Session: Proteins: General Code: MP24 Time Slot/Poster Number: 377

Top Down Analysis of Transthyretin Using ESI FTMS

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Introduction:
Top down analysis of Transthyretin (TTR) wild type and Met-30 variant was performed by ESI FTMS using Q2 CAD and SORI-CAD on our hybrid FTMS instrument. The Q2 CAD results obtained show fragmentation dominated by cleavage of the peptide bond between E42 and P43, resulting in multiply charged b and y complementary fragment pairs. Smaller fragment ions corresponding to the N- and C-termini were also observed. A TTR patient sample bearing a Val30Met mutation was analyzed in this manner. An immunoglobulin light chain was also investigated. This constitutes a useful clinical application of top down mass spectrometric analysis.

Methods:
TTR samples were obtained by immunoprecipitation. Samples were dissolved in standard ESI buffer (50:50 acetonitrile/water with 0.1% formic acid) and sprayed from home made nanoESI tip holder to a hybrid qQq ESI FTMS instrument. Ions of interest were isolated by a Q1 resolving quadrupole to undergo Q2 CAD excitation in the LINAC cell. The product fragments were preaccumulated in the hexapole and subsequently transmitted to the ICR cell as a package. The fragment ions of interest were isolated by SWIFT and if desired, were fragmented further using the SORI CAD technique in the ICR cell to improve sequence coverage or get sequence tags for the detection (or localization) of the modification site.

Abstract:
The preliminary results obtained by applying top down analysis to TTR variant characterization yield extensive sequence information. The 15+ charge state of the protein was pre-selected for CAD in the second quadrupole prior to mass analysis of the fragments in the FTMS cell. The main fragments observed were b42 and y85. These b and y fragments are pairs covering the whole protein sequence. The dominance of the fragment ion spectrum by these fragments is easily understood as b42 and y85 result from a glutamic acid-proline cleavage. Although we are exploring conditions to improve sequence coverage of TTR using Q2 CAD, we have also pursued an approach where the main fragments generated by Q2 CAD, b42 and y85, were isolated in the FTMS cell and made to undergo SORI-CAD to yield sequence information. This two stage approach was also used for a TTR sample containing a Val30Met mutation. The variant and wild type protein were pre-selected for Q2 CAD. The Q2 CAD of the protein exhibited the b42 fragment and a +32 Dalton peak not present in the wild type TTR Q2 CAD spectrum and consistent with the Val30Met mutation. The isolation and fragmentation of the b42 fragment bearing the mutation allowed a more precise location of the mutation but did not yield data that specified the mutation site. Efforts are ongoing to obtain more informative fragmentation through various conditions. An immunoglobulin light chain isolated from the urine of a patient with primary amyloidosis was analyzed. Q2 CAD followed by SORI CAD was necessary to generate fragmentation of this 23kDa protein, probably due to the presence of intermolecular disulfide bonds. This research was supported MDS/SCiex, NIH/NCRR grants P41-RR10888, P01-HL68705 and the Young Family Amyloid Research Fund.

Session: Peptides: General Code: MP25 Time Slot/Poster Number: 397

Identification of surface exposed components of MOMP of Chlamydia trachomatis serovar F

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Introduction:
Chlamydiae are obligate intracellular bacterial pathogens that cause a broad spectrum of clinically distinct diseases in humans and animals. To identify the surface exposed components of the major outer membrane protein (MOMP) of Chlamydia trachomatis is critical for modeling the 3-D structure of MOMP and, furthermore, for understanding the roles that MOMP plays in the pathogenesis of Chlamydia. Mass spectrometry was successfully applied to identify the surface exposed components and post translational modification of cysteines of MOMP, using a combination of approaches that may provide insight for structural analysis of other membrane proteins.

Methods:
1) The elementary body (EB) of C. trachomatis serovar F was hydrolyzed by trypsin to unravel the outer surface exposed amino acids. 2) Chlamydial outer membrane complex was prepared and subjected to treatment with various proteases to analyze the cysteine status. The resulting non-reduced OMC peptides were denatured and alkylated with 4-Vinyl-Pyridine(VP). After evaporation of unreacted VP, OMC was reduced with DTT and alkylated with iodoacetamide. 3) The resulting peptides were separated by HPLC followed by Matrix-Assisted Laser Desorption/Ionization-Time-of-Fflight mass spectrometry (MALDI-TOF/MS) and capillary liquid chromatography coupled to a quadrupole-othogonal-TOF mass spectrometer (capLC/MS and capLC/MS/MS) analysis. 4) Western blot and Dot blot assay using monoclonal antibodies against four VDs were also performed to identify surface exposed VDs.

Abstract:
Peptide cleavage sites at VDI and VDIII regions and peptides from the C-terminal region Lys333-Arg358 and Lys333-Lys350 were observed. Mass Spectrometry results were consistent with both Dot blot and Western blot experiments which showed that VDI and VDIII were surface-located. N-terminal amino acid sequencing of the MOMP peptides determined the exact cleavage sites on VDI and VDIII: The Glu-C cleavage sites are located between Gly 64/Glu 65 in VDI, and between Lys226/Glu 227 in VDIII; The trypsin cleavage sites were found between Lys 80/Leu 81 in VDI, and between Lys 226/Glu 227 in VDIII. 2) Post translational modification of cysteine residues were studied and Cysteine 208, 116, 337 and 26 were found to be involved in disulfide bonds, Cysteine 103 and 305 were most likely to be within the transmembrane regions of MOMP. 3) Based on these data, a new 16-transmembrane structural model was proposed.

Session: Carbohydrates/Oligosaccharides Code: MP27 Time Slot/Poster Number: 455

Controlled Depolymerization of Chondroitin Sulfate Chains and Analysis by Mass Spectrometry

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Introduction:
The study of Chondroitin Sulfate (CS), a repeating polymer consisting of GlcAβ(1,3)-GlcNAcβ(1,4) disaccharide units with various sulfation patterns at the 4- and 6-O-positions of GalNAc, and 2-O- position of GlcA (CSA, CSC, and CSB), is hindered by the large size and heterogeneity of these glycans. Enzymatic depolymerization of the CS chains has been a useful technique to obtain structural information but has proven difficult to control. The lyase cleaves smaller CS fragments faster than large fragments, producing populations containing more disaccharide than information rich oligosaccharides. In addition, each substrate has different susceptibility to the lyase, requiring different reaction conditions. In this work, controlled enzymatic depolymerization is achieved using porous chromatography beads to sequester oligosaccharides from the further degradation.

Methods:
Chondroitinase ABC (of various amounts) was added to solutions containing 100 μg CS and monitored on a Beckman DU640 UV-Vis detector at 37 ˚C until the reaction was completed. Identical reactions were exposed to varying amounts and types of hydrated size-exclusion resin to determine the optimal pore size for the desired cleavage profile. Reactions were filtered and washed to remove the resin and elute the digestion products. After lyophilization the reactions were separated by SEC (Beckman Gold 125 solvent module). The eluant was monitored at 232 nm using a Beckman Gold 166 UV detector. The fractions were analyzed on a Bruker Esquire 3000 Ion Trap mass spectrometer.

Abstract:
CS type-C was digested in the presence of various amounts of size-exclusion resin (SEC). The use of a 10,000 molecular weight cut-off dialysis membrane was also tested. The removal or sequestering of digestion fragments allows for the analysis of larger subunits than those typically observed after complete digestion. Resins were chosen by their pore sizes, with fractionation ranges from 700 to 30,000 Da, to determine the optimal pore size for an informative distribution of polysaccharide fragments, while excluding substrate (50 kDa) and enzyme (120 kDa).

Results indicate that the distribution of digested CSC is significantly be altered in the presence of resins designed to fractionate 700-7000 Da. Interestingly, the resin utilized for the separation of larger biomolecules (30,000 Da) did not have a significant effect on the digestion. In experiments where the complete digestion of CSC was observed in the control reaction, digestions performed in the presence of SEC resin possessed one-third the quantity of both disaccharide and tetrasaccharides and significant increases in the presence of hexamer, octamer and decamers species, while leaving little to no undigested CSC. The presence of these saccharides was confirmed by mass spectrometry. These results indicate that this may be useful as a general method for enzymatic depolymerization of GAG substrates.

A preliminary digestion performed using dialysis membranes (mwt c.o. 10,000 Da) did not significantly alter the distribution of the resulting fragments. However, no digestion products were observed in the buffer chamber indicating that there may not have been free exchange between chambers. This system is currently under further investigation.

Session: Proteins: Modified Code: MP26 Time Slot/Poster Number: 437

Characterization of a Kappa IV Immunoglobulin Light Chain from a Patient with Systemic Primary Amyloidosis

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Introduction:
Systemic Primary (AL, immunoglobulin light chain) amyloidosis is characterized by the deposition of immunoglobulin light chain proteins produced by a monoclonal B-cell-derived clone. Previous studies show that the amino acid replacements in the variable region and some post-translational modifications of immunoglobulin light chains may be the key factors that contribute to fibril formation by destabilizing the folding state of these proteins. To have a better understanding of the role of light chain modifications on amyloid deposition, we used a mass spectrometry based method to investigate an amyloidogenic kappa IV light chain (00-051) isolated from the urine of a patient diagnosed with AL amyloidosis, to identify amino acid sequence variations and post-translational modifications in the protein.

Methods:
The molecular masses of the intact protein, before and after treatment with dithiothreitol (DTT), were determined by nanospray ESI-MS using an Applied Biosystems/Sciex QStar-Pulsar i quadrupole orthogonal TOF mass spectrometer (QStar). The sample aliquots were proteolytically digested with trypsin, Asp-N, Lys-C, and Glu-C. The aliquots of these enzymatic digestion products were reduced with DTT. MALDI-MS and ESI-MS analyses of both reduced and non-reduced digests were performed on a Bruker Reflex IV MALDI-TOF mass spectrometer and the QStar, respectively, to generate peptide maps. Some peptides that could not be assigned by peptide mapping were sequenced using ESI-MS/MS on the QStar. These tandem mass spectrometry experiments could also be employed to acquire further information on post-translational modifications.

Abstract:
ESI-MS analysis of the intact protein was performed to obtain the molecular masses of this kappa IV light chain and its related components in the sample, in order to obtain a preliminary assessment as to whether there are modifications in the protein. Before reduction, the deconvoluted ESI mass spectrum showed two major peaks at 24,182 Da and 24,271 Da. After treatment with DTT, the peak at 24,271 Da disappeared, and a new peak at 24,154 Da was observed, which was in good agreement with the theoretical mass 24,156 Da calculated from the cDNA-deduced amino acid sequence. The 117-Da mass decrease after reduction corresponded to a loss of a disulfide-linked cysteine and reduction of two disulfide bonds in the native protein. In addition, two low abundance components in the reduced sample were observed at 24,108 and 24,139 Da, featuring a – 45 Da mass difference from the two major peaks respectively. The cDNA-deduced sequence was verified by MS peptide mapping and MS/MS sequencing with full sequence coverage obtained. Two disulfide bonds (Cys140-Cys200, Cys220-cysteine) were identified. In the reduced Lys-C digest, a doubly charged peptide (m/z 1252.14) was characterized by MS/MS analysis, and the results suggested it contained modifications at the N-terminal amino acid.. The 45-Da mass decrease from the peptide containing residues 1-24 agreed with the mass difference observed in the protein components. Another pair of peptides with the 45-Da mass difference was found in the Glu-C digest, and the unmodified one contains residues 1-17. Further experiments are underway to address the N-terminal modifications, as well as the

30-Da mass difference between the two major protein components. Acknowledgements This work was supported by NIH grants NIH/NCRR P41RR10888, S10-RR10493, S10-RR15942 (CEC), and P01-HL68705 (MS/CEC); the Gerry Foundation, and the Young Family Amyloid Research Fund (MS)

Session: Proteins: Modified Code: MP26 Time Slot/Poster Number: 442

A Mass Spectrometry and Atomic Force Microscopy Study of post-translationally modified immunoglobulin light chains from AL patients

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Introduction:
The folding stability of a light chain is generally regarded as a controlling key of its tendency to form amyloid fibrils. Some amino acid replacements and post-translational modifications of light chains may play an important role in destabilizing the folding state of these proteins, thus making them amyloidogenic. To investigate the impact of light chain modifications on amyloid deposition, a mass spectrometry based method was employed to verify cDNA-deduced amino acid sequence and to characterize sequence variations and post-translational modifications of proteins isolated from the urine of patients with AL amyloidosis. Also, the fibril formation propensity of the light chains analyzed by mass spectrometry was studied using atomic force microscopy (AFM).

