior during tetramerization in presence and absence of response element DNA. In order to discriminate between intra- and intermolecular cross-links, a mixture of ^{15}N-labeled p53 and unlabeled p53 will be used. The combination of native MS and cross-linking/MS will provide further information about the interplay between folded and intrinsically disordered domains of p53 and will delineate structural changes in p53 during the tetramerization process.

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Neue Aspekte

3D-Structural Analysis of the *N*- and *C*-terminal domains of p53

PP 51

Structural and Functional Insights into the FocA/Pyruvate Formate-Lyase Complex

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Formate is a signature metabolite of enteric bacteria which is generated by the glycyl-radical enzyme PflB under anaerobic growth conditions. As much as one third of the total carbon from glucose is converted to formate during fermentation with extra-cytoplasmic concentrations increasing to as much as 20 mM. Intracellular accumulation of formate may lead to a substantial decrease in the cytoplasmic pH resulting in destruction of the proton gradient. To counteract cytoplasmic acidification, formate is exported to the periplasm by FocA (formate channel A) [1]. PflB was identified as an interaction partner of the formate channel FocA. Chemical cross-linking and mass spectrometry in combination with the Rosetta algorithm provided structural insight in the FocA-PflB complex.

Experimenteller Teil

For structural analysis of the FocA-PflB complex, FocA and PflB were purified (by Claudia Doberenz), and chemical cross-linking was performed with the homobifunctional amine-reactive cross-linker BS^2G (*bis*(sulfosuccinimidyl)glutarate). SDS-PAGE was used to visualize cross-linked FocA complexes. Bands of interest were excised and *in-gel* digested with trypsin and GluC. Peptide mixtures were analyzed by LC/MS on an UltiMate Nano-HPLC system (LC Packings/Dionex) coupled to LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ionization source (Proxeon). Identification of cross-linked products was performed with the *in-house* software StavroX [2]. FocA and PflB were docked with the centroid-based component of the ROSETTA protein-protein docking algorithm. The resulting 50,000 models were filtered using the cross-links identified by mass spectrometry as distance constraints.

Ergebnisse und Diskussion

In this study, the interaction between PflB and the formate channel FocA was analyzed by cross-linking analysis combined with mass spectrometry. We gained insight into the topology of the 350 kDa-complex by *de novo* docking with the Rosetta software using the cross-links as experimental constraints. Fourteen intermolecular cross-links between the cytoplasmically oriented face of FocA and PflB were identified. Approximately 50,000 docking models that were generated by this approach were filtered for those that fulfilled the constraints imposed by the cutoff C_α-C_α distance of 40 Å. The majority of the cross-links identified were also satisfied below a C_α-$C_\alpha$cutoff distance of 25 Å. The fact that 11 out of 14 cross-links were found for amino acids in the *N*-terminal domain of FocA indicates a high flexibility of FocA's *N*-terminus, which enables it to react with diverse regions of PflB. Furthermore, together with the findings of *in vivo* studies, our data strongly suggests that PflB controls the bidirectional formate translocation activity of FocA through direct protein-protein interaction.

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Neue Aspekte

Structural analysis of protein complexes by chemical cross-linking and mass spectrometry in combination with Rosetta.

PP 52

Complexome Profiling – Identifizierung von zytosolischen Proteinkomplexen

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Die meisten Proteine bilden stabile und dynamische makromolekulare Komplexe mit anderen Biomolekülen um ihre biologische Funktion z.B. in der Signaltransduktion, dem Transport und der enzymatischen Katalyse ausüben zu können. Für die Proteomik stellt die Analyse der Zusammensetzung der Proteinkomplexe eine große Herausforderung dar. Am häufigsten werden Proteinkomplexe mit Co-Immunopräzipitation oder Affinitäts-chromatographie „getaggter" Proteine angereichert und mittels quantitativer Massenspektrometrie analysiert. Diese Standardverfahren können kaum Einblicke in die native Größe, Stöchiometrie, strukturellen Module und Dynamik von makromolekularen Komplexen liefern. Die kürzlich mit dem „Complexome Profiling" eingeführte Kombination von klassischer Blau-nativer Elektrophorese (BNE), quantitativer Massenspektrometrie und bioinformatischen Auswertungen erweiterte nicht nur die Möglichkeiten der Charakterisierung einzelner Proteinkomplexe sondern gibt ein Gesamtbild aller isolierten makromolekularen Komplexe in einer Probe wieder [1,2].

