1. Abstract: Trevor Adams

Site-specific cross-clade N-glycopeptide analysis of HIV-Env NFL trimers

Trevor Adams, Mukaddes Sena Cinar, Fikri Avci

The densely glycosylated Env trimer is the sole antigenic target of broadly neutralizing antibodies (bnAbs) during HIV infection. However, the glycan shield, predominantly composed of N-glycans, provides the virus with a robust defense by protecting conserved regions of the underlying protein. In previous work, our group identified a novel glycosylated epitope capable of being presented to a T cell receptor (TCR) and stimulating an immune response. This finding highlights the underappreciated potential of glycosylated peptides as novel T cell epitopes. It is therefore useful to develop an understanding of the conservation of site-specific glycoforms across clades rather than just the underlying peptide. Here, we expressed three variants of HIV-Env in Exp293 cells as native flexibly linked (NFL) trimers that represent three major clades of virus: A (BG505), B (JR-FL), and C (16055). The site-specific glycosylation of these trimers was characterized using both glycomics and glycopeptide analysis. The glycopeptide analyses utilized high-field asymmetric waveform ion mobility spectrometry (FAIMS) to enrich glycopeptides in a non-biased manner. By utilizing a variety of protease conditions, we achieved broad coverage across most sites of N-glycosylation. This work advances our understanding of the conservation of glycoforms across clades and has potential applications in the development of novel vaccines and therapeutics.

2. Abstract: Sophia Bamishaye

Identification of E. Coli Contaminant Protein During the Expression and Purification of MRI contrast agent hProCA32.Collagen1 Using Bottom-Up Proteomics Approach

Sophia Bamishaye[1,2], Md. Ackas Ali[3], Akinlotan Francis[1], Dorabadi Anita[1], Li Dongjun[1], Zongxiang Gui[1], Kirberger Michael[1], Qiao Jina[1], Wang Siming[1], Mohammad A. Halim[3], and Jenny Yang[1,2]

[1]Department of Chemistry, Georgia State University, Atlanta, GA
[2]Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA
[3]Department of Chemistry, Kennesaw State University, Kennesaw, GA

Magnetic Resonance Imaging (MRI) as a diagnostic and therapeutic tool has revolutionized medical imaging by providing non-invasive and detailed visualization of tissues and organs, providing insight into many biological processes. To overcome the limitations of low sensitivity and specificity of Clinically approved MRI contrast agents, the development of contrast agents that possess high relaxivities and targeting capabilities for molecular imaging of disease biomarkers is imperative. The protein-based MRI contrast agent, hProCA32. Collagen1 is developed from scaffold protein parvalbumin engineered to a collagen type I targeting peptide with a flexible linker. The developed MRI contrast agent was expressed in the periplasmic and cytoplasmic space of bacterial cell E. coli. However, during the purification process, additional contamination proteins are observed along with hProCA32.Collagen1. Identification of such co-purified proteins is challenging by traditional methods. In this study, we employed LC-MS and

bottom-up proteomics methods to identify the co-purified proteins. Initially, three sample fractions (A, B, and C) were characterized by LC-MS method. Sample fraction B showed a single protein of hProCA32.Collagen1 with an experimental mass of 14,394 Da which agrees with the theoretical mass. However, sample fractions A and C showed the presence of additional proteins along with hProCA32.Collagen1. To identify the co-purified protein, the samples were digested with trypsin, and peptides were separated by RP-LC using Vanquish Flex HPLC with a 90-minute gradient. An Orbitrap Exploris 240 Mass Spectrometer was used to identify the peptides employing a data-dependent analysis method. Proteome Discovery Software was used to search the LC-MS/MS data against the E. coli proteome from UniProt using the SEQUEST algorithm. Our preliminary results identified the hProCA32.Collagen1 protein with 66% sequence coverage with an additional periplasmic chaperone protein from E. Coli with high confidence. This study showed bottom-up proteomics can identify the co-purified contaminant proteins when they display a size analogous to the recombinant protein.

3. Abstract: David Brewer

Revealing the alteration of *S. aureus* and tissue-derived lipids *in vitro* using lipid-enhanced media

David Brewer and Kelly M. Hines
Department of Chemistry, University of Georgia, Athens, GA

Staphylococcus aureus can alter its native membrane composition by using exogenous fatty acids that are liberated from intact lipids by its lipase, glycerol ester hydrolase (*Geh)*. The resulting free fatty acids can then enter lipid biosynthesis through the fatty acid kinase pathway (*FakA/B1/B2*). While most research has studied incorporation of specific mammalian fatty acids, such as oleic acid, *S. aureus* has access to many different types of lipid substrates and fatty acids in the environment of a deep-tissue infection. In this study, we investigate this interplay using broth that is enriched with tissue-specific lipid extracts. *S. aureus* strain JE2 was incubated for 16 hours in tryptic soy broth (TSB) supplemented with 50 µg/mL of commercial bovine heart, brain, or liver polar lipid extracts. Lipids were extracted from both bacterial pellets and broth and analyzed by reversed-phase liquid chromatography and mass spectrometry (RPLC-MS). *S. aureus* pellets collected after growth in lipid-enriched broth contained phosphatidylglycerols (PGs) with fatty acyl tails that had one to six double bonds, indicating incorporation of bovine lipid extract-derived mono- and polyunsaturated fatty acids. Analysis of the spent broths revealed an increase in lyso-phospholipids after incubation with *S. aureus*. The majority of these lyso-lipids had the remaining acyl tail at the *sn-2* position, indicating a preference for *Geh* to act on the acyl tail at the *sn-1* position of the glycerol backbone. Our results reveal that lipids of both the bacterium and its local environment are significantly altered through the combined actions of *Geh* and *FakA/B1/B2*.

