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2015 OMSS
ORAL ABSTRACTS

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Phosphoproteomic identification of oncogenic pathways in LKB1 dependent non-small cell lung cancer by two-dimensional LC-MS/MS

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Introduction

Proteomic profiling of cancer cell lines can be used to identify potentially useful protein biomarkers and to reveal the connectivity between oncogenic pathways. Liver kinase-B1 (LKB1) is a tumor suppressor gene commonly found in 30-40% of non-small cell lung cancer (NSCLC). Somatic mutations of LKB1 lead to loss of its expression and respective key metabolic and regulatory functions. These signaling events are mediated mainly by AMP-activated protein kinase (AMPK). However, the comprehensive mechanism and relationship of LKB1 to NSCLC remains unclear. Here, we present our study on identifying these pathways based on differential regulation of phosphoproteins, and localization of phosphorylation events by label-free proteomics.

Methods

To study LKB1 signaling events we have used two LKB1-deficient cell lines HCC15, and A549 transduced with a retrovirus carrying wild-type LKB1. Controls were prepared by transducing the cell lines with a vector alone and a kinase-dead version of LKB1. Cell lysates were digested with trypsin in ProteaseMax to generate peptides. The digests were enriched for phosphopeptides with TiO_2 columns. The eluate was collected for liquid chromatography coupled to mass spectrometry analysis. The samples were fractionated online into 15-fractions in high pH reverse-phase followed by low pH gradient elution (Waters NanoAcquity). The peptides were analyzed on a Thermo Orbitrap Elite.

Preliminary Data

This reverse-phase/reverse-phase two-dimensional separation increased the coverage of the phosphoproteome by yielding approximately 4000 more phospho-modified peptides compared to a single 2hr gradient for only 4.5 µg of the sample. In each sample, we identified approximately 2000 protein groups in the phospho-modified state (1% false discovery rate). Phosphoproteins identified in this study with 90% or higher probability contained more than 10000 phosphorylation sites localized on peptides. All modified peptides contained single or multi-phosphorylation sites with phospho-serine, threonine, or tyrosine modifications.

We first compared phosphopeptides across all cell lines based on their sample type (vector, kinase-dead or wild-type), and combined phosphopeptides with the same accession number (UniProt) into one phosphoprotein group for further analysis. Based on these preliminary data sets we established our phosphoproteomic profiles and identified 51 differentially regulated phosphoproteins in wild-

type sample compared to vector. These proteins presented significantly ($p<0.05$) different protein expression levels with two or higher fold change. Comparison of wild-type and kinase-dead samples revealed 80% similarity in phosphoprotein profile indicating the importance of the LKB1 kinase domain in oncogenic signaling. In contrast, the comparison of two controls revealed no such changes in the expression levels of respective proteins.
We correlated these phospho-modified and unmodified protein changes to respective pathways (Ingenuity pathway analysis or Kyoto Encyclopedia of Genes and Genomes (KEGG)) that revealed the participation of Rho, m-TOR, AMPK, ERK/MAPK and HIPPO signaling pathways in LKB1-mediated NSCLC.

Novel Aspect
Two-dimensional separation coupled to mass spectrometry yielded more phospho-modified and unmodified peptides increasing the proteome coverage facilitating the pathway analysis.

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Combining recombinant ribonuclease U2 and protein phosphatase for RNA modification mapping by liquid chromatography–mass spectrometry

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Introduction

Ribonuclease (RNase) mapping of modified nucleosides onto RNA sequences is limited by RNase availability. Ribonuclease U2, a purine selective RNase with preference for adenosine, has been overexpressed using Escherichia coli as the host. However, RNase U2 digestion typically results in a mixture of 3'-phosphate and 2',3'-phosphate products, which lead to greater spectral complexity and difficult data analysis. To generate a homogeneous 3' -linear phosphate set of products, an enzymatic approach was investigated. The compatibility of this enzymatic approach with liquid chromatography–tandem mass spectrometry (LC–MS/MS) RNA modification mapping was then demonstrated.

Methods

All tRNAs were digested using RNase U2 at 65° C for 30 min and phosphodiesterases at various temperatures and times to identify ideal conditions. Chromatographic separation was conducted using a Thermo Surveyer HPLC system. A Waters Xbridge™ C18 column with mobile phase A of 200 mM hexafluoroisopropanol (HFIP), 8 mM triethylamine (TEA) in water, pH 6.5 and mobile phase B of 50% MPA and 50% methanol at a flow rate of 30 µL min-1 was used for separation of oligonucleotides. A Thermo LTQ-XL was used for MS and MS/MS analyses. A capillary temperature of 275 °C, spray voltage of 4 kV, and 25, 14, and 10 arbitrary flow units of sheath, auxiliary and sweep gas, respectively, were used.

Preliminary Data

RNase U2 digestion products were found to terminate in both 2',3' -cyclic phosphates and 3'-linear phosphates. Bacteriophage lambda protein phosphatase was identified as the optimal enzyme for hydrolyzing cyclic phosphates from RNase U2 products. RNase U2 digestion followed by subsequent phosphatase treatment generated nearly 100% 3'- phosphate-containing products that could be characterized by LC–MS/MS. In addition, bacteriophage lambda protein phosphatase can be used to introduce ^{18}O labels within the 3'-phosphate of digestion products when incubated in the presence of $H_2{}^{18}O$, allowing prior isotope labeling methods for mass spectrometry to include digestion products from RNase U2.

Novel Aspect

Improving MS and MS/MS characterization of RNA after digestion with RNase U2 by enzymatically removing 2',3'-cyclic phosphates.

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Integration of Electrochemistry with Ultra Performance Liquid Chromatography/Mass Spectrometry (UPLC/MS)

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Introduction

The combination with electrochemistry (EC) broadens liquid chromatography/mass spectrometry (LC/MS) applications. Previous studies show post-column electrochemical conversion in LC/MS can improve the MS detection sensitivity. We developed LC/EC/MS using ultra-performance liquid chromatography (UPLC) and adopted desorption electrospray ionization (DESI) as the coupling interface. UPLC offers fast separation, high sensitivity and low elution flow rates that fits online electrolysis better than HPLC. This new apparatus of LC/EC/DESI-MS has applications in structural analysis of proteins/peptides containing disulfide bonds. Furthermore, reactive DESI can be carried out to supercharge LC-separated proteins and online electrolytic reduction further promotes the protein charges under supercharging conditions.

Methods

In our experiment, Waters ACQUITY UPLC® System with a Waters Xevo QTOF mass spectrometer was used. Our LC/EC/MS assembly employs a thin-layer μ-PrepCell™ electrochemical flow cell. The cell was connected after the UPLC column using a piece of PEEK tubing. After UPLC separation, compounds in the eluent underwent electrochemical reduction in the electrochemical cell. The electrolyzed species flowed out of the thin-layer cell via a short piece of PEEK tube carrying a micro-orifice. Unless specified, the spray solvent for DESI was CH_3OH/H_2O/HOAc (50:50:1) and injected at 10 μL/min with a 5 kV and N_2 nebulization applied. A Roxy™ potentiostat was used to apply an optimal potential to the electrochemical flow cell to trigger redox conversion.

Preliminary Data

A disulfide-containing peptide somatostain 1-14 was first chosen for UPLC/EC/DESI-MS analysis. Tryptic digested somatostain 1-14 produced a peptide mixture. The two peptides eluted within 2.1 min. For one disulfide-containing peptide, after reduction potential applied, two new ions of *m/z* 378 and 558 were observed. Upon collision induced dissociation (CID) of the electro-generated ions provided more fragment ions that cover all of the backbone cleavage sites and the position of the cysteine residues were determined. The sum of the MWs of the two products is

higher than that of the precursor peptide by 2.0 Da, suggesting that the precursor peptide has one disulfide bond. We further analyzed the structure of insulin using this approach. All the peptides from insulin digest were subject to fast elution, in which peptides containing disulfide bonds can be recognized due to their electroactivity. Based on MS/MS analysis of reduced peptides and precursor peptides, 98% sequence and three disulfide bonds of insulin could be determined. Besides investigating protein digests, intact proteins are also tested. A protein mixture of insulin, myoglobin and α-lactalbumin was loaded onto the UPLC. Before and after electrolytic reduction, the charge state distribution shift would be used to identify the presence of disulfide bonds in proteins. Reactive DESI was employed to increase charges of the LC-separated proteins by doping spray solvent with supercharging reagents. Interestingly, online electrolytic reduction of disulfide-bond containing proteins (e.g., α-lactalbumin) in combination with supercharging leads to higher protein charges.

