Annual Spring Meeting

14-4-2015

Abstracts

Location Leiden University Medical Center, Leiden

Program

Time	
9.30	Registration / Coffee
10.00	Welcome
10.05	Dr. Christa Cobbaert – LUMC Targeted Proteomics in Laboratory Medicine: about biomarker pipelines and lifelines.
10.30	Dr. Rob Haselberg – VU University Amsterdam Simultaneous determination of protein affinity and heterogeneity by capillary electrophoresis–mass spectrometry.
10.55	Dr. Martin Giera – LUMC A role for Adrenic Acid in the resolution phase of inflammation.
11.20	Dr. Arzu Umar – Erasmus University Medical Center Rotterdam Advances in clinical proteomics in breast cancer research.
11.45	Lunch / Posters
13.15	Ledenvergadering
13.30	Wilco Duvier – Wageningen University A new approach in hair forensics: Longitudinal scanning of drugs of abuse in hair using DART-MS.
13.40	Dr. Jonathan Martens – Radboud university Nijmegen Peptide fragmentation mechanisms by infrared ion spectroscopy: recent developments.
13.50	Kees Bronsema – University of Groningen Development, validation and application of a quantitative LC-MS/MS method for insulin like growth factor-1 in human plasma.
14.15	Coffee / Posters
14.45	Dr. Manfred Wuhrer – VU University Amsterdam High-throughput clinical glycomics.
15.10	Prof. dr. Ron Wevers – Radboud University Nijmegen Next Generation Metabolic Screening.
15.35	Dr. Viollete Gautier – University of Utrecht Advanced Ti4+-IMAC (phospho)proteomics to identify novel melanoma companion drug targets and uncover phosphorylation dynamics and pathway dependence in senescence signalling.
16:00	Poster award / closing
16:05	Drinks

Targeted Proteomics in Laboratory Medicine: about biomarker pipelines and lifelines

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Abstract

The enormous potential of biomarkers to revolutionize clinical practice and improve patient care is well documented. However, the release of commercial CE-marked and/or FDA-approved IVD diagnostic tests has reached a historical nadir with an average of only 1.5 medical test per year in the period 1993-2010, and even less than 1 medical test per year beyond 2010. The low numbers of newly introduced medical tests are in huge contrast with the unmet clinical needs in several clinical domains on the one hand, and the large number of promising biomarkers detected in discovery research on the other hand.

What is needed to turn the tide? Until recently validation technologies capable of testing large numbers of candidate protein biomarkers have not been available. Often, antibody-based measurements were used. Developing a new, clinically deployable immunoassay is very expensive and time consuming which restricts its use to already highly credentialed candidates. For the large majority of new, promising candidate biomarkers enabling technology is required that has short assay development timelines, low assay cost, allows effective multiplexing of 10-50 candidates, low sample consumption and high throughput with good precision.

Technology that has recently entered academic/large medical laboratories for promising biomarker validation and/or for replacing “flawed” (immuno)assays is Stable Isotope Dilution (SID) - Multiple Reaction Monitoring (MRM) Mass Spectrometry. Use of SID-MRM-MS for protein assays is based on measurement of “signature” or “proteotypic” tryptic peptides that uniquely and stoechiometrically represent the protein candidates of interest. SID-MRM-MS based assays have several distinguishing features relative to conventional immunoassays. First, the analyte detected in the MS can be characterized with near-absolute structural specificity – something never possible using antibodies alone. This provides a critical quality advantage, especially in cases where immunoassays are subject to interferences. Second, MRM assays can be highly multiplexed such that multiple proteins can be measured during a single analysis with assay CV's of <10% demonstrated at a protein level of 1 µg/mL level or higher in plasma. Third, all of these measurements can be done in about 100 nL of serum or plasma, whereas individual immunoassays often consume 10-100 µL (i.e., 100-1,000 times more).

For clinical utility it is essential that the SID-MRM-MS technology meets quality targets based on quality specifications deduced from biological variation criteria. Using this SID-MRM-MS targeted proteomics approach, a representative example of a Lab-Developed-Test panel and the steps considered to make it useful for clinical application will be discussed.

Simultaneous determination of protein affinity and heterogeneity by capillary electrophoresis–mass spectrometry

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Abstract

The pharmacological activity of most biopharmaceuticals is based on their capacity to bind to their therapeutic target. This binding can be significantly influenced by (post-translational) modification of the drug or target. To study protein-protein interactions in detail, affinity capillary electrophoresis (ACE) holds great promise. Employing the efficient separation of intact proteins as provided by CE, ACE offers the possibility to simultaneously study the interaction of multiple proteins and/or isoforms with a target receptor under homogeneous and near-physiological conditions.

We present the hyphenation of ACE with mass spectrometry (MS) as a novel highly-selective tool for the assessment of protein-protein interactions. In a single run ACE-MS provides (a) assignment of the molecular weight of all protein sample components, including isoforms and other variants, (b) protein-variant-selective determination of affinity constants, and (c) establishment of protein-complex stoichiometries. We applied MS-compatible capillary coatings to achieve efficient protein separation and precise determination of affinity-induced changes of receptor electrophoretic mobility. The binding of the protease inhibitor aprotinin to trypsinogen was used as protein-protein affinity model; the trypsinogen sample comprised several protein modifications. ACE-MS analysis of the trypsinogen in the presence of different concentrations of aprotinin revealed that the binding stoichiometry was 1:1. With this information, binding constants for all sample components, including co-migrating compounds, could be derived based on both mobility shift and mass spectrometric data. Simultaneously, ACE-MS allowed assignment of protein modifications and enabled direct correlation with the observed affinity. In order to fully appreciate the utility and added value of the data obtained with ACE-MS, comparisons were made with affinity measurements by direct infusion MS and ACE with UV detection.

A role for Adrenic Acid in the resolution phase of inflammation

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Abstract

The resolution phase of inflammation is crucial to prevent acute inflammation from becoming chronic. It has become evident during recent years that resolution is an active process that involves several cells and molecules. Especially a novel class of specialized lipids recently emerged as potent pro-resolving mediators (SPM). Nonetheless, the molecular identity and interplay of these lipid SPM are not fully understood and novel molecules continue to be defined as important in this process. Therefore, we extensively characterized the inflammatory response in the murine zymosan induced peritonitis model using several mass spectrometry based platforms. Interestingly, we found that the n-6 polyunsaturated fatty acid adrenic acid (AdA) as well as its cyclooxygenase and lipoxygenase metabolites accumulated in the peritoneal exudate cells during the resolution phase of inflammation, suggesting a pro-resolving function of AdA. To address this possibility, we investigated the effect of AdA on immune cells. By using an LC-MS/MS based screening platform, we show that low micromolar concentrations of AdA block the formation of the potent neutrophil chemo attractant leukotriene B4 (LTB4) and its pathway marker 5-HETE, without affecting cell viability. Further investigation revealed that AdA does not block calcium influx upon ionophore stimulation, but rather blocks the release of arachidonic acid (AA) from phospholipids, suggesting an inhibitory effect on cytosolic phospholipase A2, which catalyzes this process. These findings are specific for neutrophils, as no inhibition of LTB4 production was observed in M1 macrophages. In conclusion, our data indicate that AdA selectively blocks the release of AA in neutrophils, influencing the AA cascade and thereby unveiling a novel pathway that could promote the resolution of inflammation.

