32.1
Comparison of Different SELDI ProteinChip Arrays for Proteomic Profiling of Urine

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Surface Enhanced Laser Desorption/Ionization (SELDI) is a high throughput proteomics technology that makes use of different surface arrays for fractionation and profiling of samples. We investigated the suitability of SELDI for protein profiling of urine by determining the reproducibility and number of detectable peaks for the different array types and two different matrices (SPA and CHCA). 7 Types of ProteinChips arrays were investigated: Hydrophobic chip types (SEND, H50 and H4) cation exchange (CM10) anion exchange (Q10) copper-coated IMAC chips and silica coated chips (NP20). Urine samples were applied in 8-fold to a chip (unreated or denatured in U9 buffer, depending on the chip type) using both matrices.

Data were collected in the range 1–100 kDa and analyzed for total number of unique peaks (S/N > 5), detectable in the 8 spectra.

Reproducibility was determined by calculating the percentage of peaks that were detectable in all 8 spectra and by calculating the average CV of peak intensity values. In addition, an inventory was made of the peaks detectable on each chip type with the 2 matrices.

Results: In the low mass range (1–30 kDa), the highest peak number was found for CM10-CHCA (57), CM10-CHCA also showed the best reproducibility with an average CV of 13% and percentage of peaks detected in all spectra of 54%.

For the high mass range (30–100 kDa), CM10-SPA gave the best result: total peak number is 48, 35% of peaks are present in all spectra and average CV is 26%.

Conclusion: CM10 is the best protein chip type for profiling of urine in the range 1–100 kDa, both in terms of peak number and reproducibility.

32.2
Molecular Weight Assessment of Proteins in Total Proteome Profiles Using 1D-PAGE and LC/MS/MS

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Introduction: We developed computational tools for extracting molecular weight information of intact proteins from total proteome profiles in a high throughput manner using 1D-PAGE and LC/MS/MS. We applied this technology to the proteome profile of a human lymphoblastoid cell line under standard culture conditions. From a total of 10E+6 cells, we identified 821 proteins by at least two tryptic peptides. These 821 proteins are well-localized on the 1D-SDS gel. 656 proteins (80%) occur in gel slices in which the observed molecular weight of the protein is consistent with its predicted full-length sequence. A total of 165 proteins (20%) are observed to have molecular weights that differ from their predicted full-length sequence. We explore these molecular-weight differences based on existing protein annotation.

Preliminary results: We identified 821 proteins that migrate as localized, single bands on a 1D gel. 165 proteins [20%] have molecular weights that do not fall into the range specified by our algorithm and the proteins with which it co-migrates. 88 of the 165 proteins are observed at lower Molecular Weight [MW] than predicted by the full-length sequence. These proteins are potential candidates for having alternatively spliced transcripts or may be cleaved endoproteolytically. Many proteins in this group are annotated as having signal or transit peptides. We also found a total of 77 proteins that have an observed MW that is greater than that predicted by their sequence. Several of these are likely to be glycosylated, sumoylated, or ubiquitinated. A future goal is to extend this method to greater resolution. Proteins suspected of being alternatively spliced in several conditions can be easily interrogated with RT-PCR.

32.3
Use of Adsorbed and Immobilized Trypsin in Protein Digestion

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The isolation, purification and enzymatic digestion of less abundant proteins in human tissues and fluids is still a challenging task. After the separation of such proteins by HPLC or 2D electrophoresis, tryptic digestion of such proteins can be achieved by the use of soluble or immobilized trypsin or different proteases. Here we present a new method in which trypsin is adsorbed (non-covalently bound) on the surface of chromatographic media particles, as distinguished from currently commercially available immobilized trypsin, which is embedded on the inner surface of pipette tips. The adsorption of trypsin can be on either hydrophilic or hydrophobic chromatographic media. When adsorbed on hydrophilic media, trypsin can be eluted into the solution by using aqueous buffers. In the case of trypsin adsorption on hydrophobic media, such as C-18 chromatographic media, the trypsin activity takes place in the aqueous buffer while the trypsin remains adsorbed on the media and is not eluted.

The method described above is used for the tryptic digestion of bovine serum albumin (BSA) by using: (1) trypsin adsorbed-hydrophilic and (2) hydrophobic chromatographic materials as well as (3) immobilized trypsin beads. The trypsin-digested fragments of BSA are analyzed by HPLC and AP-MALDI. The advantages of micropipette tips with trypsin adsorbed on media are that very small amounts of enzymes can be used for tryptic digestion and the enzymatic reaction can be performed with a few microliters of sample, and directly in the tip, thus resulting in less sample loss. Furthermore, when this method is used in high throughput screening, the amount of trypsin in each tip can be controlled. When C-18 absorbed trypsin is used, peptides are further purified from the buffer, by the use of the hydrophobic properties of C-18. This allows for the direct analysis of the protein samples in mass spectrometry analysis.

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Nano-LC Fraction Analysis by Chip-based Nanoelectrospray for Improved Glycopeptide Characterization

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NanoLC with 75 μm id columns and flow rates of 200 nL/min is gaining in popularity due to improved resolution, lower sample injection requirements, and better ionization efficiency leading to improved sensitivity. NanoLC peaks typically elute within 20 sec, providing most modern mass spectrometers sufficient time to perform MS/MS for simple protein ID experiments. However, for complex samples, such as glycopeptides where MS3, MS4, and MS5 experiments may be needed, nanoLC does not provide adequate analysis time. By collecting fractions from the nanoLC column, analysis times can be extended via nanospray infusion analysis. Here, we demonstrate a novel system capable of automated ultra low fraction volumes from 75 micron ID nanoLC columns followed by subsequent automated nanoelectrospray analysis for increased data content via mass spectrometry. Fractions from a nanoLC column were collected into custom tips, whose inner surface had been chemically modified to minimize peptide adsorption. These 200 nL fractions were collected every 60 sec from a column flowing at 200 nL/min with a 30 min gradient. Fractions were collected from the peak elution window of interest in an automated fashion using a robotic nanoelectrospray system (TriVersa NanoMate). The nanoLC fractions in the pipette tips dried within several minutes. Following fraction collection the residue in each tip was reconstituted in 200 nL. The sample was analyzed directly from the tip with chip-based nanoelectrospray. The chip had 2 μm id nozzles, producing flow rates of ~20 nL/min and providing 10 min analysis per fraction. Fraction collection, reconstitution and analysis steps were fully automated. System demonstration utilized Ribonuclease B (RNaseB) tryptic digest, which is known to have a single N-linked glycosylation site on the asparagine 34 residue. 100 fmol RNaseB tryptic digest was injected onto a 75 μm id, 15 cm-long column operated at 200 nL/min. The fraction analyses were performed on an LCQ Deca XP ion trap equipped with a TriVersa NanoMate. MS3, MS4, and MS5 were performed on the nanoLC fractions containing isoforms of the glycopeptide. The extended analysis time of each fraction allowed for collision energy optimization, data averaging, and multiple tandem MS experiments. The site of glycosylation was determined, the PTM was characterized as having 9 mannose and 2 N-acetylglucosamine groups, and the sequence of the peptide in which the glycosylation occurred was determined. Averaging several minutes of data was found to be necessary for obtaining interpretable tandem MS spectra. This novel approach demonstrates a useful means of extending MS analysis times for nanoLC providing more powerful mass spectrometry analysis.