Experimenteller Teil

Nach mechanischer Homogenisierung von HEK293T-Zellen wurde durch differentielle Zentrifugation die lösliche zytosolische Fraktion isoliert. Das gesamte „Complexome" des Zytosols wurde durch BNE aufgetrennt, die Gelbahn in Abschnitte aufgeteilt und tryptisch verdaut. Die eluierten Peptide wurden mittels C-18-Chromatographie aufgetrennt, durch Elektrospray ionisiert und anschließend in einem datenabhängigen Modus im Massenspektrometer analysiert. Dabei wurden aus hochaufgelösten Massenspektren Ionen ausgewählt, mittels kollisionsinduzierter Dissoziation fragmentiert und Massenspektren der Fragmente aufgezeichnet [3]. Die identifizierten Proteine wurden label-frei relativ quantifiziert. Mittels hierarchischer Clusteranalyse wurden die Proteine anhand ihres BNE-Migrationsverhaltens gruppiert und ein umfassendes Interaktionsprofil des Zytosols erstellt.

Ergebnisse und Diskussion

In der durch BNE fraktionierten zytosolischen Fraktion wurden über 2400 Proteingruppen identifiziert. Nach hierarchischer Clusteranalyse konnten Proteingruppen zu vielen bekannten Multiproteinkomplexen (Translationsinitiationskomplex, COP9-Signalosom, T-Komplex, Ribosomenfragmente u.a.) zugeordnet werden. Ein Ausschnitt des Interaktionsprofils zeigt die Untereinheiten des 26S-Proteasoms und weitere Proteasomaktivatoren (11S, PA200) [4,5]. Da die jeweiligen Module des Proteasoms auch in der BNE als Subkomplexe bzw. Assemblierungsintermediate isoliert wurden, konnten sie in Clustern gruppiert werden. Alle 14 Untereinheiten des 20S-Kernbereichs und die 3 Untereinheiten des 11S-Proteasomaktivators wurden identifiziert und in einen Block zusammengefasst. 17 von 18 identifizierten Proteinen des 19S-Haubenpartikelkomplexes wurden korrekt gruppiert. Darüber hinaus sind die verschiedenen Module zu unterschiedlich großen Proteasomen und Hybridproteasomen zusammengesetzt, deren native Masse bestimmt wurde. Weitere Multiproteinkomplexe werden gezeigt und diskutiert. Mit „Complexome Profiling" können nicht nur bekannte Proteinassoziationen sondern auch unbekannte Proteinkomplexe identifiziert werden [1]. Neben den vollassemblierten Proteinkomplexen geben die in der BNE isolierten und massenspektrometrisch analysierten niedermolekularen Subkomplexe und Assemblierungsintermediate Aufschluss zur strukturellen Zusammensetzung von makromolekularen Komplexen.

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Neue Aspekte

„Complexome Profiling" ermöglicht eine umfassende Analyse aller Multiproteinkomplexe einer Probe, ihrer Zusammensetzung, nativen Größe, Stabilität und struktureller Module.

PP 53

Structural Insight into an FMN-Dependent Ene-Reductase as a Versatile Biocatalyst Through Ion Mobility Mass Spectrometry

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In the last decades, an increasing tendency to apply biocatalysis in organic synthesis was observed. Due to advantages, such as excellent selectivity and catalytic activity, enzyme catalysis also gained tremendous interest from the chemical industry, particularly for the production of fine chemicals and pharmaceuticals.[1] Among redox enzymes, the ene reductase from *Gluconobacter oxydans* turned out to represent a versatile biocatalyst, useful for the reduction of different types of activated C=C double bonds.[2-4] Accordingly, we became interested to get an insight in the structure of this recombinant His-tagged protein. Encouraged by the success of ion mobility mass spectrometry for studying protein structures in the absence of bulk water,[5] we chose this methodology for our study.