Methods:
The urinary light chains from patients were purified using dialysis and gel filtration chromatography. The mass of intact proteins before and after reduction was determined by ESI-MS analysis on an Applied Biosystems Qstar-Pulsar quadrupole/orthogonal TOF mass spectrometer (QoTOF). Aliquots of the light chain samples were digested with trypsin, Asp-N, Lys-C, and Glu-C. The products from these enzymatic digestions were analyzed using Bruker Reflex IV MALDI-TOF as well as on and off-line reversed phase HPLC-MS/MS. ESI and MALDI tandem mass spectrometry experiments were performed on the QoTOF mass spectrometer. A Multimode AFM (Digital Instruments, Santa Barbara, CA) attached to a Nanoscope IIIa controller were used to acquire images of fibril formation under different pH conditions.

Abstract:
We have analyzed a series of urinary light chain from patients with primary (AL) amyloidosis using our published analytical template (1). Most of these light chains are post-translationally modified. The most common case is S-cysteinylation of the C-terminal cysteine. Recently we have found a light chain (00-131) whose molecular weight as determined by ESI-MS did not match the molecular weight deduced from the cDNA sequence. After reduction, two components of molecular weight 23202 and 23248 were detected. The component of molecular weight 23248 matches that deduced from the cDNA sequence whilst 23202 is 80 Da less than the main component in the non reduced ESI-MS spectrum. HPLC MSMS analysis of the Glu-C digest of the protein suggests that the C-terminal cysteine is primarily S-sulfonated, explaining the -80 Da shift observed after reduction. This is the only the second example of an S-sulfonated human protein. The first case was observed in transthyretin (2). Our AFM results show that for sample 00-131, the protein samples aggregate with time. There are also clear pH effects during the amyloid protein aggregation process which is consistent with published results. These results are being compared with those other sequenced amyloid light chains exhibiting posttranslational modifications such as homodimerization, S-cysteinylation, N-terminal modification and glycosylation. 1. Lim A, Wally J, Walsh MT, Skinner M, Costello CE. Anal. Biochem., 2001, 295, 45-56. 2. Théberge R, Skinner M, Connors LH, Skare J., Costello CE, Anal.Chem, 1999, 71 452-459. This work was supported by NIH/NCRR P41-RR10888 and NIH P01-HL68705 as well as the Young Family Amyloid Resesarch Fund

Session: Carbohydrates/Oligosaccharides Code: MP27 Time Slot/Poster Number: 446

Analysis of 2-aminobenzamide derivatives of Caenorhabditis elegans N-glycans by fluorescence detection and offline mass spectrometric analysis.

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Introduction:
Caenorhabditis elegans is an attractive model for development, innate immunity and host-pathogen interactions, all processes where carbohydrate recognition is involved. The organism is easily maintained in culture and well characterized developmentally and genetically and the genome is completely sequenced. Several groups have reported the N-glycan structures in this organism (1-3). Conserved glycans such as high mannose and short mammalian-type hybrid and complex glycans have been found. However, some higher order complex glycans predicted by the genome sequence are rare or absent in recent studies. This information coupled with the presence of some novel oligosaccharides and others that appear to be conserved in nematodes hint that the overall knowledge of the N-glycosylation repertoire of this organism incomplete.

Methods:
Reductive amination of oligosaccharides is useful for sensitivity enhancement in both HPLC and mass spectrometric analyses (4,5). Here the 2-aminobenzamide derivatives of C. elegans N-glycans from larval stages L1-4, Adult, and Dauer have been analyzed and compared. Fluorescence-detected C-18 chromatography and off-line mass spectrometric analysis using MALDI-TOF, PSD, and QoTOF MS were applied. HPLC was performed on a Waters Breeze system. MALDI-TOF MS and PSD analyses were performed on a Bruker Reflex IV instrument. CID MS/MS was performed on an Applied Biosystems/PE Sciex QStar instrument equipped with either a MALDI or ESI source.

Abstract:
The PNGaseF released glycans contained five general classes including high mannose, phosphoryl choline-substituted, Ce fucosyl, complex types, and hybrid forms of the last two. The chromatographic profiles, glycoforms detected and ion abundances were unique for each developmental stage. Phosphorylcholine-substituted and Ce-fucosyl oligosaccharides were most abundant and structurally diverse in Larva 1 and Dauer stages. Some novel structures were implied. The glycans of Adult nematodes were less complex and most closely resembled those of mixed developmental stages, a not too surprising observation since Adults are the most abundant by mass in mixed samples. Moreover, Larva 1 and Dauer larva are underrepresented in mixed samples, and this may explain why some higher order complex and phosphorylcholine oligosaccharides observed in the present study are rare or not detected in other studies since all other investigations reported to date have addressed glycans from mixed stages. These data show that fluorescence detection and off-line MS analysis of 2-aminobenzamide oligosaccharide derivatives can be a very useful approach for analysis and comparison of related biological samples. To further facilitate and enhance the method, development of strategies for online analysis is warranted and ongoing. 1. Haslam, S. M., and Dell, A. (2003) Biochimie 85, 25-32 2. Cipollo, J. F., Costello, C. E., and Hirschberg, C. B. (2002) J Biol Chem 277, 49143-49157 3. Cipollo, J. F., Awad, A. M., Costello, C. E., and Hirschberg, C. B. (2004) J Biol Chem 279, 52893-52903 4. Harvey, D. J. (1999) Mass Spectrom Rev 18, 349-450 5. Zaia, J. (2004) Mass Spectrom Rev 23, 161-227 This work was supported by National Research Service Award F32 GM66486 (to J. F. C.) and National Institutes of Health Grants RO1 GM30365 (to C. B. H.) and P41 RR10888 and S10 RR15942 (to C. E. C.).

Session: Phosphoproteome and Protein Phosphorylation Code: MP29 Time Slot/Poster Number: 488

Characterization of the Maturation-associated Dephosphorylation and Structural Changes of Hepadnavirus Nucleocapsids

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Introduction:
Dynamic protein phosphorylation is paramount to the regulation of normal cellular and viral processes, however, it presents numerous challenges for MS study due to the phosphate modification's transience and lability. Hepatitis B virus (HBV) capsid phosphoprotein serves as a useful model because dynamic phosphorylation may regulate its numerous functional roles in the HBV lifecycle, including possibly transmitting a viral maturation signal during morphogenesis that triggers envelopment and secretion of mature virions. We characterized the gross structure of purified capsids from three stages of viral maturation by atomic force microscopy (AFM), and the phosphorylation of the capsid protein by vibrational cooling (VC) MALDI-FT MS, which minimizes phosphate loss, ESI-QoTOF MS, and MALDI-TOF MS.

Methods:
Duck HBV (DHBV) nucleocapsids from three extremes of viral maturation (immature, RNA-containing, mature, DNA-containing, and virion-derived nucleocapsids) were isolated from virus-expressing cell lines and purified to homogeneity by gradient ultracentrifugation. Intact nucleocapsids were spotted directly onto AFM targets and visualized with a Veeco Multimode™ AFM attached to a Nanoscope™ IIIa controller. Denatured capsids were subjected to SDS-PAGE and digested with Lys-C or trypsin in-gel. Peptides were purified by ZipTip™ microchromatography. Phosphopeptides were subjected to MSn with a VC MALDI-FTICR MS, constructed in-house, or were subjected to tandem MS sequencing with an MDS Sciex/ABI QStar™ ESI Qo-TOF MS. Comparative analysis of phosphopeptides from different stages of viral maturation was conducted with a Bruker Reflex IV™ MALDI-TOF MS.

Abstract:
Using VC MALDI-FTICR MS, we were able to detect a novel DHBV capsid phosphopeptide, then obtain phosphosite localization by conducting successive SORI-CAD experiments (MS3) upon it. Furthermore, we obtained complete sequencing and phosphosite localization for a novel DHBV capsid proline-rich pentaphosphorylated peptide using ESI-QoTOF MS/MS, confirming four known sites of capsid phosphorylation and identifying another novel phosphorylation site. AFM analysis of the intact purified capsids from the three different stages of viral maturation demonstrated a correlation between viral maturation and a progressively increased capsid size. Comparative MALDI-TOF MS analysis of peptides derived from these same three capsid populations revealed that the capsid maturation process is correlated to a complete dephosphorylation of the capsid protein at all six phosphosites. These results, together with the known requirement of capsid phosphorylation at earlier stages in the viral lifecycle, suggest that the hepadnaviral capsid undergoes a dynamic change in phosphorylation in order to fulfill its multiple roles at different stages in the viral lifecycle. Dephosphorylation of the maturing capsids may provide the signal that triggers their envelopment and secretion, perhaps facilitated by the capsid structural changes that we detected by AFM. These maturation-associated differences in the capsids' capacity for expansion, as well as the dramatic dephosphorylation of the capsid protein, reflect previously unappreciated capsid dynamics, which we speculate may be important for capsid stability, envelopment, and/or disassembly during infection. Acknowledgements: This project was funded by NIH grants P41 RR10888 and S10RR15942 (to C.E.C.) and by NIH Grant R01 AI 43453 (to J.H.).

Session: Biomarkers and Mass Spectrometry Code: ThOAam Time Slot/Poster Number: 11:35

Proteomics Approach for Identification of Hemoglobin Variants and Post-Translational Modifications

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Introduction:
Among the more than 1200 recognized hemoglobin variants, many mutations have been found to be related to human diseases. Additionally, post-translational modifications of hemoglobin, to a large extent, may also contribute to biological function and act as a disease marker or contribute to disease pathology. In this study, a MS-based technology platform using MALDI-TOF MS, VC-MALDI-FT MS, ESI-qQq-FT MS/MS and LC-MS/MS have been explored to analyze a large number of human blood samples with diverse variants and post-translational modifications in the hemoglobin chains. While most variants have been identified consistent to their gene-based DNA sequence, PTMs and some variant types have been detected and located only by the current integrated methodology.

Methods:
Whole blood was diluted and cleaned by centrifugal filtration (Millipore) to remove cellular debris and salts. Trypsin digestion and AspN digestion of the intact globin chains were performed for peptide mass mapping and MS/MS. Intact hemoglobin chains were analyzed and top-down sequenced via ESI-qQq-FT MS/MS (quadrupole-FT hybrid constructed in-house), and the peptide mapping was performed on the same instrument. Additionally, digests were analyzed by MALDI-TOF MS (Bruker Reflex IV), MALDI-FT MS/MS (vibrational-cooling MALDI-FT constructed in-house), and online LC-MS/MS (QTOF-API-US, Waters Corporation). Data were processed and searched against SwissProt and custom programmed Hemoglobin/PTM databases using commercially available (ProteinLynx Global Server 2.1, Waters) and software written in-house.

