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| [**glycore@olemiss.edu**](mailto:glycore@olemiss.edu)[**https://tinyurl.com/hvba28bp**](https://tinyurl.com/hvba28bp)  **Program and Abstracts**  **2021 Mid-South Glycoscience Meeting**  **July 19, 2021**  **ORAL PRESENTATIONS** | |
| **Time:**  8:15 a.m | **Presentation:**  **Welcome and Introduction –Samir Ross**  **VC-ORSP**  ***Session 1: Glycobiology***  ***Chair: Josh Bloomekatz*** |
| 8:30 a.m. | **Building Lysosomes Using Sugars**  *Nancy Dahms, Medical College of Wisconsin* |
| 9:00 a.m. | **O-GlcNAc Transferase Regulates Fibroblast-to-Myofibroblast transition in**  **Idiopathic Pulmonary Fibrosis**  *Shia Vang, E. Scott Helton, Rebecca Denson, Rennan Zaharias, Stefanie Krick,*  *Jarrod Barnes, University of Alabama at Birmingham* |
| 9:30 a.m. | **Utilization of glycan-associated features contributing to prey recalcitrance to**  **myxobacterial predation for prioritization of drug discovery from novel myxobacteria**  *Cole Stevens, Andrew Ahearne, Kayleigh Phillips, and Shukria Akbar, University of Mississippi* |
| 10:00 a.m. | **Gb3-cSrc Complex in Glycosphingolipid-Enriched Microdomains Contributes to the Expression of p53 Mutant Protein and Enrichment of Cancer Stem Cells**  *Yong-Yu Liu, Kartik R. Roy, Ronald A. Hill, Yu-Teh Li, University of Louisiana, Monroe* |
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| 10:30 a.m. | Break  ***Session 2: Sulfated Polysaccharides***  ***Chair: Vitor Pomin*** |
| 10:45 a.m. | **A General Approach to O-Sulfation of Carbohydrates and Peptides via SuFEx**  **Reactions**  *Jia Niu, Chao Liu, Cangjie Yang, Boston College* |
| 11:15 a.m. | **Isolation and structural characterization of a new fucosylated chondroitin sulfate: anti-SARS-CoV-2, anticoagulant and binding properties of holothurian sulfated glycans**  *Rohini Dwivedi, Priyanka Samanta, Poonam Sharma, Fuming Zhang, Sushil K.*  *Mishra, Pavel Kucheryavy, Seonbeom Kim, AyoOluwa O. Aderibigbe, Robert J. Linhardt, RiteshTandon, Robert J. Doerksen and Vitor H. Pomin, University of Mississippi* |
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| 11:45 a.m. | **Chemical Editing of Proteoglycan Architecture**  *Meg Critcher, Timothy O’Leary, Tesia N. Stephenson, Xueyi Yang, Noah H.*  *Bartfield, Richard Hawkins, Mia L. Huang, Scripps Research* |
| 12:15 p.m.-  1:15 p.m.  1:15 p.m .-  2:15 p.m. | **Lunch and Posters**  **Poster Presentations – Odd Numbered Posters**  **Poster Presentations – Even Numbered Posters** |
|  | ***Session 3: Chemical Glycobiology and Glycoengineering***  ***Chair: Sudeshna Roy*** |
| 2:15 p.m. | **Harnessing Chemistry to Understand the Roles of Glycans in Neuroplasticity**  *Linda Hsieh-Wilson, California Institute of Technology* |
| 2:45 p.m. | **Real-time chemical tools to modulate dynamic sugar signaling in cells**  *Charlie Fehl, Wayne State University* |
| 3:15 p.m. | **Glycopolymer Engineering for Next-Generation Cancer Immunotherapies**  *Thomas A Werfel, Judith U. De Mel, Oluwaseyi Shofolawe-Bakare, Karan*  *Arora, John T. Wilson, Adam E. Smith, University of Mississippi* |
| 3:45 p.m. | **Proximity tagging identifies the glycan-mediated glycoprotein interactors of galectin-1 in muscle stem cells**  *Zak Vilen, Scripps Research* |
| 4:15 p.m. | **Break**  ***Session 4: Carbohydrate Synthesis***  ***Chair: Samir Ross*** |
| 4:30 p.m. | **Next-generation tools for the synthesis of bioactive saccharides**  *Maciej Walczak, University of Colorado, Boulder* |
| 5:00 p.m. | **1,2-Cis Selective Glycosylation through the Synergy of Trifluoromethylated**  **Benzyl Protecting Groups and Lewis-Basic Additives**  *Dancan K. Njeri,* *Erik V. Alvarez, Claude J. Pertuit and Justin R. Ragains*, *Louisiana State University* |
| 5:30 p.m. | **Human Milk Oligosaccharides as a Defense Against Group B Streptococcus**  *Steven D. Townsend, Vanderbilt University* |
| 6:00 p.m. | **Closing Remarks – Joshua Sharp**  **CERE Survey - tinyurl.com/glycosci**  cid:6c151996-586a-4c28-8658-6810f62e360c |
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**Poster Presentations**