4. Abstract: Justin Byun

Optimizing solid phase extraction to prepare wastewater samples for untargeted exposome screening

Justin Byun, Catherine Mullins, Douglas Walker
Emory University, Rollins School of Public Health, Gangarosa Department of Environmental Health, Atlanta, GA, United States

Increasing evidence suggests that environmental exposures drive risk of a number of non-communicable diseases; however, current methods to evaluate environmental factors within a population do not account for the complex chemical exposures that likely occur. With the need for a more complete environmental exposure assessment to understand the association between environmental influences and their biological response, wastewater-based epidemiology holds potential to identify the chemical exposome without requiring individual samples from a study population, avoiding the costly and logistical constraints of human biomonitoring of biofluids. The purpose of this study was to optimize and validate a solid phase extraction (SPE) method to prepare wastewater samples for untargeted screening using liquid chromatography-high resolution mass spectrometry (LC-HRMS). We evaluated how SPE can enhance determination of the composition of analytes in wastewater to potentially identify previously unrecognized exposures for a more comprehensive chemical exposome profile. To evaluate the potential of using SPE to increase detection of exposures in wastewater, samples from 27 different sites in Louisville, Kentucky were collected, including a range of industrial and residential areas and analyzed using SPE with LC-HRMS. SPE significantly increased the intensity of detected features in wastewater and the total number of detected features in wastewater compared to traditional sample preparation methods. Each site exhibited a larger number of detected features in wastewater prepared through SPE compared to wastewater prepared with traditional methods. Future steps include analyzing LC-HRMS data to determine if concentrations of certain detected chemicals differ significantly between wastewater sites with high and low median income levels.

5. Abstract: Ryan Coyle

Increased levels of Erythrocytic α-Synuclein using targeted mass spectrometry

Idiopathic Parkinson's disease (iPD) is the second most common neurodegenerative disease after Alzheimer's disease (AD). Mutations in the SCNA gene, which encodes the protein alpha-synuclein (α-syn), are associated with familial forms of Parkinson's disease (PD). Additionally, Lewy bodies (LBs) rich in α-synuclein are a hallmark of idiopathic Parkinson's disease (iPD) pathology. Unlike AD, there are no effective blood-based diagnostic assays for iPD. Recent studies show that misfolded α-syn can aid in diagnosing PD via cerebrospinal fluid (CSF) and skin biopsies, suggesting that the altered cellular processes in the brain may also occur in the periphery. However, CSF and skin biopsies are invasive, highlighting the need for a blood-based diagnostic assay. Erythrocytes (red blood cells) are the richest source of α-syn in the body, and we hypothesized that peripheral α-syn changes could be detected in erythrocytes in iPD. To test this hypothesis, we used a targeted liquid chromatography-mass spectrometry (LC-MS) assay, employing 15N-enriched recombinant α-syn as an internal standard, to compare α-syn levels in

erythrocytes from iPD patients, AD patients, and healthy controls. The results showed α-syn concentrations (standard deviation) of 42.6 μg/mL (27.8) in healthy controls, 36.7 μg/mL (19.0) in AD, and 50.3 μg/mL (29.2) in iPD. Although α-syn levels were significantly elevated in iPD, the receiver operating characteristic (ROC) analysis yielded an area under the curve (AUC) of 0.63, indicating that elevated α-syn levels alone are not sufficient for diagnostic purposes.

6. Abstract: Longping Fu

A Chemoenzymatic Method for Site-Specific Profiling of Protein O-GlcNAcylation

Longping Fu and Ronghu Wu
School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA

Protein O-GlcNAcylation plays extremely important roles in mammalian cells, and it regulates cell signaling and gene expression. Global and site-specific analysis of protein O-GlcNAcylation still remains challenging due to the low abundance of many glycoproteins and the complexity of biological samples. Proteins from human cell lysate were digested into peptides. To enrich the O-GlcNAcylation of peptides, galactosyltransferases were introduced in conjunction with UDP-galactose or UDP-GalNAc, along with $MnCl_2$, to selectively modify O-GlcNAc moieties with galactose or GalNAc. After the addition of galactose to O-GlcNAc, the C6 hydroxyl group of the galactose or GalNAc can be specifically oxidized and converted to the aldehyde group by galactose oxidase (GAO). The aldehyde group generated after the oxidation serves as a handle to enrich glycopeptides using hydrazide resins. After the removal of nonspecific binding peptides, the enriched glycopeptides were eluted with methoxyamine. The eluted O-GlcNAcylated peptides carry a relatively small mass tag, which enabled site-specific analysis of O-GlcNAcylation by LC−MS/MS. Herein, we report a chemoenzymatic method for site-specific profiling of protein O-GlcNAcylation with high specificity and sensitivity. This method was applied to analyze protein O-GlcNAcylation in MCF7 cells, and 451 glycoproteins were identified. This method is applicable to different samples, including tissues, because it has no sample restriction. Effective methods for comprehensive and site-specific analysis of protein O-GlcNAcylation will advance our understanding of this important modification and cellular activities.

7. Abstract: Jada S. Gray

A Dynamic Simultaneous Quantitation and Discovery (SQUAD) Liquid Chromatography Mass Spectrometry Approach for Inflammatory Bowel Disease Metabolomics in African Americans

Jada S. Gray, Olatomiwa O. Bifarin, Samuel Moore, David Gaul, Sana Syed, Adam Greene, Jason Papin, Subra Kugathasan, Facundo M. Fernández

School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA
University of Virginia School of Medicine & UVA Child Health Research Center, Charlottesville, VA 22903
Emory University School of Medicine, Atlanta, GA 30322

Inflammatory Bowel Disease (IBD) in African American (AA) and individuals of European Ancestry (EA) is steadily growing. Unfortunately, IBD studies underrepresent AA, compromising our understanding of disease etiology and progression in the subpopulation. IBD affects 96 of 100,000 AA every year, so generation of numerous well-balanced and matched biospecimens for metabolomic studies is challenging. Metabolomics provides the most immediate snapshot of the IBD phenotype, showing contrasting pathogenesis in AA vs. EA individuals. We here present a new Dynamic SQUAD approach where LC-MS experiments are performed in a sequential fashion as more biospecimens become available. This approach builds a targeted metabolite panel that reflects IBD pathway alterations from the non-targeted data block produced by the previous SQUAD LC-MS batch. We conducted SQUAD LC-MS experiments on biopsies from IBD patients to measure targeted and non-targeted data for different analytes using a Thermo ID-X tribrid mass spectrometer.