Novel Aspect
This is the first time to construct LC/EC/MS for structural elucidation of disulfide bond-containing proteins.

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The Use of Exhaled Breath for the Identification of Hypoxia Biomarkers

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Introduction

The identification volatile organic compounds (VOC) in exhaled breath as biomarkers of disease have been used for decades. Due to the inherent non-invasive sampling and the ability to detect several hundred compounds from a single sample, exhaled breath VOC analysis is an attractive alternative to serum or urine for biomarker discovery. This study establishes the use of novel exposure and sampling techniques combined with sensitive TD-GC-MS analysis for the monitoring exhaled breath volatile organic compounds in the context of hypoxic respiratory stress. Furthermore, an informatics workflow was established to analyze the large data sets via the Metabolite Differentiation and Discovery Lab and MatLab© software packages. The results provide a foundation for further research into hypoxia related exhaled breath biomarkers.

Methods

Human volunteer subjects were affixed with a modified flight mask in-line with a Reduced Oxygen Breathing Device. Subjects were exposed to simulated flight profiles of three, five-minute stages in sequential order, sea level O_2, O_2 equivalent of altitudes up to 25,000 feet and recovery gas, 100% oxygen. Exhaled breath was collected serially (1 minute per Tenax TA thermal desorption tube) throughout the course of the experiment using the Logistically-Enabled Sampling System-Portable. Alternatively, exhaled breath was also collected via 1L ALTEF polypropylene bags (pre, post and 2h post test) and loaded onto Tenax TA thermal desorption tubes. All thermal desorption tubes were analyzed by TD-GC-MS using 70eV electron impact ionization. Data was analyzed for variability in VOC metabolite abundances.

Preliminary Data

The ability to monitor conditions with severe cognitive consequences, such as hypoxia, is becoming increasingly necessary to ensure safety in a number of job duties. However, to date no significant effort has been made to identify biomarkers of hypoxia in humans. Using a modified

flight mask in-line with a Reduced Oxygen Breathing Device to control subject oxygen supply, we sought to identify novel volatile organic compounds associated with hypoxic respiratory stress. The TD-GC-MS analysis of the time series exhaled breath samples shows seven VOCs have a decreased log_2 abundance ratio (treated/placebo), which coincides with each subject's minimum SpO_2. Similarly, the mean log_2 abundance ratios of the seven compounds, across each exposure level (sea level, exposure and 100% O_2), exhibit a statistically significant decrease at altitude equivalent oxygen. Furthermore, six of the seven compounds return to return to sea level log_2 abundance ratios during the 100% O_2 time points, suggesting a recovery from hypoxic stress.
TD-GC-MS analysis of the bag samples, taken immediately prior to, following and 2 hours after an experimental exposure, show ten VOCs had log_2 abundance ratios that were significantly correlated with the subject's minimum SpO_2 ($p<0.01$, $n=8$). Further analysis of the bag exhaled breath data identified isoprene (2-methyl-1,3-butadiene) as the only compound with a log_2 abundance ratio that increased in more severely oxygen-deprived subjects (post-pre). Additionally when the log_2 abundance ratios were calculated for the 2h-post samples, a reversed correlation was observed.
The results from these experiments are significant for several reasons. First, we are the first to apply a novel experimental setup and informatics workflow to identify exhaled breath markers of hypoxic stress. Next, we establish seven candidate makers of hypoxic stress. Finally, we have identified methodological improvements that will be applied to further validations studies.

Novel Aspect
Our research applied novel sampling and informatics techniques to identify candidate exhaled breath biomarkers of hypoxic respiratory stress.

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Surface induced dissociation ion mobility mass spectrometry localizes multisubunit protein-ligand interactions

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Introduction

Here we assess the feasibility of surface induced dissociation (SID) ion mobility-mass spectrometry (IM-MS) as a novel, informative tool to study both protein:protein interactions and protein:ligand binding locations. The interactions between the pentameric complex cholera toxin subunit B (CTB) and GM1 sugar, pentameric C-reactive protein (CRP)/ Ca^{2+}/Phosphocholine (PC) and CRP/Fc gamma receptor IIa (FcgRIIa) were chosen for study due to their different complex:ligand binding interactions. Protein:ligand complexes were dissociated with SID and those results were compared with collision induced dissociation (CID) results.

Methods

Protein complexes and protein-ligand complexes were buffer exchanged into 100mM ammonium acetate and mixed with triethylammonium acetate at 4:1 ratio respectively. They were then analyzed using a commercial Waters Synapt G2S quadrupole / IM / time-of-flight mass spectrometer, modified to integrate a custom SID device. The Collision Cross Section (CCS) was calculated by using Concanavalin A, Transthyretin and Serum Amyloid P as calibrants.

Preliminary data

The protein:ligand and protein: protein complexes CTB/GM1, CRP/ Ca^{2+}/ PC and CRP/FcgRIIa were subjected to CID and SID. CID spectra of the three complexes show mainly ejection of monomers, ligand and ligand retained tetramers, revealing limited substructural information. In contrast, SID of CTB/GM1 yields CTB_n+(n-1)GM1, CTB_n+nGM1 and CTB_n+ (n+1)GM1 (n=1,2,3,4). Fragments with CTB_n+nGM1 are the dominant fragments, consistent with the crystal structure of the complex where the GM1 binding site is made up primarily from a single subunit but with one hydrogen bond to residue Gly33 from an adjacent subunit. Similarly, SID of CRP/ Ca^{2+}/ PC mainly generates CRP_n+nPC with Ca^{2+}, consistent with the complex crystal structure in which PC binds within each CRP monomer. Furthermore, SID of CRP/FcgRIIa yields CRP monomer and dimers plus CRP_n+FcgRIIa (n=2, 3, and 4), indicating that the ligand bridges across two CRP monomers, which matches the homology model from the SAP/FcgRIIa structure.

Novel aspect

SID IM-MS was found to be an informative tool to study protein:ligand binding locations.

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Fragmentation Analysis of Two Structural Isomers

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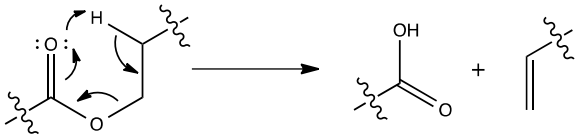
Introduction

The McLafferty rearrangement is a well-known fragmentation mechanism for polyesters. It requires a hydrogen atom on the β-carbon of the diol chain. Two polyesters will be studied in this work, CHDA.NPG and CHDA.1,5-PED. The diacid in both cases was 1,4-cyclohexane dicarboxylic acid (CHDA), and the diols were neopentyl glycol (NPG) and 1,5-pentane diol (1,5-PED). CHDA.1,5-PED can undergo the McLafferty rearrangement, while CHDA.NPG cannot. The polyesters show identical fragmentation spectra, necessitating the proposal of an alternative fragmentation mechanism for CHDA.NPG.



Methods

The samples were analyzed using both a Bruker Ultraflex III MALDI-ToF/ToF and a Synapt HDMS ESI-Q/ToF equipped with Triwave IMS. For MALDI, the samples were prepared using *trans*-2-[3-(4-*tert*-Butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) and sodium trifluoroacetate (NaTFA). The matrix, sample, and salt were dissolved in tetrahydrofuran (THF) at a concentration of 20, 10, and 10 mg/mL respectively, and then combined at a ratio of 100:50:10 (v/v) respectively. The samples were analyzed using the dry-drop method. For ESI, the samples were dissolved in THF:methanol (MeOH) (9:1, v/v) to a concentration of 0.1 mg/mL and 0.1% NaTFA (10 mg/mL) was added to the solution.

Preliminary Data

The two polyesters show identical ion masses in the mass spectra, as would be expected from structural isomers. As mentioned above, the tandem mass spectra are also identical. This requires the proposal of an alternative fragmentation mechanism for CHDA.NPG.

In order to understand the mechanism, survival yields were used to study the energetics of the fragmentation. The survival yields were calculated in triplicate, and the equation of the survival yield curve was derived. From that curve, the CE_{50} values could be determined. The polyesters showed slightly different CE_{50} values, but the difference was not statistically significant.