**Poster 1:**

**Potential Modification of Brain Aging by Receptor of Advanced Glycation Endproducts**

Brandon G. Ashmore, Cellas A. Hayes, James A. Stewart, Jr., Nicole M. Ashpole, *University of Mississippi*

**Poster 2:**

**Measuring Changes in Myxobacterial Metabolic Production Using Lipopolysaccharides of Various Prey Bacteria**

Andrew Ahearne and Cole Stevens, *University of Mississippi*

**Poster 3:**

**The GLYCORE Imaging Core: Bridging the gap between advanced imaging techniques and the glycosciences**

Ruofan Cao, Gregg Roman, *University of Mississippi*

**Poster 4:**

**Conformational Analysis of CF2-linked Flavonoid Glycosides**

Reem A. Alkhodier and David A. Colby, *University of Mississippi*

**Poster 5:**

**Mapping Interaction Domains of Cation-Independent Mannose 6-phosphate Receptor and Plasminogen through Fast Photochemical Oxidation of Proteins and High-Resolution Mass Spectrometry**

Zhi Cheng, Sandeep Misra, Richard N. Bohnsack, Linda J. Olson, Nancy M. Dahms, and Joshua S. Sharp, *University of Mississippi*

**Poster 6:**

**Coupling FDM 3D Printing with Hot-Melt Extrusion to Produce Hypertensive Dual Therapy Fixed Dose Combination Tablet**

Abdullah Alzahrani, Sagar Narala, Dinesh Nyavanandi, Mashan Almutairi, Ahmed Almotairy, Suresh Bandari, Michael Repka, *University of Mississippi*

**Poster 7**:

**Dual Responsive Glycopolymeric Nanoparticles for Neoantigen Peptide and Lipophilic Adjuvant Co-delivery**

Judith U. De Mel, Oluwaseyi Shofolawe-Bakare, Karan Arora, John T. Wilson, Adam E. Smith, Thomas A. Werfel, *University of Mississippi*

**Poster 8:**

**Synthesis of Difluorinated Benzopyrans**

Amena Begum, David A. Colby, *University of Mississippi*

**Poster 9:**

**An Immunoblot Assay to Detect Antibodies to the SARS-CoV-2 Spike Glycoprotein Antigen in Serum of COVID-19 Patients**

Wayne Gray and Shivum Desai, *University of Mississippi*

**Poster 10:**

**Chronic protein O-GlcNAcylation induces cardiac hypertrophy**

Chae-Myeong Ha, Sayan Bakshi, Luke A. Potter, John C. Chatham, Adam R. Wende, *University of Alabama at Birmingham*

**Poster 11:**

**Hot Melt Extrusion based Fused Deposition Modeling 3D Printing of Atorvastatin calcium tablets: Impact of infill density on the performance of tablets**

Preethi Mandati1, Nagireddy Dumpa1, Dinesh Nyavanandi1, Sagar Narala1, Honghe Wang1, Suresh Bandari1, Michael A. Repka1, Sandip Tiwari2, Nigel Langley,2 *1University of Mississippi, 2BASF Corporation, Tarrytown, NY*

**Poster 12:**

**Altered Mucin Sialylation Results in Delayed Mucociliary Transport in Cystic Fibrosis**

Elex Harris, E Scott Helton, Marina Mazur, Stefanie Krick, Steven M. Rowe, Jarrod W. Barnes, *University of Alabama at Birmingham*

**Poster 13:**

**Computational Chemistry and Bioinformatics Research Core**

Sushil K. Mishra, Priyanka Samanta, and Robert J. Doerksen, *University of Mississippi*

**Poster 14:**

**A novel octasaccharide isolated from mild acid hydrolysis of the Isostichopus badionotus sulfated fucan**

Seon Beom Kim, Rohini Dwivedi, Maggie C. Taylor, Pavel Kucheryavy, Vitor H. Pomin, *University of Mississippi*

**Poster 15:**

**GlyCORE: Analytical and Biophysical Chemistry Research Core**

Sandeep K. Misra, Anter Sami, Joshua S. Sharp, *University of Mississippi*

**Poster 16:**

**Polysaccharide as Colon Drug Delivery Carrier: Hot-melt Extrusion**

Sagar Narala, Dinesh Nyavanandi, Abdullah Alzahrani, Preethi Mandati, Suresh Bandari, Michael A. Repka, *University of Mississippi*

**Poster 17:**

**Carbohydrate structures shared between the Streptococcus pneumoniae capsule and common food** Moon H. Nahm and Feroze Ganaie, *University of Alabama at Birmingham*

**Poster 18:**

**NMR-based structural biology in marine medicinal glycomics**

Vitor H. Pomin, Seon Beom Kim, Rohini Dwivedi, *University of Mississippi*

**Poster 19:**

**Effects of Alginate Exposure on Metabolite Production in Novel Corallococcus Species**

Kayleigh Phillips, Shukria Akbar, Andrew Ahearne, and Cole Stevens, *University of Mississippi*