Advances in clinical proteomics in breast cancer research

Arzu Umar

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Abstract

Breast cancer (BC) is a very heterogeneous disease and consists of several subtypes, each with a distinct molecular phenotype. The most common luminal subtype is characterized by the presence of estrogen receptor (ER) and progesterone receptor (PR) proteins, which are excellent targets for hormonal therapies. Similarly, targeted treatment options are available for patients that have an amplification of the human epidermal growth factor receptor 2 (HER2) in their tumor. However, ~15% of all breast cancers lack expression of ER, PR, and HER2 and are therefore referred to as triple negative (TN). TNBCs are very aggressive in nature, therefore patients are commonly threated with adjuvant chemotherapy. Unfortunately, this results in massive overtreatment of TNBC patients. Irrespective of the molecular subtype, all patients with recurrent disease will at certain moment encounter the problem of therapy resistance. Therapy resistant metastatic tumor cells is what eventually kills the patients. Therefore, there is an urgent need for both prognostic and predictive biomarkers that can predict the natural course of disease and predict the response to treatment. In addition, new markers could serve as targets for treatment.

In our lab, we have developed a dedicated tissue proteomics pipeline that encompasses laser capture microdissection of epithelial tumor cells, nanoLC peptide separation using 50 cm reversed-phase columns and 3h gradients, online coupled to high resolution MS analysis, and label free quantification. Using this pipeline, we have analyzed TNBC patients cohorts for the identification and validation of a prognostic 11-protein signature. In addition, we have identified predictive proteins for chemotherapy resistance in TNBC, and a predictive 4-protein signature for hormonal therapy resistance in ER+ BC. For each signature, we have developed a targeted-MS assay with the purpose of introducing these protein markers into a clinical setting.

In conclusion, our tissue proteomics pipeline is very well suited for the identification and validation of clinically relevant protein markers.

A new approach in hair forensics: Longitudinal scanning of drugs of abuse in hair using DART-MS

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Abstract

Current forensic hair analysis methods are laborious, time-consuming and provide only a rough retrospective estimate of the time of drug intake. Recently, hair imaging methods using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) were reported, but these methods focus on individual hairs leading possibly to misinterpretation due to different hair growth phases. Ambient ionization of intact locks of hair would solve those issues, and could even enhance the obtained information by longitudinal scanning. Direct analysis in real time (DART), coupled to different MS instruments, has been explored as a fast screening technique for drugs of abuse in hair samples.

Initially, the optimal DART gas temperature and the accuracy of the probed hair zone were investigated for the analysis of delta-9-tetrahydrocannabinol (THC) and cocaine. After testing the method on spiked hair samples, drug user hair samples were obtained and scanned using the developed method. Next to this, the performance of different mass analyzers was evaluated regarding sensitivity and selectivity.

With the DART hair scan and an orbitrap instrument, THC and cocaine could be detected in hair samples from different drug users. Zones with different drug of abuse content could be clearly distinguished, indicating that the DART hair scan method can be used for retrospective timeline assessments.[1]

Coupling of the DART ionization source to more advanced MS instruments enhances the method: the use of a quadrupole – orbitrap mass analyzer already resulted in a more than tenfold increase in sensitivity.[1] This set-up yields full scan data enabling retrospective data analysis for other drugs, e.g., new designer drugs.

The ability of retrospective assessment of time of drug intake has been compared with that of conventional hair analysis techniques. Next to this, a critical assessment has been made of the DART-MS hair scan method versus the recommendations of the Society of Hair Testing regarding cut-off values to identify chronic drug use.[2]

[1] W.F. Duvivier et al. Rapid Commun. Mass Spectrom. 2014, 28, 682-690
[2] G.A.A. Cooper et al. Forensic Sci. Int. 2012, 218, 20-24

Peptide fragmentation mechanisms by infrared ion spectroscopy: recent developments

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Abstract

Dissociation of multiply-protonated peptide ions by electron attachment methods (electron transfer dissociation, ETD, and electron capture dissociation, ECD) is a recently developed technique to sequence peptides and proteins. This technique produces unique fragmentation patterns that complement peptide dissociation observations from the more conventional collisional or multiple-photon activation. Many appealing aspects of the technique have resulted in widespread development and implementation, however a complete description of the dissociation mechanisms involved remains under development. Recently, infrared ion spectroscopy has been used to definitively assign the structures of peptides and their fragments generated in collision induced dissociation (CID) mass spectrometry experiments. Such information lends powerful assistance to the elucidation of peptide fragmentation mechanisms and pathways. Using this technique, we are able to examine the structures of fragments generated by ETD and thus gain valuable insight into the associated dissociation mechanisms.

We report developments to provide optical access to trapped ions in a commercial quadrupole ion trap. Using the tunable infrared radiation of the FELIX free electron laser, we are then able to generate structurally diagnostic infrared spectra of peptide fragments generated by ETD. These results will be contrasted with the more conventional and better understood fragmentation that results from collisional activation of gas phase peptide ions.

Experimental results characterizing the ETD fragmentation behaviour of model peptides (e.g. AAHAR) that have previously received extensive theoretical attention will be presented and the results compared. The particular model systems were selected because they are expected to produce mainly open-shell z-type fragments upon ETD. We will characterize their structures and the radical chemistry involved in their formation.

Development, validation and application of a quantitative LC-MS/MS method for insulin like growth factor-1 in human plasma.

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Abstract

Background. Deviations in circulating concentrations of IGF-1 have been used as a biomarker in several therapeutic areas. Traditionally, IGF-1 measurements are performed by (automated) ligand binding assays, which rely on the consistency of critical reagents often resulting in poor inter-laboratory comparability.

Methods. An LC-MS/MS method was developed that discriminates between mature IGF-1 and total IGF-1, including all isotypes with extended amino acid sequences, in human plasma. The method is based on tryptic digestion of plasma and quantification of two IGF-1 specific signature peptides, one of which occurs only in mature IGF-1 while the other is present in all isotypes. Extraction was avoided prior to trypsin digestion to prevent any loss of isotypes by differences in extraction efficiency. After digestion, solid-phase extraction was applied to enrich both signature peptides.

Results. The method is capable of measuring IGF-1 concentrations over the relevant range of endogenous IGF-1 concentrations (calibration range 10 to 1000 ng/mL). The method was validated according to regulatory guidelines. Observed differences in response between several commercial IGF-1 reference standards demonstrated inconsistencies in purity and absolute amount between several commercially available IGF-1 preparations. Normalization against the WHO standard for IGF-1 resulted in a good agreement (correlation coefficient >0.97) between the LC-MS/MS method and an existing ELISA method.

Conclusions. LC-MS/MS is a reliable alternative to ligand binding assays (LBAs) for the quantitative determination of IGF-1 in plasma. Contrary to LBAs, it does not suffer from lot-to-lot variability of critical reagents, although the variable quality of reference standards proved to be a problematic factor for the absolute quantification of IGF-1. LC-MS/MS showed that IGF-1 isotypes do not constitute a significant fraction of the total plasma IGF-1 content.

High-throughput clinical glycomics

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Abstract

Glycosylation is an abundant post-translational modification on proteins. In humans, glycans are highly complex structures that are influenced by genetic as well as environmental factors. It has been widely shown that glycosylation changes are a hallmark of many disease processes, and protein-linked glycans therefore represent a rich repertoire of disease biomarkers that may be exploited for diagnosis and prognosis.

The robust analysis of large sets of well-characterized patient samples is a prerequisite for the development of reliable clinical markers. We develop high-throughput strategies for clinical glycomic studies that include automated sample preparation on a robotic liquid-handling platform, robust mass spectrometric measurement of glycans and glycopeptides, and automated data processing with embedded quality control procedures.

We apply these clinical glycomic workflows for the study of glycosylation changes in allo- and autoimmune diseases, infectious diseases, and cancer. The diagnostic and prognostic value of protein-specific glycome profiles is evaluated.