Abstract:
Minimal requirements for purification, derivatization or separation of the blood samples considerably simplified the sample preparation and reduced artifacts associated with sample purification which may perturb PTMs. MALDI-TOF MS was carried out as first-pass for peptide mapping. More accurate mass mapping has been achieved by VC-MALDI-FT MS. Nanospray ESI-qQq-FT MS was applied to the measurement of the intact hemoglobin chains. Calculation of the charge state and identification of the m/z of the monoisotopic mass was performed using software written in-house and yielded accurate mass measurement within a few ppm. Mutations and PTMs were observed at the intact protein level. Localization of the mutation(s) and PTMs was achieved using a combination of top-down sequencing, peptide mapping and MS/MS peptide sequencing. For online LC-MS/MS of the hemoglobin digests, data analysis was fully automated. By using an iterative approach to peptide sequencing, pre-programmed hemoglobin database and pre-programmed PTM database, data analysis time was dramatically reduced, and more accurate assignments were obtained. For example: an alpha chain C-terminal truncation from a clinically relevant sample was unambiguously characterized by ESI-qQq-FT MS for intact protein mass, top-down sequence analysis, AspN peptide mapping, and single peptide MS/MS sequencing. A sickle beta chain identification was achieved as well via the mass of the

intact protein and tryptic peptide mapping. Over 75 clinically interesting samples, including diverse hemoglobin variants, have been identified using this MS-based proteomics approach. The results were consistent to their DNA sequencing results. Additionally, post-translational modifications have also been revealed by this method. In summary, this MS-based platform technology allows reliable and robust detection and localization of variants and PTMs simultaneously. This method demonstrates the potential for clinical use. The strategies for the application of different kinds of MS and data analysis will be discussed, with emphasis on these clinically relevant samples.

Session: Carbohydrates/Oligosaccharides Code: ThP02 Time Slot/Poster Number: 037

Using dissociation energetics to propose a mechanism for sialylated oligosaccharide fragmentation

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Introduction:
Sialylation of oligosaccharides affects the fragmentation and the energetics of dissociation. Using dissociation energy profiles of asialo- and sialylated oligosaccharides, the mechanism of glycosidic bond cleavage was observed to depend upon the number of sialic acid residues. Analysis by negative ion nanospray-MS/MS on a quadrupole-orthogonal time-of-flight instrument revealed that sialylated ions require substantially more energy to undergo fragmentation than asialo-forms. In asialo forms, deprotonation of a hydroxyl oxygen destabilized the glycosidic bond, but with sialylated forms, the charge resided on the sialic acid, and more energy was required to fragment the glycosidic bond. The energetic nature of deprotonated asialo-glycan ions facilitates glycosidic bond fragmentation during CID, while deprotonated sialylated glycan ions are lower in energy and resist glycosidic fragmentation.

Methods:
Lewis A, Lewis X, Sialyl Lewis A, Sialyl Lewis X, LNT, LNnT, DSLNT, LST-a, LST-d, and LST-c were analyzed using negative ion nanospray on an ABI Pulsar i QStar QoTOF MS. Solutions were sprayed from 30% MeOH in water with 0.1% ammonium hydroxide at concentrations between 1 pM and 10 pM. Tandem mass spectra of either singly or doubly charged precursor ions were acquired at varying collision energies, -2.5V to -47.5V. Spectra were collected for one minute and averaged for analysis. Breakdown curves for ions of interest were plotted as the percentage of the total ion intensity versus the collision energy. The threshold for peak selection was held to 5% of the intensity of the base peak.

Abstract:
Sialylation of milk oligosaccharides significantly changes the dissociation energetics of this class of compounds. In negative ion nanospray, the energy required to deplete the precursor intensity by 50% of neutral LNT and LNnT is roughly -12V; however the sialylated forms, LST-a and LST-d respectively, are much more stable and require roughly -37V to fragment to the same extent. LNT and LNnT produce abundant Y2 and Y3 ions at low energies, indicating the destabilization of the glycosidic bond, while sialylated structures produce abundant B1 ions showing the loss of a sialic acid residue as well as higher energy C ions. When comparing the energetics of asialo- and sialylated forms of Lewis antigens, large differences were also seen in the collision energy required to fragment the precursor ion to 50% of its initial intensity. Lewis X and Lewis A required -6V, while the sialylated forms required four times the amount of energy, -27-29V. Charge is produced in neutral sugars by deprotonation of a hydroxyl oxygen during the ionization process. This anion is evidently energetic enough to undergo glycosidic bond cleavage at relatively low collision energies. As the ions have gained energy during the ionization process, the energy for complete fragmentation during CID is minimal. The sialylated sugars have the negative charge residing on the terminal sialic acid, remote from the glycosidic oxygen; therefore, to cause glycosidic bond fragmentation energy needs to be added to the system in order to induce homolytic bond cleavage or deprotonate one of the hydroxyl oxygens. Sialylated sugars thus require more energy during the CID process to induce glycosidic cleavages.

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Session: MS Contribution to Immunology Code: ThP26 Time Slot/Poster Number: 422

Novel machines that control cyclin A and the cell cycle of B cells through chromatin remodeling and transcription factor recruitment

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Introduction:
We describe mass spectrometry (MS) identification of participants in a newly described multiprotein complex associated with the double bromodomain protein Brd2, to explore B cell proliferation. Brd2 is related to the basal transcription factor TAFII250. In transgenic mice, B cell-restricted constitutive expression of Brd2 transcriptionally activates cyclin A, causing B cell leukemia and lymphoma. Brd2 partitions between free and complexed forms in a regulated manner. Brd2 complexes recruit E2Fs and histone H4–directed histone acetyltransferase to the cyclin A promoter, contributing to cell cycle control in normal B cells. Hypothesis: Brd2-containing proteins provide a "scaffolding" or platform function for transcription or chromatin remodeling machines, anchoring them to promoter nucleosomes. The time-order and identity of Brd2 associated proteins reveals functions.

Methods:
B cell extracts were subjected to anti-Brd2 antibody affinity chromatography. Columns were washed extensively and pH drop eluted complexes. After pH neutralization, complexes were dialyzed against ammonium bicarbonate. Trypsin digestions were performed, then quenched by Speedvac drying. On-line capillary LC-MS with automatic tandem mass spectrometry (MS/MS) was performed on a QTOF-API-US quadrupole-time-of-flight mass spectrometer coupled with capillary LC system. Reversed phase capillary HPLC column chromatography with linear gradient elution was performed over 60 minutes. MS and MS/MS were acquired in positive polarity mode with mass accuracy within 10ppm (lockmass) 70ppm external calibration. Data from LC-MS/MS were analyzed against Swiss-Prot (SP)/TREMBL and user-programmed databases. Protein-Lynx Global Server 2.05 and 2.1 were used for proteomics data analysis and protein identification.

Abstract:
We describe the first affinity purification and MS analysis to identify components of a mammalian Swi/Snf complex. We identify components of Brd2 transcription complexes, including endogenous histones (also the first such report), as expected from association between bromodomains and histones. The Brd2-associated set of Swi/Snf components we report may define a specific Brd2-dependent chromatin-remodeling complex that regulates transcription. Innovation: The cell cycle of B cells has never before been analyzed at this level of detail. Comparative analyses of the Brd2 complexes in B cell lymphoma are likely to be informative of mechanisms of proliferation and lymphomagenesis. The results have broad significance for our understanding of adaptive immunity and B cell malignancy.

Session: Bioinformatics Code: TP22 Time Slot/Poster Number: 402

MASSPIKE (Mass Spectrum Interpretation and Kernel Extraction) for Biological Samples

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Introduction:
A suite of data reduction algorithms, called MASSPIKE has been developed for improving, enhancing, integrating and applying recently developed methods for isotopic cluster identification, charge state determination, deisotoping, and deconvolution for isotopically resolved mass spectra. The final output is shown to be an accurate and complete list of the monoisotopic mass information in the spectrum, which can be used to search the protein databases with high mass accuracy.

Methods:
Previously developed algorithms for isotopic cluster identification were improved in order to identify overlapping clusters of very low and high charge states. For a given molecular mass, elemental composition was determined using the averagine model for proteins. Rockwood's Mercury6 algorithm was incorporated into the program to generate theoretical isotopic distribu tions for a given elemental composition. The previously reported charge state determination routine (Matched Filter approach) was modified using improved normalization of the experimental and theoretical isotopic distributions before determining the charge sta te. Special focus has been given to resolving overlapping isotopic distributions with different charge states. All these methods w ere integrated and are being incorporated into the BUDA (Boston University Data Analysis) open source software.

Abstract:
MASSPIKE was successful in resolving 9 overlapping isotopic distributions, with multiple distributions sharing isotopic peaks, in the spectrum of a biologically derived sample (Ubch10 protein from an immunoprecipitated whole cell lysate). The complete analysis of Ubch10 spectrum elucidated 75 isotopic distributions which are being assigned to the corresponding theoretical masses. The Matched Filter approach used for charge state determination was found to work correctly in 91% cases when a total of 775 isotopic distributions of myoglobin with charge states ranging from 8-22 were investigated. A deisotoping module has been developed to align the experimental and theoretical distributions and was tested using 3150 Monte Carlo simulations. These tests revealed that the Maximum Likelihood alignment method works better, correctly aligning 85% of the time, as compared to the least squares error method which gave 76% correct results. This comparison was done using a distribution was generated with only 100 ions. MASSPIKE was applied to the intact protein and top-down ESI spectra obtained from a number of samples obtained from biological sources. Alpha chain of Hemoglobin from human blood samples was used to distinguish between a mutated alpha variant and a normal person. Beta chain of Hemoglobin revealed differences between a beta sickle and normal beta chain. Work is in progress to use this method for samples of the Amyloidogenic TTR protein. Monoisotopic masses for large intact proteins (20-23 kDa) were determined from a single isotopic cluster with an accuracy of less than 5 ppm.

Session: Instrumentation: FTMS Code: TP11 Time Slot/Poster Number: 208

Design of the Cryogenic Fourier Transform Mass Spectrometer

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Introduction:
Fourier Transform Mass Spectrometry is limited in its base performance characteristics (resolution, sensitivity, mass accuracy, etcetera) by three fundamental instrumental design considerations, pressure, magnetic-field strength, and preamplifier noise. A new FTMS design has been developed and is being constructed in which the FTMS is built inside a narrow, cold bore magnet. Because the cold bore acts as a cryopump, the ion transfer vacuum chamber can be made substantially narrower than normal, allowing use of narrower bore magnets than are normally used in FTMS while still achieving the needed <1e-9 mbar pressures. The cold surfaces can also be used to chill a preamplifier to cryogenic temperatures for improved signal/noise performance.

Methods:
A specially designed magnet has been designed to couple with a standard external source MALDI FTMS design. The FTMS consists of an external MALDI source, an accumulation hexapole, a transfer hexapole, and a capacitively coupled open cylindrical cell, pumped by two ~200 l/sec turbomolecular pumps. The titanium in-bore vacuum tube is designed to have high radiative heat coupling to the interior of the magnet chamber, but low radiative heat transfer down the tube. Furthermore, the tube walls are as thin as possible to decrease conductive heat transfer to the 4K region. The data system is a modular FTMS data system based on National Instruments boards.