MALDI Imaging of Zebrafish Sections

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The zebrafish (Danio rerio) is a well established model animal for the investigation of mammalian development. Many knockdowns mutants are available. The effect of gene knockdowns is usually evaluated by light microscopy or by Laser-Scanning-Microscopy. MALDI-imaging is a method that can add additional information to an optic image, because MALDI has the intrinsic possibility to detect proteins, peptides, and lipids that may go undetected by other imaging techniques. In this study, we evaluated the possibilities of obtaining MALDI-images from zebrafish sections. Different MALDI-matrices and preparation methods as well as the suitability of nitrogen and solid-state lasers were compared. Sagittal cryosections of zebrafish have been prepared with a cryomicrotome onto conductive, indium-tin-oxide coated glass slides, and microscopic images of the samples were taken. The MALDI-matrix was prepared by standard dried droplet methods or by pneumatic spray. Different matrices were compared. The slides were introduced into the mass spectrometer with a slide adapter sample carrier. Selected mass signals could be displayed as coloured pixels underlaid with the optic image.

While it was possible to obtain mass spectra with dried droplet preparation on the tissue, this led to lateral delocalisation of the analyte molecules. By spray-preparation with an airbrush, thin matrix-layers could be applied on the sample. No relevant delocalisation of the analyte was observed. It was possible to localize different internal organs of the zebrafishes by the MALDI-images, so MALDI imaging will indeed help in the analysis of gene knockdown mutants in zebrafish.

On-membrane Direct MALDI-TOF MS Identification of Proteins Detected by Western Blotting and Lectin Blotting

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We have developed a new procedure to identify proteins using mass spectrometry (MS) after the detection by immunostaining on the membrane in conjunction with piezoelectric chemical inkjet technology. For the first time, this methodology has enabled the whole procedures from Western blotting to mass spectrometric identification to be completed directly on the identical membrane. Effectiveness of this technology was confirmed using the two-dimensionally separated Escherichia coli blot on a polyvinylidene difluoride (PVDF) membrane. GroEL, FtsZ, DnaK and GroES proteins were immunostained by its corresponding antibodies eliminating the use of blocking agent with inhibitory effect on the mass spectrum, subjected to on-membrane digestion using the chemical inkjet printer without multiple liquid-handling steps for digestion and extraction, and successfully identified by direct MALDI-TOF MS from the membrane. This strategy could be adopted to the first development of direct identification of counterpart proteins under molecular interactions on the membrane. Taking the lectin staining of glycoprotein as a model system, ovalbumin was run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a PVDF membrane, and subjected to the lectin staining and on-membrane MS identification. Direct MALDI-TOF MS analysis of the glycoprotein detected by Alexa488-conjugated concanavalin A on the membrane were successful after removal of the lectin, combined with the tryptic digestion using the chemical inkjet printer. Through these procedures, newly developed on-membrane MS/MS analysis using the MALDI quadrupole-ion-trap TOF MS was effective for the identification of blotteded proteins. This novel methodology described here opens a new phase on current proteomics, making direct connections to well-established “classic” protein sciences.
Sample Preparation for Mass Spectrometry Using Magnetic Bead Technology

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The identification of disease-specific protein and/or peptide biomarkers in body fluids could provide a means to detect and diagnose disease and allow the assessment of disease severity, progression, and the effectiveness of treatments. Mass spectrometry (MS) is the analytical method of choice for biomarker discovery whereby protein and/or peptide profiles need to be obtained from large sample numbers. The complex nature of biological fluids, dynamic range constraints associated with MS, and the sensitivity of MS to salts and detergents means that reproducible sample preparation procedures reducing sample complexity and removing contaminants are required. We have developed ion exchange and reversed phase magnetic beads for protein and/or peptide isolation and for the fractionation of complex sample mixtures. These can be utilised as sample preparation techniques for mass spectrometry.

Weak cation exchange magnetic beads can be used to fractionate complex mixtures of proteins, consequently increasing the amount of information that can be obtained from these samples. To illustrate this, proteins from a cancer cell lysate were able to be split into three fractions whereby only 2% of the proteins were common to all three fractions. The total number of proteins identifiable from the lysate was increased more than three fold in the three fractions compared to the crude lysate.

Reversed phase chromatography (RPC) magnetic beads have been developed for the fractionation, desalting and concentration of protein and peptide mixtures. We have data that shows that samples containing salts and contaminants that prevented the attainment of MS spectra could be used to obtain excellent MS spectra after using simple automatable experimental protocols involving RPC magnetic beads.

An Integrated Approach for Intact Protein Analysis and Protein Identification by LC/MS for Complex Biological Samples

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An integrated method for analyzing human serum and other biological fluids using the complementary strategies of in gel-based quantitation and shotgun analysis has been developed. This approach combines the strengths of classical gel-based approaches with the analytical vigor and reach of shotgun proteomics. Poly-acrylamide gel electrophoresis (PAGE) in conjunction with gel-appropriate labeling allows the visual confirmation of protein representation, aids in target identification, serves as an excellent profiling tool, and provides an additional level of complex mixture fractionation before LC-MS. On the other hand, LC-only approach is met with the challenges of accurate peak identification and accurate profiling, while producing a high level confidence in reproducibility and sample identification. We have implemented a scheme of intact-protein analysis system (IPAS) which includes tagging of proteins with Cy dyes two-dimensional chromatographic fractionation followed by 1-D SDS gel analysis of individual fractions for relative protein ratio determination, followed by LC-MS analysis of selected gel spots. The pre-fractionation scheme gradually reduces the complexity of the sample by first removing the most abundant proteins in the mixture (immunodepletion), then separating the proteins on the basis of charge (strong anion exchange), and subsequently separating proteins on the basis of hydrophobicity (reversed phase). A portion of the fractions subjected to 1-D SDS analysis is subjected to LC-MS analysis to determine the full repertoire of proteins in each fraction. The integrated approach is applied to the analysis of serum to detect cancer biomarkers. Our findings indicate a high level of resolution and quantitative accuracy in the detection and identification of circulating proteins derived from cancer cells.
32.9
Improving the Confidence of Protein Identification by Combining MALDI-MS and -MS/MS Data
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The objective of the present study was to optimize the interplay between searches using MALDI-MS and -MS/MS to increase the confidence of protein identification. MALDI-MS is especially advantageous in our hands if only low sample amounts are available and high sequence coverage is desirable. A reference set of MALDI-MS/MS data was constructed to investigate the information obtained from MALDI-MS and -MS/MS of singly charged peptides. This reference set was used to study which missed cleavages were observed and to investigate the abundance of the charge remote fragments ions observed after fragmentation of peptides. More than 95% of the missed cleavages were found to follow simple rules similar to those previously reported. Charge remote fragmentation was found to be highly abundant compared to charge induced fragmentation. In addition, intense signals directed by the presence of some modifications are reported. From the data obtained two strategies were used; i) to improve the confidence level of PMF-based protein identification by taking into account the type of missed cleavages observed and ii) to predict which intense ions are expected upon MALDI-MS/MS analysis. Both strategies are being incorporated into a newly developed search program VEMS v3.1. By combining these two strategies it was possible to obtain confirmatory MS/MS data by selecting peptides, which were predicted to yield intense fragment ions and subject these peptides to MS/MS analysis in order to verify the search result. The overall confidence of the result is considerably improved and the sample amount used is minimized, leaving a larger amount of sample for analysis of potential post translational modified peptides.