**Poster 20:**

**Synthesis of ROS-Responsive Mannose Glycopolymers for Macrophage-Targeted Drug Delivery** Oluwaseyi T. Shofolawe-Bakare, Judith U. De Mel, Adam E. Smith, Thomas A. Werfel, *University of Mississippi*

**Poster 21:**

**Anti-SARS-CoV-2 and binding properties of fucosylated chondroitin sulfates**

Priyanka Samanta, Rohini Dwivedi, Poonam Sharma, Fuming Zhang, Sushil K. Mishra, Pavel Kucheryavy, Seonbeom Kim, AyoOluwa O. Aderibigbe, Robert J. Linhardt, Ritesh Tandon, Vitor H. Pomin, Robert J. Doerksen, *University of Mississippi*

**Poster 22:**

**N262-Associated Glycan Microdomain of HIV-1 Env gp120 Exerts a Ripple Effect on Env Glycan-Shield Density, Function, and Antibody Recognition**

Qing Wei, Audra A. Hargett, Barbora Knoppova, Alexandra Duverger, Reda Rawi, Chen-Hsiang Shen, S. Katie Farney, Stacy Hall, Rhubell Brown, Brandon F. Keele, Sonya L. Heath, Michael S Saag, Olaf Kutsch, Gwo-Yu Chuang, Peter D. Kwong, Zina Moldoveanu, Milan Raska, Matthew B. Renfrow, Jan Novak, *University of Alabama at Birmingham*

**Poster 23:**

**Hydroxyl Radical Carbohydrate Footprinting for Probing Protein-Binding Components of Oligosaccharides**

Hao Liu, Lianyan Xu, Steven D. Townsend and Joshua S. Sharp, *University of Mississippi*

**Poster 24:**

**An evaluation of the Glycoscience Center of Research Excellence (GlyCORE)**

Shannon R. Sharp, Sarah Mason, Anna T. Beavers, Olivia Melvin, *Center for Research Evaluation, University of Mississippi*

***ABSTRACTS***

***Oral Presentations***

**Building Lysosomes Using Sugars**

**Nancy M. Dahms**

*Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226*

**Introduction:** Lysosomes perform degradative metabolism critical to many endocytic, phagocytic, and autophagic processes. The activity of lysosomes depends upon a collection of over 60 soluble acid hydrolases that must be continually delivered from their site of synthesis in the endoplasmic reticulum to lysosomes. The acid hydrolases’ *N*-glycans undergo unique posttranslational modifications in the Golgi that results in the acquisition of phosphorylated mannose, either as a phosphomonoester (mannose 6-phosphate, M6P) or a phosphodiester (M6P-GlcNAc), that marks them for delivery to lysosomes by specific receptors, M6P receptors (MPRs). The biogenesis of lysosomes is dependent upon two MPRs, 46kDa cation-dependent (CD-MPR) and 300kDa cation-independent (CI-MPR), that carry M6P-tagged acid hydrolases from the trans Golgi network to endosomal compartments where the acidic environment causes the MPRs to release their cargo. Failure to mark acid hydrolases with M6P results in developmental defects and the lysosomal storage diseases (LSDs), mucolipidosis II and III. Most LSDs are caused by mutations in lysosomal proteins, mainly enzymes, that result in defective catabolism and substrate accumulation. Characteristic of the family of ~70 LSDs is their progressive and debilitating nature due to their impact on multiple organ systems. Treatment is symptomatic for most LSDs, with only a dozen having FDA-approved therapies. CI-MPR’s ability to internalize recombinant M6P-containing enzymes delivered to patients by bi-weekly intravenous infusion forms the basis of enzyme replacement therapy (ERT) for 9 of these therapies. However, structural knowledge of the interaction between CI-MPR and its cargo of ~60 different hydrolytic enzymes is lacking.

**Methods:** We have taken multiple approaches, including glycan microarrays, SPR-based solid phase binding assays using glycosylated acid hydrolases, mutagenesis, NMR spectroscopy, X-ray crystallography, electron microscopy and hydroxyl radical protein footprinting to 1) identify structural elements essential for the trafficking of acid hydrolases to lysosomes by MPRs, and 2) evaluate the impact of ligand binding and pH on the conformation of these receptors.

**Results:** We identified the phosphorylated glycans that serve as high affinity ligands for the MPRs and showed that the individual glycan-binding sites of the two PMRs differ in the glycan structures recognized. Our structural studies reveal four residues (Gln, Arg, Glu, Tyr) are conserved among the MPRs’ binding sites and are located in identical positions within the carbohydrate binding pocket. Furthermore, pH and ligand binding affect the overall conformation of the MPRs.