Experimenteller Teil

Preparation of purified recombinant His-tagged ene reductase from *Gluconobacter oxydans* was done as described earlier and contained transformation of *E. coli* Bl21(DE3) cells with the expression plasmid pGOX, subsequent fermentation and purification after cell disruption via immobilized metal affinity chromatography IMAC (Ni-NTA).[2,4] Biochemical characterization using trans-hex-2-enal showed an enzyme activity of 3.06 U/mg protein. This *N*-terminal hexahistidine-tagged fusion protein was analysed using a nanoESI-Q-IMS-ToF mass spectrometer (Synapt G2S, Waters, Manchester). Both denaturing and native conditions were employed during the analysis of primary sequence and conformation of the protein. Additionally, Top-Down fragmentation of this ene reductase was achieved using CID and ETD. Data analysis was performed using and Driftscope™ and BioLynx™ (Waters, Manchester).

Ergebnisse und Diskussion

Intact ene reductase from *G. oxydans* was analysed both under denaturing and native ionisation conditions. When denaturing conditions were employed, the non-covalent bound co-factor FMN was not bound to the protein, as expected. Top-down fragmentation of the denatured protein enabled the unambiguous identification of the ene reductase from *G. oxydans*. With CID, the *C*-terminus of this enzyme was identified while ETD resulted in the identification of the *N*-terminus.

However, when native conditions were employed, ene reductase from *G. oxydans* was also detected, but only in complex with the co-factor FMN. This finding also clearly indicates that binding of the cofactor FMN by the ene reductase from *G. oxydans* is complete. When the ion mobility data was evaluated, several drift time species of this enzyme were visible. All these species had FMN bound to the protein but their drift times varied significantly. It was also visible, that the protein species with the lowest drift time exhibited lower charge states (11-14), whereas the species with the longer drift times exhibited higher charge states (13-18). This finding is of high biochemical interest since it reveals the presence of different types of folded species of the ene reductase from *G. oxydans* in solution, which all contain the required cofactor FMN. Clarification of the reasons behind this different folding as well as the catalytic activities of the different folded protein species represents a task for future work. In contrast, the driftplot (drift-time vs *m/z*) of the protein acquired using denaturing conditions is typical for a uniform conformation.

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Neue Aspekte

Ion mobility mass spectrometry enables a rapid identification of soluble cofactor-containing enzymes, which differ only by their protein folding.

PP 54

Using a benchtop quadrupole-Orbitrap mass spectrometer to evaluate the effects of gradient length on identified peptide/protein.

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The ability to sequence and identify large number of proteins from semi- to complex samples has significantly increased with advances in biological mass spectrometry. The shot-gun methodology is widely used in proteomics. The workflow is based on proteolytic digestion of complex protein mixture and analysis of resulting peptides by liquid chromatography coupled to a tandem mass spectrometer. The success of shot-gun experiments are highly dependent on the sample preparation, chromatography/MS settings and data mining capabilities [1,2]. Reverse phase chromatography (RPLC) with a linear acetonitrile gradient is by far the most preferred separation technique due to its easy coupling and the solvent compatibility with mass spectrometer. Here we evaluate the effect of gradient length on the number of overlapping peptides/proteins identified.

Experimenteller Teil

Lyophilized proteolytic HeLa lysate was dissolved in 0.1 % TFA and separated on the Thermo Scientific Ultimate 3000 nano LC using different gradient lengths of 30, 60, and 90 minutes, and subsequently analyzed on the Thermo Scientific Q Exactive Plus mass spectrometer. The instrument was operated in the data-dependent acquisition mode selecting the top 20 most intense precursor per spectrum for HCD fragmentation. The acquired raw files were analyzed using Thermo Scientific Proteome Discoverer™1.4 software with SEQUEST HT® search. The identified proteins were filtered using high confidence on the peptide level, peptide mass deviation of 10 ppm and peptide rank one.