Next, ion mobility mass spectrometry was used to calculate the collisional cross sections (CCS) in order to determine what effect the size of the molecules might have on the fragmentation mechanism. The CCSs were calculated by comparing the drift time of the polyesters to the drift time of the standard, polyalanine. While slight differences were seen in the CCSs, the difference

was not significant enough to be considered relevant. Based on the energetics and size information, a mechanism is proposed for the CHDA.NPG fragmentation.

Novel Aspect
Different structures, with indistinguishable size and energetics, lead to identical fragmentation spectra.

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Modified Guanosine at position 34 of placental human tRNA Asp(GUC) and tRNA Asn(GUU)

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Introduction

Transfer ribonucleic acids (tRNAs) are abundantly represented in the human genome with over 450 genes that have been annotated. More than 270 unique sequences exist encompassing 51 possible anticodons for 21 amino acids. These genes are further subdivided into isodecoders, tRNAs, which have the same anticodon but sequence difference elsewhere. Isolating a specific tRNA from a human placenta is challenging due to the complexity of the sample matrix. Here we show the successful isolation of tRNA Asp (GUC) and tRNA Asn(GUU) from human placenta by the use of an oligonucleotide affinity probe.

Methods

A 20-mer biotinylated oligonucleotide probe was designed complimentary to a localized region in tRNA Asp and tRNA Asn. Hybridization was performed at 37.0 °C. The purified tRNA was digested with RNase T1 (1 µg tRNA to 50 U enzyme ratio). Waters Xbridge™ C18 column with mobile phase A (MPA) of 400 mM hexafluoroisopropanol (HFIP), 16.3 mM triethylamine (TEA) in water, pH 7.0 and mobile phase B (MPB) of 50% MPA and 50% methanol was used to separate the oligonucleotides. LTQ XL mass spectrometry conditions: negative polarity, capillary temperature of 275°C, spray voltage of 4 kV, and 25, 14, and 10 arbitrary flow units of sheath, auxiliary and sweep gas, respectively.

Preliminary data

The enzymatic digestion of a tRNA using selective RNases, such as RNase T1, will generate a series of a unique digestion products that can be used to identify a specific tRNA.3 A particular advantage of RNase T1, which cleaves specifically after unmodified guanosines, is the ability to detect modifications at G34 in tRNA anticodons. When G34 is not modified, RNase T1 will cleave at that location generating oligonucleotides that do not contain the entire anticodon region (tRNA positions 34-35-36). However, if G34 is modified, RNase T1 cannot cleave those modifications yielding a much longer oligonucleotide that will contain the entire anticodon region.

Using the standard RNA mass mapping approach, the anticodon containing sequence for tRNA Asp (GUC) contains two modifications: mannosyl/galactosyl Queosine at position G34 and a base methylated Cytosine at position 38. To our knowledge, this is the first report identifying the anticodon region modifications in htRNA Asp. tRNA Asn (GUU) has Queosine at position G34 and N6-threonylcarbamoyl adenosine (t6A) for position 38. This finding verifies the previously reported anticodon sequence of tRNA Asn(GUU), which was obtained using 2-Dimensional

acrylamide gels. Efforts are currently on the way to get a complete sequence coverage and identify all the modifications on the tRNAs.

Novel Aspect
Isolation of a human tRNA, identification and mapping of modifications using mass spectrometry.

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Surface-Induced Dissociation/Ion Mobility of protein complexes: Interface Area and Subunit Packing

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Introduction

Numerous biophysical methods have been developed with the aim of understanding biological complexes. Mass spectrometry (MS) coupled with ion mobility (IM) is emerging as a new bioanalytical method. The dissociation of protein complexes in the gas-phase via MS/MS gives information on protein complex stoichiometry and geometry. Surface induced dissociation (SID) has been shown to give rise to products more reflective of solution topologies of protein complexes, in comparison to unfolded monomer products obtained from conventional collision induced dissociation (CID). IM-MS can provide an extra dimension of information separating proteins based on charge, shape, and size. Here, we describe a comparative study of tetramers, pentamers, and hexamers using CID and SID to understand the role of interface area and subunit packing in the dissociation.

Methods

All studies presented here performed on an in-house modified Waters Synapt G2 (Manchester, UK) mass spectrometer with a custom SID device. The SID device is located between a truncated trap traveling wave ion guide (TWIG) and the IM cell enabling the additional separation of MS/MS fragment ions based on charge, shape, and size.

Complexes investigated were tetramers- Pyruvate Kinase (PK) and Streptavidin (STR), pentamers- CTB and SAP, and hexamers- bacterial urease and bovine GDH. Protein complex interfaces were analyzed using PISA web service and compared with tandem MS dissociation pathways.

Preliminary Data

Native-like conditions were employed using 20 % (v/v) triethyl ammonium acetate (TEAA) in 100 mM ammonium acetate buffer. The addition of TEAA allows the study of lower charge states, which are thought to help preserve native-like conformations of protein complexes. Complexes were subjected to both SID and CID for comparison of dissociation and disassembly pathway.

Pyruvate kinase and streptavidin are homotetramers packed in a **dihedral fashion**. PISA (PDBe service) analysis provides interfacial area, number of hydrogen bonds and salt bridges at the interface. Interfacial area and number of non-covalent bonds between subunits gives an estimate of strength of the interface. Homotetramers, PK and STR, show two strong and two weak interfaces. Combined weak interfacial area between STR dimers is 831 $Å^2$ while between PK dimers is 2356 $Å^2$. SID and corresponding Energy Resolved Mass Spectra plot (ERMS) of

homotetramers show **dimer as a dominant product** in the low SID energy which reflects subunit arrangement of PK and STR which are **dimer of dimers.** Strength of PK and STR interfaces and onset energy of dissociation (dimer formation) also show good correlation. Bacterial urease and bovine GDH are two hexameric proteins arranged in dihedral fashion. SID gives trimers as a major dissociation product which reflects subunit arrangement of bacterial urease and bovine GDH which are **dimer of trimers**.

CTB and SAP are homopentamers which are **arranged in cyclic fashion**. PISA analysis shows only **one type interface** as subunits are arranged in a cyclic manner. SID gives monomers as a dominant product followed by dimers, trimers, and tetramers. Another advantage of SID over CID is that products (e.g. monomers) produced from pentamers are more folded and native-like which reflects solution phase native structure.

Hence, disassembly of protein complexes while maintaining native-like conformation would possibly be used to confer assembly of protein complexes. SID also can be used to understand stoichiometry and assembly of unknown protein complexes.

Novel Aspect

SID-IM is a novel tool to determine the subunit assembly of protein complexes.

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Improving RNA Modification Mapping by Mutagenesis of RNase U2

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Introduction

Mass spectrometry is commonly used as a way of detecting short oligonucleotides for RNA sequencing as well as for locating post-transcriptional modifications in those sequences. Ribonuclease U2 is an enzyme used to generate purine-ending products for the analysis of RNA sequences. However, due to the lack of specificity of this enzyme, mass spectrometry-based sequencing becomes tedious due to a large variety of possible digestion products. In order to simplify the expected digestion products, we aim to modify the affinity of RNase U2 to cleave only after adenosines.

Methods

The available crystal structure of RNase U2 bound to 3'-Amp (PDB ID:3AGN) was used to determine possible mutations in the binding site of RNase U2. Synthetic genes coding for mutant enzymes were cloned into pET22b plasmids, which were then transformed into *E. coli* cells.
Cells were grown at 37^{o}C where expression of the mutant proteins was induced by addition of IPTG. The enzymes were then purified through a nickel-Nta column.
Enzyme activity was determined by UV absorbance monitoring at 260nm of poly As incubated with the mutant enzymes where wild type RNase U2 served as a positive control. To determine the specificity of the mutant enzymes, optimization of the digestion conditions of *S. cerevisiae* $tRNA^{Phe}$ was performed. The Thermo LTQ-XL mass spectrometer was used to analyze digestion products generated from mutant digests of yeast $tRNA^{Phe.}$

Preliminary Data

Here, we are able to generate two mutants: E46H-E49Q (mtQ) and E46H-E49K (mtK). The activity of the mutant enzymes were reduced to 65% and 53% for mtQ and mtK, respectively, relative to the wild type enzyme. Our initial mass spectrometry results suggest that one of our mutants, mtQ, was able to produce similar digestion products with slightly enhanced activity when compared to wild type RNase U2.
By treating the protein as an adenosine-specific enzyme, we were able to generate theoretical *S. cerevisiae* $tRNA^{Phe}$ digestion products ending in adenosine. The m/z values of the theoretical digestion products were sought from the raw data. As peak area indicates the intensity of each digestion product, we found that mtQ generated more abundant A-ending products than the wild type enzyme. However, in order to determine nonspecific cleavage, de novo sequencing is currently ongoing. Further optimization of this mutant type is in progress with hopes of improving its activity and specificity over wild type.