A literature metanalysis indicated specific metabolic reactions that can differentiate Crohn's disease (CD) from healthy patients. Pathways altered included amino acid metabolism, tryptophan metabolism, microbial metabolism, and energy metabolism, indicating dysbiosis of the gut microbiome, showing a decrease in the diversity of microbes/metabolites in the gut[1]. Additionally, metabolic modeling indicated that inflamed CD patient ileal tissue displays a distinct metabolic signature compared with non-inflamed tissue[2]. Subsequently, we sought to experimentally confirm whether the CD ileum contained metabolic changes versus controls using non-targeted LC-MS of a small external pilot cohort, complementary to *in silico* metabolic modeling to characterize whether the changes in metabolic pathways correlate to specific changes in specific lipids and/or metabolite composition. The top discriminant analytes were also added to the first targeted SQUAD panel. These ongoing studies will help us probe metabolomics alterations in the mucosal tissue to further our insights on IBD differences across populations.

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8. Abstract: Alexandria Van Grouw

Automated Single Cell Lipidomics for Mapping MSC Heterogeneity

Alexandria Van Grouw[1], Samuel Erlich[2], Alexandre E. Dunnum[2], Rahul Deshpande[3], Joseph Corstvet[1], Johnna S. Temenoff[4], Craig R. Forest[2], Facundo M. Fernández[1]

1. School of Chemistry and Biochemistry, Georgia Institute of Technology
2. GW Woodruff School of Mechanical Engineering, Georgia Institute of Technology
3. Thermo Fisher Scientific, Inc.
4. Wallace H. Coulter Department of Biochemical Engineering

Mesenchymal stromal cells (MSCs) are multipotent stem cells with anti-inflammatory properties that show promise in treating various immune disorders and injuries. However, their therapeutic potential has been difficult to harness due to individual cell heterogeneity and relatively rapid progression to senescence compared to other cell types. A novel single cell selection method paired with ultra-sensitive high resolution mass spectrometry permits metabolomic analysis at the single cell level.

In order to obtain an initial sensitivity assessment, cell lysate from HEK cells at a concentration of 10,000 cells/µL that was diluted 10x, 100x, 1000x, and 10,000x so that the lowest calibration level was at a concentration of 1 cell/µL. These samples were analyzed using multiple techniques in order to compare sensitivity between multiple mass spectrometry methods. They were analyzed using microflow reverse phase liquid chromatography (LC) at 400 µL/min and 50 µL/min, as well as using nanoflow reverse phase LC at a flow rate of 500nL/min. Samples were also analyzed on multiple mass spectrometers including a Thermo Orbitrap Astral and Thermo Orbitrap Exploris 240. All samples were run in positive ion mode with DDA for MS2 spectra collection. Data was processed and analyzed using Thermo Compound Discoverer 3.3 and Lipid Search.

Nanoflow LC separation combined with analysis on a Thermo Orbitrap Astral utilizing MS1 data collection in the Orbitrap with simultaneous MS2 scans in the Astral mass analyzer achieved the greatest sensitivity with 172 lipid annotations at the single cell level. With such appropriate sensitivity achieved, we will employ a single cell selection method using a robotic automatic single cell patch clamping setup that leverages a machine vision algorithm for cell detection detection. Once the single cell sample preparation has been fully optimized, profiles of single cell lipidomes of MSCs will be measured and used to map colony heterogeneity.

9. Abstract: Jiangpeiyun Jin

Deciphering the Mechanism of Antibiotic Response in Cystic Fibrosis Bacteria and Their Crosstalk with Fungal Pathogen Using Metabolomics

Jiangpeiyun Jin, Neha Garg
School of Chemistry and Biochemistry, Georgia Institute of Technology,
901 Atlantic Drive, Atlanta, GA 30332, USA

Burkholderia cenocepacia can cause chronic infections in immunocompromised individuals, such as cystic fibrosis (CF) patients. Due to the inherent resistance to many commonly used antibiotics in clinical treatment, infections by *B. cenocepacia* are challenging to treat and life-threatening for CF patients. *B. cenocepacia* is known to produce a variety of natural products, some of which serve as virulence factors during infections, providing a fitness benefit to survive and adapt to the environmental changes. Production of natural products is dependent on the expression of their biosynthetic gene clusters (BGCs), which are typically silent under normal growth conditions. Antibiotics, as an important constituent of the CF lung environment, can act as external stimuli to activate the production of natural products in *B. cenocepacia* via an unknown mechanism. In addition, the presence of several different pathogen species in CF lungs provides a great opportunity for polymicrobial communication. We utilized mass spectrometry based untargeted metabolomics to investigate the response of *B. cenocepacia* to clinically used antibiotics at sublethal concentration and leveraged mutations in quorum sensing genes to mechanistically characterize such response. Using the metabolomics approach, we linked QS-controlled gene

regulation to the antibiotic mediated metabolic response in *B. cenocepacia*. Furthermore, we revealed the chemical interactions between *Burkholderia cenocepacia* and *Aspergillus fumigatus*. This work emphasized that antibiotics can act as signaling molecules at sublethal concentration to mediate microbial BGCs expression that can involve in cell-to-cell communication. This work enabled us to improve our understanding on pathogenic physiology of *B. cenocepacia* under clinically relevant conditions.