Abstract:
The complete design for the system is finished and will be presented. The magnet is designed to hold ~100 l of liquid helium, which will be shielded from direct radiative exposure to room temperature by an aluminum radiation baffle which will be held at ~50k. Both the 4K region and the shield will be cooled by a two stage cryorefrigerator which can cool ~1.5W at 4K and >50W at 50K. Due to the narrower 3” bore, the magnet will be designed as a 15 Tesla magnet which will achieve acceptance specifications at 14 Tesla. The bore is designed with two copper mounting brackets which will function both to align the inner FTMS vacuum chamber with the magnet and as thermal anchors. However, due to the large surface areas involved, the primary heat transfer will be due to direct radial radiative heat transfer, which will allow most of the heat flowing down the vacuum chamber walls to be transferred to the 50K shield before it reaches the 4K region. Full drawings of the system will be presented. Thermal analysis of the vacuum chamber and ion optics will be presented. These analyses show that it is possible to build a vacuum chamber which can transmit less than 0.5W of heat from the source to the cell. The instrument is currently being constructed, and if possible, initial spectra will also be shown in this presentation. This work is supported by NIH/NCRR P41RR10888 and by NIH/NHLBI N01HV28178.

Session: Carbohydrates Code: TOEpm Time Slot/Poster Number: 03:00

The role of mobile protons in negative CID tandem MS of sulfated oligosaccharides

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Introduction:
Most classes of animal glycans are acidic, and the use of negative ionization is a natural choice. The aim of this work is to explain product ion fragmentation patterns for negatively charged acidic glycans. Such an understanding is important to develop tandem MS strategies in glycomics. This work shows how the presence of uronic acid residues influences the product ion patterns of sulfated glycosaminoglycan oligosaccharides. Uronic acid protons becomes mobile during the CID process and destabilize glycosidic bonds. In the absence of such mobile protons, the ions are significantly more stable and require more energy for glycosidic cleavage. Both charge location and potentially mobile protons strongly influence product ion pattern for acidic oligosaccharides.

Methods:
Oligosaccharides were produced by digesting chondroitin sulfate with chondroitinase ABC or testicular hyaluronidase and purified by size exclusion chromatography using a Superdex Peptide column (Amersham Pharmacia). Oligosaccharides were methyl esterified using methanolic HCl. All mass spectra were acquired in nano-electrospray mode using a Applied Biosystems/Sciex Qstar Pulsar-i mass spectrometer. Samples were dissolved at a concentration of ~1 micromolar in 30% methanol and sprayed through uncoated borosilicate glass tips pulled to a 1 micrometer diameter orifice. Steady signals were typically observed with -1100 V spray potentials.

Abstract:
The presence of acidic groups (sulfate, phosphate, sialic acid) strongly influences the pattern of product ions resulting from CID of negative oligosaccharide ions. Tandem MS of chondroitin sulfate oligosaccharides of the form (ΔHexA)(HexA)n-1(GalNAcSulfate)n, where ΔHexA = 4,5-unsaturated HexA, were acquired before and after methyl esterification of uronic acid residues. The native structures fragment under significantly less energetic conditions than do the methyl esterified glycans. Abundances of cross-ring cleavage ions increase for the methyl esterified relative to the native glycans. Chondroitin sulfate oligosaccharides lacking a Δ-unsaturated uronic acid residue do not have cross-ring cleavage as a fragmentation channel. In their methyl esterified forms, these ions require approximately -22 V collision energy to reduce the precursor ion intensity by 50%. For the native structures, only -15 V collision energy is required.

These results are consistent with the conclusion that, despite the deprotonated precursor ion, remaining carboxyl protons become delocalized as the ion temperature rises during the CID process and associate with glycosidic oxygen atoms. This association predisposes the glycosidic bond to scission, in a manner similar to that described for positively charged glycans. Methyl esterified precursor ions, lacking carboxylic protons, require significantly higher fragmentation energies than do native ions. The abundances of cross-ring cleavages increase because of the lack of mobile protons to destabilize glycosidic bonds.

Both the location of negative charge and the presence of protons that become mobilized during the CID process strongly influence the observed product ion patterns. In the absence of mobile protons, acidic glycans resist glycosidic bond cleavage because charge is located distant from the glycosidic bonds. These results have clear implications regarding the design of on-line LC MS/MS conditions for glycomics experiments.

Financial support from NIH/NCRR P41RR10888 is gratefully acknowledged.

Session: Proteomics: Biomarkers Code: WP25 Time Slot/Poster Number: 481

Mass Spectrometric Analysis of Eicosanoids and Proteins in Exhaled Breath Condensate

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Introduction:
Exhaled breath condensate (EBC) harbors a rich mixture of volatile species, lipids, and proteins. Among these are the eicosanoids, such as prostaglandins and leukotrienes, and cytokine proteins, such as the interleukins, which have previously been associated with impaired pulmonary function. Collection of EBC is non-invasive and can be performed easily, even by patients with pulmonary disease. It has been suggested that EBC reflects the composition of the airway lining fluid (ALF); therefore, measurement of these markers in EBC may improve our understanding of the pathogenesis of diseases such as chronic obstructive pulmonary disease and asthma. 1-2

Methods:
Condensate was collected from both control subjects and asthma clinic patients by having each subject breathe tidally for 15 minutes into an R-tube that was cooled to -20 °C. After collection, the samples were stored at -80 °C, and then were prepared using several methods, including lyophilization, separation on 1D electrophoretic gels, solvent extraction, and solid-phase extraction. After one or more of these procedures, samples were analyzed by MALDI-TOF-MS and LC/MS/MS.

Abstract:
Previous reports on ELISA measurements of individual eicosanoids in EBC indicate that these compounds are present at levels in the range of pg/mL to ng/mL3. Our total protein assays performed on individual control samples of EBC indicated the concentration of total protein ranged from 10 to 20 μg/mL. To compensate for the low concentrations, EBC samples were pooled in groups of 2-20 and concentrated 10-100-fold using the methods described above. Several eicosanoids associated with inflammation and oxidative stress were tentatively identified in the EBC of asthma clinic patients using a microbore HPLC column and online ESI-MS. With MALDI-TOF-MS, intact proteins were detected over the range from m/z 5000-50,000 in the spectra obtained for samples of EBC obtained from control subjects. Separation on 1-D gels, in-gel protease digestion and capillary LC/MS/MS analyses of the resulting peptides has enabled identification of multiple proteins in EBC of control subjects. We are investigating the occurrence and relative abundance of these proteins in the EBC of patients. This research is supported by NIH P41 RR10888, S10 RR15942 and S10 RR10493 and the Flight Attendants Medical Research Institute's Young Clinical Scientist Award.

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Session: Proteins: General Code: MP24 Time Slot/Poster Number: 373

Characterization of Distinct E-cadherin Containing Protein Complexes

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Introduction:
E-cadherin is a membrane glycoprotein that provides strong, Ca2+-dependent epithelial intercellular adhesion. Through mediation of cell-cell contacts, E-cadherin functions in various morphogenic processes as well as in cell transformation (1-4). E-cadherin cell-cell contacts are dynamic and undergo continuous rearrangement depending on cell context. Therefore, the recruitment of various proteins to the adhesion complex define the overall stability of E-cadherin mediated adhesion. Separation and identification of individual adhesive complex components provide insight on E-cadherin cellular activities.

Methods:
Ion Exchange chromatography with Q Sepharose is used to separate several classes of E-cadherin protein complexes from cancerous (A253) and normal (MDCK) epithelial cells. Immunoprecipitation to capture E-cadherin complexes followed by immunoblotting and protein analysis by LCMS-MS/MS, using an Applied Biosystems Sciex i QoTOF mass spectrometer (QStar) coupled to a Waters CapLC, is also done.

Abstract:
Our results indicate that E-cadherin exists in different types of protein complexes that vary among cell types (i.e., normal and cancerous epithelial tissue) and, over time, even within the same cell type; such changes are accompanied by differences in the aggregation properties of the cells. These findings suggest that the dynamic balance among such complexes governs the overall stability of E-cadherin mediated cell-cell contacts.

This research is supported by NIH grants P41 RR10888 and S10 RR15942 (CEC) and R01 DE10183-11 (MAK). KEB-R receives support from AG00115 (PI P. Polgar).

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Session: Instrumentation: FTMS Code: TP11 Time Slot/Poster Number: 211

Use of the Filter Diagonalization Method in the Study of Space Charge Related Frequency Modulation in FTMS.

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Introduction:
The Filter Diagonalization Method (FDM) is a recently developed signal processing algorithm based on quantum mechanic's mathematical formalism of the harmonic inversion problem. FDM is shown to provide extremely high precision in finding resonance frequencies with < 1ppm accuracy on small number of transient data points e.g. 10k. It was used in frequency shift chasing experiments for the purposes of determining intra-transient frequency shifts and using them for reference deconvolution and study of space charge effect.

Methods:
In this study we used in-house C++ implementation of the FFT Square Window FDM, which will be available as open source software in an upcoming release of the Boston University Data Analysis (BUDA) system. Theoretical spectra were generated by using in-house simulation software with 1 mega-point length and 1MHz sampling rate. Real spectra of Ubiquitin, Myoglobin, Calmodulin, Cytochrome C as well as Substance P, and C60 acquired on in-house built MALDI and ESI FTMS instruments were also 1 mega-point length with 1MHz acquisition rate. Frequency chasing experiments were performed on transient domains ranging from 256 to 16384 data points starting with the 0 offset and shifting 1/8 of the domain into the transient. Kwin used ranges from 4-11 points.

Abstract:
In the course of the study we have observed FDM frequency precision of < 10 ppb on the theoretical transients and transients with no apparent space charge. Frequency shift plots for real spectra of biological compounds (see methods) were obtained and results were tested against the theoretical spectra with modeled frequency modulation. It is possible to use these findings for reference deconvolution to improve resolution and accuracy of the spectra. Compared to FFT, FDM proved to be superior in terms of precision but much slower, even though it has the same quasi-linear time complexity, so that the FDM is more appropriate for frequency chasing experiments rather then direct competition with the FFT. However, in spite of FDM's superior precision, mass accuracy analysis showed no improvement over FFT derived peak centroids. The FDM results show that space-charge frequency shifts in a typical transient are surprisingly high (over 20ppm's), but the FFT effectively averages these shifts away to give close to 1ppm overall error. These could be potentially corrected by reference deconvolution using FDM to generate frequency shift plots. Acknowledgements: This work was supported in by NIH/NCRR P41-RR10888 and NIH/NHLBI NO1 HV28178. We'd like to acknowledge Bogdan Budnik, Jason Cournoyer, Parminder Kaur and, Cheng Lin for experimental assistance and helpful discussions.

Session: FTICR Instrumentation and Methods Code: WOFam Time Slot/Poster Number: 11:35

Identification, Localization and Differentiation of O-GlcNAc and O-Phosphoryl Modified Peptides Using ECD and SORI CAD

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Introduction:
Substitution with O-GlcNAc and O-phosphoryl groups are both reversible post-translational modifications found on many nuclear and cytosolic proteins. The occurrence and location(s) of these modifications change in response to extracellular glucose concentrations, morphogens and the cell cycle and, hence, they are implicated in various signal transduction pathways. Sites in several proteins that carry this modification, such as the C-terminal domain of RNA polymerase II, exhibit, under varying conditions, either the phosphate or the O-GlcNAc modification, and the status of these modifications governs the activity of the protein (Yin-Yang relationship). Thus, in order to understand their functional significance, it is important to know, for each state of the proteins, the precise sites and level of occupancy by both phosphorylation and glycosylation.