Ergebnisse und Diskussion

For our experiments a proteolytic digest of HeLa lysate was used to evaluate the mass spectrometer's performance. Different gradients were used to determine the number of proteins identified in different separation times. Comparisons were performed by evaluating the overlaps in identified peptides and proteins obtained at the same and also at different chromatographic separation times. In a data dependent TopN experiment combined with a short chromatographic separation time the mass spectrometer is expected to select and fragment the highest abundant peptides only due to fast elution of the peptide mixture not allowing for digging deeper into low abundant species. We assumed that most if not all of the proteins identified in a shorter gradient should also be identified using the longer gradient. Comparing the unique peptides and proteins identified in triplicate runs of each gradient length, less than 5 % of the identified peptides and proteins were solely found in the runs applying the short gradient. As expected the total number of identified peptides and proteins continuously increased with increased gradient lengths. However, with increasing separation times the overlap in identified peptides and proteins compared to the shortest gradient decreased.

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Neue Aspekte

In-depth analysis of the degree of overlapping protein/peptides identified with different gradient length using a benchtop Orbitrap mass spectrometer.

PP 55

Microcolumn SCX displacement chromatography for phosphopeptide enrichment

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Many efforts have been taken to develop methods for phosphor-peptide enrichment prior to their mass spectrometry analysis. Although a huge number of papers with different methods were published, phosphopeptide enrichment still represents a big challenge.[1] For global phosphoproteomic approaches the combination of SCX chromatography followed by TiO_2 or IMAC was found to be well suited.[2] However, this approach suffers from limitations like huge starting amounts and expensive HPLC systems. We therefore developed an SCX displacement chromatography for phosphopeptide pre-fractionation. Displacement chromatography offers the opportunity to minimize the column dimension.[3][4] Here, self-packed microcolumns were applied offering the chance to use low starting amounts, no need for HPLC systems and buffers compatible with subsequent enrichment strategies like IMAC or TiO_2.

Experimenteller Teil

SCX micro-columns were prepared using GELoader tips. SCX bulk media was dissolved in methanol and placed on top of a SCX frit until a length of 10 mm. The column was equilibrated with loading buffer (0.1% TFA, 20% ACN in MS-water) and binding capacities were determined with pulses of both tryptic BSA peptides and spermine. For SCX chromatography tryptic casein peptides (alpha- and beta-casein) were loaded. Elution was carried out with 13 injections of 3 µg spermine (dissolved in loading buffer) followed by one injection of 20 µl 1 M NaCl. The flow-through and each elution fraction were measured by MALDI-TOF and ESI-IT-MS/MS. From LC-MS-experiments EICs from distinct peptides were made to characterize the elution profile.

Ergebnisse und Diskussion

The majority of phosphopeptides were identified by ESI and MALDI mass spectrometry in the flow-through and in the following three fractions including hydrophilic peptides, whereas most unphosphorylated peptides eluted in the posterior fractions. The results show that with the displacement approach an experiment can be designed in which one fraction with practically no affinity (flow-through fraction) containing almost phosphorylated peptides togehter with a fraction of low affinity (fraction 2-3) containing phosphopeptides and none phosphorylated peptides with low net charges (+2) and a fraction of peptides with a high affinity (net charges >+2, later fractions) towards the SCX material is obtained. The number of fractions can be controlled by the amount of displacer (spermine) used for elution. This micro column approach offers a flexible and inexpensive method since no HPLC systems or columns are needed and the number of fractions can be controlled by the concentration of the displacer used for the pulsed elution. Furthermore salt free buffers can be used thus making buffer exchange steps dispensable for preparing the sample for IMAC or TiO_2 enrichment.

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Neue Aspekte

We present a simplified, inexpensive method for phosphopeptide prefractionation based on SCX displacement chromatography in a multidimensional phosphopeptide enrichment approach.

PP 56

Monitoring Conformational Changes in PPARα by Photo-Affinity Labeling and Mass Spectrometry

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Chemical cross-linking, combined with an enzymatic digestion and mass spectrometric analysis of the reaction products, has evolved into an alternative strategy to identify protein-protein and protein-ligand interactions. Peroxisome proliferator-activated receptors (PPARs) belong to a subfamily of nuclear receptors that are involved in metabolic processes. One subtype, PPARα, plays a crucial role in lipid metabolism and presents an important target for designing antidyslipidemic drugs for treatment of the metabolic syndrome [1]. Conformational changes in PPARα upon ligand binding were investigated by photo-affinity labeling (PAL) studies combined with mass spectrometry [2].