(NIH R01DK042267)

**O-GlcNAc Transferase Regulates Fibroblast-to-Myofibroblast transition in Idiopathic Pulmonary Fibrosis**

**Shia Vang1, E. Scott Helton1, Rebecca Denson1, Rennan Zaharias1, Stefanie Krick1, and Jarrod Barnes1**

*Division of Pulmonary, Allergy, and Critical Care Medicine, University of Alabama at*

*Birmingham, Birmingham, AL*

**Introduction**-:Idiopathic pulmonary fibrosis (IPF) is a chronic disease that affects the lungs. The scarring and progressive fibrosis, mediated by communication from the intracellular network and the extracellular matrix (ECM), results in impaired lung function causing respiratory failure and mortality. IPF has been documented as a metabolic and age-related disease with the evidence of several hallmarks of aging including dysregulated nutrient sensing. Interestingly, dysregulated nutrient sensing, via altered glucose utilization, has been shown to drive metabolic reprogramming in myofibroblasts. O-linked N-Acetylglucosamine (O-GlcNAc) transferase (OGT) is a metabolic ‘stress sensor’ and is responsible for the O-GlcNAc modification of proteins involved in many cellular processes including metabolism, intracellular communication, and growth factor signaling. However, the potential role of OGT/O-GlcNAc on growth factor signaling, intracellular communication, and ECM changes during transdifferentiation has not been studied.

**Methods**: Lung tissue from individuals with IPF and controls were subjected to immunohistochemistry (IHC) staining to assess O-GlcNAc modification. In addition, the role of OGT on transforming growth factor-beta 1 (TGFβ1) induced fibroblast-to-myofibroblast transition (FMT) was assessed on primary human lung fibroblasts cultured in the presence or absence of an OGT inhibitor, OSMI-1. Alpha smooth muscle actin (α-SMA), type 1 collagen (COL1α1), and Smad3 phosphorylation following TGFβ1 stimulation with and without OSMI-1 were examined by Western blot analysis.

**Results**: In human lung tissue sections, O-GlcNAc levels were increased in IPF lung tissue compared to control human lungs as determined by IHC staining. In primary human lung fibroblast, OGT inhibition by OSMI-1 followed by TGFβ1 (up to 2 hours) was shown to alter Smad3 phosphorylation. Furthermore, TGFβ1 administration resulted in an increase in fibroblast transdifferentiation, which was determined by higher COL1α1 and α-SMA expression. Interestingly, treatment with OSMI-1 followed by TGFβ1 stimulation reduced expression of COL1α1 and α-SMA in the human lung fibroblasts.

**Conclusion**: Our data suggest that the metabolic sensor, OGT, and the O-GlcNAc protein modification may have a role in regulating the expression of ECM proteins including COL1α1 by altering TGFβ1 signaling during FMT. More importantly, the OGT/O-GlcNAc axis may be directly involved in the pathogenesis of IPF and regulation of cellular transdifferentiation through TGFβ1 signaling and overall ECM composition. Determining the mechanistic role of the O-GlcNAc modification of proteins on these processes is needed, and may help to identify novel therapies for the treatment of IPF or any other fibrotic diseases.

**Utilization of glycan-associated features contributing to prey recalcitrance to myxobacterial**

**predation for prioritization of drug discovery from novel myxobacteria**

**Cole Stevens, Andrew Ahearne, Kayleigh Phillips, and Shukria Akbar**

*Department of BioMolecular Sciences, University of Mississippi*

**Introduction:** Predatory myxobacteria have served as prolific sources of novel specialized metabolites for scrutiny as potential therapeutics. Unique from other “gifted” natural product-producing bacteria, the currently accepted taxonomic position of the order *Myxococcales* includes a variety of genera across 3 sub-orders; of which almost all include large genomes (~9-13 mb) that are replete with specialized metabolite biosynthetic pathways. Ubiquitous to soils and marine sediments, predatory myxobacteria have been recently identified as a keystone taxa within microbial communities contributing to nutrient cycling as generalist predators.1 However, prey range does not correlate with phylogeny and genetic indicators of predatory capacity are not currently known. This work seeks to identify predatory myxobacteria that capably overcome mucoid phenotypes of *Pseudomonas putida* and *Pseudomonas aeruginosa* for prioritization of subsequent drug discovery efforts.

**Methods:** Discovery of novel metabolites is more likely from novel species and underexplored genera

of myxobacteria.2 Thus, initial efforts focused on isolation, sequencing, and taxonomic classification of

novel myxobacteria from North American soils. Using these discovered myxobacteria, we concurrently

conducted predation assays with a previously generated mucoid phenotype of *P. putida* that overproduces alginate as well as *P. aeruginosa*. Concurrently, extracts from each novel myxobacteria were fractionated and utilized in antimicrobial assays against *P. putida* and *P. aeruginosa*.