Methods:
Both of these modifications are difficult to study as they occur in substoichiometric concentrations and are labile under collisional activation conditions, the O-GlcNAc being especially labile, and therefore difficult to detect using conventional tandem MS strategies. In order to employ mass spectrometry for the detection of these extremely labile modifications, both the ionization methods and fragmentation methods used during analysis have to be "soft" and specifically directed to keep the modifications intact. Therefore, to study these modifications with maximum retention of specificity and sensitivity, we chose to avoid derivatization methods and, instead, used instrumentation recently developed in-house, a Vibrational Cooling MALDI FTMS (VC MALDI FTMS) and an ESI-qQq FTMS.

Abstract:
Our FTMS instruments have proven to be ideal for the analysis of both O-GlcNAcylation and O-phosphorylation analysis, as both instruments transfer the modified peptides into the ICR cell in an intact state. On both systems, an element essential for the success of the analyses was the sensitivity of the instruments. Sensitivity on the ESI qQq FTMS is attributed to use of the Q1 for isolation and Q2 for the accumulation of the intact modified peptide, whereas, with the VC MALDI instrument, the ionization process and efficiency of ion transfer into the ICR cell were important. A universal method of sequence analysis applicable to both of these post-translational modifications that avoids the requirement for any derivatization step was preferred and thus the use of different fragmentation techniques in the ICR cell was investigated. It was found that both Electron Capture Dissociation (ECD) and low-energy tandem SORI-CAD experiments show a remarkable ability to extensively fragment O-GlcNAc modified and phosphorylated peptides, yielding almost complete sequence information without causing the loss of the labile modifications. The low energy tandem SORI CAD experiments were explored as a means to fragment singly charged ions in conjunction with MALDI ionization, since ECD cannot be performed on singly charged ions. Both C-Myc and C-terminal RNA polymerase peptides containing both O-GlcNAc and O-phosphoryl modifications were investigated. Fragmentation data was obtained for these peptides and it allowed identification of the peptides and the localization of both O-GlcNAc and O-phosphoryl groups. This study indicates that it is possible to investigate these modifications, using the methods described here, in further proteomic type studies designed to explore the biology related to these modifications. Acknowledgements: This

research was supported by NIH/NCRR P41RR10888, NIH/NHLBI N01HV28178, and by a research collaborative agreement with MDS Sciex

Session: Proteomics: Sample Prep & Methodologies Code: ThP33 Time Slot/Poster Number: 553

DIRECT PROTEIN 2D-LC MALDI WITH ON-TARGET DIGESTION FOR HIGH-THROUGHPUT PROTEOMIC ANALYSES

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Introduction:
Large-scale proteomic analyses necessitate high-throughput sample preparation techniques. However, highly complex mixtures require multi-dimensional fractionation prior to MS analysis to maximize the yield of useful MS data. This geometrically expands sample numbers, dramatically intensifying processing load, commonly (e.g., HPLC) involves dilution, often demanding sample concentration, and typically requires multiple steps of sample handling, including transfer to different reaction vessels, that are time consuming, lead to sample losses and potential contamination. We have explored the use of a novel, simple, inexpensive (non-robotic) 96-well array technology, the BD™ MALDI Concentrator, to conduct one-pot on-target sample preparation for MALDI-MS analysis. We have applied this technology with transfers from 2D protein LC direct-to-target for peptide mapping by MALDI-TOF MS and MALDI-FT MS.

Methods:
Protein standards were digested in-solution on-target/in-well using the BD Biosciences MALDI Concentrator™, concentrated under vacuum and co-crystallized with matrix under differing conditions. Two-dimensional HPLC fractionation of protein mixtures and human plasma was conducted with the Beckman PF2D™. Eluate fractions were collected directly into the wells of the BD device, and optimized conditions were used to concentrate, digest in-solution on-target/in-well, and co-crystallize the samples with matrix. MALDI mass spectra were obtained with a Bruker Reflex IV MALDI-TOF MS and a home-built VC MALDI-FTICR MS. Results were compared, with fractionated protein mixtures and peptide standards that had been digested, concentrated and co-crystallized with matrix by conventional methods. Results were further compared with other methods for LC MALDI deposition.

Abstract:
One-pot on-target/in-well digestion, concentration and sample/matrix co-crystallization under optimized solvent conditions readily yielded MS analyses with minimal sample loss from 1 pM protein standard and as little as 10 fM of peptide standard from up to 50 µl starting solution. This amounted to good recovery of MS signal from picomolar and sub picomolar peptide concentrations. The coupling of 1D and 2DLC to MALDI MS through the collection of LC eluate fractions directly into the 96-well array MALDI target affixed with the BD Biosciences MALDI Concentrator™ enabled rapid, high-throughput protein fractionation, digestion, peptide matrix co-crystallization, and MALDI MS analyses with minimal sample handling. Coupling of 1D RP LC of peptides obtained from protein digests enabled the use of MALDI as an off-line method for MS analysis. The implementation of this technology upon the fractionation and MS analysis of human plasma led to the identification of several human plasma proteins. Sensitivity and robustness of this methodology will be discussed and compared with other methods of 1D and 2D LC MALDI methods.

Session: Instrumentation: FTMS Code: TP11 Time Slot/Poster Number: 218

Design of a preamplifier for Cryogenic Fourier Transform Mass Spectrometry using GaAs High Electron Mobility Transistors

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Introduction:
Noise in an FTICRMS preamplifier circuit can be minimized in the following ways: 1. Building a fully - differential amplification circuit to cancel correlated electrical noise on the two input channels. 2. Using modern low noise active devices. 3. Cooling the circuit to low temperatures to reduce Johnson noise in the input resistors. GaAs is a compound semiconductor material which has higher electron mobility and low power consumption compared to silicon and can be operated at cryogenic temperatures (~4K). Here we report ongoing progress in the developments of cryogenic low noise preamplifier based on these devices for FTMS.

Methods:
The designed preamplifier is mounted on the detection plates of the cell. This preamplifier used GaAs HEMTs which have a Noise Figure of 1.2 dB at 12 GHz. One limiting factor in the detection circuit is the intrinsic cell capacitance, which is ~12 pF per plate for the open cylindrical cell used. This capacitance of the plates together with the input resistance in the amplifier circuit adds a high frequency pole which defines the bandwidth of the preamplifier. The GaAs HEMT differential preamplifier circuit is mounted on a specially designed PCB for in-vacuum operation.

Abstract:
The current preamplifier design has achieved a voltage gain of 5 and a 3 dB bandwidth of 1.6 MHz, using a 1 Mohm input resistor at room temperature. This design should allow about a five-fold improvement in signal/noise ratio compared to current designs, and has been created using components that are compatible with cryogenic temperature operation, which will potentially reduce the noise another ~8 fold. It is expected that the bias point of the transistors will change with temperature, thus requiring adjustment of the input bias potential at low temperatures. To test this, a simple cryostat is being constructed for optimizing this amplifier at low temperatures. Generally, a preamplifier should be designed to achieve maximum gain, but the power and bandwidth constraints limit this value. In order to minimize heat generation in a cryogenic environment, the HEMT operating point is set at IDS = 2 mA and VDS = 2 volts. This has kept the power dissipation in the circuit to a maximum of 10 mW. The input capacitace of the HEMTs used at the input of the preamplifier is around 20 fF, which is insignificant compared to the cell capacitance. For the m/z range of > 100 Da, the preamplifier needs a bandwidth of ~1 MHz. The achievable 3 dB bandwidth with a 1 Mohm input resistor is calculated to be ~800 KHz, which agrees with spice simulations. Thus, ions with m/z values < 100 Da can also be detected, as the roll off after 800 KHz is ~3 dB/decade. While testing on bench, the cell capacitance is modeled by a 20 pF capacitor to ground at each of the inputs. Acknowledgements: This work was sponsored by NIH/NCRR P41RR10888 and NIH/NHLBI N01HV28178.

Session: Proteomics: New & Improved Methods Code: TP29 Time Slot/Poster Number: 492

Comparative Proteomics of Abundant Protein-depleted Plasma from Patients with Sickle Cell Disease-related Pulmonary Hypertension

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Introduction:
Human plasma proteomics is becoming a powerful means to investigate the molecular etiology and pathogenesis of disease, as well as to identify disease biomarkers. Separation of plasma proteins by successive chromatofocusing and reversed-phase HPLC (2DLC), particularly following abundant plasma protein depletion, greatly facilitates MS analyses and can provide differential protein expression maps that direct research into proteins displaying significant disease-related changes. We explored a comparative proteomics approach based on 2DLC followed by ESI-FT MS, MALDI-FT MS, MALDI-TOF MS and LC MS/MS analyses of abundant protein-depleted plasma from patients with sickle cell disease (SCD), one of the most common human genetic diseases, and its accompanying complication of unknown etiology, pulmonary hypertension (PH).

Methods:
Plasma, obtained from age, sex and racially-matched patients in each of the following groups, SCD with PH, SCD without PH, PH without SCD, and healthy controls, was albumin-depleted, or was abundant plasma-protein depleted by off-line HPLC, then was separated by two-dimensional HPLC on a Beckman PF2D™. Differential 2D expression maps were generated with the Beckman software ProteoVue™ and DeltaVue™, and fractions showing appreciable differences in protein expression levels, pI, or reversed-phase retention time, were targeted for MS analyses. Intact protein and tryptic peptides were analyzed using a home-built ESI-FTICR MS, a home-built MALDI-FTICR MS, and a Bruker Reflex IV MALDI-TOF MS. Further analysis was performed on a Waters LC QTOF MS.

Abstract:
Coupling 2DLC of abundant protein-depleted plasma to MS, we have conducted a comparative proteomic analysis of the plasma of SCD, PH, and healthy patients. Differential 2D expression mapping has allowed us to target only those proteins in the plasma that changed significantly in abundance, pI, or retention time in correlation with disease. Ongoing MALDI-TOF MS, ESI-FTICR MS, VC MALD-FTICR MS, and LC QTOF MS analyses have allowed us to identify several abundant and medium-abundance proteins that appear to vary with disease. Among others, we have identified two proteins from SCD patients with PH, transferrin and apolipoprotein A1, which exhibit pronounced changes in post-translational modifications. Modifications of transferrin, an important molecule in iron metabolism and transport, and apolipoprotein A1, a molecule required for HDL-mediated activation of endothelial nitric oxide synthase, could play a role in the pathogenesis of PH in SCD, or could serve as important biomarkers of disease. Further MS analyses of these and other differentially expressed proteins will be instrumental in determining the role of oxidant stress in the pathogenesis of PH in SCD patients. Differential plasma proteomics such as this study may have a significant impact on the detection and treatment of SCD, PH and other human diseases.