Novel Aspect
Simplifying data analysis of RNase U2 digestion products by improving its capability to cleave only at adenosines.

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Development of Electrochemical Cross-Linking Mass Spectrometry for Probing Protein Three-Dimensional Structures and Conformational Changes

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Introduction

Chemical cross-linking combining with mass spectrometry (MS) is a powerful method providing low-resolution protein structural information. However, challenges still hamper this approach, such as the identification of target cross-links and the existence of native disulfide bonds. We first time describe an electrochemistry-assisted isotope-labeled chemical cross-linking method involving a disulfide-containing cross-linker, dithiobis[succinimidyl propionate] (d_0-DSP) and its isotopic form (d_8-DSP). The twin-peak characteristics displayed by cross-linkers helps pinpoint cross-link candidates among digest mixture. Electrolytic reduction brings pronounced intensity drop to disulfide bond-containing peptides, including cross-links and native disulfide-containing peptides. Reduced products from cross-links are rapidly recognized as they remain twin-peak characteristic and matched with their precursors according to the particular mass relationships, while others are related to native disulfide bonds.

Methods

d_0/d_8-DSP were pre-mixed at 1:1 ratio with access amount, reacted with intact proteins in buffer, and followed by trypsin digestion. The resulted mixture flowed through a thin-layer μ-PrepCell™ electrochemical flow cell for sample reduction at rate of 5 μl/min by syringe pump. A magic diamond electrode was equipped as the working electrode, and reduction potential was applied by a Roxy™potentiostat (AntecBV, Netherlands). The reduced species flowed out via a piece of fused silica capillary and underwent ionization by DESI. H_2O:MeOH (v/v 50:50) containing 1% FA was used as DESI spray solvent at a flow rate of 5 μl/min. A Waters Xevo QTOF mass spectrometer was employed for ion detection and tandem MS analysis.

Preliminary Data

SLIGKV-NH_2, somatostatin-14, α-lactalbumin and calmodulin-melittin, calmodulin-mastoparan complexes were chosen as model samples. Samples were dissolved in PBS buffer and reacted with access amount of pre-mixed d_0/d_8-DSP (1:1). After desalting and trypsin digestion, resulted peptide mixture containing cross-links was monitored by online electrolytic reduction coupling with DESI-MS. At the first sight of injection, twin-peak characteristics displayed by cross-links was recognized as a distinguish sign to help narrow down the number of cross-link candidates among the mixture. Followed by electrolytic reduction, disulfide-containing peptides would suffer a large relative intensity change. Therefore, disulfide-free peptides were excluded from peptide mixture and the rest included possible cross-links as well as peptide containing native

disulfide bonds. With the assistance of isotope-labeling, reduced products from cross-links were rapidly differentiated from native disulfide bonds, as former ones remained isotope-labeled characteristic. In addition, types of cross-links were determined according to their electrochemical behaviors and specific mass interval changes. Upon electrolytic reduction, the mass interval for products of dead-end cross-links would change to 4 Da with a mass decrement of 88 Da. The interpeptide cross-links would yield two linear peptides separately due to the cleavage of disulfide bond from DSP, and each product peptide would display 4 Da mass interval. Intrapeptide cross-links would result in adjacent reduced products with +2 Da mass shift. As the cleavage of intrapeptide disulfide bond would not have any tag lose nor peptides separately, the mass interval would remain 8 Da. Furthermore, the electrolytic reduction resulted in linear peptides, which benefited MS/MS analysis by providing more sequence information compared with their precursors. Thus, the modification positions were pinpointed easily, which served as a high-throughput method for proteomics studies.

Novel Aspect

Electrochemistry-assisted isotope-labeled chemical cross-linking method offers rapid identification of cross-links and is value for high-throughput protein 3D structural analysis.

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Structural identification by Mass Spectrometry of Cyclic Rhamnosylated EF-P

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Elongation factor P (EF-P) is a ubiquitous bacterial protein that is required for the efficient synthesis of poly-proline motifs during translation. In Escherichia coli and Salmonella enterica the post-translational β-lysylation of Lys34 by the PoxA protein is critical for EF-P activity. PoxA is absent from many bacteria such as Pseudomonas aeruginosa, prompting a search for alternative EF-P post-translation modification pathways. ETD-HCD MS3 analyses of P. aeruginosa EF-P revealed the attachment of a single cyclic rhamnose moiety to an Arg residue at the equivalent position where β-Lys is attached to E. coli EF-P. Analysis of the genomes of organisms that both lack *poxA* and encode an Arg34 containing EF-P (*efpR*) revealed a highly conserved glycosyltransferase (EarP) encoded adjacent to *efpR*. EF-P proteins isolated from P. aeruginosa *ΔearP*, or from a Δ*rmlC::aac1* strain deficient in dTDP-L-rhamnose biosynthesis, were unmodified. In vitro assays confirmed the ability of EarP to use dTDP-L-rhamnose as a substrate for the post-translational glycosylation of EF-P. The role of rhamnosylated EF-P in translational control was investigated in P. aeruginosa using a $(Pro)_4$-GFP *in vivo* reporter assay, florescence of which was significantly reduced in *ΔefpR*, *ΔearP*, and *ΔrmlC::acc1* strains. *ΔrmlC::acc1*, *ΔearP* and *ΔefpR* strains also displayed significant increases in their sensitivities to a range of antibiotics including ertapenem, Polymyxin B, cefotaxim, and piperacillin, consistent with a role for modified EF-P in virulence. Taken together our findings indicate that post-translational rhamnosylation of EF-P plays a key role in P. aeruginosa gene expression and virulence.

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Development and validation of a tandem mass spectral library for modified oligonucleotides from transfer RNAs

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Introduction

Transfer ribonucleic acids (tRNAs) play a crucial role in protein biosynthesis. The main purpose of tRNAs is to decode messenger RNA (mRNA) during protein synthesis. Biologically active tRNAs are post-transcriptional modified, and these modified nucleosides are important for tRNA structure, stability and decoding. Tandem mass spectrometry is a powerful tool for mapping the sequence locations of modified nucleosides on tRNAs. A typical experimental approach involves digestion of total tRNAs with a base-specific ribonuclease, and these digestion products are separated and analyzed by LC-MS/MS. One challenge of this strategy arises in the interpretation of tandem mass spectra of modified digestion products. Here we exam the utility of a tandem MS spectral library for annotating and identifying modified digestion products.

Methods

Escherichia coli total tRNA was prepared and digested by RNase T1, and LC-MS/MS analysis of the resulting digestion products was performed by using a Thermo LTQ XL™. Mongo Oligo Mass Calculator was used to calculate precursor mass and fragment ions (c- and y-type ions) for each expected digestion product. The matching spectra were extracted from the datafile and then all spectra were merged into one library file that could be searched using NIST MS Search V2.2. Scoring of MS/MS matches to the library was done using dot products and score of hits.

Preliminary Data

An *Escherichia coli* tRNA RNase T1 MS/MS library was derived from multiple tandem mass spectral scans of a precursor ion of tRNA digestion product. The peak*m/z* and ion abundances in multiple spectra of one precursor ion were averaged and then combined to form a consensus spectrum. Any *m/z* peak in a consensus spectrum that was less than 0.5% of the base peak was removed. Individual spectra in the library were identified by the charging amino acid, anticodon and sequence, so that it was straightforward to find the origin of each sequence. All the consensus spectra were imported into NIST MS Search to generate the final library. At present, the *E. coli* T1 MS/MS library consists of 180 spectra. MS Pep Search is used for MS/MS batch searching. The search result report lists the matched spectrum in the library, and shows the score, dot product and probability of each hit. Tests were performed to confirm the validity of this library, and matched spectral hits had a relatively high dot product and score.

Novel Aspect
A new MS/MS library matching strategy to identify modified RNAs was developed.