10. Abstract: Katherine J. Kenney

An Optimized MALDI Assay for Tree Gum Discrimination in Works of Art

Katherine J. Kenney[1], Daniel D. Vallejo[1], Katell Bathany[2,3], Caroline Tokarski[2,3], Facundo M. Fernandez[1]

[1] School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA, USA
[2] University of Bordeaux, CNRS, Bordeaux INP, CBMN, UMR 5248, F-33600 Pessac, France
[3] Bordeaux Proteome Platform, University of Bordeaux, F-33000 Bordeaux, France

Gums are naturally occurring polymers formed by plant cell walls and trees. Due to their chemical and mechanical properties many plant gums are ubiquitously employed in industry as binding media and adhesives. However, they are also found in cultural heritage objects (CHO). Discrimination between these gums is significant for determining appropriate conservation efforts for such objects; however, confident characterization is challenging due to their complex polysaccharide structural motifs, high molecular weight, and high polydispersity. Recent efforts have been made to improve discrimination of gums through fingerprinting approaches with mass spectrometry (MS). By optimizing this technology, we will not only improve analysis throughput, but also establish the level of confidence that can be achieved with identifying plant gums. We reproduced a MALDI MS assay for characterizing three different gums: Arabic Gum(AG), Tragacanth Gum(TG), and Locust Bean Gum(LBG). The current assay requires 5-hour digestion; mass spectral fingerprints to differentiate each gum can be observed at 1-hour of digestion, and further optimization of this assay will be tackled to further improve results. Enzymatic cleavage breaks down polysaccharidic gums into hexose units of varying length to be analyzed using matrix-assisted laser desorption/ionization (MALDI) MS. Notably, each gum is composed of distinct and repeating oligosaccharidic chains. Every polysaccharidic sugar chain is composed of one base sugar and its respective residue. Gums can be identified by the range of characteristic monosaccharides and oligosaccharides signals that lead to a fingerprint for each gum. With further optimization of the enzymatic digestion, we plan to improve throughput of analysis by 5-fold. By implementing an automated data analysis package, we hope to further improve throughput of analysis and generate a mass spectral library for plant gums.

11. Abstract: Jessica Eyram Kugblenu

Histidine[421] mutation alters catalysis and metal content of *Pseudomonas aeruginosa* D-2-Hydroxyglutarate Dehydrogenase

Jessica Eyram Kugblenu[§], [§]Joanna A. Quaye[§] and [§¤‡]Gadda, Giovanni[§¤‡].
[§]Department of Chemistry, [¤]Department of Biology, [‡]The Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30302-3965, United States

P. aeruginosa is an opportunistic gram-negative, aerobic, and non-spore-forming bacterium found in soil, water, and skin flora[1–4]. It causes infections in immunocompromised individuals with ailments such as cystic fibrosis, cancer, AIDS, lung diseases[5,6]. In 2017 alone, *pseudomonas* infections caused 3000 deaths and 30,000 nosocomial infections[5]. *P. aeruginosa* depends on D-2-hydroxyglutarate dehydrogenase (*Pa*D2HGDH), which oxidizes D-2-hydroxyglutarate to 2-ketoglutarate, to drive L-serine biosynthesis for survival[1,2,7]. Knockout of the *Pa*D2HGDH gene inhibits *P. aeruginosa* growth, making *Pa*D2HGDH a therapeutic target against *P. aeruginosa*[2,8]. Upon recombinant expression of *Pa*D2HGDH, there was only a 15% flavin reduction. Previous studies on closely related FAD-dependent dehydrogenases showed increased enzyme activity upon exogenous metal addition[6]. When *Pa*D2HGDH was analyzed using ICP-MS, significant but non-stoichiometric amounts of Mg^{2+} and Zn^{2+} were bound to *Pa*D2HGDH; however, only zinc could increase *Pa*D2HGDH activity[1]. Upon *Pa*D2HGDH purification in 1 mM $ZnCl_2$, flavin reduction increased to 92% and zinc was identified as a required metal cofactor that binds, orients, and activates the D-2-hydroxyglutarate substrate for catalysis[1,7]. However, in closely related flavin-dependent enzymes such as the glucose-methanol-choline (GMC) enzymes, a catalytic base is responsible for activating the substrate for catalysis[9]. Amino acid sequence comparison of *Pa*D2HGDH with other GMC enzymes reveals a fully conserved histidine[421] residue, whose topology in the *Pa*D2HGDH active site is conserved for catalytic bases in other GMC protein structures[2]. However, the role of H[421] in *Pa*D2HGDH has not been established.

In this study, site-directed mutagenesis was employed to replace H[421] with glutamine, asparagine, phenylalanine, or cysteine. The variant enzymes were purified in the presence of 1 mM $ZnCl_2$ and investigated for their kinetic and biophysical properties. ICP-MS was used to detect the metal content of the variant enzymes. The ICP-MS data showed that all variants retained significant amounts of the zinc metal while Mg^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Fe^{2+} occurred in trace amounts. The kinetic data for the variant enzymes showed a 400-fold decrease in activities in comparison to the turnover rate of the wild type suggesting that though the zinc metal was present in the variants, the fully conserved H[421] residue is important for *Pa*D2HGDH catalysis.