Session: Proteomics: Sample Prep & Methodologies Code: TP31 Time Slot/Poster Number: 546

Proteomic approach to mapping the stimulus-specific signaling pathways in periodontitis

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Introduction:
Periodontitis is an inflammatory disease with host-parasite interactions which contribute to connective tissue destruction and alveolar bone resorption. Porphyromonas gingivalis (P.g.) a black-pigmented Gram-negative anaerobic bacterium, is a major pathogen in the development and progression of periodontitis. Cell wall and appendage structures, such as lipopolysaccharide (LPS) and fimbriae, play important roles in the induction of innate immune responses, including cytokine production by localized and circulating monocyte/macrophage. Based on preliminary data, live P.g. stimulates unique pro-inflammatory signal transduction pathways in human peripheral blood monocytes (PBM) as opposed to bacterial components, such as LPS or fimbriae. In these experiments we test at the level of protein expression that unique signaling pathways are differentially induced by live P.g.

Methods:
Human PBM (95% pure) freshly elutriated from healthy donor and monocyte-like THP-1 cells were exposed to live P.g., P.g. LPS and P.g. fimbriae.1-dimensional (1-D) and 2-dimensional gel electrophoresis (2-DGE) were performed on the cell lysate proteins of PBM and monocyte like THP-1 differentially produced. The expressed proteins were excised and subjected to tryptic in-gel digestion. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) and reversed phase high performance liquid chromatography (RP-HPLC) in combination with MS were performed. MS/MS will be performed to characterize novel proteins.

Abstract:
2-DGE was performed on cell lysate proteins differentially produced by PBM when challenged by live P.g. vs. unchallenged conditions. The 2-DGE profiles identified several differentially expressed protein spots. To rule out protein spots resulting from P.g. proteins we performed also 2-DGE of P.g. and used it against the experimental gel. Differentially expressed proteins for P.g. challenged PBM were excised, in-gel digested with trypsin and mass spectra were obtained to characterize them. Results show that specific proteins are induced or down-regulated by live P.g.

Similar procedures were implemented for LPS and fimbriae induced PBM. 1-D gel electrophoresis was performed on the PBM challenged by live P.g., LPS and fimbriae. This approach allowed for identification of biologically significant proteins that might be in low abundance. The separated protein bands were subjected to in-gel tryptic digestion and MALDI-MS spectra were obtained. On-line data base search was undertaken to identify the proteins. This approach may still miss many proteins of biological significance. Thus, the resulting peptides were subjected to RP-HPLC separation prior to introduction into the MS. To improve separation during isoelectric focusing stage of 2-DGE, novel buffer conditions were used for extraction of proteins from the cell lysates of PBM and THP-1. The extraction buffer avoids the use of SDS, which hampers separation of proteins in the first dimension. The 2-DGE profiles show similar patterns of protein separation for control, live P.g.. LPS and fimbriae induced THP-1, making it easier for identification of differentially expressed protein spots by direct visualization. Analysis of the 2-DGE profiles identified several differentially expressed protein spots. MALDI spectra were acquired and protein identification was performed using online protein database search. Similar approach will be undertaken for human PBM. This work was sponsored by NIH/NCRR P41RR10888.

Session: Mechanisms of Peptide Fragmentation Code: WOEam Time Slot/Poster Number: 10:55

Free Radical Driven Isotopic Scrambling in the Electron Capture Dissociation of Linear Peptides

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Introduction:
Since its introduction as a tandem mass spectrometry (MS) technique, electron capture dissociation (ECD) has proven to be a valuable tool in the structural analysis of peptides and proteins, in that it preferentially breaks the backbone N-Cα bonds while keeping the labile post-translational modifications largely intact. These unique features lead to the proposed "nonergodic" mechanism, in which single N-Cα cleavage takes place prior to energy randomization. However, in recent ECD studies, extensive secondary cleavages in both cyclic and linear peptides were observed, suggesting a second, ergodic fragmentation pathway which appears to be free-radical driven. Here, we present a further investigation of the ECD process by studying the deuterium scrambling in the ECD fragments of several selectively isotope-labeled linear peptides.

Methods:
All experiments were performed on a home-built 7-Tesla Fourier transform ion cyclotron mass spectrometer (FT-ICR-MS) equipped with an external electrospray ionization (ESI) source. The electrosprayed peptide ions were mass selected via a front-end resolving quadrupole and externally accumulated for ~100 msec before being introduced into the ICR cell. Electrons were generated using an indirectly heated dispenser cathode. Both low energy ECD and hot ECD were performed by biasing the center potential of the dispenser cathode at either -0.2 V or -9 V relative to ground. Collisionally activated dissociation (CAD) experiments were performed both in the Q2 cell and in the ICR cell (SORI-CAD). The spectra were zero filled twice and Fourier transformed without apodization.

Abstract:
The free radical cascade mechanism requires that the radicals formed from the primary cleavage have time to migrate prior to the final, observed fragmentation, with the glycine alpha carbon positions being the preferred migration sites. If such is true, isotopic scrambling in some of the secondary fragment ions would be observed when the alpha hydrogens are replaced by deuteriums. To investigate these possibilities, two sets of peptides, with the sequence of RAAAGADGDGAGADAR and RAGADGDADGDAGAAR, respectively, were synthesized in both the normal isotope form and with the alpha carbon sites of the four glycine residues fully deuterated.
In the ECD spectra of all four peptides, both the radical and the even electron forms of the fragment ions were observed, with the radical form being 1 Da lighter. In the ECD spectra of deuterated peptides, however, additional peaks about 2 Da lighter than the even electron species can be seen in almost all isotopic distributions detected. These are the radical fragments that have lost a deuterium and gained a hydrogen, thus the ratio of their abundances to those of the corresponding non-scrambled radical fragments should indicate the degree of isotopic scrambling for individual fragmentation channels. It is found that cleavages at sites C-terminal to

aspartic acid positions showed effectively no scrambling, which likely results from these being the primary cleavage sites.

As a control experiment, Q2-CAD and SORI-CAD of these same peptides were performed. With CAD, scrambling still occured, but to a much lower degree compared to ECD. SORI-CAD performed with electron gun on and the cell, therefore, hot, showed no observale increase in deuterium scrambling, ruling out the possibility that the increased scrambling in ECD experiments occured prior to MS/MS.

Acknowledgments: this work was sponsored by the ACS Petroleum Research Fund and by NIH/NCRR P41RR10888

Session: Lipidomics Code: WP13 Time Slot/Poster Number: 217

LC-MS based method for the qualitative and quantitative analysis of complex lipid mixtures

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Introduction:
While nanospray MS is a good choice for the characterization of simple lipid mixtures, it is often not sufficient for the qualitative and quantitative analysis of highly complex samples1. Most separation methods described are limited, in that they either target only specific classes of interest2, or are not well suited for MS detection, the superior detection method especially when it comes to small amounts. We are developing a simple and reproducible three-step system for lipid analysis by adapting separation systems described earlier for LC-MS3,4. After an initial fractionation, we use normal phase HPLC-MS first, then optional reversed phase LC-MSMS. The system is used here for the analysis of human low density lipoprotein (LDL) lipids.

Methods:
Isolated and extracted LDL lipids and lipid standards are passed stepwise onto and eluted off Silica 60 resin with MTBE (methyl t-butyl ether) or chloroform, followed by methanol. Using two different solvent systems, these two fractions are further separated on a Waters/YMC microbore PVA-Sil column on a Agilent HP1090 HPLC, and detected by a Waters Quattro II mass spectrometer in positive and negative ion modes. Quantification is based on this step. Fractions obtained can be further characterized by reversed phase LC-MSMS using a C18 Atlantis capillary column on a Waters CapLC system interfaced to an Applied Biosystems Sciex Pulsar i QoTOF mass spectrometer (QStar), or by nanospray MSMS and precursor ion scanning on either mass spectrometer.

Abstract:
Lipid standards containing diverse nonpolar, phospho- and glycolipids have been reproducibly separated on the basis of polarity by elution with MTBE and methanol from Silica 60 resin. This step, when used for biological samples, also serves to protect the following column. The two fractions can be further separated on a PVA-Sil normal phase column with a heptane/MTBE and an MTBE/MeOH based gradient. These separations on the normal phase column allow for an at least semi-quantitative detection. The quality of the quantification depends mostly on the quality of internal and external standards available. Nanospray MS (MSMS, precursor ion scanning and neutral loss scanning) of the fractions collected from the PVA-Sil column allows the determination of the molecular species present. We currently are working on improvements of these results by reversed-phase LC-MS/MS, and we are searching for optimal external and internal standards for quantification. We recently used the system as described for the semiquantative analysis of human apolipoprotein B-675 LDL samples and compared them to standard LDL.
This research is supported by NIH grants P41 RR10888 and S10 RR10493 to CEC.
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Session: Lipids: Structural Analysis Code: WP13 Time Slot/Poster Number: 229

Structural characterization of toxin-binding gangliosides by TLC/VC-FTMS

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Introduction:

GM1 and GD1a gangliosides serve as trafficking receptors for the cholera toxin and related LTIIb toxin, respectively. LTIIb is not active in human intestinal cells because the LTIIb-GD1a complex does not move retrograde from the plasma membrane into the endoplasmic reticulum (1). Here, we test the idea that structural variation in the GD1a lipid anchor explains the failure of this ganglioside to act as a trafficking receptor. To address this problem, we are using our previously developed method of direct coupling of TLC plates with vibrationally cooled (VC) MALDI-FTMS. This allows direct TLC-MALDI-FTMS without adversely affecting the FT high resolution by the irregular surface of the TLC plate. Collisional cooling is necessary for stabilization and detection of intact gangliosides.

Methods:

We are using polarized intestinal epithelial cell line T-84 and monkey kidney Vero cells for ganglioside purification and functional studies on the mechanism of toxin biology (2). We have described ganglioside separations and instrumental parameters for VC MALDI-FTMS (3). In the current study, the samples are MALDI-desorbed directly off TLC plate surfaces with ~0.5 mm sampling steps and thermalized by a pulse of the cooling gas. Subsequently, fragmentation is performed by SORI-CAD (1.5% &Delta&omega, 500 ms, 12V) and IRMPD techniques (10.6&mu CO2 laser, 200-400 ms).

Abstract:

Preliminary results showed that, in addition to a variety of oligosaccharide headgroup compositions, the ceramide structure in gangliosides from both cell lines exhibit substantial heterogeneity. The high separation efficiency of the HP-TLC plate allowed for observation of numerous homologs following each scanning step. This was demonstrated by analysis of the "pure" synthetic gangliosides and whole brain extract. A high level of ganglioside fucosylation was observed in both cell lines. Vibrational cooling resulted in stabilization of the labile sialic acid and fucose glycosidic linkages, and this feature was highly advantageous for the analysis of the heterogeneous mixtures. Mass accuracy and resolution were not affected by desorption from the uneven TLC plate surface. Compared to SORI-CAD, IRMPD demonstrated more efficient fragmentation of both parent and product ions. Further analysis will focus on structural elucidation of the ceramide moiety in terms of length, degree of saturation, hydroxylation and branching. These factors are assumed to be responsible for the toxin traffic in an infected cell. Characterization of the individual long-chain base and fatty acid within each ceramide is required. Previous experiments have shown that cleavage of a ceramide is feasible by optimization of SORI-CAD/IRMPD techniques and/or doping the samples with alkaline salts.

Acknowledgement The project has been funded in part with Federal funds from the National Institutes of Health, under grant No. P41 RR10888 from the National Center for Research Resources and contract No. N01-HV-28178 from the National Heart, Lung, and Blood Institute to CEC; NIH DK48106 and DK57827 and DK34854 to WIL and DK02934 and an Investigator Development award from the CDHNF to AW. 1) Fujinaga, Y., et.al. (2003) Mol. Biol. Cell. 14, 4783-4793. 2) Wolf, A. A., et.al. (1998). J. Cell. Biol. 141, 917-927. 3) Ivleva V.B., et.al. (2004) Anal. Chem. 76, 6484-6512.