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Surface induced dissociation utilized to characterize protein complexes trapped in the trap cell of a Q-TOF instrument

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Introduction

Mass spectrometry has been shown to be a useful tool in the analysis of proteins and protein complexes, enabling intact non-covalent complexes to be transferred into the gas-phase. For the study of native-like proteins, quadrupole-time-of-flight (Q-TOF) instruments are most frequently used. The fast transmission through such instruments, however, complicates fragmentation by methods such as electron transfer dissociation (ETD) which can provide additional structural information on proteins. In this study, proteins and protein complexes have been trapped for extended periods of time from 5 s to 120 s and then characterized by surface-induced dissociation (SID) to monitor structural changes induced by trapping.

Methods

Cytochrome C (10 μM), beta-lactoglobulin (40 μM) and streptavidin (40 μM) were prepared in 100 mM ammonium acetate and were ionized via nano-ESI. All studies were performed on an in-house modified Waters Synapt G2S mass spectrometer (Waters, UK), with a customized SID device installed before the ion mobility cell. Protein ions were trapped in the trap T-wave cell by rapid electrode voltage control via a WREnS (Waters Research Enabled Software) script. Trapping conditions were then optimized for each protein or protein complex. The effect of trapping conditions on the structure of protein complexes was further studied by fragmenting trapped ions with SID, in which the precursor ions are fragmented by a fast single step collision with a surface.

Preliminary data

The +7 charge state ion of cytochrome C monomer (12 kDa), the +13 charge state ion of beta-lactoglobulin dimer (37 kDa) and the +11 and +15 charge state ions of streptavidin tetramer (53 kDa) have been successfully trapped in the trap T-wave cell of a Waters Synapt G2S instrument for more than one minute without a significant change in trapping efficiency. As would be expected, the studies presented here reveal that the electrode voltages and the pressure in the trap T-wave cell can have a significant effect on the trapping efficiency. Currently, these parameters have to be tuned for each system of study for maximum trapping efficiency but it is anticipated that as more systems are run, a general set of operating conditions tailored to groups of charge states, m/z values, etc will be developed.

It was observed that charge stripping of precursor ions occurs with increasing trapping time for all the systems studied to date. Furthermore, the streptavidin tetramer with +11 charge state

undergoes less extensive charge stripping in comparison with the +15 charge state of this protein complex.
Following trapping for varying lengths of time, the +13 charge state ion of beta-lactoglobulin dimer, the +11 and +15 charge state ions of streptavidin tetramer were subjected to SID. The resulting spectra demonstrate that with trapping times of greater than 5 s, the conformation of the precursor has been altered resulting in different dissociation patterns.

Novel aspect
The protein complexes (12 kDa - 53 kDa) can be successfully trapped in a Q-TOF instrument.

2015 OMSS
VENDOR WORKSHOP ABSTRACTS

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Laserless Matrix-Assisted Ionization-Mass Spectrometry: Rapid Measurements of Small and Large Compounds on Low and High Performance Mass Spectrometers

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Introduction

Laserless matrix-assisted ionization (MAI) (Inutan and Trimpin MCP 2013) is rapid, requires minimal training, and reduces the energy requirements for ionization making it potentially ideal for use in clinical, forensic, and homeland security applications where mass spectrometers capable of simultaneous detection and quantification of a wide array of chemical and biological agents in a non-laboratory setting are likely to find a home.

Methods

The Thermo LTQ Velos, LTQ-Orbitrap Elite, and Orbitrap Fusion mass spectrometers with the ESI ion source open and interlocks overridden were used. The ion source temperature was maintained between 50-200 °C. The matrix 3-nitrobenzonitrile was prepared in acetonitrile (40 mg mL^{-1}) and 1,2-dicyanobenzene (100 mg mL^{-1}) in 70% acetonitrile:water. Analyte was dissolved in water, 50% methanol, or 50% methanol with 1% acetic acid, and the matrix and analyte solutions were mixed 1:1 and allowed to dry before being introduced to the mass spectrometer inlet. Biological fluids, as obtained, were mixed 1:1 with matrix solution. The matrix:analyte solution (1 µL) was on various sample holders such as paper and pipet tips was touched dry against the inlet aperture.

Preliminary Results

MAI is applicable to ionizing analytes with sensitivities in the low femtomole range for drugs and proteins. We show reproducibility of <10% for detection of illicit drugs such as morphine using direct analysis with internal standards. This performance is competitive with ESI but at much higher speed (~4 sec per sample). Surfaces can be readily investigated such as paper and TLC plates even in an automated fashion using a xyz-stage typically used for imaging applications. We demonstrate the feasibility of identifying proteins, peptides, drugs and metabolites directly from complex biological fluids including saliva, whole blood and urine using MAI-MS. Contrary to ESI, MAI is robust towards salts, buffers, and detergents. Furthermore, we demonstrate its

applicability to high performance mass spectrometers for rapid analysis of proteins with high resolution (>100,000) at concentrations as low of 25 fmol (25 nM). MAI-MS/MS using electron transfer dissociation (ETD) is obtained at ultra-high resolution of ubiquitin charge state +11. A prototype MAI source is introduced that is rapid and requires minimal training, making it ideal for use in clinical, forensic and homeland security applications.

Novel aspects
We demonstrate the utility of laserless matrix-assisted ionization for addressing analytical issues associated with rapid and simple measurement technology applicable with low and high end mass spectrometers.

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Novel Tools to Facilitate Translational Medicine Research; Application to an Investigation of the Effect of Prolonged Glucose Stimulation on the Lipid Profile of Mouse Heart Muscle.

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Translational Medicine has been defined in *Science* as an effort to carry scientific knowledge "from bench to bedside". Translational medicine builds on basic research advances, studies of biological processes using cell cultures, or animal models and uses them to develop new therapies or medical procedures.

Metabolomics, lipidomics and proteomics are key players in translational medicine. They represent a paradigm shift in research, away from approaches that focus on a limited number of enzymatic reactions or single pathways, to approaches that attempt to capture the complexity of biological networks. The high-throughput nature of these approaches is also well suited to potential biomarker discovery. Mass spectrometry is highly discriminatory for a large range of pathological processes making it the principal analytical method for these studies. The large amount of data derived from “omics” based mass spectrometry studies requires a workflow employing novel methods and computational tools. We will demonstrate the utility of our workflow with an example employing animal models to better understand the metabolic syndrome.

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Revisiting the Single Quad Mass Spectrometer. A compact, robust, straightforward and versatile, work horse platform.

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Introduction
Mass Spectrometry has been at the pinnacle of analytical techniques for many years but is expensive and to the uninitiated shrouded in mystery requiring expert knowledge and understanding. The degree of complexity in use manifest in earlier days has diminished over time, but the care required in sample preparation, set up and time to obtain results still pose frustrations for many. In developing a novel compact mass spectrometer (CMS), Advion has developed a simple to operate, multi-technique platform designed to be a robust, general purpose, multi-user, walk up system requiring little expertise in operation or maintenance yet uncompromising in performance.

Methods
Design specifications of the system will be briefly reviewed and examples of the most impactful uses of the CMS in a chemistry setting will be discussed. These include the use of a TLC plate reader to directly sample spots from developed plates. The direct sampling of solids (and liquids) using a direct analysis probe, ASAP and the mass directed fractionation of Flash Chromatography samples and the implications of this on the quality of the drug discovery/chemical synthesis process viewed from a Pharmaceutical industry perspective.

Preliminary data
Results from the detection and quantification of chemical compounds related to cannabis plants using both the TLC reader and the ASAP probe are reviewed along with comparisons between approaches for the monitoring of reactions and the analysis of nutritional supplements and toxins.

Novel aspect
Compact design and using a turbo pump specified from the semi conductor industry define the performance characteristics of the system.

2015 OMSS
POSTER ABSTRACTS

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Alternative Gas-phase Chemistry with the Flowing Atmospheric-Pressure Afterglow (FAPA) Ambient Ionization Source

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Introduction

Plasma-based ambient desorption/ionization techniques, which employ a low-energy plasma as a means to desorb and/or ionize molecular species, have attracted considerable attention in last few years. These plasma-based ambient desorption/ionization sources are versatile in that they enable desorption and ionization of analytes from gaseous, liquid, and solid samples. However, ionization matrix effects caused by competitive ionization processes can worsen sensitivity and inhibit quantitative capabilities. Additionally, much remains to be learned about the fundamental mechanisms which govern desorption and ionization processes. In the present study, we aim to expand the analytical capability of the flowing atmospheric-pressure afterglow (FAPA) ambient ionization source by exploring additional types of ionization chemistry.