**Results**: To date we have isolated and sequenced a total of 6 novel myxobacteria from the genera *Myxococcus*, *Corallococcus*, *Pyxidicoccus*, *Stigmatella*, and *Nannocystis*. Aside from overproduction of alginate, our generated *P. putida* predation-resistant phenotype also produced detectible quantities of phenazine-1-carboxylic acid not observed from the parent strain of *P. putida.* Increased production of phenazines and alginate have previously been identified as predation resistant features as well as features associated with clinical isolates of *P. aeruginosa*.3 Comparative genome analysis, taxonomic placement, and assessment of biosynthetic capacity informed our initial prioritization of novel myxobacteria from the genus *Corallococcus* due to the presence of annotated alginate lyases and phenazine degradation pathways. Ultimately, we anticipate exploration of novel myxobacteria combined with consideration of prey range and features associated with resistances to myxobacterial predation will provide an ecology-informed approach for natural product discovery while also uncovering genetic determinants associated with prey range.

1 Petters S., *et al. ISME J*. 2021 Mar 21. doi: 10.1038/s41396-021-00958-2.

2 Hoffmann T., *et al. Nat Commun*. 2018 Feb 23;9(1):803. doi: 10.1038/s41467-018-03184-1.

3 Rossi E., *et al. Nat Rev Microbiol*. 2021 May;19(5):331-342. doi: 10.1038/s41579-020-00477-5.

**Gb3-cSrc Complex in Glycosphingolipid-Enriched Microdomains Contributes to the Expression of p53 Mutant Protein and Enrichment of Cancer Stem Cells**

**Yong-Yu Liu\*, Kartik R. Roy\*, Ronald A. Hill\*, Yu-Teh Li§**

*\*School of Basic Pharmaceutical and Toxicological Sciences, College of Pharmacy, University of Louisiana Monroe, Monroe, Louisiana, USA §Department of Biochemistry and Molecular Biology, Tulane University School of Medicine, New Orleans, Louisiana, USA*

**Introduction**: Glucosylceramide synthase (GCS) is a key enzyme catalyzing ceramide glycosylation to generate glucosylceramides (GlcCer), which in turn serve as precursors for cells to produce an array of further-elaborated glycosphingolipids (GSLs). In cell membranes, GSLs serve as essential components of GSL-enriched microdomains (GEMs), thereby modulating membrane functions and cell behaviors. GSLs, including globotriaosylceramide (Gb3) and ganglioside GD3, are involved in regulating pluripotency of embryonic stem cells and cancer stem cells. Recent studies showed that ceramide glycosylation by GCS correlates with upregulated the expression of p53 mutant R273H

and cancer drug resistance. Yet, the underlying mechanisms remain elusive.

**Methods**: HPLC-based in-vivo enzyme activity assay, GEMs separation and Gb3 dotblot assay, RT-qPCR of RNA methyladenosine, LC-MS and imaging flow cytometry were applied to assess alterations of GCS activity, Gb3 levels, mutant protein expression and cancer stem cell abundance in the genetically modified cancer cell lines and model mice.

**Results**: Gb3 is associated with cSrc kinase in GEMs and plays a crucial role in modulating expression of p53 R273H mutant and enrichment of cancer stem cells, causative of dug resistance. Colon cancer cell lines, either WiDr homozygous for missense-mutated *TP53* (R273H+/+), or SW48/TP53-Dox heterozygously bearing *TP53* mutant (R273H/+, knock-in via CRISPR/Cas9 gene editing), display drug resistance with increased ceramide glycosylation. Under treatments with doxorubicin, SW48/TP53-Dox cells presented epithelial-mesenchymal transition and significantly increased cancer stem cells (CD44v6+/CD133+) either in cell culture or in animal studies. Inhibition of GCS with Genz-161 (GENZ 667161) re-sensitized cells to apoptosis in these p53 mutant-carrying cancer cells. Genz-161 effectively inhibited GCS activity, and substantially suppressed the elevated Gb3 levels seen in GEMs of p53-mutant cells exposed to doxorubicin. Complex formation between Gb3 and cSrc in GEMs to activate β-catenin was detected in both cultured cells and xenograft tumors. Suppression of ceramide glycosylation significantly decreased Gb3-cSrc in GEMs, β-catenin, and methyltransferase-like 3 (METTL3, which catalyzes m6A RNA methylation at the mutant codon), thus altering premRNA splicing, resulting in upregulated expression of wild-type p53 protein, but not

mutants, in cells carrying p53 R273H. Altogether, increased Gb3-cSrc complex in GEMs of membranes in response to anticancer drug induced cell stress promotes upregulated expression of p53 mutant proteins and accordant cancer drug resistance.