Experimenteller Teil

Cross-linking reactions were performed using a home-built UV irradiation chamber. After *in-solution* or *in-gel* digestion, cross-linking reaction mixtures were analyzed by nanoHPLC/nano-ESI-MS/MS with a nano-HPLC system (Dionex, RP C18 column 75 µm * 250 mm) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nano-ESI source (Proxeon). Cross-links were evaluated with the *in-house* Software StavroX [3], checked manually, and visualized by PyMol. Intact proteins were desalted with C4-ZipTip columns and analyzed by MALDI-TOF-MS (Ultraflex III, Bruker Daltonik).

Ergebnisse und Diskussion

For PAL studies, the photoreactive amino acid *para*-benzoylphenylalanine (Bpa) was incorporated at specific positions into the ligand binding domain of PPARα [4]. The incorporation of Bpa instead of the naturally occurring amino acids, Leu-258 and Phe-273, was confirmed by MS/MS. Both PPARα variants were employed for PAL studies in the absence and presence of either the PPARα antagonist GW6471 or the agonist GW7647 [2]. For deriving 3D-structural information, the position of the amino acid to be exchanged by Bpa is of great importance as Bpa is rather bulky. In the absence of ligands, a higher number of cross-linked products were identified in both PPARα variants than in the presence verifying that the conformation of PPARα is stabilized upon ligand binding. By a detailed inspection of MS/MS data, we were able to confirm mixed cross-linked species between Bpa-258 and Phe-218 / Arg-226 and Ala-233 / Pro-238. The fact that some cross-links are found for free and ligand-bound PPARα alike indicates that PPARα exists in a number of conformations simultaneously and that the nature of the ligand determines, which of the coexisting conformations is preferred.

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Neue Aspekte

photo-affinity labeling, *para*-benzoylphenylalanine

PP 57

Human serum is a peptide biomarker gold mine and an artefact snakepit – good that there is mass spectrometry!

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With its convenience of sample collection and due to its organism-wide circulation, serum offers a valuable resource for biomarker discovery [1]. Yet, mass spectrometric peptide profiling of human serum is challenging because of protein complexity and huge dynamic ranges [2]. Also, since human serum and plasma contains many (unintentionally) activatable proteases and peptidases [3,4], robustness of biomarker peptide candidates needs to be validated before clinical application of peptide-based screening assays. We investigated stability of peptides in cord blood serum samples [5] and monitored protease activation under different but thoroughly controlled storage and sample work-up conditions using MALDI ToF MS and nanoESI Q-ToF MS, respectively.

Experimenteller Teil

Cord blood serum samples were diluted 1:10 with solvents of different pH and temperatures. Samples were incubated for extended periods of time before and after heat-denaturation, and with or without addition of protease inhibitors. MALDI matrix was prepared by suspending solid ferulic acid in 30 % ACN / 0.1% TFA, 0.5 µl of diluted serum solution and 1 µl matrix solution were deposited on a MALDI target and dried on air. Serum samples were analysed by MALDI-TOF MS and MALDI FT-ICR MS, respectively. Peptides of interest were further analysed and fragmented by nano-LC ESI-MS and nano-LC ESI-MS/MS.

Ergebnisse und Diskussion

We investigated time courses of sample incubations with different but typical blood serum sample preparation conditions using mass spectrometry. A mass range of m/z 1000 to m/z 20,000 was investigated. Changes in peptide ion intensities were recorded as functions of incubation time and in dependance of pH. Changes of cord blood protein ions were determined by interrogating abundances of hemoglobins [*m/z* 15,129 hemoglobin α, *m/z* 15,308 α^{glyc} hemoglobin, and *m/z* 16,001 hemoglobin $^{G}\gamma$] to estimate hemolysis. In addition, ion signals of chemokines CXCL4 and CXCL7 [CXCL4 aa32-101 at *m/z* 7766 CXCL7 aa44-128 at *m/z* 9292] were assumed indicative for platelet activation.