Next Generation Metabolic Screening

Wevers Ron A.[a]

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Abstract

The body fluid metabolome of a patient at any point in time is influenced by the metabolism of the patient, by the gut microbiome, by food intake and also by the medication given by the clinician. The analytical challenge is to find the relevant biomarker(s) that reflect the disease of the patient and provide information about the course of the disease. We have shown that our approach with LC-Qtof MS detects a very broad range of small molecules in the complex matrix of the body fluid. Sensitivity limits are in the nanomolar range. Our work shows that even in an individual patient the relevant biomarker profile can be picked up without prior knowledge of the underlying disease. The basis for this is a robust liquid chromatography system with the Qtof as a sensitive detector providing the accurate mass. From the complexity of 10000 signals that are detected in a single plasma sample it requires our in house developed chemometric pipe-line and access to relevant small molecule data-bases to find the relevant biomarkers. Since November 2013 this technique is used in the patient care in Nijmegen for diagnosis and follow-up of patients with inborn errors of metabolism. We have introduced the technique on worldwide congress platforms as “Next Generation Metabolic Screening”. Although we have developed this technique for the purpose of diagnosing inborn errors of metabolism the further perspective surely reaches out to the diagnostics of other diseases like infectious disorders or cancer diagnostics and further. Using this approach we enter the era of personalised medicine and make a big next step in biomarker use in Laboratory Medicine.

Advanced Ti^{4+}-IMAC (phospho)proteomics to identify novel melanoma companion drug targets and uncover phosphorylation dynamics and pathway dependence in senescence signaling

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Abstract

Malignant melanoma is mainly fuelled through the ERK pathway, mainly due to oncogenic mutant $BRAF^{V600E}$. Development of targeted therapy against $BRAF^{V600E}$ and MEK gives unprecedented clinical benefits. However, eventually most melanomas become resistant to inhibition of the ERK pathway, and patients succumb to the disease. Therefore, there is a dire need to find alternative drug (co)-targets or activate preventative mechanisms such as oncogene-induced senescence (OIS). Here we use MS-based (phospho)proteomics, using Ti^{4+}-IMAC, and targeted phospho-SRM approaches, to study melanoma cell response to $BRAF^{V600E}$ inhibitor and investigate mechanisms underlying acquired drug resistance, but also to describe the dynamics of signaling pathways involved in OIS.

In our drug sensitizer screen, we identified ~11,500 phosphosites and ~5,700 proteins. As expected, exposure to PLX4720 led to down-regulation of the phosphorylation state of kinases within the MAPK pathway: phospho-MEK, phospho-ERK1/2 and phospho-p90RSK. Moreover, the phosphorylation state of RPS6 was significantly down regulated, indicative of an inactive state of the mTORC1 pathway. Using our integrated proteomics and kinome *sh*RNA genomics screening platforms, we identified a novel kinase, whose interference generated an additive effect in vemurafenib therapy against melanoma. Next, we studied the molecular mechanisms of acquired drug resistance in patient-derived, treatment naïve, sensitive and resistant melanoma cells. Our single-shot analysis allows us to quantify around 4500 phospho-sites and 3400 proteins. Interestingly, together, these data revealed recently proposed combination drug targets but also pinpoint involvement of several promising novel pathways containing potential drug co-targets. Finally, to gain more insight into the vital tumour suppression mechanism OIS, we performed a MS-based screening in cycling and OIS cells as well as cells that have abrogated senescence (OISb). Using phosphopeptide enrichment by Ti^{4+}-IMAC and phosphotyrosine antibody enrichment, we identified over 15,000 phosphorylation sites. Among the regulated phosphorylation sites, we encountered components of the interleukins, BRAF and CDK-retinoblastoma (Rb) pathways. Next, we monitor phosphorylation dynamics of the regulated PI3K-mTOR and MAPK signaling networks, by combining SRM with our highly selective phosphopeptide enrichment. Previously uncharacterized phosphorylation changes in OIS, associated with diverse biological phenotypes and pharmacological intervention of the PI3K-mTOR pathway were highlighted.

Abstracts poster presentations

Accurate measurement of the essential micronutrients methionine, homocysteine, vitamins B_6, B_{12}, B_9 and their metabolites in plasma, brain and maternal milk of mice using LC/MS ion trap analysis

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Abstract

Methionine, homocysteine, vitamins B6, B12, B9 and their metabolites are crucial co-factors and substrates for many basic biological pathways including one-carbon metabolism, and particularly important for brain function and development and epigenetic mechanisms. These are essential nutrients, which cannot be synthesized endogenously and thus need to be taken in via the diet. A novel method was developed enabling simultaneous assessment of the exact concentrations of these essential micronutrients in various matrices including maternal milk, plasma and brain of neonatal mice. The protocol for analysis of these components in the various matrices consists of a cleanup step, lipid extraction followed by protein precipitation, combined with a liquid chromatography mass spectrometry (LC/MS) ion trap method with high sensitivity and selectivity (SRM mode). This novel method enables the measurement of these essential nutrients with good recoveries (69–117%), and high intra-day (<10%) and high intra-day precision (<15% for compounds with an isotopologue as internal standard and <20% for compounds without an isotopologue as internal standard) in plasma, maternal milk and brain of mice at low and high levels. Degradation of vitamins and oxidation of homocysteine is limited to a minimum and only small sample volumes (30 µL plasma, 20 mg brain and maternal milk) are needed for precise and simultaneous measurement. This method allows to study how these nutrients are transferred from mother to offspring via maternal milk, and into how these nutrients are being absorbed by the offspring and eventually taken up in various tissues amongst the brain in preclinical and clinical research settings exploring critical periods in lactating mothers and developing offspring.

Benchmarking fragmentation methods on an Orbitrap Fusion for top-down phospho-proteoform characterization

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Abstract

Top-down analysis of intact proteins by mass spectrometry provides an ideal platform for comprehensive proteoform characterization, in particular, for the identification and localization of co-occurring post-translational modifications (PTM). One of its main bottlenecks is insufficient sequence coverage caused by incomplete fragmentation. Based on previous work on peptides, increasing sequence coverage and PTM localization by combining sequential ETD and HCD fragmentation in a single event (EThcD), we hypothesized that protein sequence coverage and phospho-proteoform characterization could be equally improved. Here, we systematically benchmark the performance of several fragmentation methods for intact protein analysis on an Orbitrap Fusion, using as a model system the mitotic regulator Bora. During cell division Bora becomes multiply phosphorylated by the cell cycle kinases Aurora A and Plk1, albeit at distinctive sites. We monitor Bora phosphorylation by Aurora A and Plk1, analyzing the generated distinctive phospho-proteoforms by top-down MS. We show that EThcD and ETciD are capable of providing richer fragmentation spectra compared to HCD or ETD alone, increasing protein sequence coverage, and thereby facilitating phosphosite localization and the determination of kinase specific phosphorylation sites in these phospho-proteoforms.

A Comprehensive Analysis of the Mouse Brain Proteome Sampled in Mass Spectrometry Imaging.

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Abstract

On-tissue enzymatic digestion is performed in MALDI mass spectrometry imaging (MSI) experiments to access larger protein species and to assign protein identities through mass matching to the results from an LC-MS/MS analysis of tissue extracts. Proteome coverage in on-tissue digestion MALDI-MSI is hampered by both sub-optimal conditions for proteolysis, and the bias exhibited by MALDI towards the detection of Arg-terminated tryptic peptides. Previous LC-MS/MS experiments have demonstrated that combining the data from a cohort of complementary proteases increases protein sequence coverage as well as the number of protein identifications. Here, we have investigated if proteome coverage in MALDI-MSI may be similarly increased by using a cohort of proteolytic enzymes, specifically (i) trypsin, (ii) Lyc-C, (iii) recombinant Lys-N, (iv) Arg-C, and (v) a mixture of trypsin and Lys-C (trypsin/Lys-C).