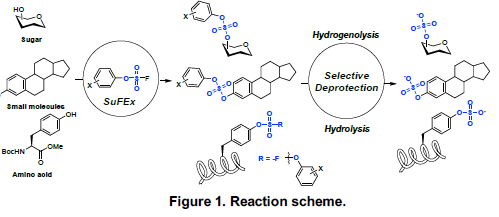
**A General Approach to *O*-Sulfation of Carbohydrates and Peptides via SuFEx Reactions**

**Jia Niu\*, Chao Liu, Cangjie Yang**

*Boston College, 2609 Beacon Street, Chestnut Hill, MA 02467*

*Correspondence:* [*jia.niu@bc.edu*](mailto:jia.niu@bc.edu)

**Introduction:** *O*-Sulfation is an important chemical code widely existing in bioactive molecules such as small molecule metabolites, peptides, proteins, and carbohydrates, but the scalable and facile synthesis of complex bioactive molecules carrying *O*-sulfation remains challenging. There are mainly two classes of existing strategies for introducing *O*-sulfation to a variety of substrates including carbohydrates and peptides: late-stage sulfation and early-stage sulfation. While late-stage sulfation methods often suffer from challenging purification of the highly polar *O*-sulfated products and their chemical instability, existing early-stage sulfation methods are limited by the incompatibility with common reaction conditions and the deactivating effect on the modified carbohydrate substrates in glycosylation. In this presentation, we will discuss a general earlystage *O*-sulfation approach to sitespecifically install sulfate diesters onto carbohydrate and peptide substrates via the sulfur(VI)-fluoride exchange (SuFEx) reaction (**Figure 1**).



**Method:** The SuFEx reaction between an aliphatic or aromatic silylether and an aryl fluorosulfate were used to install sulfate diesters onto carbohydrate and non-carbohydrate substrates. Subsequent selective deprotection of the aryl sulfate monoester then led to the desired *O*-sulfated compounds. Compared to the existing *O*-sulfation strategies, this approach allowed for balance stability and reactivity to accommodate different substrates and achieve efficient deprotection.

**Results:** Our results showed that efficient sulfate diester formation was achieved through systematic optimization of the electronic properties of aryl fluorosulfates. The versatility of this *O*-sulfation strategy was further demonstrated in the scalable syntheses of a variety of complex molecules carrying sulfate diesters at various positions, including monosaccharides, disaccharides, amino acid, and small molecules. Fmoc-protected tyrosine sulfate diesters were efficiently prepared and incorporated into synthetic peptides through solid-phase synthesis. Selective deprotection of the aryl masking groups from sulfate diesters via hydrogenolysis or hydrolysis yielded the corresponding *O*-sulfated carbohydrate and peptides. This strategy provides a powerful tool for the synthesis of *O*-sulfated bioactive compounds and the investigation into the functional roles of the sulfation pattern in carbohydrates and peptides.

**Isolation and structural characterization of a new fucosylated chondroitin sulfate: anti-SARS-CoV-2, anticoagulant and binding properties of holothurian sulfated glycans**

**Rohini Dwivedi1, Priyanka Samanta1, Poonam Sharma2, Fuming Zhang3, Sushil Mishra1, Pavel Kucheryavy1, Seonbeom Kim1, AyoOluwa O. Aderibigbe1, Robert J. Linhardt3, Ritesh Tandon2, Robert J. Doerksen1,4, Vitor *H. Pomin1,4,***

*1Department of BioMolecular Sciences, University of Mississippi, Oxford, Mississippi, USA*

*2Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York, USA*

*3Department of Microbiology and Immunology, University of Mississippi Medical Center, Jackson, Mississippi, USA.*

*4Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, Oxford, MS, USA*

**Introduction**: Sulfated glycans can display multiple biological actions and the effects are related to their structures. Action against SARS-CoV-2 has been attributed to a competitive binding onto the glycosaminoglycan**-**binding**-**like motifs present in the receptor binding domain (RBD) of SARS**-**CoV**-**2 S**-**protein. This molecular competition prevents viral attachment to the host cell surface heparan sulfate. Here, we report the structural elucidation of a new fucosylated chondroitin sulfate isolated from *Pentacta pygmaea* (PpFucCS). We further studied both the anti-SARS-CoV-2 and anticoagulant activities of PpFucCS along with two other known holothurian glycans, sulfated fucan (IbSF) and fucosylated chondroitin sulfate (IbFucCS) isolated from *Isostichopus badionotus*.

**Methods:** Sulfated polysaccharides were isolated by proteolytic digestion of the sea cucumber body wall, and fractionated by anion exchange liquid chromatography. PpFucCS was structurally characterized by multiple NMR methods, and its molecular weight distribution was determined by PAGE. Anticoagulant activity was measured by aPTT and serpin-mediated inhibitory assays. Anti SARS-CoV-2 activity was examined using a

pseudotyped SARS-CoV-2 baculoviral vector in HEK293T cells monitored by technique of green fluorescence protein. Anti-SARS- CoV-2 action was mechanistically investigated by SPR experiments using wild type and mutant S-protein RBD’s. Computational MD simulations and docking were carried out to map the molecular interactions between glycans and S-protein RBD’s.