Methods

The FAPA source, which is based around an atmospheric-pressure glow discharge sustained in helium, was used. A negative potential is applied to the cathode through 5 kΩ ballast resister, from a high-voltage, dc power supply, while the anode was connected to the ground terminal. The plasma produces highly excited helium atoms (He*), He^+, He_2^+, He_2^*, and electrons which then exit the source and interact with atmospheric gases to form reagent ions. Analytes are desorbed from the sample, by hot gas in the afterglow region, which are subsequently ionized through chemical reactions with the aforementioned reagent ions. Ions created at atmospheric pressure enter the mass spectrometer and are separated according to their mass-to-charge ratio (*m/z*).

Preliminary data

We have found that the abundance of certain reagent ions produced by FAPA source, and the corresponding ionization pathways of analytes, can be altered by changing the source working conditions (e.g., discharge power, gas flow rate, gas composition, etc.). To examine what operating conditions drastically influence the discharge and, hence, the ion chemistry, reagent-ion signals were monitored while changing the discharge current and gas flow rate. High abundance of charge-transfer reagent ions were preferentially found at low helium flow rates and high discharge currents whereas high flow rate and low discharge current produced more proton transfer reagent ions showing two different operating regimes. A model analyte, biphenyl, has shown to significantly change ionization pathway based on source operating parameters, which demonstrates that different analyte ions can be formed under a unique set of operating condition. This mode of operation enhances selectivity leading to more efficient ionization of analytes and,

in turn, enhanced sensitivity for the analytes with minimal effect on speed of detection. Furthermore, this alternative gas-phase chemistry has the potential to reduce ionization matrix effects.

Novel aspect
Added dimensionality of ambient MS through alternative gas-phase chemistry increases the range and number of detectable analytes.

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Applied Mass Spectrometry in Veterinary Diagnostic Testing

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Introduction

Mass spectrometry is an important analytical tool in veterinary diagnostic toxicology.. Inductively coupled plasma mass spectrometry (ICP-MS) and gas chromatography-mass spectrometry (GC-MS) are instruments commonly used in veterinary toxicology. New analytical methods are continuously developed to meet needs within the laboratory.

Two new methods using mass spectrometry were developed for the UKVDL. One method using ICP-MS was developed to quantitate lead and mercury in eggs at trace levels. For commercial and free-range chickens, toxic metal exposure is a concern. Routinely testing for lead and mercury in eggs indicates exposure to environmental sources and prevents toxicity in animals and humans.

Another method, using GC-MS, was developed to identify specific plant alkaloids in gastrointestinal contents. Some alkaloid-containing plants in Kentucky can be acutely toxic in small doses (*i.e. Taxus spp*, or yew) to grazing and companion animals. A method targeting nine toxic plant alkaloids or unique markers was developed to identify these compounds in gastrointestinal (GI) contents: coniine, γ-coniceine, nicotine, 3,5-dimethyoxyphenol, anabasine, atropine, scopolamine, deltaline and aconitine. These alkaloids are present in a variety of toxic plants.

Methods

Toxic metals in eggs: Aliquots of homogenized egg (yolk and albumin), from a commercial source, were digested with nitric acid at 100°C then diluted with purified water. The sample was further diluted with an internal standard solution containing cleaning materials and analyzed by ICP-MS (Agilent 7500ce) with a quadrupole mass analyzer. Along with standards, negative and positive controls were prepared in parallel to the samples for quality control purposes. Single ion monitoring (SIM) of *m/z* 206, 207, and 208 signals were combined for total lead detection with an internal standard of bismuth (*m/z* 209). Bismuth-209 was also used as the internal standard for the SIM of *m/z* 202 for mercury detection.

Plant alkaloid screen in GI contents: The alkaloids in the screen included: coniine, γ-coniceine, nicotine, 3,5-dimethyoxyphenol, anabasine, atropine, scopolamine, deltaline and aconitine. Gastrointestinal contents were extracted with 5% ethanol in ethyl acetate at pH 9-10. A 2µL analysis by GC-MS (Agilent 7890A) with electron ionization and a quadrupole mass analyzer was performed. A single ion monitoring (SIM) program was utilized for each target alkaloid to increase sensitivity. In parallel with the SIM program, a full mass scan was acquired from *m/z* 45-710. Qualitative results were confirmed by comparison to a standard overspike sample.

Preliminary Results

Metal analyses in eggs: Linearity was established for lead over 0-1000ppb and for mercury over 0-100ppb. Using the ICP-MS method, lead instrument limits of detection and quantitation were 1.58ppb and 5.26ppb, respectively. For mercury, the instrument limits of detection and quantitation were 3.00ppb and 10.0ppb, respectively. Method recoveries of overspiked egg samples ranged from 94-105% with acceptable relative standard deviations.

Alkaloid identification in GI contents: Using the GC-MS SIM method, limits of detection ranged from 0.5-2.5 ppm for the naturally occurring alkaloids or unique markers and 0.1% (w/w) for γ-coniceine. Detection of targeted alkaloids, at low concentrations, was achieved where traditional full scan and NIST database referencing methods could not because of the full scan elevated background.

Novel Aspect

These two new methods are now being used at the UKVDL to diagnose toxic metal exposures in chickens and alkaloid intoxication in livestock and companion animals.

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Rapid Screening of Raw Materials to be Used As Natural, Herbal Supplements with Direct Analysis Mass Spectrometry

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Introduction

The United States Food and Drug Administration (FDA) is responsible for monitoring the safety and efficacy of pharmaceuticals, food supply, cosmetics, as well as other biological products. While the production of manmade drugs is strictly governed by FDA regulations, dietary supplements, including homeopathic remedies, are not subject to the same rigorous rules and guidelines because they are classified as foods, not drugs. Supplement companies are tasked with developing tools and methods for evaluating the identity and safety of their products, with little guidance from the US FDA or US Pharmacopeia for how to screen materials. In the present study, we demonstrate the use of direct, high-resolution mass spectrometry approaches to screen raw materials to be used in natural, dietary supplements.

Methods

Direct Analysis in Real Time (DART) ion source was used with an Orbitrap mass spectrometer to rapidly screen raw botanical materials to be used as natural supplements. A small amount of sample was placed on wire mesh and subsequently desorbed from the mesh via resistive heating. Compounds that are characteristic of each raw material were identified via high mass accuracy and tandem mass spectrometry. The type, absolute abundance, and relative distribution of the species will be used to screen the legitimacy and efficacy of bulk imports of these raw materials.

Preliminary Data

Thus far, 21 individual raw botanical materials to be used as natural supplements have been analyzed and 62 characteristic compounds within those supplements have been identified. For example, the compounds characteristic of turmeric that were detected and identified with mass spectrometry include Ar-tumerone, tumerone, bidemethoxycurcumin, demethoxycurcumin, and curcumin. Identities of some compounds have been confirmed with MS/MS on a linear ion trap mass spectrometer to obtain structural information. In addition, authentic saw palmetto was compared to saw palmetto from brands that were recently recalled for not containing authentic ingredients. Each recalled brand contained significantly less lauric acid, oleic acid, and myristic acid, and contained no oleic acid, compared to the authentic material. Lastly, use of this method to detect trace amounts of pesticides in these raw materials will also be explored.

Novel Aspect
DART mass spectrometry to measure identity and purity of raw materials to be used in natural, herbal supplements.

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Titan's Tholins: How can we determine the components of organic "Paradise"? A comprehensive FT-ICR study

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Introduction

Saturn's largest moon, Titan, is often described as an "organic paradise". The term is used not for catching the attention but mostly because Titan's atmosphere resembles the early (reductive) atmosphere of Earth. The great success of the Cassini-Huygens mission allowed us to directly detect ions and neutrals in Titan;s atmosphere by using, e.g., the ion-neutral mass spectrometer (INMS) mounted on the Cassini spacecraft. Complex organic materials found on the surface and within the haze layers of Titan are attributed to chemistry occurring in Titan's tick N_2/CH_4 (ca., 98:2) atmosphere. Unfortunately, the "in-situ" measurements on Titan have limitations due to limited mass resolution or mass range. Thus, high resolution mass spectrometers that are necessary to reveal important details of chemical composition and reliably distinguish "isobaric" ions are still to be build and sent to orbit. This is an enormous technical and engineering (and costly) challenge that will be overcome easily in near future. Therefore, using model reactions and ultrahigh resolution mass spectrometers in laboratories on Earth are, currently, the only way to obtain detailed and comprehensive information on ion-molecule processes and their products. In our presentation, we show some selected results from our long term collaboration projects.