32.10
Rapid and High Quality Peptide Mapping Using Silica Nano-Monoliths in On- and Off-line Capillary LC-ESI- and LC-MALDI-MS/MS
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Proteomic-based studies require the design of rapid, highly sensitive, selective and reproducible separations suited for mass spectrometry (MS) as the preferred detector, to gain high quality data for fast peptide and protein identification. Capillary and nano-flow HPLC techniques in combination with on-line electrospray and off-line MALDI MS find increased usage in these studies, where often only limited sample amounts are available. The limitations of existing particulate capillary- and nano-columns, has driven us to develop 100/μM and 200/μM ID monolithic silica columns.

In this study, standard proteins in solution and proteins from 2DE-gels were reduced, alkylated and digested with trypsin. Mixtures were separated on 100 μM and 200 μM capillaries with the tradename Chromolith®CapRodTMRP18e and eluted peptides were analyzed on-line by ESI ion trap MS or off-line by MALDI-MS. The unique monolithic silica structure results in enhanced mass transfer efficiency and reduced back pressure allowing mobile phase flow rates up to 8 μl/min in commercially available HPLC and MS systems. We could demonstrate that the capillaries are very robust and especially suited for rapid and high quality peptide mapping in the low fmol range. The silica-based monolithic columns allow for a sensitive and highly reproducible separation of biomolecules under ESI MS compatible formic acid conditions with an excellent protein coverage rate in the MS/MS database search in significantly reduced analysis time. Furthermore, the CapRodTM capillary facilitates simplified robotic spotting on MALDI targets, providing increased sensitivity in LC-MALDI compared to standard dried droplet preparation.
Microwave-assisted Chemical Digestion; a Promising Technique for Rapid Protein Identification

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Mass spectrometry-based identification and characterization of proteins at a proteomic scale has hitherto been achieved predominantly with the bottom-up approach. A typical bottom-up experiment necessitates the enzymatic digestion or chemical degradation of proteins to peptides prior to mass analysis. Some of these reactions and processes can be accelerated or made more selective when microwave, rather than heat, is applied [1, 2]. In this work, we report a rapid microwave-assisted digestion approach for protein identification by mass spectrometry based on chemical cleavage using dilute formic acid. A 2% formic acid aqueous solution was chosen as the hydrolysis medium, followed by microwave irradiation. The induced peptide fragmentation was directly analyzed by matrix-assisted laser desorption ionization mass spectrometry. The proteins were cleaved specifically at the C-terminal of the aspartyl residues within 10 min of exposure to microwave irradiation. Interestingly, we found that most of the intact proteins, myoglobin for example, were cleaved only once at the C-terminal of the Asp residues if microwave irradiation was applied for only up to 2 min. The efficacy and simplicity of this technique for protein mapping was demonstrated by the mass analyses of the in-gel digestion of myoglobin and bovine serum albumin, as well as proteins isolated from *Escherichia coli* K12 cells. After database query with MASCOT, we were able to obtain 100% sequence coverage for myoglobin. For BSA, the sequence coverage was significantly improved to 46% after 0.5 mg DTT was included in the reaction mixture. Furthermore, our approach allows clear identification of gel-purified proteins isolated from *Escherichia coli* K12 cells. We believe that further development of this technique may offer a potential solution for proteomics applications, such as online digestion coupled with mass spectrometry LC-MS/MS.

References


A Novel High Speed Duty Cycle of Ion Traps for a More Detailed Analysis of Protein Mixtures

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A major advantage of ion traps in comparison to other mass analyzers is their high duty cycle for a combined MS and MS/MS analysis. Together with the unsurpassed MS/MS sensitivity that was achieved by the high ion storage capacity two years ago, ion traps are ideal for complex proteomics analyses. Complete proteomes analyzed in 2D nanoLC-MS/MS runs may contain several thousands of proteins. A major question is here if low abundant peptides are still MS/MS analyzed to a sufficient extend. To get down to low level peptides the achievable number of MS/MS spectra per time should be as high as possible.

Careful investigation of performance factors allowed dramatic improvements of the duty cycle for a high capacity trap. Digested *E. coli* cell lysate and depleted human serum samples of high complexity and dynamic range were separated by a single 2h gradient with a capillary LC and analyzed by the improved ESI-ion trap MS. For these samples the effect of different AutoMS(n) modes and pre-cursor ion selection criteria has been investigated systematically. As a result of the improvements based on a new hardware platform and new software the number of MS/MS spectra per unit time in data dependant AutoMS(n) experiments could be multiplied even compared to previous fast speed designs of high capacity ion traps. This led to a significant increase of identified peptides. Additionally intelligent precursor selection criteria for selecting peptides in the chemical background have been used. Thus low abundant peptides have been detected more confidentially as well as the sequence coverage of the entity of proteins in these extremely complex mixtures could be further increased.

Proteomic Analysis Based on pH Elution and Integrated Multiple Dimensional Liquid Chromatography Coupled to Mass Spectrometry

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Traditional 2D-LC-MS combining the ion exchange and reverse phase was based on salt elution. We test the pH step elution to wash out the peptide in the SCX column, in which the peptide can be eluted according to their pI distribution. Furthermore, a novel integrated multi-dimensional liquid chromatography (IMDL) method is demonstrated for the separation of peptide mixtures by two dimensional HPLC coupled with ion trap mass spectrometry. The method uses an integrated column, containing both strong cation exchange and reverse phase sections for two-dimensional liquid chromatography. The peptide mixture was fractionated by a pH step gradient using a series of pH buffers, followed by reverse phase chromatography. Since no salt was used during separation, the integrated multi-dimensional liquid chromatography can be directly connected to mass spectrometry for peptide analysis. The pH buffers were injected from an auto-sampler and the method can be carried out on a one-dimensional liquid chromatography system. In one analysis, the IMDL system, coupled with linear ion trap mass spectrometry, identified more than 2000 proteins in mouse liver. The peptides were eluted according to their pI distribution. The resolution of the pH fractionation is about 0.5 pH units. The method has low overlapping across pH fractions, good resolution of peptide mixture, and good correlation of peptide pIs with pH steps. This method provides a technique for large scale protein identification using existing one-dimensional HPLC systems.
Dynamic MALDI Targets
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We describe here functionalized monolithic surfaces for single droplet handling. At the sensitivity level routinely achieved by mass spectrometry, monolayer surface chemistry does not allow to reach a sufficient concentration for real world analysis. So we decided to use polymer monolithic devices we had previously developed for capillary columns dedicated to proteomics applications, which gives access to 1 micron thick film. These methacrylate-based monoliths were anchored on a gold surface using a linker bearing at one end a disulfide moiety and at the other end a methacrylate group and prepared by an original living polymerisation method. Non reactive methacrylate like butyl methacrylate (BMA) or reactive like glycidylmethacrylate (EGDMA) may be used and mixed together in any proportion. Most of the experiments described here were carried out either on plain gold surface or 1.5 mm gold spots. We first tested the desalting efficiency using a lauryl methacrylate phase. A 1/MH2O2 droplet of a salted solution containing various peptides (1 picomolar) was placed on the surface and exchange was forced through repeated aspiration-deposition cycles. The spot was then washed with water, extracted with acetonitrile/water and analyzed off line either by ESI or MALDI. In both cases, no salt adducts were detected in mass spectra using solutions up to 1M NaCl!