For this study a C57BL/6J mouse was sacrificed by cervical dislocation, the brain was excised and flash frozen on dry-ice. Consecutive, 12 µm thick cryo-sections were washed with aqueous/organic solvents and homogeneously covered with one of the previously listed proteases. After overnight digestion, the sections were covered with MALDI matrix (α-cyano-4-hydroxycinnamic acid for analysis by MALDI-TOF/TOF-MS, and 2,5-dihydroxybenzoic acid for MALDI-FTICR-MS) before MSI analysis and peptide extraction. The matrix extracts were analyzed by LC-ESI-MS/MS, using the SwissProt database (bold red-requirement, score ≥25, peptides ≥1, FDR 1%) to provide protein identifications. Datasets from the tested enzymes were compared using ClinProTools, flexImaging, Mascot, and Excel. Combining datasets from different proteases resulted in 5337 peptide identifications and 1198 protein identifications, an increase of respectively 179% and 110% compared to a trypsin digestion (1913 peptides, 570 proteins). To connect the confidently identified peptides and proteins from the matrix extracts to the on-tissue digestion MALDI-MSI experiments, high mass resolution MALDI-MSI data was acquired on a 9.4T MALDI-FTICR instrument. A combined total of 633 peptides, originating from 280 proteins, were assigned to these high mass resolution datasets. This work reports the peptides identified from the on-tissue digestion matrix proteome as well as the peptides detected by high mass resolution MALDI-MSI, for multiple proteolytic enzymes. The results demonstrate the ability to increase proteome coverage by using different enzymes and indicate that on-tissue digestion MALDI-MSI analysis of the mouse brain can be applied in a wide range of disease related, biomarker discovery or other neuroscience applications.

A novel method for simultaneous measurement of concentration and enrichment of NO synthesis-specific amino acids in human plasma using stable isotopes and LC/MS ion trap analysis

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Abstract

Stable isotope studies offer the opportunity to study the in-depth metabolic pathway of glutamine, citrulline, and arginine amino acids involved in NO synthesis. The use of multiple stable isotopes can be used to elucidate the exact transformation of glutamine to citrulline and arginine de novo synthesis. This novel method provides a purification step using cation exchange resin in combination with a rapid and easy derivatization procedure for a precise and robust measurement of the concentration and isotopic enrichments of NO synthesis-specific amino acids using a liquid chromatography mass spectrometry (LC/MS) ion trap system with high sensitivity and selectivity. The ethyl chloroformate derivatization procedure is beneficial in terms of robustness, velocity, simplicity, and derivative stability. In addition, the ethyl chloroformate derivatization can be performed at room temperature in an aqueous environment without incubation and the isolation of the derivatives from the reaction mixture also serves as a purification step. The concentration and enrichment of NO synthesis-specific amino acids as well as phenylalanine and tyrosine to determine protein turnover, were measured with good inter-day precision for the concentration (< 7.4%) and enrichment (< 12.7%) in plasma samples at low and high levels. The low limit of quantification was 0.2 µmol/L for most of the amino acids and the purification method showed to have good recoveries between 78–98%. No ion-suppression was observed by post-column infusion experiments. In order to develop new nutritional strategies, this novel method can be used to support the elucidation of the effect of administration of specific supplements on the glutamine-citrulline-arginine pathway by using stable isotope studies.

Disulfide bond mapping using online LC–Electrochemistry–MS

Characterization of Notch3 protein fragments

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Abstract

Disulfide bonds (DSBs) are important for the stabilization of the three-dimensional structure and biological function of proteins. Alterations in the number of cysteines and DSBs, e.g. due to genetic mutations, may affect protein function. For example, in Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), mutations in the NOTCH3 gene disrupt the triply-paired DSB structure of the Notch3 protein. This leads to aggregation of Notch3 in vascular smooth muscle cells, which is believed to cause dementia and stroke in CADASIL patients. Such cases and others have increased the interest in characterization of protein DSBs.
The analysis of proteins is traditionally done using a bottom-up or top-down proteomics approach. In bottom-up proteomics, the use of reducing and alkylating agents increases the number of protein identifications, but also results in a loss of information regarding DSBs. In top-down proteomics, electrochemistry has been used for online reduction of DSBs in intact proteins, which is not only beneficial for sequence coverage, but also allows for disulfide mapping. Due to mass limitations and complexity of the generated data, this approach has only been applied to peptides and small proteins, such as oxytocin and lactoglobulin.

In the work presented here, electrochemistry (EC) has been implemented in a bottom-up proteomics workflow. For this, an electrochemical cell was connected to a LC system and an ESI-FTICR MS instrument to allow for online reduction and characterization of DSBs in disulfide-linked peptides from a protein digest. Proteins were digested with trypsin and endoproteinase GluC without prior reduction and alkylation to keep the DSBs intact, and at low pH to minimize disulfide reshuffling. The digests were first analyzed with the LC–EC–MS system without electrochemical reduction (CellOFF mode) to detect the disulfide-linked peptides. In the second analysis, the DSBs were reduced using electrochemistry (CellON mode) to detect the disconnected peptides. The DSBs are mapped based on MS2 data and the retention times of the disulfide-linked peptides and disconnected peptides. The approach was evaluated with a standard protein, ribonuclease B (14 kDa) of which all 4 DSBs were characterized with LC–EC–MS. The methodology was then applied to a Notch3 protein fragment (20 kDa, 15 DSBs) expressed in a cell culture system and purified using HaloTag® technology. Despite the low concentration and polymer contamination, several Notch3 peptides were detected in CellON mode. Work is ongoing to identify the disulfide-linked peptides in the CellOFF analysis.

Deciphering the proteome dynamics during neural development

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Abstract

Recent studies have revealed the importance of brain development in many neurological disorders. Neural development describes the cellular and molecular mechanisms involving initial polarization, neurite outgrowth, axon formation, dendrite formation, synaptogenesis and further maturation. However, studying human neural development suffers from severe cell or tissue heterogeneity in combination with complex developmental and environmental factors .Alternatively, human induced pluripotent stem cells (iPSC) can rapidly differentiate towards functional neurons by forced expression of a single transcription factor. This approach exhibit rapid and reproducible production of homogeneous population of glutametergic neurons with high yield which can be used as a model system to study the molecular mechanisms during development. Resolving the molecular mechanisms requires a quantitative assessment of associated proteins. Proteins are the final molecular effectors of cellular processes and perturbation at protein level is linked to pathological states. Here, we use stable isotope labeling-based quantitative mass spectometry to establish an inventory of neuron-specific proteome dynamics during differentiation.

Influence of glycoforms on the tryptic digestion efficiency of immunoglobulin G based biopharmaceuticals

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Abstract

Bottom-up proteomics is employed frequently in biological and clinical research. Nowadays, it is becoming increasingly popular for the analysis of biopharmaceuticals in discovery, development and batch control. The digestion of the target protein by proteases, generally by trypsin, into more easy to analyse peptides is a key step in bottom-up proteomics. Thus, a robust analysis needs to incorporate an efficient and unbiased tryptic digestion step. It has been observed previously that incomplete tryptic digestion can introduce biases into glycoform profiles.
Therefore, we investigated the preferential tryptic digestion of glycoforms of two biopharmaceutical formats: 1. an immunoglobulin G1 (IgG1) monoclonal antibody (mAb) and 2. Flebogamma, an intravenous IgG (IVIG) from healthy donor pools. By studying the digestion on both intact as well as denatured protein, we collected evidence for the 3D structure dependence of the biases.
The tryptic digestion of the target protein into glycopeptides was followed over time. The digestion of protein with an intact 3D structure was compared to that of protein denatured by various methods. Glycopeptides from the conserved glycosylation site in the F_C part of IgG were detected by a nanoLC–ESI-q-TOF-MS method. By relative quantitation of the glycopeptides and estimation of their absolute abundances, we could visualize a preference of trypsin for the digestion of certain glycoforms.
In general, we proved that the glycoforms of the IgG1 mAb containing high mannose and hybrid type glycans in the Fc portion were preferentially digested into the glycopeptides compared to those glycoforms containing complex type glycans. The main bias in IVIG was created by preferential digestion of bisected species, though biases were generally smaller. Interestingly, the α2-3 linked sialic acid containing species in the IgG1 mAb were amongst the first to be digested while the α2-6 linked sialic acid containing species in IVIG were not preferred. The biases are much less pronounced in denatured samples und this effect is independent of the increased digestion efficiency. A significant part of the digestion biases must therefore be caused by the differences in antibody 3D structure induced by the different glycoforms.
In conclusion, proper denaturation and digestion completeness are key to avoiding biases in bottom-up proteomics based glycoform profiling. Beyond glycoproteomics, for example, relative protein quantitation (even with isotopically labelled standards) in two samples could be biased by differences in glycoform profiles, if complete tryptic digestion is not achieved.