Methods

Laboratory tholin samples were generated in an airtight, recently designed HV ultrahigh vacuum chamber mimicking the atmospheric conditions on Titan (at the altitude of ca. 1,000 km). The atmospheric composition and pressure are well known due to the Cassini-Huygens probes' measurements. Because there is no oxygen containing molecules (> ppm level) in Titan's atmosphere the laboratory tholin samples were harvested anaerobically. Ions from tholin samples were generated by using laser desorption/ionization (LDI) and electrospray ionization in both the positive and negative modes. A Bruker 9.4 T Apex Qh FT-ICR ultrahigh resolution was used to obtain LDI and ESI spectra.

Preliminary Data

Tholins are organic polymers the components of which have a general formula of $C_xH_yN_z$. More saturated compounds (imines, amines) are detected in the positive mode, while more unsaturated compounds indicating compounds rich in CN group contribution. These differences can be easily visualize on van Krevelen plots. Many of the $C_xH_yN_z$ can be hydrolyzed with water and/or

ammonia/ice water that leads to $C_xH_yN_zO_n$ molecules. Hydrolysis kinetics was measured from which Arrhenius activation energies have been determined indicating that oxygen incorporation can occur in a 3,000-10,000 year time frame even at around 100 K (surface temperature). Our recent study showed that, overall, both ESI and LDI resulted in complex negative ion MS spectra that contains several hundreds of ions in the m/z range of 50-300. LDI produced more $C_xN_z^-$ ions, such as $C_{10}N_5^-$, which we assigned as the pentacyanide cyclopentadiene ion that was supported by MS/MS measurements (CID, QCID, SORI, and IRMPD). In addition to the ultrahigh resolution MS and MS/MS results we will show supporting information obtained by variable wavelength IRMPD experiments and quantum chemical calculations. To our best knowledge this is the first time when the characteristic νCN stretching vibration band was detected for a deprotonated tetracyanide. Quantum chemical calculations predict the formation of small anion-neutral complexes that can be considered as "seeds" for larger covalent and/or non-covalent complexes.

Novel Aspect
The applied experimental techniques (tholin production and ultrahigh resolution FT-ICR) provide detailed insight in the formation and structures of several pre-biotic molecules formed by gas-phase reactions.

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On-line Manipulation of Protein Charge State by Contained-electrospray Ionization

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Introduction

It is well established that highly charged unfolded proteins yield better sequence information but the current methods of producing highly charged protein ions, including solution-phase protein denaturation (via addition of acid, soap, or supercharging reagents) and ion/ion reactions are not amendable with liquid chromatographic (LC) systems. The motivation behind this study is to develop analytical devices that are capable of protein denaturation and ionization in a single experiment step, and can be coupled to LC-MS instrumentation. In pursuit of this larger aim, the current study describes a new electrospray ionization (ESI) device (contained-electrospray apparatus) that achieves protein charge state manipulation (CSM) through the exposure of the electrospray (ES) droplets containing the protein to acid vapor at atmospheric pressure. The droplet modification occurs within milliseconds; this is something that can allow the coupling of the contained-ES device to LC (separation occurs in minutes time scale). Tetralysine peptide (K_4, *MW* = 530.7 Da), myoglobin (*MW* = 17.6 kDa) and trypsin (*MW* = 23.8 kDa) have been analyzed using the contained-ES apparatus, in a direct infusion experiment, and shown that on-line CSM can be achieved simply by changing the electrospray nebulizer gas pressure. For myoglobin, we did not detect the presence of the heme cofactor in the resulting mass spectrum. This observation suggests that the shift to higher charge states occurred quicker than the time (<100 ms) required for the heme group to escape the unfolding protein in the presence of acid.

Methods

The contained ESI apparatus is made from a cross Swagelok element with three inputs and one outlet; the three delivering inputs and the exit channel are arranged at 90° to each other. The electrospray (ES) emitter (100 µm ID fused silica (FS) capillary) is inserted into a second/outer capillary (250 µm ID FS capillary). For these two concentric capillaries, the emitter is placed slightly outside of the outer capillary and delivers the sample from the syringe pump to the exit channel where the derived droplets interact with headspace vapor of HCl or acetic acid. The acid vapor is carried by a nitrogen nebulizer gas, brought in from the top input. The bottom input is connected to a container in which the volatile reagents can be placed. Non-volatile reagents can be used by replacing the 200 µm FS outer capillary with a 0.8 mm (ID) theta glass capillary; in our experiments, we insert the electrospray emitter into one chamber and the second remaining chamber is filled with the non-volatile reactive reagent. Mixing of the analyte from the electrospray emitter and the reagent occurs inside the Taylor cone before the release of charged micro-droplets.

Preliminary Data

The main advantage associated with the contained-ES ion source is the ease with which CSM can be achieved – simply by changing nebulizer gas pressure. For example, conventional ESI analysis of Lys-Lys-Lys-Lys prepared in MeOH/H_2O (50:50, in the absence of acid) showed the presence of singly (mostly) protonated cation. In a separate experiment, the same Lys-Lys-Lys-Lys solution was analyzed using the contained-ES ion source at 30 psi nebulizer gas pressure and in the presence of acetic acid. The results of analysis showed an increased charged state from +1 to +2 (minor), and +3 (major). At higher nebulizer gas pressure (100 psi), however, the dominant charge state for Lys-Lys-Lys-Lys was observed to be the doubly charged state. This result signifies pH effects in which a high concentration of headspace vapor is allowed to interact with the droplets at low N_2 gas pressure compared with higher pressures. In separate experiment, we exposed the electrospray droplets to ammonium acetate (10 mM) using the theta capillary arrangement and observed the packing of the different charge states into a single doubly charged K_4 species. For the acid vapor exposure experiments, similar pH effect was observed for myoglobin prepared in MeOH/H_2O (80/20). Conventional ESI (and in the absence of acid) analysis of this solution produced myoglobin cations at +8 and +9 charge states. To further increase the charge state, we exposed the electrospray droplets to headspace vapor of acetic acid. At 30 psi nebulizer gas pressure, we observed a shift to +9, +10, +11 charge states, with the +10 cation being the most intense signal. The signal-to-noise ratio for myoglobin analysis was increased (~10%) in the presence of acetic acid vapor. As expected, increasing the nebulizer gas pressure from 30 to 100 psi reduced the abundance of the acid vapor, and shifted the ions to relatively lower charge state compared with low pressure. At 100 psi, we observed +8, +9 (major), +10, +11 charge states. In all cases, detectable amount of heme cofactor from the myoglobin was not observed. Contained-ES-MS data for trypsin will be discussed.

Novel Aspect

A contained ESI apparatus allow on-line protein charge states manipulation at atmospheric pressure

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On-line Modification of Charged Droplet Environment with Acid Vapor Eliminates Matrix Effects in Electrospray Ionization Mass Spectrometry

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Introduction

There is significant demand for accurate qualitative and quantitative analysis of complex mixtures by electrospray ionization mass spectrometry (ESI-MS). Nevertheless, ESI-MS is frequently complicated by ion suppression effects among the different type of analytes (e.g., polar, non-polar, surface active, etc.), which cause difference in their ionization response. As a consequence, accuracy, precision and sensitivity of ESI-MS analysis are greatly affected, irrespective of the choice of a mass analyzer. The objective of this study is to reduce ion suppression effect in ESI. This aim was achieved through the development of a new contained-electrospray apparatus/emitter. The new contained-ESI ion source provides mass spectrum in which the relative ion intensities of all analytes reflect actual concentration of each analyte in the original solution mixture. The mechanism for this effect is elucidated and fully discussed. In addition, lower limits of detection are achieved when the apparatus is operated in the reactive mode.

Methods

To construct the contained-ES apparatus, the electrospray (ES) emitter (inner capillary) is inserted into an outer capillary and linked to a cross Swagelok element. There are three inlets in the apparatus (nebulizer gas, analyte solution and head space vapor of reactive gas) and only one outlet for continuous droplets releasing and modifying by applying DC voltage to the ES emitter.The apparatus can be operated under two operating modes: (i) analytical mode - the ES emitter is slightly (~ 1 mm) pushed outside of the outer capillary for the short chemical droplet modification (within milliseconds) with the reactive gas headspace vapor, and (ii) reactive mode - the ES emitter is pulled inside of the outer capillary to make a cavity, where extended droplet modification occur.