A second series of tests involved a functionalized glycidyl methacrylate on which was first linked immobilized trypsin. After washing of the surface, one microliter of a 1 picomolar Cytochrom C solution was deposited on the surface for a quarter of hour. More DI water was added and the sample desalted. The resulting mass spectra showed a very clean digestion allowing a high score identification on proteomics search engine. Analysis on crude biological samples like human plasma will be also presented.

Multiplexed Screening for Posttranslational Modifications by Tandem Mass Spectrometry
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The use of mass spectrometric peptide sequencing for protein identification has become commonplace in proteomics. Post-translational modifications (PTMs) pose a challenge to this non-targeted approach, however, since peptides are selected for fragmentation based on their abundance, thus PTM analysis is random. Targeted approaches for individual PTMs demand modification-specific isolation techniques prior to, or dedicated scan types during MS analysis, while targeted multiplexed methods for PTM analysis did not exist, as they would be too time-consuming or computationally expensive to be performed by LC-MS/MS. We have developed a multiplexed method that utilizes the unique trapping capabilities of a hybrid triple quadrupole/linear ion trap instrument (Applied Biosystems/MDS Sciex QTRAP) to rapidly screen for PTMs in parallel. In this novel scan strategy, all peptides are transmitted through Q1, fragmented in parallel in the collision cell, and their fragment ions are trapped in Q3 and scanned out to determine their m/z values. For PTMs producing characteristic fragment ions Multiple Precursor Ion Monitoring (MPM) is used, in which the low-mass region that contains these modification-specific marker ions is trapped and analyzed after high-energy collisions. Since ion lineage is lost, repeated MPM with decreasing m/z windows is used to locate the modified precursor ion. PTMs causing neutral losses are analyzed by Multiple Neutral Loss Monitoring (MNM), in which the high-mass region is trapped after low-energy collisions, and collision energy profiling and autocorrelation analyses are employed to reveal the modified precursor ion. The combination of MPM and MNM allows for comprehensive PTM screening, and many PTMs, including tyrosine phosphorylation/sulfation, serine/threonine phosphorylation/glycosylation, and lysine acetylation/trimethylation have been studied successfully using this method.
MassPective; a Graphical Tool to Validate Posttranslational Modification Identification from Tandem Mass Spectra
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Tandem mass spectrometry is one of the popular methods for peptide sequencing. It is difficult to unambiguously determine the complete sequence because information in tandem mass spectra is not complete and fragmentation may occur not only at amide bonds but also at other random positions. Various efforts have been made to interpret tandem mass spectra automatically, but the results provided by peptide sequencing algorithms depend heavily on the quality of spectrum data, in terms of both mass accuracy and resolution of the mass spectrometers as well as the information content of tandem mass spectrum, thus requires validation by human experts. Manual inspection is most required when the peptide contains post-translational modifications (PTMs), as the interpretation of mass spectrum becomes a lot harder than in the case of simple peptide identification.

We have proposed an efficient algorithm called MOD' that interprets a tandem mass spectrum of a peptide having multiple PTMs while taking into account hundreds of modification types published on www.unimod.org. MassPective can be used to display PTM interpretations generated by MOD'. It first displays a list of candidate peptides that may match a given tandem mass spectrum. For each candidate sequence, chains of partial sequences, called sequence tags, and in-between gaps are listed, where a gap represents a peptide segment suspected to contain PTMs. Based on MOD' results, MassPective not only visualizes the spectral alignment of b-ions and y-ions for sequence tags, but also displays theoretical fragment ion peaks for each PTM interpretation for gap. MassPective can be used to manually complete sequencing in the gap by inferring all possible sequence tags of length one from fragment ion pairs in the designated area of spectrum and helps a user to sequence a peptide manually so that complete peptide and PTM identification can be augmented to the MOD' interpretation.

Polymeric Microfluidic Devices for Proteomic Analysis
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As part of our U.S. National Heart, Lung and Blood Institute-sponsored Cardiovascular Proteomics Center efforts to develop improved technologies for proteomic analysis, we are working on inexpensive, disposable microfluidic devices for sample preparation prior to mass spectrometric (MS) analysis. As the initial step toward more complex devices, we are developing arrays of capillary reversed phase HPLC columns in cyclolefin copolymer (COC) chips that interface to MS via either matrix assisted laser desorption ionization (MALDI) or electrospray ionization (ESI). The microfluidic chips are prepared by hot embossing in COC (Zeonor®) wafers using photo-lithographically formed masters of SU-8 photoresist on silicon wafers or electroformed nickel masters. Cover wafers are attached using solvent-enhanced thermal pressure bonding utilizing a new vapor phase solvent enhancement method. The microfluidic channel walls are functionalized to facilitate bonding of the monolithic columns using photoinitiated graft polymerization via a photomask. Acrylate-based porous monolithic chromatography columns are then prepared in the channels using photopolymerization via a photomask. Samples and mobile phase are applied via an external pumping system. The MALDI interfacing utilizes simultaneous electrostatic transfer of fractions from multiple columns onto MALDI plates with pre-applied matrix. ESI interfacing utilizes planar carbon electrode connections to the channels formed by embossing traces of a carbon ink, which was formulated to resist the solvents used in column preparation. To confine the ESI Taylor cone to the diameter of the column exit (to maximize sensitivity and avoid postcolumn mixing), the edges of the chips are coated with a fluorocarbon layer using radio frequency chemical vapor deposition. A second approach to ESI interfacing uses carbon fiber ESI emitters. These reversed phase column arrays with MS interface comprise one module of the microfluidic devices for proteomic analysis.
32.18

New Developments in Direct Analysis by MALDI Mass Spectrometry for the Study of Ovarian Cancer

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Understanding cancer’ mechanisms is fundamental for discovered new potential targets, for innovation of therapeutics agents or for early detection of diseases and especially in case of ovarian cancer. Direct MALDI mass spectrometry on tissue samples has the advantage of obtaining peptides/proteins profiling on a tissue section (Caprioli et al., Anal. Chem. 1997; 69(23): 4751–4760, Fournier et al., Neuro. Endocrinol. Lett. 2003; 24(1–2): 9–14) as well as the ability to reconstruct molecular maps giving molecule repartition (Stoeckli 2001; 7(4): 493–496). However, at this time, all studies have been performed on fresh frozen tissues which have its limitations, since all pathology tissue archives (eg. up 30 years) kept in the hospitals are embedded in paraffin, and it is clear that a major challenge is the possibility of peptides/proteins profiling on that type of tissue. In the present work, we studied peptide profiling on ovarian fresh biopsy samples, using direct MALDI analysis technology, and compare results with peptide profiling of ascite liquid after extraction. By this strategy, we discovered several potential biomarkers and have shown that these ones are also present in the peritoneal fluid reflecting the ability of this technique to follow the migration of biomarkers from the tumour to the ascite liquid. Moreover, we focus on new developments for MALDI tissue direct analysis and imaging in order to improve signal and/or detection, and especially we report here, for the first time, direct MALDI analysis of tissue sections embedded in paraffin.

32.19

Combining Novel Fragmentation and Front End Enrichment Techniques for Highly Increased Sensitivity and Selectivity of Phosphopeptide Detection

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The characterization of serine and threonine phosphorylation is usually challenging. The commonly applied collision-induced dissociation (CID) results in neutral loss of the labile phosphate group with often insufficient further fragmentation of the peptide chain itself, and subsequently very limited sequence information. We will present a novel concept of MS/MS in the ion trap by combining CID and electron transfer dissociation (ETD), which is particularly suitable for phosphorylation identification due to its non-ergodic nature: the prompt fragmentation along the amid backbone following the electron capture leaves the amino acid-phosphate bond intact.