Native mass spectrometry: what can it bring to you?

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Abstract

Native mass spectrometry (native MS) is an analytical technique, which underwent quite some technical progress over the last decade. The key feature that makes native MS unique is the retention of molecular non-covalent interactions during the ionization process and the subsequent mass analysis. This particular feature allows the mass spectrometric analysis of intact proteins and protein complexes under their native folded state providing a wide range of information, beside others: structural information, complex stoichiometry, sample composition and purity. Over the years, the progressive development has been pushing the limits of this technique, today allowing the measurement of protein complexes up to mega Daltons. Moreover, the recent implementation of the Orbitrap analyzer significantly extended the number of applications enabling in-depth analyses of post-translational modifications (PTMs) directly at the intact protein level. Here we describe a variety of examples of native MS applications.

If on the one hand the extremely simplified sample preparation makes this technique amenable for high throughput analysis, on the other hand the need of advanced mass spectrometers specifically optimized for native MS still limits the uptake of this technique in research & development.

Therefore, we provide such services and fit-for-use research in the field of (native) mass spectrometric protein characterization and analysis to the life sciences academic and industrial community. Currently services are built around therapeutic antibodies and antibody mixtures. In the coming years the portfolio will be expanded to include characterization of ADC's and other new antibody platforms. This is based on the extensive knowledge and experience of the Heck-lab who have been in the forefront of the field of native MS for over a decade.

Total plasma N-glycome changes in Diabetes mellitus type 2

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Abstract

Diabetes mellitus type 2 (T2D) is a global public health problem affecting over 300 million people. T2D is characterized by high blood glucose levels due to insulin resistance and may lead to numerous complications, including cardiovascular disease and increased risk for blindness and death. Whereas the non-enzymatic glycation of plasma proteins in T2D has been investigated extensively, little is known about enzymatic protein glycosylation in T2D. Recently, changes in serum *N*-glycome between T2D patients and healthy controls were found by analyzing 10 *N*-glycan species *via* CGE-LIF after desialylation [Testa et al. 2015 PLoS ONE 10(3):e0119983]. However, sialylation of protein *N*-glycans is known to be implicated in various (patho)physiological conditions. Therefore, we used a recently developed approach, which allows sialic acid stabilization and differentiation of its linkages in MALDI-TOF-MS, to analyze differences in the plasma *N*-glycome between T2D and healthy controls. Plasma samples from a sex- and age-matched sub-cohort of the DiaGene study, comprising 221 T2D patients and 113 controls were analyzed in high-throughput manner, and more than 100 *N*-glycan species were detected. Among others, multiple glycans bearing fucose and/or bisecting *N*-acetylglucosamine were found to be decreased significantly in T2D patients vs. controls. Sialylation of various glycans increased in male and female patients, driven by an increase of α2,6-sialylation, while α2,3-sialylation decreased with T2D. The latter might be explained by elevated fibrinogen levels known to be present in T2D patients. Various *N*-glycans were correlated with the body mass index and/or age of patients, and showed sex-specific variations.

In summary, our data indicate that – among others – specific changes in *N*-glycan sialylation occur in T2D. New advances in analytical MS-techniques as demonstrated by our new method will enable unravelling even isomer-specific glycomic changes in disease and, thus, promote clinical research. Further studies are needed to elucidate the possible pathophysiological role and clinical usefulness of glycomic changes in T2D with and without complications.

Exploiting colon cancer signaling crosstalk for therapy

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Abstract

Colonrectal cancer (CRC) is a major cause of cancer morbidity and mortality in developed countries. BRAF(V600E) mutation is present in ~10% of CRCs but its inhibition shows only a very limited response to targeted drug treatment. This resistance is caused by the activation of the epidermal growth factor receptor (EGFR) after BRAF inhibition (BRAFi) in CRC[1]. Therefore, BRAF(V600E) has been indicated as a viable therapeutic target in combination with EGFR inhibitors (EGFRi) and therapeutic strategies for patients carrying a BRAF mutation are now in an early stage of clinical development.

In this study an onco-proteogenomics approach is used to explore CRC signaling and define the mechanisms of the resistance to drug therapy in CRC cells. Moreover, we aim at identifying novel feedback loops which enable a better tailored treatment for individual patients.

We set out a large-scale (phospho)proteomics and transcriptomics workflow to monitor WiDr cells after EGFR stimulation, in four different conditions (control, BRAFi, EGFRi and a combinatorial therapy of the two drugs) and at multiple time points (0h, 2h, 6h, 24h, 48h). The (phospho)proteomics study was carried out using a label-free LC/MS/MS approach combined with Ti^{4+}-IMAC phosphopetide enrichment. This platform enabled to gain insights on the full proteome and temporal phosphorylation dynamics upon drug treatment. As far as transcriptomics is concerned, expression was measured using ribosomal depleted whole RNA Sequencing.

The first analytical assessment of the transcriptomics and phophoproteomics data revealed strong concordance among both technical and biological replicates. A proteomics analysis will be performed before starting the -OMICS integration.

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Hinge-region O-glycosylation of human immunoglobulin G3 (IgG3)

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Abstract

Immunoglobulin G (IgG) is one of the most abundant proteins present in human serum and a fundamental component of the immune system. IgG3 represents approximately 8% of the total amount of IgG in human serum and stands out from the other IgG subclasses because of its elongated hinge region and enhanced effector functions. This study reports partial O-glycosylation of the IgG3 hinge region, observed with nanoLC-ESI-IT-MS(/MS) analysis after proteolytic digestion. The repeat regions within the IgG3 hinge were found to be in part O-glycosylated at the threonine in the triple repeat motif. Non-, mono- and disialylated core 1-type O-glycans were detected in various IgG3 samples, both poly- and monoclonal. NanoLC-ESI-IT-MS/MS with ETD fragmentation and CE-MS/MS with CID fragmentation were used to determine the site of IgG3 O-glycosylation. The O-glycosylation site was further confirmed by the recombinant production of mutant IgG3 in which potential O-glycosylation sites had been knocked out.

For IgG3 samples from six donors we found similar O-glycan structures and site occupancies, whereas for the same samples the conserved N-glycosylation of the Fc CH2 domain showed considerable inter-individual variation. The occupancy of each of the 3 O-glycosylation sites was found to be approximately 10% in six serum-derived IgG3 samples and approximately 13% in two monoclonal IgG3 allotypes.

A quantitative proteomics approach to identify newly synthesized proteins in a VWM mouse model

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Abstract

Vanishing white matter disease (VWM) is a severe inherited childhood disease. It leads to slowly progressive white matter degeneration that is aggravated by small head injuries or fever. Patients suffer from various neurological symptoms, such as ataxia and spasticity and they usually die within a few years after onset. It is already known that either one of the five subunits of eukaryotic initiation factor 2B (eIF2B) is affected in the patient. eIF2B normally regulates protein synthesis rate under different stress conditions throughout the body. Surprisingly the mutation in VWM only affects oligodendrocytes and astrocytes in the brain white matter, while other cell types are spared. The underlying molecular mechanism for this selective vulnerability of the white matter is still elusive.

In this study a recently developed VWM mouse model is used to identify differently expressed proteins in astrocytes of wild type and mutant (homozygous for the R191H mutation in eIF2Bε) mice. We used stable isotope labeling with amino acids in cell culture (SILAC) to distinguish between the two genotypes of astrocytes and pulsed L-azidohomoalanine (AHA) to pulldown newly synthesized proteins.