Finally, analysis of sample preparation time courses with diluted serum kept under defined conditions showed that two ion signals at m/z 2753.45 and m/z 2937.56 were changing in abundance as well. Since the nature of these ions was unknown, we performed high-resolution FT-ICR mass spectrometry and determined the accurate masses to be m/z 2753.437 and m/z 2937.558; the mass difference of was indicative of the dipeptide "IA". Further analysis by nano LC ESI-MS under standard conditions (pH 3) showed a high intensity of the quadruply charged ion signal at m/z 689.15. Only in basic solutions (pH 9.5) an intensive doubly charged ion signal was obtained at m/z 1377.23 for which CID fragmentation had been successful at last. Sequence analysis showed that the peptides of interest were N-terminal fragments of human serum albumin encompassing amino acids 1-14 and 1-16, suggesting that intrinsic serum protease and/or peptidase activities had caused variable ex-vivo proteolysis.

Consequently, only samples that were processed using inert conditions passed all quality criteria and were forwarded to multiplex serum protein profiling analysis. Assay performance was tested with the sample sets, finally resulting in statistically highly significant differentiation of patient samples from those of healthy donors and controls.

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Neue Aspekte

A systematic investigation on potential artefacts in mass spectrometric blood serum protein profiling enables biomarker assays with high confidence.

PP 58

Amide-bound advanced glycation end-products as prospective type 2 diabetes mellitus biomarkers

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Glycation refers to non-enzymatic reaction of aldoses and ketoses with amino groups of proteins [1] yielding Amadori and Heyns compounds, respectively, which readily can undergo further rearrangement reactions and oxidative degradation yielding highly reactive deoxysone intermediates. The latter have been shown to form amide-advanced glycation end-products (amide-AGEs) with lysine residues [2]. As this compound class was described only few years ago, their patterns in human plasma under hyperglycemic conditions are still not characterized. Here we describe the mechanisms of peptide amide-AGE formation from corresponding Amadori compounds and their potential value as novel plasma type 2 diabetes mellitus (T2DM) biomarkers.

Experimenteller Teil

Peptides formylated, acetylated, or glycerinylated in position 2 of the model sequence Ac-AKASASFL-NH_2 were synthesized on solid phase by Fmoc/^{t}Bu-strategy [2], purified by RP-HPLC, and characterized by ESI-QqTOF-MS and -MS/MS. The glycated model peptide was incubated in 100 mmol/L sodium phosphate buffer (pH 7.4) containing 18 µmol/L iron(II) at 37°C for 14 days and the product mixture was analyzed by LC-ESI-QqTOF-MS. The AGEs at Lys were annotated by exact mass, co-elution with authentic standards and MS/MS. Four groups of pooled plasma samples (75 ng), i.e. lean and obese healthy individuals and T2DM patients with good or poor glycemic control, were analyzed by nanoUPLC-ESI-LIT-Orbitrap-DDA-MS using a gas phase fractionation approach. AGE-modified peptides were identified and label-free relatively quantified (nanoUPLC-ESI-Orbitrap-MS).

Ergebnisse und Diskussion

Peptides carrying AGE-modifications at Lys-2 were obtained in reasonable yields (~10%) and high purities (>90%). ESI-QqTOF tandem mass spectra of the corresponding quasi-molecular ions were dominated by intense b fragment ion series, covering the full peptide sequence, which were accompanied by signals indicating one or two water molecules depending on the number of serine-residues. However, no mass losses from the AGE moiety were observed, except for a low-abundant carbonyl loss from the formyl-group.

As the formation of amide-bound AGEs was previously studied only at the amino acid level by incubating them with reactive intermediates [2], we investigated whether these modifications may result from early glycation peptide products. Therefore, the Amadori-modified model peptide was incubated in sodium phosphate buffer (100 mmol/L, pH 7.4) at 37°C in the presence of iron(II) (18 µmol/L) simulating plasma conditions. Indeed, acetyl and formyl lysine-modified peptides were detected after 14 days of incubation.