Since phosphopeptides are often present at very low concentrations and more difficult to ionize, they are prone to suppression by other peptides in nanoESI, in particular in highly complex mixtures. Therefore, a front-end enrichment is highly recommended. Here, various methods are presented and discussed, ranging from functional surfaces on magnetic beads to TiO2 columns.

Digests of standard proteins spiked with sub-stoichiometric phosphopeptide amounts and phosphopeptide enriched digests from Arabidopsis were analyzed using nanoLC-MS/MS.

The comparison of ETD and CID spectra shows the benefits of ETD, where dephosphorylation of the parent ion was not observed. CID in contrast shows generally the phosphate loss as the most abundant signal, indicating the phosphorylation presence. The combination of both fragmentation methods within one acquisition cycle provided improved fragmentation data and enabled the unambiguous determination of sequence and phosphorylation site.

32.20

Nano-ESI-IT, AP-MALDI-IT, MALDI-IT/RTOF, and MALDI TOF/Curved Field RTOF MS for de Novo Sequencing of the Elucitator of Type I Allergy in Elderberry (Sambucus nigra)

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Products of elderberry trees are widely used in herbal medicine or as foodstuff and has been suspected to induce type I allergy, but has never been verified. In our study gel electrophoretic techniques pointed out that a predominant human IgE binding protein found in elderberry flowers, consisting of two subunits having identical MWt. and isoforms scattered between pl 5 and 7, was the responsible agent. Edman sequencing of this allergen resulted in an amino acid sequence giving first - but insufficient - indication for a type 2 ribosome-inactivating protein (RIP). MS was a very powerful tool to substantiate this finding. In-gel digestions after SDS PAGE of the 33.2 kDa and 66.6 kDa protein were performed and the resulting peptides were further sequenced by low energy (LE) CID experiments. Amino acid sequences could be assigned by means of a hybrid multistage MALDI-IT/RTOF instrument. These results were supplemented by CID on a standard nanoESI-IT MS providing thereafter partial as well as complete sequences of some peptides with high confidence. Furthermore AP-MALDI-IT LE CID experiments were carried out to introduce a different soft ionization technique to gather information on fragile post-translational modifications. A newly developed instrument facilitating MALDI-TOF/ RTOF with high energy CID was implemented in these studies to smooth out amino acid uncertainties (e.g. I /L) and to further improve sequence coverage for protein identification. Bioinformatics showed that a high homology to lectins, in particular to type 2 RIPS, is given. In consideration that dietary lectins can induce histamine release this has been an interesting hint for future research.
Novel Biochip Platform for High-throughput MALDI MASS Spectrometry of Peptides

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Mass spectrometry needs to improve the disparity between the concentration of proteins in the human proteome (sub-atto moles, $10^{-18}$) and the current level of achievable sensitivity (high-atto moles, to femtomoles, $10^{-15}$) to provide greater insight into understanding the human proteome. In addition, to achieve this goal there is the need to develop techniques that minimize ion suppression caused by contaminants and sample complexity.

We outline a chip-based technology that increases sensitivity of detection and removes contaminants with reduced sample loss. This results in low atto mole sensitivity, coupled with the removal of contaminants to produce the availability of more ions for detection by the mass spectrometer.

A new biochip architecture has been created to address the above issues for matrix assisted laser desorption ionisation (MALDI) mass spectrometry. This architecture is called surface tension segmented biochips (STS-Biochips™). These biochips are constructed with self assembled monolayers (SAM) containing various chemically active functional groups exposed on the surface. These chemically defined virtual wells (CDVW) employ a series of differentially wettable surfaces arranged geometrically in concentric zones. Both the chemistry and the geometry of the wells enable the addition of 10 to 100 times more analyte to each well compared to standard MALDI (up to 50 uL of sample). Coupled with the drying fluidic forces that concentrate the sample into a small analysis zone more than half the size of standard MALDI (up to 0.6 mm diameter) at the centre of the well, we have observed greater than a 50 fold increase in the sensitivity of detection of peptides compared to standard MALDI (less than 100 atto moles). We have also observed the detection of peptides from proteins separated on acrylamide gels at amounts beyond current staining detection limits (less than 0.1 nanograms of protein loaded).

Identification of Glomerular Proteins Involved in a Renal Disease by MALDI High Energy CID (TOF/RTOF) and Atmospheric Pressure MALDI Low Energy CID (3D IT)

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The puromycin aminonucleoside nephrosis (PAN) model in the rat is widely used to study the cellular mechanisms involved in diseases with an altered glomerular filtration barrier. The phenotype of PAN resembles closely the histological and ultrastructural changes of the human minimal change nephropathy. But the exact mechanisms leading to alterations of the glomerular filtration barrier and to glomerular injury remain still unclear. In view of this, we applied a proteomic based approach to profile glomerular proteins during puromycin aminonucleoside induced glomerular injury. One strategy was that glomerular proteins were labelled by in vivo perfusion of the kidney with CyDye DIGE Fluor minimal dyes and subsequent Triton X-114 fractionation. The second, more common strategy was that proteins from isolated glomeruli were Triton X-114 extracted/fractionated and labelled applying the DIGE technique. Both ways are based on pre-electrophoretic labelling. Solubilized proteins were separated by 2D PAGE and detected by fluorescence imaging and subsequent silver staining. For the purpose of final protein identification and characterization a vacuum MALDI TOF/curved field reflectron mass spectrometer equipped with a high energy CID cell was used in the MS mode for PMF and in the MS/MS mode for sequence determination. In parallel, MSh ($n \geq 2$) analysis of tryptic peptides in the low energy CID mode was performed on a 3D ion trap instrument equipped with an AP-MALDI ion source. High energy CID MS/MS and AP-MALDI MS/MS (MS/MS/MS if necessary) analysis of tryptic peptides provided partial sequence tags, even of low abundant protein spots. A comparison of the data sets will be given. Up to now 25 pathological relevant glomerular proteins were analysed and could be unambiguous identified. Furthermore, proteins which were fluorescent dye labelled by the in vivo perfusion experiments could be assigned by the CID experiments with high confidence to proteins present in the SwissProt/NCBI database, allowing us to get a deeper insight in the in vivo situation of glomerular cells and the localization of certain proteins.
Improved Protein Identification Using a Microfluidic Chip-based System

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Nanospray LC/MS/MS is widely used for protein identification in order to achieve the highest sensitivity and greatest number of protein identifications. However, nanoscale systems can be more challenging to use and maintain. With nanoflow systems, the numerous connections in the fluidic path must be made carefully as dispersion from poor connections leads to peak broadening. Improved chromatographic performance, resulting in sharper peaks and better resolution, can enhance the data-dependent tandem MS and result in a greater number of peptides detected.

A microfluidic chip-based system has been developed that integrates columns and connections on the chip thus minimizing connections and delay volume. This work compares the performance of this device against the traditional nanospray approach. A proteome sample of medium complexity (1D gel band) was chosen to compare the two technologies. The microfluidic device was shown to identify significantly more peptides resulting in more confident protein identification and an increased number of proteins identified.