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12. Abstract: Yi Lasanajak

Advanced LC-MS Capabilities for Protein, Glycan, and Oligonucleotide Analysis at the Emory EGMIC Core

Yi Lasanajak[1], Afzal Yacoob[1], Xuezheng Song[1,2], Blaine Roberts[1,2]

1. Emory Glycomics and Molecular Interaction Core, Atlanta, GA 30322
2. Department of Biochemistry, Atlanta, GA 30322

Emory Glycomics and Molecular Interactions Core (EGMIC) is equipped with top-of-the-line LC-MS instruments. Our core provides extensive support to researchers through a broad range of mass spectrometry tools and techniques. This includes the options to learn to conduct LC-MS analysis for trainees or to have our dedicated staff run the samples. We are unique in the world by offering ion mobility mass spectrometry with electron capture dissociation. These techniques allow characterization of the shape and quaternary structure of proteins, and resolve glycan, and lipid isomers. In addition, we conduct validation of reagents especially recombinant proteins and synthetic peptides. This allows the user to confirm they received what they ordered or made the correct protein. We find 10% or more of synthetic peptides from reputable suppliers are incorrect and recombinant proteins contain surprising additions or truncations ~25% of the time. LC-MS offers precise molecular mass measurements and comprehensive analyses of proteins, peptides, glycans, and oligonucleotides. For antibodies, proteins and peptides, LC-MS provides insights into purity, folding stages, dimer configurations, protein interactions, post-translational modifications, and top-down sequencing. In glycan analysis, LC-MS enables the study of various glycoforms and glycosylation in monoclonal antibodies, utilizing workflows such as Agilent InstantPC tagging for rapid and high-throughput N-glycan composition analysis. Validation of synthetic oligonucleotides, LC-MS delivers critical data on sequence confirmation, purity, and structural modifications. Our team is dedicated to helping you leverage LC-MS to gain deeper insights into your samples, ensuring high-quality data and impactful scientific outcomes.

13. Abstract: Mónica Monge-Loría

Wielding untargeted metabolomics to explore marine bacteria community interactions

Mónica Monge-Loría, Neha Garg
School of Chemistry and Biochemistry, Georgia Institute of Technology,
901 Atlantic Drive, Atlanta, GA 30332, USA

The ocean covers the majority of Earth's surface and hosts an immense diversity of organisms. Particularly, coral reefs harbor 25% of the ocean's biodiversity[1] rendering them an ecosystem of interest. This diversity translates into a wide variety of compounds with unique molecular scaffolds, functions, and bioactivities. Such variation stems, in part, from the intricate relationships formed in the reef, as some of these compounds are useful for intra- and inter-species communication, pathogenesis, nutrient acquisition, and defense[2]. However, this full array of

molecules is often not detectable under normal laboratory conditions, as the genes that encode for their production are commonly silent[3]. In this work, we aim to induce these biosynthetic gene clusters through bacterial cocultivation and leverage the untargeted metabolomics workflow to survey the resulting interactions. We specifically cocultured marine bacteria with the coral pathogen *Vibrio coralliilyticus* Cn52-H1, as it produces the secondary metabolite elicitor andrimid[4]. Using this approach, along with NMR characterization, we detected the degradation of amphibactin siderophores from *V. coralliilyticus* Cn52-H1 by *Microbulbifer* sp. CN-SA-002. Further experiments have revealed this to be an enzymatic process, leading to protein isolation and analysis. Determining the enzyme responsible for this degradation will allow us to parse out the interactions at play between these bacteria and gain insight into iron acquisition by *Microbulbifer*.

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14. Abstract: Lester S. Manly

Application of ion mobility and electron capture dissociation for the characterization of peptides for bottom-up proteomics

Lester S. Manly[1,3], Yury V. Vasil'ev PhD[4], Joesph S. Beckman PhD[5], Blaine R. Roberts PhD[1,2,3]

[1]Department of Biochemistry, Emory University, Atlanta, Georgia, USA
[2]Department of Neurology, Emory University, Atlanta, Georgia, USA
[3]Molecular and System Pharmacology Graduate Program, Emory University, Atlanta, Georgia, USA
[4]Agilent Technologies, Corvallis, Oregon, USA
[5]Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon, USA

Ion mobility (IM) and electron capture dissociation (ECD) offer unique advantages for peptide identification during bottom-up proteomics analysis. IM separates ions based on their collision cross-section (CCS), which relates to an ion's shape, size, and charge. In proteomics, ECD is a fragmentation method that introduces low-energy electrons to multiply charged peptide ions, causing selective fragmentation along the peptide backbone and resulting in a high population of c- and z-type fragment ions. CCS provides an additional dimension for peptide identification, while ECD offers high sequence coverage and can preserve labile bonds of side chains and post-

translational modifications (PTMs). In this presentation, we highlight the utility of both methods as demonstrated using an Agilent 6560 IM Q-TOF retrofitted with Agilent's latest ExD cell. Substance P was used as a model peptide, directly infused into the instrument via a syringe pump. Data were analyzed using Agilent IM-Browser and ExDViewer. IM revealed distinct conformations between the observed 1+, 2+, and 3+ charge states: the 2+ state had at least five conformations (range: 344–509 Å), the 3+ state had two conformations (range: 479–518 Å), and the 1+ state had one conformation (range: 371 Å). We then compared traditional collision-induced dissociation (CID) to ECD targeting the 2+ charge state. Respective optimized CID and ECD fragmentation methods both resulted in complete sequence and fragmentation coverage of Substance P. However, CID spectra had a higher percentage of peaks that could not be annotated compared to ECD. With these findings, we aim to evaluate the performance of both IM and ECD for the characterization of peptide-level PTMs and peptide identification in complex mixtures. One PTM of particular interest is residue isomerization, such as differentiating aspartate from isoaspartate, as this PTM cannot be characterized by traditional CID-based LC-MS/MS methods.