**Results:** The structure of PpFucCS (MW~10-60 kDa) is composed specifically of two types of monofucose branches (α-Fuc2,4S and α-Fuc4S) and one difucose branch (α-Fuc2,4S(1→4)-α-Fuc), attached to the C3 position of the glucuronic acid linked predominantly at a 4-sulfated N-acetylgalactosamine unit. All three holothurian glycans exhibit anti-SARS-CoV-2 activity, ~12 times higher than heparin. Competitive SPR inhibition analysis of glycans against heparin binding to SARS-CoV-2 wild type S-protein shows IC50 values at least 6 times more efficient than heparin. Computational docking suggests similar binding site of all sulfated glycans on S-protein RBD and reveals conformational changes of some oligosaccharide structures upon binding. Among all holothurian glycans, IbSF has presented the best anti-SARS-CoV-2 candidate in light of its negligible anticoagulant effect.

**Chemical Editing of Proteoglycan Architecture**

**Timothy O’Leary\*, Meg Critcher\*, Tesia N. Stephenson, Xueyi Yang, Noah H. Bartfield, Richard Hawkins, Mia L. Huang (\* Equal authorship)**

*Scripps Research*

Proteoglycans are heterogenous macromolecular glycoconjugates that have diverse structures and functions, orchestrating many important cellular processes. Current research has focused on the poly-sulfated glycosaminoglycan chains that decorate proteoglycans, whilst their other key architectural elements, namely their anchoring core protein and their membrane localisation, have received less attention. Because they are difficulty to study as replete glycoconjugates, comprehensive structurefunction relationships are currently limited. We present an inclusive approach toward studying proteoglycan functions by generating semi-synthetic modular proteoglycans that can be tailored for cell surface display. By incorporating unnatural amino acids in

proteoglycan core proteins, we created chemical handles for the conjugation of sulfation-defined glycosaminoglycans, which were produced by metabolic oligosaccharide engineering. These glycoconjugates, generated by biorthogonal click chemistry, permit the methodical dissection of requirements for optimal binding and function of various proteoglycan-binding proteins, both in solution and on living cells through glycocalyx remodelling. We demonstrate these replete proteoglycan ectodomains can recapitulate the functions of native proteoglycans in both the differentiation of mouse embryonic stem cells and spreading of mammary carcinoma

cells. Our modular approach allows for the identification of contributing elements towards each function, permitting structural resolution towards the investigation of proteoglycan structure-function relationships in cell biology.

**Harnessing Chemistry to Understand the Roles of Glycans in Neuroplasticity**

**Linda Hsieh-Wilson**

*Division of Chemistry and Chemical Engineering, California Institute of Technology*

**Introduction:** The field of chemical neurobiology is providing insights into the molecules and interactions involved in neuronal development, sensory perception, and memory storage. In this talk, I will describe our efforts to understand how chondroitin sulfate glycosaminoglycans (GAGs) contribute to neuroplasticity – the ability of the brain to adapt and form new neural connections.

**Methods:** Using a combination of synthetic organic chemistry, biochemistry, computational chemistry, cell biology, and neurobiology, we have developed a diverse set of chemical tools for studying the biology of GAGs.

**Results:** Our results indicate that GAG activity is driven by the presence or absence of specific sulfation motifs. These motifs mediate the interactions of GAGs with particular protein partners and thereby regulate signaling events that underlie processes such as axon regeneration, synaptic plasticity, and the formation of neural circuits. The ability to identify and modulate these sulfation motifs opens up new therapeutic opportunities for neuronal regeneration and repair after stroke, central nervous system injury, and neurodegenerative diseases.

**Real-time chemical tools to modulate dynamic sugar signaling in cells**

**Charlie Fehl**

*Department of Chemistry, Wayne State University*

# Introduction: All cells use glucose. One important role of this ubiquitous sugar is to chemically regulate cellular metabolism by remodeling intracellular signaling proteins with glycans. Hyperglycemic conditions are promoted by obesity and are strongly correlated with risk factors for cancer and metabolic disease, which are promoted by glycosylated signaling proteins. However, intracellular glycosylation processes are intrinsically challenging to study in real-time due to the rapid and variable rate of sugar metabolite utilization.

# Methods: We approach this challenge by building and refining tools to allow chemical control over sugar-based signaling pathways, especially the glucose-sensing O-linked N- acetylglucosamine modification of proteins (O-GlcNAcylation). We use two strategies to enable chemical control over O-GlcNAc labeling: photochemistry and synthetic biology. Our photochemical methods include photocaged-GlcNAc chemical reporters and photoredox-based protein modification methods. Our synthetic biology methods create protein biosensors that can be initiated to “tag” O-GlcNAc proteins in living cells by fusing lectin- or glycosidase-based O-GlcNAc binding domains with chemical or enzymatic labeling systems, a strategy we call “GlycoID.”

# Results: Initial versions of our photocaged-GlcNAc and GlycoID platforms are being applied to study the effects of aberrant sugar metabolism on diseases. Disease targets include TET1 O-GlcNAc-regulated epigenetic programs that drive breast cancer, the metabolic sensing protein NF-kB, and insulin signaling. Our real-time and spatially-controlled tools enable accurate correlation of metabolic activity and signaling across different time scales of these disease processes, from minutes to hours to days, providing real-time insight into glycobiological regulation of cellular signaling pathways by glucose metabolism.