Enhanced Dynamic Range and Improved Mass Accuracy for Proteomics Analysis Using a Hybrid Linear Ion Trap; FTICR Mass Spectrometer

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Recent work in proteomic research demonstrates the need to identify proteins in mixtures at extremely low abundance levels with the highest possible confidence. Analysis of complex mixtures by mass spectrometry requires both sensitivity and high dynamic range and benefits from high mass accuracy. Moreover, for MS/MS experiments, fast scan repetition rates are a necessity. All of these method parameters involve trade-offs - dynamic range can be gained by sacrificing resolution and mass accuracy. Here we describe different instrument methods to overcome these limitations. A critical parameter influencing mass accuracy and dynamic range on ion trap based hybrid FTICR mass spectrometers is the number of ions trapped in the ICR cell. When a moderate number of ions are introduced for analysis, the resulting full scan yields ions with mass accuracy below 2 ppm. This setting, in combination with parallel data dependent ion trap MS/MS experiments, leads to the identification of 7 out of 20 proteins in a protein mixture. Deliberately increasing the number of ions to overfill the ICR cell results in a higher dynamic range but, due to space charging effects, mass accuracy is decreased, increasing the probability of false positive identification. To improve the mass accuracy while increasing the dynamic range, the scan cycle is extended by adding a data dependent single ion monitoring scan. The complete method setup is a full scan followed by 3 pairs of data dependent SIM and ion trap MS/MS scans resulting in a scan cycle time of about 3 seconds. The analysis using this technique leads to the identification of all 20 proteins with greatly improved sequence coverage. The observed mass accuracy for precursor ions derived from the SIM scan is better than 0.5 ppm.

Imunochemistry with Direct Mass Spectrometric Detection

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Objectives: The study of intact protein complex by mass spectrometry stills challenging because of the tendency of the complexes to dissociate during the ionization step. The detection of an intact complex by mass spectrometry is also limited by the saturation observed with MCP detectors when analysing complex mixtures. We present a new method combining cross-linking chemistry to stabilize protein complexes before ionization and MALDI mass spectrometry equipped with a superconducting tunnel junction detector. As a first model to evaluate this new method, we have analyzed Antibodies/Antigen complexes, performing sandwich assays, competition assays for epitope mapping and binding kinetics.

Method: Different complexes antibody/antigen have been analyzed with our new method. We have also evaluated our method to detect specific Ab/Ag complexes in biological matrices such as blood serum. A MALDI ToF mass spectrometer equipped with a superconducting tunnel junction (STJ) detector has been utilized for all mass measurements.

Results: Only 10 minutes after starting the binding reaction between the antibodies and the antigens, we have detected the specific complexes (AntiHSA/HAS, AntiBSA/BSA, 6H4/bPrP). The mass spectra of the reaction 6H4/bPrP is constituted of three major peaks in the range 150–210 kDa representing the unbound monoclonal antibody (m/z 150 kDa) and two peaks with highest intensity (m/z 175 kDa and 200 kDa) representing the monoclonal antibody interacting specifically with one or two prion proteins. Our new method has been successfully tested for epitope mapping b competition assay, sandwich assay and binding kinetics.

Conclusion: Combining cross-linking chemistry and STJ detection offers high detection sensitivity (fmol quantities of antigen), high specificity (detection of antigen directly in serum), high accuracy, the possibility for epitope mapping, kinetics studies and sandwich assays. This opens the way for higher throughput protein complex analysis with direct mass spectrometric detection.
32.26
Comparative Analysis of Ovarian Cancer Plasma Proteome Using SELDI-TOF MS and FTICR MS with Different Ionization Methods

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Usage of mass spectrometry as a tool for early disease diagnostics is complicated by large differences in mass spectra from plasma/serum samples obtained same protocol. It is getting clear now that it is almost hopeless to find single molecular biomarker of particular disease at its early stage, and research is directed to the disease response of the whole proteome system. Mass spectrometry gives us the information on changes of proteome profile through changes in mass spectra of specially treated plasma samples. The main two sources of differences in mass spectra of the same samples measured in different laboratories are sample preparation and mass discrimination of mass spectrometers. Mass discrimination depends on the ionization method and type of mass spectrometer used in analyses. We investigated the same serum samples from healthy and ovarian cancer patients using SELDI approach in combination with different types of ionization methods including SELDI/MALDI in different pressure conditions, i.e. at atmospheric pressure, at intermediate pressure (1 Torr) and in high vacuum. Ovarian cancer biomarker, serum amyloid A (11.7 kDa), that was found by SELDI using original Ciphergen linear TOF was investigated on different types of mass spectrometer, such as Bruker Ultraflex TOF, Bruker ApexQ FT ICR MS and Finnigan LTQ FT MS. The results obtained are discussed and recommendations on the choice of ionization method and mass spectrometer type are made.

32.27
Improved Peptide Identification and Protein Coverage for Proteomic Samples Using Novel Alternative 2D-HPLC MS/MS Approaches

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Currently the most popular 2D-HPLC approach for separation of peptides is a combination of strong cation exchange (SCX) and reversed-phase (RP) HPLC. In this work we developed two alternative strategies for 2D-HPLC: a combination of two RP-RP modes and hydrophilic interaction chromatography (HLIC) with RP-HPLC. The off-line 2D-HPLC methods were evaluated using mixtures of 5 or 17 tryptic digested proteins containing 250 –2000 peptides and then applied to human serum samples. The proposed RP-RP and HLIC-RP 2D-HPLC schemes compared favorably to traditional SCX-RP; separation orthogonality was found to be comparable or greater. The orthogonality in the RP-PR approach was achieved by altering the pH of mobile phases in the separation dimensions. In addition, we utilized a novel alternate scanning mode (MS³) for MS/MS data acquisition. A novel MS/MS method using alternate scans at low and elevated energy has an increased duty cycle over traditional DDA methods and increased MS/MS spectra quality. These new 2D-HPLC approaches and MS/MS method allowed for a significantly increased number of identified peptides and proteins with significantly better confidence from highly complex sample.

32.28
Long Term Archiving of Proteomic Samples on Disposable Prespotted MALDI Targets

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Archiving of MALDI sample preparations is an important issue not only with regard to classical long-term storage triggered by legal or GLP demands. It becomes relevant, whenever there is a significant time lag between sample preparation and mass spectrometric analysis and whenever one has to revisit selected samples under different aspects or to verify results. Any kind of long-term archiving requires a cost-effective, reliable platform technology and dedicated storage conditions. Recently a new disposable plastic MALDI target was introduced. These have been used for archiving proteomics samples under different storage conditions. 250 amol and 5 fmol tryptic BSA digest were prepared on the HCCA matrix anchors of a number of prespotted AnchorChip targets (Bruker Daltonik, Germany). Right after preparation a first set of MS and MS/MS reference data was acquired on a MALDI-TOF/TOF. Then the targets were sealed in plastic bags with different gas fillings (vacuum vs. air vs. nitrogen) and were stored at different temperatures (20 °C vs. 4 °C vs. −18 °C). We revisit the samples monthly under well defined experimental conditions in order to check their mass spectrometric performance (sequence coverage, intensity coverage, number of matching peptides). So far we have reached nine months storage time. After that period of time no significant sample degradation is observed for samples stored at room temperature under nitrogen. Although S/N slightly decreases with time, a safe protein identification is still possible even at the 250 amol level. The sequence coverage remained unchanged at about 40%.
32.29

Analysis of Post-translationally Modified Peptides and Proteins by High Resolution Electron Capture Dissociation (ECD) FTICR-MS

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Proteomics research has evolved rapidly from simple identification of proteins to detailed analysis of their exact structure, including all possible modifications. Post-translational modifications (PTMs) have been shown to play an extremely important role in determining the regulation, function and activity of proteins. As such, it is extremely valuable to have a method for their characterization.