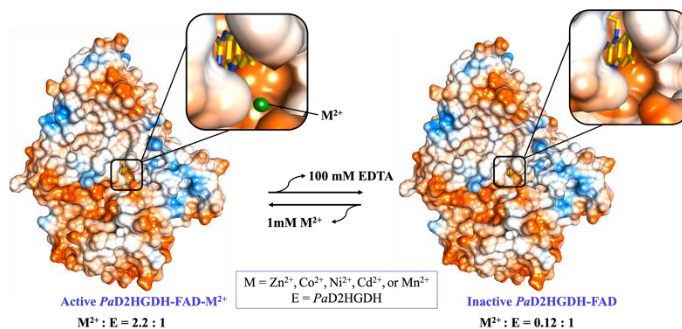
15. Abstract: Bilkis Mehrin Moni

Assessment of Alternative Metal Binding to D2-Hydroxyglutarate Dehydrogenase Using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Bilkis Mehrin Moni[1], Joanna A. Quaye[1] and Giovanni Gadda[1,2,3]

[1]Departments of Chemistry, [2]Biology, and [3]The Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30302-3965

The enzyme D-2-hydroxyglutarate dehydrogenase (*Pa*D2HGDH) is a metallo-flavoenzyme, primarily relying on Zn^{2+} to catalyze the oxidation of D-2-hydroxyglutarate to a-ketoglutarate. (1,2) The enzyme can also function with Co^{2+}, Ni^{2+}, Mn^{2+}, and Cd^{2+} as alternative metal cofactors, (2) though their roles in the catalytic mechanism are less explored. In this study, Zn^{2+} was substituted with Co^{2+}, Ni^{2+}, Cd^{2+}, or Mn^{2+} to investigate the significance of alternative metals on the rate of flavin reduction and catalysis of *Pa*D2HGDH. Recombinant His-tagged *Pa*D2HGDH was treated with 100 mM EDTA to create the metallo-apo-enzyme (E-FAD) or purified with 1 mM chloride salts of various metals. The M^{2+} to protein ratio was determined by ICP-MS to assess metal binding to the enzyme. The mol M^{2+}:protein stoichiometry for E-Zn^{2+} was 2.2:1, E-Co^{2+} was 0.6:1, E-Ni^{2+} was 2:1, E-Cd^{2+} was 1.2:1 and E-Mn^{2+} 0.3:1. The anaerobic reductive half-reaction showed a ≤ 2-fold increase in k_{red} values (85-160 s^{-1}) for Co^{2+}, Ni^{2+}, and Mn^{2+} compared to Zn^{2+} (70 s^{-1}), indicating that alternative metals modulate the rate of flavin reduction in *Pa*D2HGDH. K_d values for D-malate were similar for Co^{2+} and Mn^{2+} (~10 mM) compared to Zn^{2+} (8 mM) but decreased by ~2-fold with Ni^{2+} (4 mM), suggesting a higher binding affinity of E-Ni^{2+} for the substrate. Steady-state



kinetics of *Pa*D2HGDH loaded with different metal ions at varying D-malate revealed a ≤6-fold variation in k_{cat}/K_m (950 - 40,000 $M^{-1}s^{-1}$) and ≤4-fold in k_{cat} values (10-45 s^{-1}), indicating that alternative metals alter substrate capture and the slowest step of catalysis. Notably, the k_{cat} for E-FAD with 400 μM Zn^{2+} was 16 s^{-1}, increasing to 34 s^{-1} with 800 μM Zn^{2+}. For Co^{2+}, k_{cat} was 20 s^{-1} at 250 μM Co^{2+}, rising to 50 and 60 s^{-1} at 450 and 800 μM Co^{2+}, respectively, suggesting different enzyme conformations at different metal concentrations. Steady-state kinetics of E-FAD (0.20 μM-25 μM) with 800 μM Co^{2+} at varying D-malate concentrations showed a sigmoidal increase in k_{cat} and k_{cat}/K_m with increasing the ratios of Co^{2+} to E-FAD enzyme, suggesting that the binding of Co^{2+} to the active site cooperatively influences the binding of additional Co^{2+} ions to the other sites, enhancing the enzymatic activity in a cooperative manner. Collectively, these findings indicate that the alternative metal ions alter the rate of flavin reduction and catalysis of *Pa*D2HGDH.

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16. Abstract: Shaima Muhammed Nazaar

Development of red blood cells and plasma-based diagnostic lipid biomarker panel for Parkinson's disease

Shaima Muhammed Nazaar[1], Anne M. Roberts[1], Ankit Jain[1], James Doecke[3], Malcolm Horne[4], Stephan Klatt[5], and Blaine Roberts[1,2]

[1]Department of Biochemistry, Emory University, Atlanta, Georgia, [2]Department of Neurology, Emory University, Atlanta, Georgia, [3]CSIRO Health and Biosecurity, Herston, Australia, [4]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Victoria, Australia, [5]Institue for Vascular Signaling, University of Frankfurt-Goethe University, Frankfort, Germany

Parkinson's disease (PD), a common neurodegenerative disorder, is characterized by the loss of dopaminergic neurons and the formation of Lewy bodies. While motor symptoms are typically used for diagnosis, early detection is critical. Lipids, essential for various cellular functions, are implicated in PD pathogenesis. Genetic mutations in lipid-producing enzymes and genes related to lipid metabolism have been associated with sporadic PD and interactions between α-synuclein and lipids are believed to influence disease progression.

This study employed LC-MS/MS to analyze lipid profiles in red blood cells (RBCs) and plasma to discover potential lipid biomarkers for PD and explore disrupted lipid regulatory pathways. A total of 286 patient samples (140 PD patients and 146 control subjects in total) from the Victorian Parkinson's Disease Registry were analyzed using butanol/methanol extraction. The samples were processed using a Bruker timsTOF flex mass spectrometer with a data-dependent method. Feature extraction was performed using MS Dial, followed by verification and relative quantification using Skyline.

Multivariate analysis with the least absolute shrinkage and selection operator (LASSO) identified 12 lipid biomarkers from RBCs with a receiver operating characteristic (ROC) of 0.84. In plasma, 10 lipid biomarkers were identified with an ROC of 0.85. A combined panel of RBC and plasma lipids achieved a higher ROC of 0.89 with 12 lipids. The WGCNA co-expression network revealed specific modules enriched in pathways such as ferroptosis, sphingolipid metabolism, glycerophospholipid metabolism, and sphingolipid signaling.