As glycation is highly abundant in plasma proteins [3], we extended our study to the above mentioned four groups of pooled plasma samples. NanoUPLC-ESI-LIT-Orbitrap-MS/MS DDA analyses of the pooled diabetic plasma samples revealed 2 glycerinylated, 10 formylated, and 7 acetylated lysine residues in 2, 6, and 4 proteins, respectively. Among them, one acetylated and one formylated peptide were significantly more abundant in plasma samples obtained from T2DM patients with poor glycemic control than in diabetic patients with good glycemic control or healthy persons.

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Neue Aspekte

Amide-AGE peptides were mass-spectrometrically characterized and detected *in vitro* as products of Amadori peptide degradation as well as *in vivo*.

PP 59

High-throughput/high accuracy protein biomarker discovery by application of isobaric reporter ion quantification (TMT10plex) in combination with HCD-MS3 acquisition

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In biomarker discovery studies, appropriate throughput is a crucial requirement. Mass spectrometric quantitation upon labeling with isobaric reagents (e.g. TMT reagents) allows multiplexing at reasonable costs. TMT6plex reagents allow the simultaneous analysis of up to six samples. Here, we expand the throughput of the TMT-based experiments to 10-plex as hypothesised recently [1,2].
Isobaric MS2-based quantition in complex samples may be compromised by coisolation of unrelated analytes in the MS precursor ion selection window. Fragmentation of contaminating analytes results in ratio distortion and causes an underestimation of differences in expression. Triple stage MS3 (HCD-MS3) almost completely eliminates interference [3].
We show proof of performance data with confected samples and the application of the TMT10plex HCD-MS3 workflow of 200 rat brain samples.

Experimenteller Teil

Rat brain samples were homogenized, reduced and alkylated, endopeptidase digested, and labeled with TMT6plex or TMT10plex reagents.
LC-MS was acquired using 115 and 225 minutes gradients, respectively. MS measurements were performed on an Orbitrap Velos. MS survey scans were acquired at the Orbitrap. The 10 most intensive signals were selected for further fragmentation for peptide identification and reporter ion quantitation.
For MS2 quantitation, HCD-MS2 was acquired at the Orbitrap.
For MS3 quantitation, MS2 was acquired in the ion trap using CID for peptide identification. An on-line algorithm (multi-notch, see [1]) further selects 6-9 MS2 fragments ions for HCD-MS3 reporter ion signal acquisition in the Orbitrap.
MS data was processed with Proteome Discoverer and applied to statistical analysis.

Ergebnisse und Diskussion

We investigated various parameters of the TMT10plex MS workflow as proof of performance.
A TMT6-labeled digest of seven proteins with known reporter ratios was spiked into a complex matrix (TMT6-labeled hippocampus digest) and quantitatively analysed by MS2 and MS3. Quantitative values for MS3 (4.299) are closer to theoretical values of 5 compared to MS (2.725). By this we verified the increased dynamic range/less isolation interference of the MS3 method.
Although stated otherwise, quantifiable peptide numbers in HCD-MS3 spectra using trypsin (84%) digestion instead of Lys-C (87%) are similar. This can be explained by the amplified intensity of the b-ion series of TMT labelled peptides.
The online multi-notch algorithm selects multiple product ions of the MS spectrum for further fragmentation in MS3 resulting in higher reporter ion intensity. Use of this algorithm resulted in 54% increase of quantified peptides.
A TMT6-TMT10 comparison was achieved by application to four individual rat brain tissues exposed to different chemicals. The TMT10 reagents performed similar in comparison to the actual TMT6 reagent using multi-notch MS3 for rat hippocampus. The total number of identified peptide groups was 3,352 ±169 for TMT6 and 3,164±80 for TMT10 labeled samples. On protein level, the number was 877±36 for TMT6 and 891±2 for TMT10. Furthermore, reporter ions presence in HCD-MS3 spectra was similar for TMT6 and TMT10 labeled samples. 84.4% (TMT6) and 84.2% (TMT10) contained the complete set of reporter ions.
This TMT10-based workflow enables rapid cycle times: the total experiment time for 200 samples from sample preparation to statistical analysis was ~25 days. Total MS time was ~14 days. Using PLS and ANOVA, we identified 33 to 80 putative biomarkers for each tissue with a significant *p*-value below 0.05.