Mass spectrometric characterization of proteins represents some unique challenges:
1) many PTMs are extremely labile - when using vibrationally activating fragmentation techniques such as collisionally induced dissociation (CID) or infrared multiphoton dissociation (IRMPD), the PTM is the favored initial site of fragmentation and information about it is consequently lost.
2) the common method of bottom up protein sequencing requires enzymatic digestion and analysis of the proteolytic fragments. While it is an effective way to determine sites of modification on peptides, one loses the overall context in which the PTMs exist in various protein isoforms.

Top down protein analysis with electron capture dissociation (ECD) is a solution to both of these problems. ECD uses low energy electrons inducing a radical-driven fragmentation process involving the peptide bond between amino acids exclusively. This process preserves PTM's. Likewise, top down sequencing utilizes fragmentation of the intact protein, not its component peptides. In this way, the entire protein structure can be characterized, including sites of modification. We have used ECD for analyses of various peptides and proteins to show that sites of modifications can be unambiguously determined. We applied ECD in different proteomic approaches, including data-dependent LC-ECD-FTMS and top-down characterization of intact proteins with a hybrid linear ion trap Fourier transform mass spectrometer. Multiply charged precursor ions were selected using the linear ion trap, and isolated ions were either fragmented by CID and passed to the FTICR analyzer for analysis, or passed directly to the ICR cell for activation by ECD before mass analysis. The resulting mass spectra are deconvoluted and compared to simulated spectra of these proteins to demonstrate protein identification and determination of PTM sites.

32.30

Simplified 2D LC-MS/MS Method for Proteomic Analysis

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Bottom up LC-MS/MS analysis of proteomics samples is complicated by the sheer complexity of the analysis - there are peptides from a huge number of proteins and they are present over a large dynamic concentration range. Two-dimensional LC/MS methods are routinely used for complex peptide mixtures because they offer the enormous peak capacity of orthogonal chromatographic separations based on strong cation exchange (SCX) in the first dimension and reversed phase (RP) in the second. Most two-dimensional LC techniques employ two columns and two HPLC systems so that each column can be conditioned and eluted separately. They commonly use salt to elute peptides from the SCX column onto the RP column. There are several drawbacks to such systems: 1) plumbing and programming complexity, 2) salt used for peptide elution can suppress peptide ionization and can plug the system if mistakes are made, and 3) salt steps often elute the same peptide in multiple fractions. In this study, a single capillary column with both SCX and RP regions, referred to as a “biphasic” column, was utilized. In addition, to elute peptides captured on the SCX resin, a series of pH steps, using buffers that are compatible with mass spectrometry, was used. Benefits of this method include: 1) system simplification - only one HPLC pump is needed, 2) only one biphasic column is used, improving peptide recovery relative to multi-column systems, and 3) peptides are eluted from the SCX by pH according to their pl, leading to less peptide carry-over into multiple RP analyses. Results are shown for separations of samples varying in complexity from simple digests to cell lysates. This system appears to be maximally advantageous for highly complex samples.

32.31

Automation of In-gel Digestion and MALDI Spotting of Two-dimensional Electrophoresis Separated Rat Liver Proteins

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Proteomic approaches are widely explored in short term toxicological tests and for gaining insight into toxic and carcinogenic effects of chemicals. Since emerging proteomic technologies still depend on data generated by two-dimensional electrophoresis (2-DE), our aim was to establish a new 2-DE based map of male Wistar rat liver proteins in the context of a molecular toxicological joint research project. At present, the map contains 621 proteins and could be the basis for a publicly accessible HTML database, providing quick information to identify protein expression patterns of toxicological relevance. As an alternative to our approved manual procedure (optimized at the FMP) high performance sample preparation requires a robot system capable of spot picking, digesting and spotting directly onto MALDI target. The best results has been delivered by the ProPic spotpicker in combination with the ProGest digester and ProMS spotting station (all from Genomic Solutions) allowing simultaneous processing of 96 gel spots within 12 hours. The ProMS is compatible with the most commercially available MALDI targets. We have successfully transferred our manual protocol to the automation system and tested strongly as well as weakly stained spots and both led to confidential spectra with high peak intensities and identification scores.
Evaluation of Solvent-free MALDI-MS for the Analysis of Proteins via the Mini-ball Mill Approach

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Recently, an efficient, low sample load mini-ball mill (MBM) sample preparation procedure was developed for solvent-free MALDI analysis of peptides and proteins. [Trimpin, S., Deinzer, M. L., J. Amer. Mass Spectrom. 16:542–547, 2005.] These promising results encouraged us to more thoroughly investigate the practical benefits of this new solvent-free MBM MALDI-MS method. Thus, the aim of this contribution is to evaluate the advantages of MBM MALDI-MS as compared to conventional solvent-based MALDI-MS towards i) the characterization of hydrophobic peptides, melittin and β-amyloid peptides, as well as bacteriorhodopsin as a membrane protein, hence special emphasis is given to its applicability to problematic compounds and reaction mixtures, irrespective of solubility; and additionally, ii) the analysis of H/D-ratio measurements from exchange experiments (e.g. MW determination and peptide mapping investigations of stathmin prior and after H/D-exchange experiments) to secure sufficient retention of label to ensure accurate measurements applying the MALDI-TOF/TOF high energy collision fragmentation, hence, measurements that show undesired proton back-exchange in conventional MALDI and ESI-MS approaches also due to solvent-sensitivity.

Overall, a solvent-free MBM MALDI-MS method was developed that meets important criteria for the analysis of hydrophobic peptides and membrane proteins, including intact molecular weight measurements (global information) and peptide mapping investigations (local information). Improvements are found, e.g. only the MBM method determined β-amyloid peptide (1–42) in a defined β-amyloid peptide mixture of soluble and insoluble constituents. MBM also appears to be an improvement over standard solvent-based MS procedures for the determination of H/D-ratio measurements due to reduced back-exchange. [NIEHS ES10338]

Complementary Analysis of Human CSF Proteins by Nano LC MALDI and ESI/MS/MS

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Electrospray and MALDI ionization are both commonly used to generate biomolecular ions for mass spectrometry analysis. Findings from previous studies have shown that both ionization techniques can be complementary and, when combined, can greatly improve proteome coverage and overall protein identification in complex protein mixtures.