Preliminary Data

The contained-ES apparatus was employed under analytical mode to analyze steroids (e.g., cortisone, corticosterone, etc.) in the presence of commonly accounted matrices: (1) quaternary ammonium species (e.g., Girard reagent T) was selected to represent species that do not compete with the analyte for protons, (2) long chain alkyl amines (e.g., dodecyl amine) as an example of matrices that compete for protons, and (3) phospholipids (e.g., 2-Oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine) to represent typical matrices found in biological samples. The response from a typical ESI-MS for cortisone shows significant suppression from Girard reagent T (GRT) and dodecyl amine, regardless of the amount of cortisone used. By employing the contained-ES apparatus in which we exposure the electrospray droplets to HCl headspace vapor, the ion

intensities for all analytes were adjusted according to their real abundances in solutions, including cortisone-GRT molar ratios of 1:1, 1:2, 1:3, 2:1, 3:1, and cortisone-dodecyl amine (1:1) mixtures. This result is ascribed to the combined effect of proton abundance and the production of ultrafine charged droplets under specific conditions described in this project. Furthermore, equal relative ion intensities were accordingly adjusted (at an optimized pressure and heat) for a methanol/water (50/50) solution consists of equimolar (0.5 μM) mixture of methamphetamine, benzoylecgonine, and delta-9-THC. In the reactive mode, a condensation reaction between cortisone and GRT was performed, producing a charged-labeled product. The observed signal enhancement under this reactive mode of the contained-ESI will be also presented for different steroids.

Novel Aspect

A new electrospray apparatus is described that eliminates ion suppression effects in ESI-MS analysis of complex mixtures without prior extraction or chromatographic steps.

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FT-ICR combined with gas phase HDX, IRMPD, and IM to distinguish between isomeric structures of peptide ion fragments

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Introduction

Studies on peptide fragmentation mechanisms have been an important area of fundamental mass spectrometry that led to the better understanding the role of the mobile ionizing proton (mobile proton model [1]). Although initial work has been performed on low resolution (e.g., Q-type hybrid instruments), more recently FT-ICR instruments with ultrahigh resolution have extensively used to confirm and/or correct chemical compositions of (isobaric) fragment ions. (For example, the b2 ion from protonated GnR peptides ($C_5H_9N_2O$, m/z 115.05020) is "isobaric" with an R related fragment ($C_5H_{11}N_2O$, 115.08659), see Ref. [2]).

FT-ICR instruments allow us not only to determine ion masses (chemical compositions) with sub ppm accuracy but also to perform MS/MS fragmentation and ion-molecule reactions by efficiently trapping ions in the ICR cell. A relatively simple hydrogen/deuterium exchange (HDX) method was implemented in our laboratory with only minor hardware and software additions. The degree and kinetics of HDX depend on the structure of the trapped ions, thus structural isomers can be distinguished in the gas phase. HDX kinetics can be controlled by using different reagents, such as D_2O (slower rates) or ND_3 (faster rates).

Methods

Model peptides were either purchased or synthesized by conventional solid phase methods. 10-50 μM peptide solutions were prepared in H_2O:ACN 1:1 (containing 0.1% formic acid) and were spray by applying regular electrospray conditions. Gas-phase HDX experiments were performed on a Bruker 9.4T ApexQh FT-ICR instrument with minimal hardware modifications. HDX kinetics was controlled by using different reagents, such as D_2O (slower rates) or ND_3 (faster rates). Variable wavelength IRMP experiments were carried out in the CLIO laboratory (Paris Sud, Orsay, France). Ion mobility (IM) experiments were performed in the Wysocki lab on a Synapt G2 Q-TOF instrument.

Preliminary Data

In our presentation, we show some selected examples for using FTICR HDX experiments to distinguish between oxazolone (up to 3 exchanges) and diketopiperazine (only one exchange) structures of b_2 and b_3 fragments [3, 4], amino acid (i.e., potential metabolites) fragments [5]. We will also demonstrate the usefulness of FTICR HDX reactions for specific gas-phase "guest-host" chemistry that can be used to distinguish between larger cyclic or linear b_n type ions [6]. Related

and supporting results from variable wavelength IRMP and ion mobility (IM) will also be presented.

Novel Aspect
The combination of gas phase HDX, IRMPD and IM experiments provide information on isomeric ion structures and peptide fragmentation mechanisms.

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Investigating Crystallization Mechanisms of Microporous Materials Using Ion Mobility-Mass Spectrometry (IM-MS)

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Metal organic frameworks (MOFs) have drawn immense attention in past two decades because of their interesting structure and significant potential for membrane and adsorption applications. The key challenges for broader utilization of MOFs remain achieving high yields and synthesizing novel structures. These deficiencies originate from a poor understanding of the detailed crystallization mechanism responsible for the different structures.

This project seeks to determine the species critical for nucleation and growth of metal-organic frameworks (MOFs) using ion mobility-mass spectrometry (IM-MS). Several other methods including NMR, WAXS/SAXS, and AFM have provided important insights on the crystallization process, but none of the studies have identified the molecular species responsible for the supramolecular structures. We hypothesize that with sensitivity and resolution of ion mobility-mass spectrometry (IM-MS) we can monitor the dynamics of crystallization and identify the critical species. This novel application of IM-MS will enable discovery of new crystal structures and modification of synthetic conditions to improve crystal phase purity and overall yield. The preliminary data shows existence of various species with high *m/z* at residence times on the scale

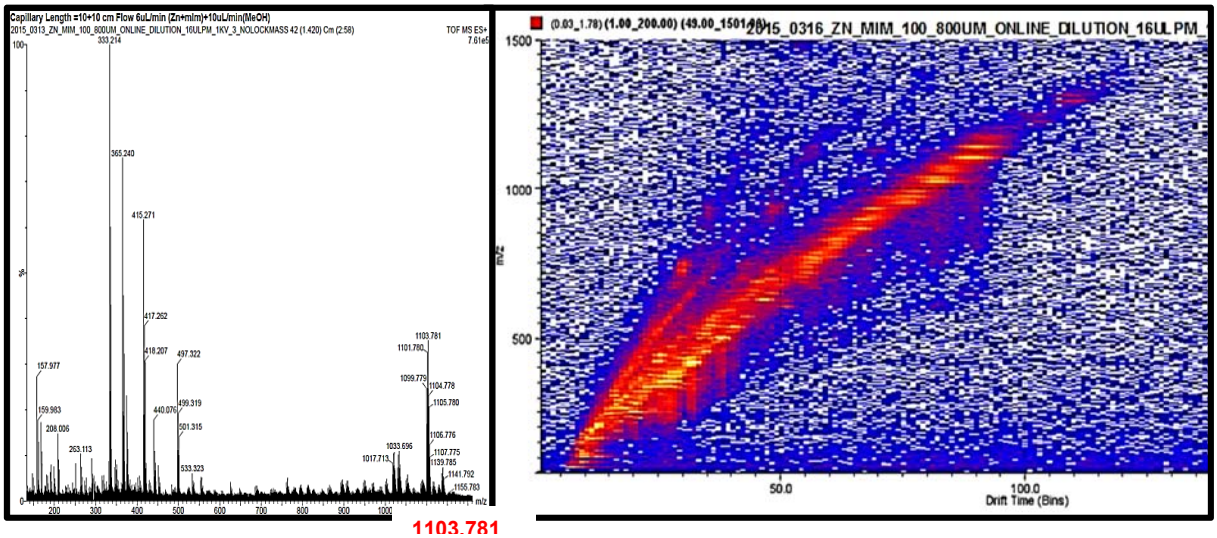

Figure 1: Spectra and mobilogram for online mixing of 100μM $Zn(NO_3)_2.6H_2O$ and 800μM 2-methylimidazole with online dilution using methanol. The data was acquired using Waters Synapt G2-S with ESI source.

of 10s. The long-term objective of this work is to establish IM-MS as an important characterization method for organic-inorganic material crystallization. The system will be expanded to study crystallization of aluminum species for thin film dielectrics, crystallization of aluminum and silicon species for zeolites, and assembly of catalytic intermediates.