Session: Carbohydrates/Oligosaccharides Code: WP14 Time Slot/Poster Number: 259

An LC/MS/MS platform for glycoform quantification of chondroitin sulfate

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Introduction:
Glycomics, known as the study of the structure and function of glycans, is a rapidly growing field. Glycosaminoglycan (GAG) chains are made of repeating disaccharide units that are attached to proteoglycan core proteins on adherent animal cell surfaces and in extracellular matrices. Chondroitin sulfate (CS) is a glycosaminoglycan that consists of repeating disaccharide units of [(GlcAβ(1-3)GalNAcβ(1-4)]. Three types of CS exist, CS-A, CS-B, and CS-C. Presently, the field of glycomics lacks an effective analytical method for the isomeric differentiation and relative quantification of GAGs in small (1-10 microgram) biological samples. This work describes the development of a method for quantification of glycoforms using a stable isotopic labeling technique, and its application to the sulfated GAGs.

Methods:
CS samples, both standard and unknown, were partially depolymerized by chondroitin lyase ABC. The standard CS was then derivatized via a reductive amination reaction with 2-anthranilic acid, while the unknown CS was derivatized with 2-anthranilic-3,4,5,6-d4 acid. The derivatized CS samples were cleaned and the excess reagent removed via a cellulose microspin column. Equimolar mixtures of the standard and unknown CS samples were made. The isotopically labeled CS mixture was subjected to size exclusion liquid chromatography in a 10% acetonitrile, 50 mM ammonium formate buffer with on-line electrospray ionization mass spectrometrometric detection in the negative mode. Automated tandem mass spectrometry was acquired and quantification of unknown samples was found using relative ion abundances of diagnostic ions.

Abstract:
A sample of lyase digested CSA was used as a reference against which all unknown CS samples were compared. The reference sample was reductively aminated with d0-anthranilic acid and the unknown CS samples with d4-anthranilic acid. The samples were mixed and separated using SEC with on-line negative ESI MS/MS detection. The HPLC flow was split prior to the sample inlet, allowing 10 microliters/minute of flow into the mass spectrometer. Tandem MS was performed using the automated MSn feature of the ion trap. The isolation and fragmentation windows were set to 12.0 u so that CID spectra of both heavy and light forms were acquired simultaneously. Tandem MS resulted in Y ions and [M-H-SO3] ions containing the reducing end and differing by four mass units, and B ions that are isobaric for both heavy and light forms of AA-labeled CS oligosaccharides. The abundances of Y and [M-H-SO3] heavy and light ions were used for glycoform distribution predictors for unknown CS samples. The method is validated by acquiring automated tandem mass spectra on several isotopically labeled CS mixtures in triplicate. The mixtures were analyzed and the percent total ion abundances of light and heavy predictive ions containing the reducing end were calculated. Light and heavy predictive ion contributions from the unknown were then put into a set of three equations. The three equations were solved for three unknowns that represent the percentage of CSA, CSB, and CSC in a mixture. The results demonstrate that tandem mass spectrometry can be used for the isotopic quantification of glycoforms of chondroitin sulfate.

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Session: Proteins: Glycoproteins Code: WP15 Time Slot/Poster Number: 269

Analysis of N-glycosylation pattern of glycoprotein, UP Ia and UP Ib -a better understanding of urinary tract infection mechanisms

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Introduction:

Two structurally related glycoproteins, the uroplakins (UP) Ia and Ib, interact with UP II and III, to form 16 nm particles hexagonally packed to form 2D crystals that cover almost the entire apical surface of mammalian bladder epithelium. It has been proposed that glycosylation patterns of the UPs determine the binding efficiency of bacteria that cause urinary tract infections. A rapid and sensitive MS strategy has been utilized in this study for the structural determination of the glycans and the identification of occupied glycosylation sites. The results should contribute to a better understanding of the mechanism of urinary tract infection and to improvements in its diagnosis and treatment.

Methods:

Bovine and murine UPs Ia and Ib were purified by SDS-PAGE. The excellent resolution between murine 24kDa UP Ia and 29kDa UP Ib contrasted with the poor resolution between bovine 27kDa UP Ia and 28kDa UP Ib. Bands of interest were excised and deglycosylated in-gel with PNGase F, and the extracted glycans were subjected to permethylation. Tryptic digestion of the proteins was performed in-gel, after release of the N-glycans. The peptides and the permethylated oligosaccharides were characterized using a Bruker Reflex IV matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer, and further analyzed using a QSTAR Pulsar i quadrupole-orthogonal TOF mass spectrometer (QoTOF MS). The carbohydrates and peptides of interest were sequenced by MS/MS.

Abstract:

Using a combination of glycosidase and protease in-gel digestion, MALDI-MS, and ESI MS/MS, we verified the amino acid sequences of the proteins and determined the pattern of glycan heterogeneity at the single glycosylation site in UPs Ia and Ib. Bovine UP Ia/Ib were found to contain a series of high mannose type N-linked glycans at Asn131 of UP Ib and Asn170 of UP Ia. The N-linked glycan at Asn169 in murine UP Ia was determined to be a series of high mannose glycans, while murine UP Ib was found to contain a series of multiple-antennary complex, high mannose, and hybrid N-linked glycans at Asn131.

The permethylated glycan pool generated in this study allowed relative quantification of glycan constituents. The survey on the distribution of glycoforms in UP Ia and Ib was carried out using MALDI-TOF-MS. The main glycoforms of murine UP Ia were found to be the high mannose glycans containing 7, 8 and 9 mannose residues, while those of murine UP Ib were found to be mainly complex glycans (more than 85% of the glycoforms) with small amounts of high mannose and the hybrid glycans. MALDI-MS profiles of the native and permethylated glycans from bovine UP Ia/Ib suggested that the observed profile of native glycans is in good agreement with the results obtained after permethylation, in terms of both the identities and distributions of glycoforms. These results are consistent with the electrophoretic mobility changes of UPs Ia and Ib after they are treated with endo H and F glycosidases. Our results provide a biochemical explanation for the observation that the type 1-fimbriated, uropathogenic E. coli binds selectively to uroplakins Ia but not to the closely related uroplakin Ib. This work was supported by NIH grants P41 RR10888, S10 RR15942 (to CEC) and P01-DK52206 (to TTS).

Session: Computer Applications: General Code: WP20 Time Slot/Poster Number: 359

A Software Shell for MS Data Conversion and Database Submission of MS Data

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Introduction:
With a multitude of different MS instrumentation and data analysis software platforms available for MS and proteomics it becomes difficult to manipulate and manage various data sets. Recently we presented a software application that will allow the conversion of processed MS data files obtained on a variety of instruments into several common formats accepted by different software applications. We have further developed the program to add support for mzXML format and incorporate a front end interface linked to a few database searching engines including ProteinProspector and BUPID (a peptide mass fingerprinting program based on a log-likelihood ratio model developed in-house).

Methods:
The data processing software was developed using Microsoft Visual Basic 6.0. To add support for mzXML format, we adopted the mzXML2other converter developed by the Institute for Systems Biology and converted it to a Visual C++ library that can be accessed by the main Visual Basic program. Other supported data formats are intermediate files converted from raw data files using manufacturers' software: LC MS/MS data with Analyst QS (ABI/Sciex), MassLynx/PLGS2.1 (Waters); MALDI MS data with MOverZ (Proteometrics LLC); and FTMS data with BUDA (Boston University). The BUPID program is developed in C under Linux and made accessible to main program through a CGI based web interface.

Abstract:
The shell data conversion program was written to implement a user friendly GUI interface which may be operated in an unattended batch processing mode. Testing of the program was performed on existing MALDI-TOF MS, MALDI-FT MS and LC MS/MS data sets obtained in house. The program allowed the conversion of large volumes of data obtained on different instruments to the formats of several commercially and publicly available search engines. Files were then submitted for protein identification to the search engines with the search settings specified by the user. For a batch of files, the search setting only needs to be specified once, thus allowing unattended operation. Results files obtained are formatted depending on the type of database search engine employed. When used in conjunction with the BUPID server, the search results can then be viewed in HTML format and saved in a user-specified location. Implementation of the mzXML format introduced by ISB affords the benefits of a common data format for summation of results obtained on different MS platforms, comparative analysis of MS methodology and archiving of data such that it may be analyzed at a later date in-house or at a different facility.

Session: Bioinformatics Code: WP21 Time Slot/Poster Number: 388

BUPID: Probability-Based Protein Identification by Searching Sequence Databases Using Peptide Mass Fingerprint Data

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Introduction:
Mass spectrometry (MS) has become a standard method for protein identification. Several programs have been developed for MS data interpretation by querying a sequence database with peptide masses obtained from the MS experiment. We present results from a new search algorithm BUPID, a robust and accurate statistical model for protein identification using MS data. The algorithm offers a number of new features: 1. Using log likelihood ratio as scoring function, the algorithm can best distinguish correctly assigned peptides from incorrect assignments. 2. Matching peaks with a background-dependent threshold offers more flexibility and accuracy than the traditional mass window. 3. The statistical model outperforms conventional database search algorithms in sensitivity and specificity.

Methods:
We use log-likelihood ratio to calculate the probability that a protein is present in the sample. The model distinguishes two hypotheses – H0: That a set of peaks in the spectrum is generated by the random background; and HA: That the same set of peaks is generated by peptides corresponding to a specific protein. Peak assignment is also based on log-likelihood ratio, thus abandoning the traditional concept of "matches" and "miss-matches". A peak is included in the set if the probability that it is produced by the protein is more significant than that it is otherwise produced by the random background. Final results are ranked by the p-value of their probability score using the sequence information of the protein.

Abstract:
We compared the performance of the BUPID web server and the MASCOT web server. Peptide map data sets were obtained from existing ongoing projects. Both web servers were used to identify proteins in the samples by searching the PMF data against SwissProt. The following parameters are used in the search - Taxonomy: Human; Enzyme: Trypsin; Maximum Missed Cleavage: 1; Peptide Mass Tolerance: 0.2 Dalton. In an example data set obtained during analysis of human blood samples, MASCOT reported over 100 incorrect isoforms of human hemoglobin proteins in the database search. BUPID only reported the correct wild-type sequence. After redundant variations were removed, BUPID outperformed MASCOT by ~25% regarding both sensitivity and specificity using these data sets. Within the top 10 predictions, BUPID database search results have, on average, 27% more sensitivity and 21% more specificity in comparison to MASCOT results. In additional, BUPID was able to find all five human hemoglobin proteins in 5 out of 30 cases, whereas MASCOT only succeeded in one case. Within the top 100 predictions, BUPID shows 27% more sensitivity and 22% more specificity as compared to MASCOT. When using peaks with higher than 5% relative intensity, BUPID outperformed MASCOT by 24~30%. Within top 10 predictions, BUPID in average has 28% and 24% more in sensitivity and specificity, respectively. Within the top 100 predictions, BUPID, on average, has 30% and 24% more sensitivity and specificity, respectively. BUPID also provides various data visualizations tools that are found useful by many users, including combined view of a protein mixture, mass spectrum of shared or similar peptides in different proteins, etc. A typical BUPID run takes 2~3 minutes on a Pentium IV PC.