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Neue Aspekte

TMT10plex labelling reagents allow high through-put for biomarker discovery experiments using high accuracy MS3 multi-notch online algorithm.

PP 60

Structure elucidation and insights into posttranslational processing and sequence evolution of dimeric neuropeptide gene products in insects

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Neuropeptides are the structural most diverse messenger molecules within the nervous system. In insects, which belong to the most attractive model organisms for studying neuropeptide processing and function, more than 100 neuropeptides are known from single species. Most of these peptides can easily be identified by direct tissue or cell profiling using MALDI-TOF mass spectrometry. Protein hormones or larger precursor peptides (> 50 AA) with intermolecular bonds are usually missed in these studies. Here we present an approach for *de novo* sequencing and characterization of homo- and heterodimeric products from *adipokinetic hormone* (*akh*) genes using single insect specimens.

Experimenteller Teil

The corpora cardiaca were dissected and either directly transferred on a MALDI target (direct tissue profiling) or homogenized in 50% methanol / 0.5% formic acid (tissue extract). MALDI-TOF analysis was performed on an UltrafleXtreme (Bruker Daltonik) using 1,5-diaminonaphthalene (DAN) and 2,5-dihydroxybenzoic acid (DHB) as matrix substances. DAN was dissolved in 0.1% TFA containing 50% acetonitrile at a final concentration of 10 mg/ml. DHB was dissolved in 1% formic acid containing 20% acetonitrile. DAN preparations were well suited for the identification of monomers but less suitable for *de novo* sequencing. Therefore, additional experiments were performed with tissue extracts. In these experiments, peptides with disulfide bonds were reduced and alkylated with dithiothreitol (DTT) and iodoacetamide (IAA) following standard protocols.

Ergebnisse und Diskussion

Detection of dimeric AKH precursor-related peptides (APRPs)

A mass spectrometric analysis of corpora cardiaca (the functional analog to pituitary glands in vertebrates) from different cockroach species revealed several distinct ion signals in the mass range of 8000-9000Da. Since a number of protein hormones in these tissues were suggested, but not yet identified by MS-based proteomics, these signals were of interest for further analysis. Application of disulfide-reducing methods (DAN;IAA/DTT) revealed the dimeric structure of these proteins.

Protein identification

MALDI-TOF-MS/MS experiments of tissues from single insects enabled the identification as APRPs which are encoded by *akh*-genes, also encoding the adipokinetic hormones - functional analogs to glucagon in insects. Surprisingly, the number of detectable monomers always exceeded the number of respective *akh*-genes. Therefore a diversification based on posttranslational processing was suggested.

Structure elucidation and posttranslational processing

MALDI-TOF-MS/MS experiments with high sequence coverage enabled sequence confirmation of APRP-monomers [1,2] but also *de novo* sequencing in species where genomic data were not available.

Beside the 38 amino acid-long full-length monomers others were identified as C-terminal truncated forms generated by exoproteolytic activity. Cross-linking of these forms leads to a complex pattern of hetero- and homodimers, resulting in a similar complex processing as it was described for locusts [3]. However, the products of the *akh*-genes are remarkably different between both insect groups.

Population variability

In the American cockroach our proteomic approach revealed four different alleles of *akh-II* gene. In contrast to *akh-I*-derived APRPs no truncated forms were detectable. Instead of that, paternal and maternal gene products of *akh-II* are cross-linked with each other and with *akh-I* monomers multiplying proteomic diversity to 28 predictable dimeric species. More than a third were confirmed by mass matches so far.

The complex processing pattern and the highly conserved sequence between distant insect groups suggest a physiological role of APRPs in insects.

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Neue Aspekte

Comparative analysis of endocrine tissue from insects enabled insights into hormone precursor sequence evolution, processing and population variability.