17. Abstract: Joanna A. Quaye

Identification of *Pseudomonas aeruginosa* PAO1 D-2-Hydroxyglutarate Dehydrogenase cofactors using Mass Spectrometry

[1]Joanna A. Quaye and [1,2,3]Giovanni Gadda

[1]Department of Chemistry, [2]Department of Biology, [3]The Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30302-3965, United States

Mass spectrometry (MS) is a powerful tool for characterizing various inorganic and organic biomolecules, and its precision allows for the quantification and distinction of similar enzyme cofactors.[1–3] Recent enzyme studies have revealed a new class of flavin-dependent enzymes, known as metallo flavoproteins, with the ability to use both a metal and flavin for catalysis.[4] Flavin Mononucleotide (FMN) and Flavin Adenine Dinucleotide (FAD) are two forms of the three-ringed biological molecule flavin incorporated into enzymes as cofactors.[5] Metallo enzymes use various metals for catalysis, including Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+}. Since the identity of the flavin and metal cofactors dictates the chemistry employed by an enzyme, characterizing the flavin and metal cofactors is essential in understanding the mode of action and catalytic mechanism of metallo flavoproteins. *Pseudomonas aeruginosa* D-2-hydroxyglutarate dehydrogenase (*Pa*D2HGDH) is a recently identified therapeutic target against *P. aeruginosa* due to its ability to regenerate 2-ketoglutarate form D-2-hydroxyglutarate to drive the L-Serine biosynthesis for bacterial survival. *Pa*D2HGDH is also active with D-malate as an alternative substrate.[6] The enzyme has been annotated as a flavin-dependent enzyme. From sequence alignment and homology model prediction of the *Pa*D2HGDH structure, a unique and conserved metal binding motif has been identified in the enzyme's active site.[6] However, the identities of the *Pa*D2HGDH cofactors are unknown, preventing the elucidation of the enzyme's catalytic mechanism for new therapeutic development against *P. aeruginosa*. This study uses mass spectrometry to identify the flavin and metal cofactors in *Pa*D2HGDH. The flavin cofactor was extracted by heat denaturing *Pa*D2HGDH at 100 °C for 30 min, followed by characterization using MALDI-TOF MS and confirmation by fluorescence spectrofluorometry.[6] The *Pa*D2HGDH metal cofactor has been identified by ICP-MS and verified by enzyme activity assays. The MALDI and fluorescence data demonstrated the presence of FAD, and the ICP-MS data showed Zn^{2+} in high quantities, and the enzyme assays confirmed Zn^{2+} as the *Pa*D2HGDH metal cofactor. *Pa*D2HGDH had a Zn^{2+}:protein stoichiometry of 2:1, yielding an enzyme with ~40 s^{-1} k_{cat} for D-malate. Upon mild treatment with 1 mM EDTA, the Zn^{2+}:protein ratio decreased to 1:1 without changing the kinetic parameters with D-malate. However, upon complete removal of Zn^{2+} using 100 mM EDTA, complete enzyme inactivation was observed for the metallo apoenzyme. The data demonstrate that *Pa*D2HGDH uses FAD and Zn^{2+} as required cofactors.

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18. Abstract: Phuong Tran

EThcD-MS/MS method application towards the analysis of complex N-glycans in biological samples

Phuong Tran[1], Betlehem Mekonnen[2], Christopher A. Haynes[2], Zsuzsanna Kuklenyik[1], John R. Barr[1,2], Anna A. Ivanova[1].

(1) Cardiovascular Disease Biomarker Discovery and Translation Laboratory and (2) Protein Structure Laboratory, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA 30341, USA.

Glycosylation is one of the major protein post-translational modifications which covalently attaches a carbohydrate to the biomolecules. It plays a critical role in protein structure, stability, and functions. Numerous studies have shown key role of glycosylation in various disorders, including cancer, liver, cardiovascular, and Alzheimer's diseases. Nevertheless, a detailed analysis of protein glycosylation is still required to establish molecular linkage between glycosylation and human disease states and promote new diagnostic biomarkers. To address this need, we examined the N-glycosylation status of the human plasma proteins using a bottom-up mass spectrometry that implements the electron-transfer/high-energy collision (EthCD) method. This study utilized the commercially available recombinant human purified proteins and a non-disease human plasma pool. The samples were subjected to reduction by dithiothreitol and alkylation by iodoacetic acid in the presence of 0.05% RapiGest (Waters), followed by digestion with various enzymes. Then, Liquid Chromatography–Mass Spectrometry (LC-MS/MS) analysis was performed on a Thermo Fisher Orbitrap Eclipse Tribrid mass spectrometer. Collected MS/MS data were processed using the Byonic node (Protein Metrics) within the Proteome Discoverer 3.0 (Thermo) software. To ensure the most complete coverage of detected N-glycosylation sites, we analyzed various combinations of different digesting enzymes. This method not only applies to purified protein but also to complex biological fluid samples, such as human plasma. For example, the glycosylation status of the proteins was monitored in the plasma pool sample. Our results demonstrate the method's effectiveness in mapping protein glycosylation, providing critical insights into protein function and interactions in metabolism.

Disclaimer. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. The use of trade names is for identification only and does not imply endorsement by the CDC.