In this study, we utilized the combination of nano LC/MS/MS with LC-MALDI/MS/MS to characterize the human cerebrospinal fluid (CSF) proteome. CSF proteins were denaturated with 8 M urea and separated into 6 fractions using a strong anion exchange spin column with pH elution steps. Each fraction was digested and reduced or reduced, alkylated and then digested. Finally digests were analyzed by reversed phase nano LC with a post column flow split in two equal parts providing 1) online (ESI) and 2) offline sample spotting for MALDI/ MS and MS/MS analyses. Mass spectrometry was performed on a vMALDI LTQ system or by nano ESI LTQ. Results from both approaches, including numbers of unique peptides or proteins found, sequence coverage and general measures of data quality, were determined and compared. Generally about 50% of identified proteins were identical between two techniques with higher sequence coverage from nano LC/MS/MS probably due to greater amount of performed MS/MS scans: 4500 vs. 1500 for the typical run. Superior sequence coverage and the highest number of identified peptides and proteins for the analysis of CSF was found by combining data from ESI-MS/MS and MALDI-MS/MS experiments rather than the use of either technology alone.
Strategies for Generating Sequence Information from High Mass Peptides/Low Mass Proteins Using Novel Tandem TOF Technology and iTRAQ™ Reagent Chemistry

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Analysis of high mass peptides/low mass proteins has been a reoccurring problem in proteomics arena. This is particularly the case for biomarker profiling experiments that often require the identification of peptides in excess of 10 kDa. Recent improvements in TOF/TOF technology have led to improved detection and fragmentation efficiency of high mass peptides and small proteins. However large gaps in the sequence coverage still create problems with de novo sequencing software tools. One way to circumvent the problem is to generate sequence tags to identify the proteins but this is limited to proteins in databases. Another approach is to induce better fragmentation by labeling the peptides/proteins with commercially available reagents such as iTRAQ™ reagent chemistry. iTRAQ™ reagent chemistry has already proved to be extremely useful in generating improved sequence information data from tryptic and non-tryptic peptides up to 4 kDa. The strategy would impact the field of proteomics especially in the diagnostic and biomarker discovery applications. Several examples of high mass peptides/low mass proteins have been labeled with iTRAQ™ reagent chemistry to evaluate the potential of this workflow. The advantages of iTRAQ™ reagent chemistry are two fold. It not only facilitates better fragmentation but also enables quantitation of the peptides/proteins in the samples. Preliminary analysis of unlabeled proteins using novel tandem TOF technology has shown improvements in fragmentation that may well be enhanced by the use of iTRAQ™ reagent chemistry. A wide range of standard and real life high mass peptide/low mass proteins samples will be analyzed to ascertain the suitability of the iTRAQ™ reagent labeling workflow. These include amyloid peptides (AB40 and AB42) that have been implicated as causative agents for Alzheimer’s disease (AD). An analysis strategy based on high mass peptides/low mass proteins would enable development of better diagnostic tools for diseases such as Alzheimer’s.

New Approaches to Full Characterization of Cancer Biomarkers at the Trace Level; Epidermal Growth Factor Receptor

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With a diversity of potential biomarkers, there is considerable interest to fully characterize such markers. For example, epidermal growth factor receptor (EGFR) is an important biomarker and drug target for lung and breast cancers. When activated, the cytoplasmic kinase domain of EGFR triggers signaling cascades within the cell that are implicated in the above diseases. The capability to analyze such receptors at the trace level (fmol), including the posttranslational modifications which are indicative of their activation states, could offer important insights into a number of disease processes. Because of the large size and heterogeneous modifications of EGFR (a 180 kDa transmembrane glycoprotein with multiple PTM sites), the comprehensive characterization (high sequence coverage plus glycosylation and phosphorylation) with fmol amounts of material has been very difficult. We have developed a new and sensitive LC-MS platform, Extended Range Proteomic Analysis (ERPA), that is capable of achieving very high sequence coverage (>95%) and comprehensive characterization of glycosylation and phosphorylation modifications of EGFR from crude cell lines or tissues. This new platform achieves the analysis of large fragments (up to 10 kDa) with a new data acquisition strategy using the hybrid LTQ-FT mass spectrometer. A strategy to determine glycosylation and phosphorylation sites and the nature of the attached glycans will be demonstrated including the identification of both the glycosylated and phosphorylated peptides of EGFR upon stimulation with epidermal growth factor at different time intervals. This capability can address important biological issues such as the relationship of changes in extracellular glycosylation that are related to intracellular activation (phosphorylation) states, upon stimulation with different ligands. In addition, the ability to observe simultaneously the paired isoforms of EGFR, such as phosphorylated and nonphosphorylated as well as glycosylated and nonglycosylated peptides, in a single analysis provides a powerful means to quantify the dynamic changes of the activation states.
Mesoporous Material as MALDI-matrix and Enzyme Reactor for Proteomics

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The organic matrixes in MALDI suffer from some problems and are not always effective. As an alternative, mesoporous material has been drawing considerable attention to develop mainly on its analytical sensitivity and spectra quality. This presentation will focus the investigation on the proteomic application with highly-ordered mesoporous material.

The mesopore powder was suspended in water/methanol (1:1, v/v) and sonicated for 5 min. The analytes were dissolved in water. The 1 \( \mu l \) of the suspension was pipetted onto the target plate and then 1 \( \mu l \) of analyte was deposited on them. For a micro-reactor experiment, the trypsin in solution form was immobilized in the hole area of mesopore material with various methods for proteomic analysis.

Special experiments showed that mesoporous material has a stronger tolerance towards potassium salts than CHCA. A serial of reproducible spectra with reasonable S/N at pico-mol level were easily obtained using the mesoporous matrix, with a significant improvement compared with the nano-mol detection level previously reported.

Experiments with mesoporous matrix as matrix were performed on a model protein system (MYO tryptic digests, 15 pmol), and the protein searched from SwissProt database is consistent with the standard MYO with a sequence coverage of 68%. Experiments also show that the mesopore is suitable as a nano-enzymatic-reactor. With immobilized trypsin, the protein can be digested within a very short time of 2–20 seconds, which shows a faster kinetic reaction time compared to the conventional solution tryptic digestion. In the proteolysis process, the mesoporous material has exhibited a capability to unfold protein partially, helping a more effective digestion.

An Automated Top-down LC/MS\textsuperscript{n} Approach for Identification and Characterization of Unknown Yeast Proteins

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In top-down proteomics the intact protein molecular ions are fragmented inside the mass spectrometer without prior proteolysis, allowing not only their direct identification but also characterization of posttranslational modifications. The probability of a correct identification increases significantly when MS\textsuperscript{2} fragments are formed by the sequential loss of amino acid residues, thus comprising a sequence tag. Here, an automated top-down strategy, including MS\textsuperscript{3} experiments for efficient sequence tag generation, is described. The mixture of unknown yeast proteins has been separated on-line using reverse-phase HPLC and analyzed using ESI on an LTQ FT mass spectrometer, where the most intense molecular ions were automatically selected, isolated and fragmented. The deconvoluted MS/MS spectra were searched using ProSight PTM search engine to identify three proteins, two of which were found to be modified. Further off-line MS\textsuperscript{3} analysis pinpointed the location of these modifications. Alternatively, an additional MS\textsuperscript{3} stage was introduced into the standard top-down experiment to reliably generate sequence tags from MS\textsuperscript{2} fragments. The experiment was performed with standard and unknown proteins using a stand-alone linear trap mass spectrometer. Initially, the most intense molecular ions were fragmented, followed by dissociation of selected MS/MS fragments. The resulting spectra were automatically processed to identify sequence tags of 8 to 16 amino acids in length. The following hybrid search using both the mass of the MS\textsuperscript{2} fragment and the MS\textsuperscript{3} sequence tag provided unambiguous identification of the standard and unknown proteins when searched against a modified human database. This additional MS\textsuperscript{3} step can be reliably used to generate sequence tags from MS\textsuperscript{2} fragments, thus greatly improving the confidence of the database retrieval and further characterizing the primary sequence of the protein of interest.