We found that AHA is incorporated in wild type and mutant astrocytes at a similar efficiency rate, without affecting cell viability. Therefore AHA incorporation could be used in combination with click chemistry for the pulldown experiments. A total of 1241 protein groups was identified in at least 3 replicates after LC-MS data-analysis and 80 of the protein groups were differently expressed between mutant and wild type astrocytes. A strong enrichment of signal peptide-containing proteins was found in this last group. Signal peptide-containing proteins are transported to the ER during translation. They then remain in the ER or are sent towards the secretory pathway, to end up in the extracellular space, lysosomes or the plasma membrane.

This finding will be further explored to unravel the potential link between the secretory pathway and the selective disruption of white matter astrocytes and oligodendrocytes in VWM.

Bortezomib resistance increases the need for extracellular nutrients

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Abstract

Bortezomib is the first clinically approved proteasome inhibitor and is used in the treatment of multiple myeloma. The proteasome is responsible for the degradation of intracellular proteins and is involved in a wide range of cellular processes. Proteasome inhibition interferes with cellular homeostasis and causes apoptosis in malignant cells. Despite its clinical success, the use of bortezomib is hampered by the occurrence of primary and acquired resistance. Different mechanisms for bortezomib resistance have been described, but many factors in the occurrence of resistance remain unknown. We hypothesize that bortezomib resistance is associated with metabolic remodeling of the cell. Therefore, we aim to combine mass spectrometry based metabolomics with chemical biology approaches to elucidate the metabolic pathways involved in bortezomib resistance.

Metabolic screens of bortezomib sensitive and –resistant cell lines showed differences in the metabolic profiles of these cells, suggesting that bortezomib resistance is coupled to metabolic adaptations. Further studies showed that bortezomib resistant cells had an increased uptake of nutrients from the growth medium. A large fraction of these nutrients was used for anti-oxidant purposes. Cell viability assays under different conditions revealed that bortezomib resistant cells are less viable at lower concentrations of specific extracellular nutrients compared to bortezomib sensitive cells. Furthermore, we found that starving the cells for these nutrients re-sensitized bortezomib resistant cells to the drug.

These findings indicate that screening for metabolic adaptations in cells that are resistant to proteasome inhibitors may help to uncover new therapeutic strategies to overcome resistance to this type of treatment in multiple myeloma patients.

Development of a gel-based quantitative mass spectrometry assay for determining the fragmentation of cardiac troponin T in serum

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Introduction

Cardiac troponin T (cTnT) is an important biomarker for the diagnosis of acute coronary syndromes like acute myocardial infarctions (AMI) and myocardial ischemia. Previous research in our laboratory has shown that cTnT is present predominantly in fragmented forms in human serum. The precise molecular structure of these fragments, as well as their effect on the clinical immunoassay, is still unknown. In this study, we have developed a gel-based, targeted, mass spectrometry assay to identify and quantify changes in the molecular structure of cTnT from the serum of patients suffering from AMI.

Methods

cTnT and cTnT-fragments were isolated from human serum using an immunoprecipitation technique employing the M11.7 catcher antibody by Roche Diagnostics and fractionated with SDS-PAGE. Coomassie stained bands at 37, 29, 19, 18 and 16 kDa were carefully excised from the gel and digested with trypsin in the presence of ProteaseMax™ surfactant (Promega). The digests were subsequently analysed on a Q Exactive instrument (Thermo Scientific) set on targeted Selected Ion Monitoring (t-SIM) mode. As a second scan event, data dependent tandem-MS (dd-MS2) was employed to identify the selected peaks. Retention times and optimal collision energies were determined by measuring synthesised cTnT peptides of interest using a data-dependent method. Database searching was performed using SEQUEST and intensity chromatograms were analysed with the targeted proteomics software platform Skyline.

Results

Linearity of the assay was shown for all peptides of interests (r^2 >0.95) with a linear dynamic range of 140 – 1.2 *10^3 ng/L cTnT (consistent with the common cTnT serum-concentration found in AMI-patients). Serum with cTnT was next incubated at 37 degrees Celcius for up to 48 hours to induce fragmentation. Western blots employing the antibodies of the clinical cTnT assay show the presence of these fragments. The area under the curve (AUC) of the selected precursor chromatograms were calculated and normalised. Next, ratios were calculated of the normalised AUC of each precursor with that of a reference peptide. These ratios were compared between the different fragments, indicating the removal of the N-terminus from the 29 kDa fragment, followed by the progressive removal of the C-terminus from the smaller cTnT fragments.

Conclusions

A quantitative targeted mass spectrometric method was developed to identify the extent of fragmentation of cTnT in human serum. In principle, this method can be expanded to include other proteins and modifications.

Diagnostic and Prognostic Biomarker Discovery of Soft Tissue Sarcoma by Mass Spectrometry Imaging

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Abstract

The combination of high heterogeneity, both intra-tumoral and inter-tumoral, with their rarity (comprising just 1% of all malignant tumors) has made diagnosis, prognosis, staging and treatment of soft tissue sarcomas (STS) difficult [1]. There is an urgent need for more objective molecular and biochemical protein biomarkers, to differentiate between the many different subtypes, and to also provide new treatment targets.

Mass Spectrometry Imaging (MSI) has been previously used to investigate the molecular make-up of STS [5] and to investigate molecular markers to aid their differential diagnosis [6]. For other tumors MSI has amply demonstrated its ability to identify markers for patient survival, metastasis and response to therapy [7]. These examples indicate that MSI has great potential for providing novel abilities for improved differential diagnosis and patient management [2-4].

In this study we investigated the ability of MALDI MSI to distinguish between the most encountered (still infrequently) but clinically challenging high grade STS, including osteosarcoma (OS, n=16), leiomyosarcoma (LMS, n=12), myxofibrosarcoma (MFS, n=13) and undifferentiated pleomorphic sarcoma (UPS, n = 12). We also investigated if there are individual proteins or protein signatures that are statistically associated with patient survival and development of metastases, and thus may be prognostic biomarkers.

Two distinct approaches were used: the first follows the established clinical MSI protocol of using virtual-microdissection of the histological image to extract the average mass spectral profile of histologically specific tumor areas. The second approach was recently reported by Balluff et al.[8] and incorporated intratumor heterogeneity into the discovery pipeline.

Twenty protein peaks were found that were characteristic for specific tumors ($p < 0.05$). They were all highly expressed in LMS patients and lowly expressed in MFS patients. Acyl-CoA-binding protein (*m/z* 11161), Macrophage migration inhibitory factor (*m/z* 12349), Thioredoxin (*m/z* 11608) and Galectin-1 (*m/z* 14631) were tentatively assigned based on comparisons with databases of protein ions commonly detected by MALDI MSI.

Fourteen protein peaks were found to be associated ($p < 0.05$) with clinical outcome. Based on their intensities, 9 peaks displayed significant differences for overall survival while the rest showed significant differences for metastases free survival. Tentative assignments were Thymosin beta-10 (*m/z* 4942, OS patients with metastasis), Proteasome activator complex subunit 1 (*m/z* 9753, non-OS patients with poor survival), modified Histone H4 (*m/z* 11314, *m/z* 11355, LMS patients with poor survival).

Thus, our results confirm intratumor heterogeneity in high grade STS. And using statistical methods, we identified diagnostic markers that could distinguish between the different entities, and prognostic biomarkers that were found statistically associated with patient survival and metastasis.