19. Abstract: Jose Villalobos

Comparison of colostrum and colostrum products utilizing glycomic techniques

Jose Villalobos, Stephanie Archer, Parastoo Azadi

Colostrum is a milk-like, nutrient-dense substance produced during the first few days after birth. It is crucial for developing the newborn's immunity and contains a wealth of immune factors, growth and tissue repair elements, immunoglobulins, and natural antimicrobial agents such as milk oligosaccharides (MOs). In recent years, several companies have started marketing bovine colostrum—a byproduct of the dairy industry—as a supplement for adults, claiming benefits for digestion, skin, hair, nails, and post-exercise recovery. While much attention has been given to the immune factors in bovine colostrum, there has been less focus on comparing the MOs present.
We have recently developed methodologies to profile and perform detail characterization of milk oligosaccharide by a process of isolation, purification, and permethylation (Porfirio et al (2020) Glycobiology 30:10, 774-786). Prior to permethylation, proteins are precipitated out and lipids are removed by solid phase extraction. Reducing ends of purified MOs are then reduced yielding a more stable product. Our improved permethylation method stabilizes the structure of the oligosaccharides, enhances sensitivity, and enables detailed sequential structure analysis to identify potential isomers, providing a level of analysis deeper than that of most underivatized protocols. This study aims to provide detailed comparisons of milk oligosaccharides in human and bovine milk, including comparisons of MOs in colostrum versus mature breast milk, human versus bovine MOs, and fresh bovine colostrum MOs versus those in three commercial supplement products.

20. Abstract: Mujeeb A. Wakeel

Investigating Enzyme-Substrate Interactions in Proteusin Ribosomally Synthesized and Post-translationally Modified Peptides Biosynthesis

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are natural products with varied chemical structures and biological activities, making them valuable for drug discovery. RiPP precursor peptides consist of an N-terminal leader peptide that binds to RiPP precursor peptide recognition elements (RRE) of modifying enzymes and a C-terminal core peptide that undergoes enzymatic modifications. After modifications, a protease cleaves the mature C-terminal core peptide from the N-terminal leader peptide.

The RiPPs group of natural products consists of various sub-families often defined based on the nature of the chemical modifications afforded by the modifying enzymes on the mature natural products (e.g., linear azol(in)e-containing peptides). Among the well-characterized family of enzymes that modify RiPPs are the YcaO-like cyclodehydratases that catalyze (methyl)oxazoline/thiazoline heterocycle formation by cyclodehydrating Thr/Ser/Cys residues in the core peptide. Most RiPPs, such as cyanobactins, derive from precursor peptides with a short, unstructured leader peptide (about 30 amino acids). Cyanobactins have been reported to use a conserved C-terminus LXXXXL motif in the unstructured leader peptide for binding YcaO-like cyclodehydratases.

However, proteusins, another RiPP family, feature a much longer (about 75 amino acids) leader peptide with a highly structured N-terminus. The relevance of this structured region in the

posttranslational modification of proteusins is yet to be described. Our research involves mining biosynthetic gene clusters (BGCs) containing putative proteusin precursor peptide(s) and using biophysical and biochemical tools to investigate protein-protein interactions between the precursor peptide and the RRE of modifying enzymes.

Our results suggest that proteusin precursor peptides require their structured N-terminus region and the conserved LXXXXL motif of the unstructured region in binding YcaO-like cyclodehydratases for efficient processing of the core region. These findings indicate another distinct binding site/mode for proteusin precursor peptides and offer new insights into the protein-protein interactions essential for proteusin biosynthesis, potentially aiding in developing innovative combinatorial biocatalysis approaches.

21. Sponsor Abstract: Affinisep

Development and optimization of a method for automated peptide desalting on the DigestPro MSi robot using AttractSPE® C18 tips

Célia JARDIN[1], Soumia HAMADA[1], Cédric PIONNEAU[1], Martin TECHNAU[2], Mana SHAFAEI[3], Florine HALLEZ[4], Michel AROTCARENA[4], Sami BAYOUDH[4], Kaynoush NARAGHI[4], Solenne CHARDONNET[1]

1. Sorbonne University, Inserm, UMS Production et Analyse des données en Sciences de la vie et en Santé (PASS), Plateforme Post-génomique de la Pitié-Salpêtrière (P3S), F-75013, Paris, France
2. CEM GmbH, Carl-Friedrich-Gauß-Straße 9, D-47475 Kamp-Lintfort, Germany
3. Affinisep USA, 25 SE 2nd Ave. Suite 1016, Miami, Fl 33131, USA
4. Affinisep, 10 rue Richard Dufour, 76770 Le Houlme, France

Bottom-up approaches are commonly used for proteomics analysis in LC-MS/MS. However, salts and digestion buffers can generate ion-suppression that limits peptide detection and greatly impacts protein identification. Therefore, peptide desalting is an indispensable step. The P3S proteomics facility is equipped with a nanoElute – timsTOF Pro LC-MS/MS system and has chosen to perform the desalting step prior to LC-MS/MS analysis with direct injection on the analytical column.

With the aim to automate peptide desalting, P3S has asked CEM (previously Intavis) to create a custom needle to run home-made StageTips on the DigestPro MSi robot. The program was adapted from manual StageTips protocol and further optimized by playing with liquid aspirate and dispense speeds and volumes. Moreover, P3S has evaluated AttractSPE® C18 Tips (Affinisep) compared to home-made StageTips (with Empore C18 SPE disks) for 2µg and 100ng of protein digest. The AttractSPE® Tips are packed with small sorbent beads embedded in a thin and mechanically stable membrane that combines high capacity and small dead volume and are adapted for centrifugation or positive pressure assays. The comparison showed little difference between the two kinds of tips when working with 2µg of peptides but showed a 10% increase in the number of identified proteins with the AttractSPE® C18 Tips when working with 100ng of peptides. Moreover, the peptide intensities were higher with the AttractSPE® C18 Tips. In both cases, 2µg and 100ng starting material, the AttractSPE® C18 Tips performed better in the hydrophilic range.

Our results show that AttractSPE® C18 sorbent offers a wider spectrum of interactions with a broad range of peptides, from the most hydrophilic to the most hydrophobic ones, compared to home-made StageTips. In addition, the P3S team has optimized the program on the DigestPro MSi robot to reduce the time from 40min down to 10min per sample.