Session: Peptides: Posttranslational Modifications Code: WP23 Time Slot/Poster Number: 452

Detecting isoaspartyl residues in proteins by electron capture dissociation

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Introduction:
Deamidation of asparaginyl residues in proteins is a post-translational modification resulting in a mixture of aspartyl and isoaspartyl residues and thought to be responsible for the inactivation and misfolding of proteins. Of the two isomeric products, isoaspartyl is thought to be the most damaging to protein activity because the primary structure is shifted by the insertion of a methylene group into the protein backbone thus making differentiation of the two forms important. ECD (electron capture dissociation) has been shown to differentiate the two forms in synthetic peptides based on characteristic fragment ions of each form. Data presented here shows that these ECD fragment ions are reproducible in tryptic peptides from a deamidated protein proving the method's applicability to protein analysis.

Methods:
ECD analysis was performed on a home built qQq-FTMS (Fourier transform mass spectrometer with mass filtering front-end quadrupoles and CAD cell) equipped with a nano-spray source and 7T actively shielded magnet. For each experiment, the multiply charged precursor ions were isolated in Q1, externally accumulated in Q2 and then transmitted to the ICR cell for ECD and subsequent detection. A tryptic fragment of cytochrome C (H-TGPNLHGLFGR-OH, m/z = 584.8153, 2+) was fully deamidated overnight at 80°C and pH 12 indicated by a mass shift of approximately 1 dalton. Calmodulin was incubated at 37°C and pH 8 for two weeks then digested by tryspin and a tryptic peptide (H-VFDKDGNGYISAAELR-OH, m/z = 585.6290, 3+) was shown to be completely deamidated.

Abstract:
The ECD spectrum of the cytochrome C deamidated tryptic peptide showed all z• ions within the range of detection although only four c ions were detected (c7-c10) and were in general of lower abundance due to the N-terminal arginine residue. A peak corresponding to the z8-57 (<1 ppm) fragment indicated the presence of the isoaspartyl residue. No complimentary fragment ion (c3•+58) was found in the spectrum. The deficiency of this ion is most likely due to the position of the arginine residue in concurrence with the fact that the diagnostic isoaspartyl ions are typically of lower abundance than the c/z• series ion. A peak corresponding to the neutral loss of 60 daltons, the loss of the aspartic acid side chain from the reduced precursor ion, was not found indicating that the aspartyl product was of much lower abundance than the isoaspartyl form.
The ECD spectrum of the calmodulin tryptic peptide showed 12 c and 12 z• ions, all of which are in similar abundances most likely due to the N-terminal arginine residue and the lysine residue close to the C-terminus. Both the c6•+58 and z10-57 ions were found (<1 ppm) indicating the presence of the isoaspartyl residue substituted for the arginine residue. The peak corresponding to the loss of the aspartic acid side chain from the reduced precursor ion for this peptide was of considerable abundance but cannot provide unambiguous evidence of the aspartyl form because of the two aspartyl residues in the peptide.

The results above show that the isoaspartyl product from deamidation of asparaginyl residues in peptides and proteins can be detected based on the presence of the c7•+58 and z8-57 diagnostic ions.

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Session: Peptides: Post-translational Modifications Code: WP23 Time Slot/Poster Number: 453

Tandem excitation technique for sequencing biomolecules with labile post-translational modifications in Vibrational Cooling (VC) MALDI FTMS.

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Introduction:
Vibrational Cooling (VC) MALDI-FTMS generates ions by desorbing/ionizing analytes under 1-10 mbar of collision gas. This method allows for detection of labile species with FTMS resolution and accuracy, making this instrument highly applicable for proteomic experiments, particularly for the analysis of labile post-translational modifications (PTMs). However, once the ions are generated, it is necessary to perform MS/MS experiments on them. This presentation concerns investigation of the use of multiple collisional activation events as a tandem mass spectrometry method that provides nearly full sequence coverage for these molecules.

Methods:
VC MALDI FTMS has previously been described [1]. In the current experiments, multishot accumulation was used to acquire a high abundance of ions in the hexapole. Subsequently, ions were transferred to the cell, where precursor ions were isolated by SWIFT. These ions were then subjected to a series of on- and off- resonant excitation events similar to the MECA technique [2] and SORI [3]. Synthetic peptides were used to probe the fragmentation of unmodified and post-translationally modified peptides with varying lability.

Abstract:
Experiments using a series of SORI- and MECA- type collisional activation events were preformed on peptides formed by VC MALDI. As expected, SORI-CAD of these peptides generated a few specific cleavages. However, use of multiple SORI and MECA events for activation yielded high quality CAD spectra of these peptides, generating b/y type cleavage at almost every peptide bond. Furthermore, these CAD spectra, when generated on the VC-MALDI-FTMS instrument, preserved the information regarding the position of labile post-translational modifications such as phosphorylation and O-glycosylation. The same peptides, when analyzed with ESI-FTMS using the same multi-CAD approach, tended to preferentially lose both the labile PTM and the positional information. A number of synthetic peptides were analyzed to confirm this unexpected observation. For example, VC-MALDI-FTMS of ACTH 18-39 peptide ion under standard SORI produced only 8 fragment ions in total; whereas the multi-CAD conditions produced 10 b- and 10 y- types of fragments, providing sequence information for 18 out of 21 amino acid residues. More interestingly, when applying multi-CAD to peptides with labile side chain modifications such as O-glycosylation on Ser and/or Thr residues, and single and multiple phosphorylation on Ser and Thr, information-rich spectra are generated. Most of the fragment ions in the product ion spectra still carry the labile side chain modification(s), providing nearly complete sequence information and unambiguous localization of the sites of modifications. Ongoing work is investigating the application of this technique to sulfated peptides and to classes of carbohydrates that are normally extremely difficult to analyze by CAD-based MS/MS methods. 1. O'Connor PB, Budnik BA, Ivleva VB, Kaur P, Moyer SC, Pittman JL, Costello CE JASMS 2004 15(1): 128-132 2. Lee SA, Jiao CQ, Huang YQ, Freiser BS RCM 1993, 7(9), 819-821 3. Gauthier JW, Trautman TR, Jacobson DB 1991, Analytica Chimica Acta 246, 211-225

Session: Quantitation Code: WP27 Time Slot/Poster Number: 510

ICAT (isotope-coded affinity tag) -based quantification of reversible oxidative post-translational thiol modifications of H-ras that accompany its activation

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Introduction:
We recently described an approach for identifying and quantifying oxidant-sensitive protein thiols using a cysteine-specific, acid-cleavable isotope-coded affinity tag (ICAT) reagent (Applied Biosystems, Foster City, CA). We are now using this approach to explore the relationship between redox sensitivity of individual cysteine residues and physiologically significant oxidative post-translational modifications, as well as irreversible thiol oxidation by oxidant stress associated with disease. As a proof of principle, we are quantitatively evaluating reversible (e.g. S-glutathiolation) and irreversible (e.g. S-sulfonylation) modifications of the recombinant protein H-ras.

Methods:
H-ras (1-10 μg) was treated with 100 μM peroxynitrite for 5 min. at 37 oC in the presence or absence of reduced glutathione. Ras activity was assayed by association with Raf-1 and by GTP/GDP exchange. The peroxynitrite-treated samples were labeled with heavy ICAT reagent by incubating at 37 oC for 30 min. The heavy isotope-labeled protein was mixed in equal amounts with the untreated H-ras which had previously been labeled with light ICAT reagent. The light and heavy labeled proteins were digested with trypsin, desalted, and affinity-purified using an avidin cartridge (Applied Biosystems, USA). The samples were concentrated and dissolved in 0.1% TFA for MALDI TOF MS or 1% formic acid for analysis with a QoTOF LC-MS (Micromass).

Abstract:
The amino acid sequence of H-ras contains six cysteine residues. Of the six, four (118, 181, 184 and 186) are surface-exposed, as determined by structural, chemical and mutational studies. Although Cys-181, Cys-184 and Cys-186 are known to be modified by prenylation in intact cells, all of the reactive cysteines are potentially oxidized during normal and pathological conditions and this oxidation could alter the cellular function of the protein. We recently demonstrated that Ras was S-glutathiolated and activated by oxidants generated from NADPH oxidase in smooth muscle cells stimulated with angiotensin II. This result now makes it imperative to quantify the thiol modifications in the protein associated with its oxidant-mediated activation. The activity of Ras was significantly increased 2- to 3-fold following exposure to peroxynitrite and glutathione, but not to peroxynitrite alone. We are therefore applying our ICAT approach to identify and quantify cysteine modification that occur upon treatment with peroxynitrite in the presence and absence of glutathione. MALDI-TOF MS of the ICAT-labeled peptides of H-ras showed 15-20 ICAT-labeled peptides with appropriate 9-Da differences between the light and heavy isotope labeled peptides. LC MS/MS is being carried out to quantitify the cysteine oxidation on the basis of the change in signal intensity for the heavy isotope labeled peptide. ICAT-labeling, before and after treatment with glutaredoxin-1 is being performed to specifically reduce S-glutathiolation, to specifically quantify this reversible modification. We anticipate that quantitative evaluation of the extent of modification of individual cysteine residues can be correlated to the activation or inactivation of H-ras when subjected to reactive oxygen/nitrogen species. Acknowledgements: This project is funded by NHLBI contract N01-HV-28178 and HL 68758.

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Development of a protein 3-nitrotyrosine identification method using solid phase capture

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Introduction:
3-Nitrotyrosine, formed by the reaction of various redox reactive nitrogen species, notably peroxynitrite, is produced under normal physiological conditions and in increased amounts under conditions of oxidant stress and is thus a potential marker for oxidative stress induced modifications in proteins. Most MS identifications of nitrated proteins have resulted from mass fingerprint identification of gel spots showing a positive signal from Western blots with an anti-nitrotyrosine antibody. Where sequence specific information has been obtained, most proteins have been nitrated chemically or were in high abundance. Here we present a method to detect nitrotyrosine-containing proteins in a proteomic manner, via a functional group conversion and solid phase capture followed by MS analysis, with the goal that sequence-specific information can be obtained.

Methods:
A model peptide and bovine serum albumin (BSA), used as models for in vitro protein nitration, were nitrated with tetranitromethane and dialyzed. The modified products were reduced and alkylated. After desalting, the sample was reduced with sodium hydrosulfite, desalted, and exposed to an amine-reactive substrate (a modified commercial resin) at low pH. The captured protein was proteolytically digested on the substrate. After extensive washing, the retained peptides were eluted from the beads by reduction of a disulfide containing linker on the substrate. The captured peptides were characterized by MALDI using a Bruker Reflex IV MALDI-TOF MS and by LC–MS/MS using a Waters QTOF API-US MS. Data was analyzed using the ProteinLynx Global Server 2.1 proteomics suite.

Abstract:
The chemistry for nitrotyrosine reduction to aminotyrosine, and solid-phase capture have been demonstrated using nitrated angiotensin II, a synthetic, nitrotyrosine containing BSA tryptic peptide and BSA itself. Nitration of BSA was confirmed by an absorbance shift to the visible range, MALDI-TOF MS, and Western blot analysis (with a decrease in signal seen after reduction by sodium hydrosulfide).

Preliminary testing on the amine-reactive substrate was carried out with a nitrotyrosine-containing synthetic peptide. The peptide recovered from the substrate had the expected mass increase. Application of this method to nitrotyrosine-containing BSA yielded three tryptic fragments by MALDI–TOF MS. All assigned peptides correspond to previously known sites of in vitro nitration in BSA. Sensitivity has been measured to at least the 10 μg level. Current work is focused on increasing by optimizing the chemistries for substrate attachment and product isolation.

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