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Differential Mobility Separation of Leukotriene Isomers

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Abstract

Eicosanoids are very low abundant (EC_{50} pM-nM) but highly bioactive lipid mediators (LM) with highly specific structural features like double bond geometry and stereo-centers playing a crucial role in defining their bioactivity. Many eicosanoids are isoelemental and structurally closely related diastereomers and/or geometric E/Z-isomers, which make characterization by high resolution ESI-MS or even ESI-MS/MS difficult or impossible. We have developed an ion mobility method to separate isoelemental eicosanoids using an AB Sciex QTrap 5500 system equipped with a SelexIon™ differential mobility unit. LC-MS/MS measurements were conducted using a µHPLC system. Data was acquired in negative SRM mode using the transition *m/z* 335->195, analyzing the following compounds: Leukotriene B_4 (LTB_4), 6-trans-LTB_4, 6-trans-12-epi-LTB_4, 12-epi-LTB_4 and 5S,12S-diHETE. Initially direct infusion experiments of four compounds were conducted for optimizing the minimum separation voltage (SV). It was found that near baseline separation of these compounds is achievable at 4200V -4500V. Next, the SelexIon™ parameters were translated to a µHPLC method. To this end, the compensation voltages (COV) of the compounds were mapped at a SV of 4500V. A minor shift in COV compared to direct infusion was observed, which may be due to a slightly differing solvent composition of the µHPLC method. Our results highlight the possibility of mapping the COV of isomeric compounds without the need for direct infusion, thus allowing the SelexIon™-separation of endogenous compounds where no synthetic standard is available. Finally, we conducted the SelexIon™-based separation of LTB_4 and its diastereomer 5S, 12S-diHETE, which are extremely difficult to resolve using LC-MS/MS. LTB_4 is produced by activated neutrophilic cells, whereas the formation of 5S,12S-diHETE results from a neutrophil/platelet interaction. Moreover, LTB_4 is one of the strongest known chemotactic substances while 5S,12S-diHETE is almost inactive. Hence, a differentiation of both isomers is of fundamental importance to the understanding of inflammatory processes. Using SelexIon™, we show that 5S,12S-diHETE is present in residential murine peritoneal cells, while LTB_4 is produced after zymosan A challenge.

In conclusion, we showed that differential mobility is a powerful tool for adding an additional dimension of selectivity to chromatography for difficult separations of isomeric eicosanoids even for compounds present only at very low levels in biological samples.

Multidimensional mass spectrometry-based nitrosative stress lipidomics

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Abstract

Nitrosative stress is closely linked to oxidative stress and is mediated by nitric oxide (·NO), nitrogen dioxide (·NO_2), dinitrogen trioxide (N_2O_3) and peroxynitrite (ONOO-). These reactive nitrogen species (RNS) can react with a broad range of molecules, including unsaturated fatty acids to form nitro-fatty acids (NO_2-FAs). After the *de novo* synthesis of NO_2-FAs they can be find found either in a free signalling form and excreted from cells, or esterified to phospholipids in the plasma membranes. Using the Shimadzu LCMS-8050 we will use a multidimensional mass spectrometry-based (MDMS) lipidomics approach to investigate and identify the presence of *de novo* synthesized NO_2-FAs in biological matrixes, including plasma and urine. As a technique, MDMS is a combination of product ion scans, precursor ion scans, neutral loss scans and selected reaction monitoring. The generated molecular mass and fragmentation information will be used to detect and identify the synthesized NO_2-FAs. As a complement, high-resolution mass spectrometry will be applied to determine the accurate masses of the identified NO_2-FAs, after which they will be included in our oxidative and nitrosative stress screening platform.

On-tissue derivatization for the visualization of brain amino metabolites by mass spectrometry imaging

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Abstract

Neurotransmitters such as catecholamines, amino acids and their metabolites are implicated in many neurological diseases. For this reason, the ability to directly measure the spatial localization of these molecules on tissue is indispensable for precise pathological analysis of disorders like migraine, the exact cause and mechanism of which is still not completely understood. The detection of small polar compounds using MALDI-MSI has been hindered due to their low ionization efficiency, background spectral interference from MALDI matrix, and highly variable detection sensitivity. To enhance amino metabolite detection by MALDI-MSI we have developed a sample-treatment approach based on on-tissue derivatization of amino metabolites.

For that purpose, three different derivatization reagents (*p*-*N,N,N*-trimethylammonioanilyl *N'*-hydroxysuccinimidyl carbamate iodide (TAHS), 4-hydroxy-3-methoxycinnamaldehyde (CA), and 2,3-diphenyl-pyranylium tetrafluoro-borate (DPP-TFB)) were tested and compared. Several reaction parameters were optimized in order to improve sensitivity, maintain spatial localization and at the same time minimize molecular interferences from the matrix and the derivatization reagent. The length of the incubation time, the amount of derivatisation reagent applied on tissue, the co-spraying of the derivatization reagent and the matrix, and the necessity of using an organic matrix were investigated. Compounds were assigned on the basis of MALDI-FTICR-MS high resolution/accurate mass analysis. For many amino-metabolites the spatial localization was confirmed by the three derivatization reagents and consistent with the gene expression data for their receptors (as provided the Allen Brain Atlas).

The optimized method was applied to the visualization of metabolome changes after cortical spreading depression (CSD), the electrophysiological equivalent of migraine with aura. Coronal brain sections of SHAM and CSD wild-type mouse were derivatized and analysed using MALDI-FTICR-MSI. Thanks to the application of derivatization reagents it was possible to observe the distribution of 21 different compounds. Several differences in concentration of amino metabolites were observed in the cortex between the control and CSD hemisphere, highlighting the decrease of excitatory neurotransmitters like glutamate and aspartate, together with an increase of the inhibitory neurotransmitter GABA.

The permeation of caffeine through skin studied by MALDI-MS imaging

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Abstract

To study the permeation of exogenous compounds in tissue, commonly used techniques include extraction and horizontal sectioning methods, both of which can be used for quantification but which lack spatial information. Alternatively, spectroscopic techniques such as Raman imaging can be used for localization, but they generally lack selectivity. Autoradiography is a selective and sensitive method giving both quantitative and spatial information, but requires the use of radiolabels. In addition, information on endogenous compounds is absent.

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) is a powerful label-free technique that enables determination of the distribution of a large range of biomolecules directly in tissue sections. Here, we use MALDI-MSI supported by quantitative LC-MS/MS to assess the permeation of caffeine into *ex-vivo* skin and to determine co-localization with endogenous skin lipids.

As a model, a static diffusion cell with porcine skin was used. A formulation containing 2.5% caffeine was topically applied and was incubated at 37˚C for 0, 1, 4 and 24 hours. Samples were taken from the excess formulation (donor solution), and acceptor solution to determine the flux through the skin by LC-MS/MS. The skin tissue disks were snap-frozen in liquid nitrogen and cryo-sectioned (10 µm). A-Cyano-4-hydroxycinnamic acid was applied as MALDI matrix both the caffeine and skin lipids were localized. MS images were obtained using a Synapt G2 HDMS Q-TOF-MS (Waters) at 50 µm spatial resolution. In conjunction, the accumulated caffeine levels in tissue were determined by LC-MS/MS after tissue homogenization and extraction.

By using MALDI-MSI we show that topically applied caffeine accumulates mainly in the epidermis layer over time. Furthermore, in the same imaging experiments lipid markers were detected which were co-localized with the different compartments of the skin *e.g.* hair follicles, sebaceous glands, epidermis- and hypodermis layer. We show that the local MALDI-MSI intensities of accumulated caffeine in tissue are strongly correlated with the concentration levels found by skin extraction measurements. We have shown that MALDI-MSI can act as a powerful complementary technique to study the permeation of exogenous compounds in skin. This work provides new insights into the application of MALDI-MSI in skin research.

Glycopeptide analysis at enhanced sensitivity with CESI-MS

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Abstract

The combination of capillary electrophoresis (CE) and high resolution TOF-MS forms a powerful analytical platform for the analysis of biomolecules. CE–MS is known for its high separation efficiency and sensitivity, especially in combination with the innovative sheathless CE – electrospray ionisation interface (CESI). We investigated the potential of CESI-MS(/MS) for the analysis of protein glycosylation in tryptic digests of the glycoproteins immunoglobulin gamma (IgG) and prostate specific antigen (PSA).

The CESI-MS/MS analysis revealed a separation of glycopeptides mainly on the basis of peptide moieties as well as on the number of sialic acid residues. Sialic acids are often part of the binding motifs of human lectins, toxins and pathogens. A growing body of evidence shows that the linkage of sialic acids on glycoproteins is a valuable pathology marker in various diseases, for example α2,3- vs α2,6-linkage [1]. Interestingly, for PSA we observed isomer separation of sialylated glycopeptide species, suggesting that our CE method separates α2,3- and α2,6-linked sialic acid isomers. The analysis of tryptic IgG Fc glycopeptides confirmed the separation of α2,3-sialylated glycopeptides, derived from recombinant IgG, from α2,6-sialylated glycopeptides, derived from human plasma IgG.

In addition, to boost the sensitivity and robustness of the CESI interface, we implemented the use of acetonitrile enriched nitrogen dry gas which has been proven to be beneficial in nanospray LC-ESI-MS [2]. For this purpose, we used the well characterized glycoprotein IgG which contains one *N*-linked glycosylation site in its conserved domain [3, 4]. IgG was treated with porcine trypsin and glycopeptides were measured using CESI-MS/MS with and without applying a nebulizer gas. Preliminary results showed a 2-fold increase in the total intensity of 32 target glycopeptides (including subclasses IgG1, IgG2/3 and IgG4) using the acetonitrile-enriched nebulizer gas, next to a reduction of chemical background noise and consequently an increase in signal-to-noise. Further analysis is ongoing to investigate the reproducibility of the enhanced glycopeptide ionization.

Our CESI-MS/MS platform shows great potential for studying glycosylation of proteins at ultrahigh mass sensitivity. This allows the analysis of samples available in only minute amounts with resolution of sialic acid isomers, providing valuable information on glycosylation microheterogeneity. This approach is useful for both the analysis of clinical biomarkers and biopharmaceutical samples.

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Unpublished Figures

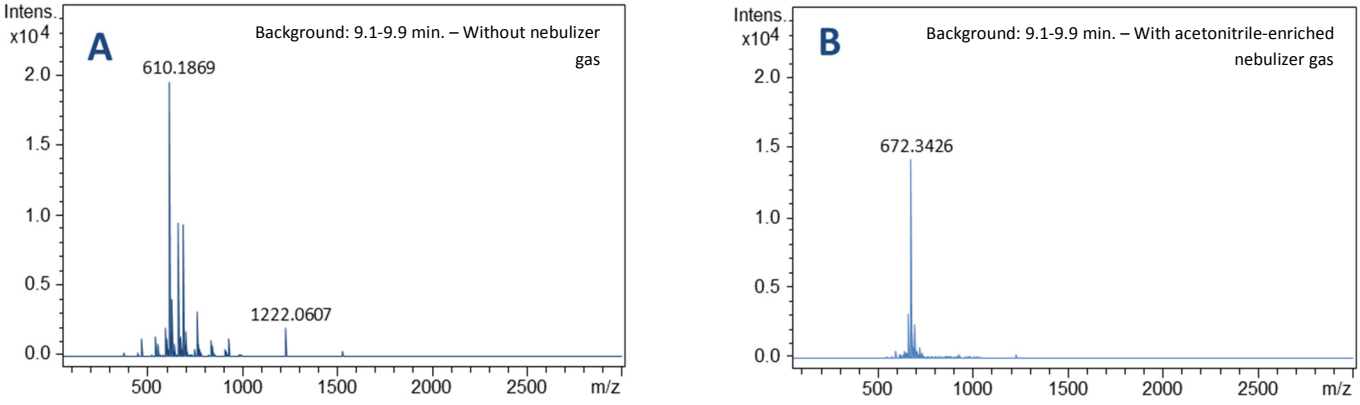

Figure 1: Background in MS signal using CESI-MS with or without an acetonitrile-enriched nebulizer gas. **(A)** Background region with a conventional CESI-MS platform. **(B)** Background region of the CESI-MS when acetonitrile-enriched nebulizer gas is employed**.** No clear difference in the intensity of the major background peak was observed although the overall background above *m/z* 700 is lower with the acetonitrile-enriched nebulizer gas.

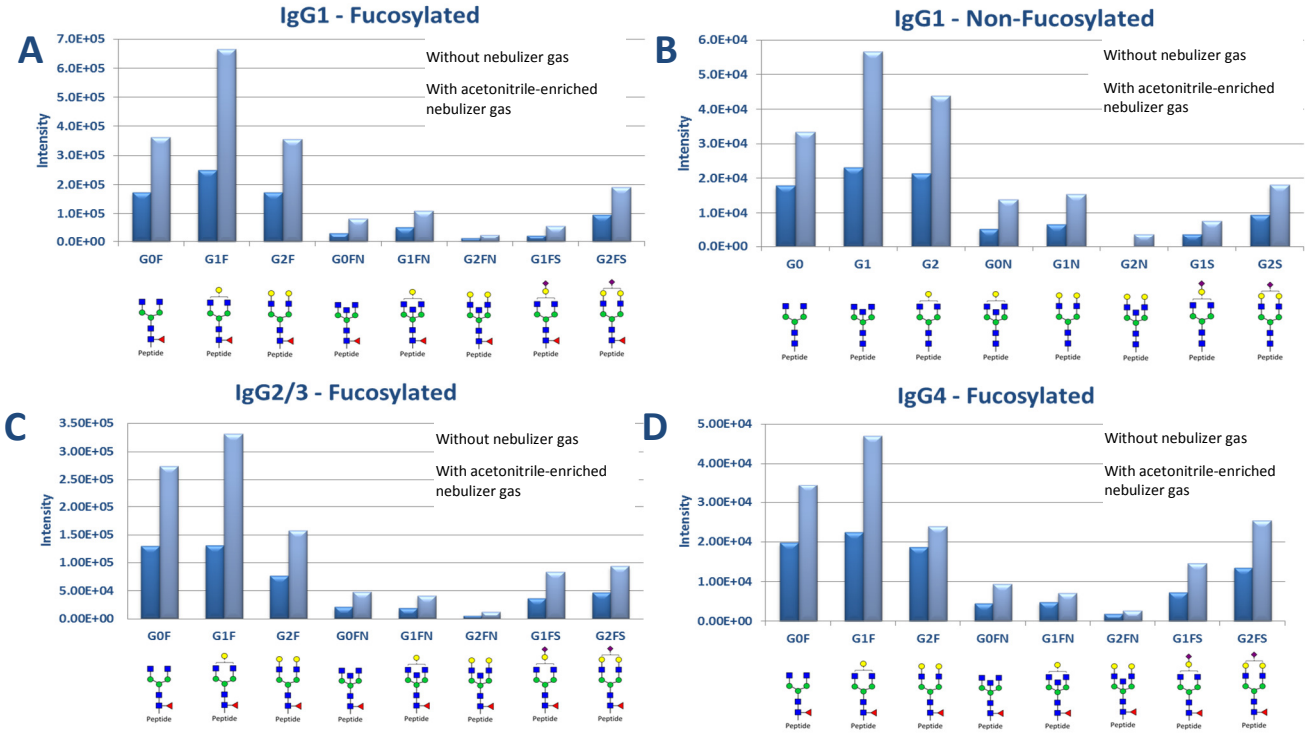

Figure2: Extracted intensities of IgG glycopeptides **(A)** Data from the fucosylated glycopeptides of the IgG1 subclass. **(B)** Data from the non fucosylated glycopeptides of the IgG1 subclass **(C)** Data from the fucosylated glycopeptides of the IgG2/3 subclass. **(D)** Data from the fucosylated glycopeptides of the IgG4 subclass. Numbers shown are acquired by listing the maximum intensity as observed by summing the first three isotopes of doubly and triply charged species in a smoothed spectrum. The figure illustrates an improved sensitivity when using the acetonitrile-enriched nebulizer gas.