**Glycopolymer Engineering for Next-Generation Cancer Immunotherapies**

**Thomas A. Werfel1, 2, 3 , Judith U. De Mel1\*, Oluwaseyi Shofolawe-Bakare2, Karan Arora4, John T. Wilson4, and Adam E. Smith1,2**

*1Biomedical Engineering, 2Chemical Engineering, and 3BioMolecular Sciences, University of Mississippi, University, MS, USA; 4Chemical and Biomolecular Engineering, Vanderbilt University, Nashville, TN, USA*

**Introduction:** Here, we present multiple strategies to improve the efficacy and safety of cancer immunotherapies by engineering glycopolymers – synthetic polymers composed of carbohydrate building blocks – for targeted drug delivery. In one approach, nanoparticles (NPs) composed of poly(propylene sulfide)-*b*-poly(methacrylamidoglucopyranose)-*b*-poly(methacrylomidomannose) [**PPS-*b*-PMAG-*b*-PMAM**] triblock glycopolymers were produced to load hydrophobic drug (PPS) which is targeted to macrophages via high-mannose decoration on the nanoparticle corona (PMAM) and released preferentially in response to elevated ROS levels within macrophages (PPS). In another approach, dual stimuli-responsive poly[2-(diisopropylamino)ethyl methacrylate]-b-poly[(pyridyl disulfide ethyl methacrylate)-*co*-(methacrylamidoglucopyranose)]

(**PDPA-*b*-P(PDSMA-*co*-MAG)**) glycopolymers were produced to improve the delivery of neoantigen cancer vaccines by incorporating a “pH-responsive switch” (DPA) for endosomolysis and reversible peptide linkage (PDSMA) for reduction and release in the cell’s cytosol.

**Methods:** Glycopolymers were synthesized by reversible addition-fragmentation chain-transfer (RAFT) polymerization, characterized by NMR, GPC, FT-IR, and LC-MS. Nanoparticles (NPs) were assessed for size, surface charge, and stability using DLS. Drug loading and release was observed using Nile Red-loaded NPs in buffer (pH 7.4) on a fluorescence plate reader.

**Results:** Successful synthesis of PPS-*b*-PMAG-*b*-PMAM was confirmed, and the resulting polymers formed NPs of ~98 nm diameter with a slightly negative surface charge (-13.3 mV). These NPs will enable targeted delivery of immune agonists to tumor-associated macrophages in order to boost immunogenicity and limit systemic exposure of the immune agonists. NPs formed from PDPA-*b*-P(PDSMA-*co*-MAG) (PDPA: Mn 20 kDa, Đ 1.07 and PDSMA-*co*-MAG: Mn 10 kDa Đ 1.09) in neutral pH (7.4) were 33.5 ± 0.4 nm in diameter with a surface charge of 3.59 ± 0.29 mV. Upon loading the model drug Nile Red, NPs expanded to 38.8 ± 0.5 nm with no significant change to the surface potential. These NPs exhibited pH-dependent release of Nile Red, where rapid drug release was triggered below pH 6.5, and were stable through lyophilization due to the presence of MAG in the NP corona. Collectively, our efforts engineering novel glycopolymer structures have produced rationally-designed nanoparticles capable of both overcoming the drug delivery barriers that limit immunotherapy success and improving the efficacy and safety of immune agonists and neoantigen cancer vaccines.

**Proximity tagging identifies the glycan-mediated glycoprotein interactors of galectin-1 in**

**muscle stem cells**

**Zak Vilen, Eugene Joeh, Meg Critcher, Christopher G. Parker, and Mia L. Huang**

*Scripps Research*

**Introduction:** Glycosylation and glycan-protein interactions regulate many important events in mammalian development, including myogenic differentiation, the process by which precursor muscle myoblasts are irreversibly converted to contractile myotubes. Galectin-1, a b-galactoside glycan binding protein, is a potent activator of myogenic differentiation and is currently being explored as a potential therapeutic in the context of muscular repair. Despite galectin-1s important role in myogenesis, a comprehensive understanding of how galectin-1 activates myogenic differentiation is lacking, and it is currently unknown which glycoproteins at the cell surface and extracellular matrix interact with galectin-1 to orchestrate this process.

**Method:** We present a proximity tagging strategy paired with quantitative mass spectrometry-based proteomics to capture and identify glycan-mediated interactors of galectin-1 in live mouse myoblast precursor cells.

**Results:** We identify several known, as well as uncover several novel glycan mediated galectin-1 ligands and validate that galectin-1 binds them in a glycan-mediated manner. Given the importance of three-dimensional glycan presentation towards capturing physiologically relevant glycan-protein interactions and the capability of this approach to tag interactomes in live cells, the resulting dataset may serve as a resource to aid investigations into the mechanism by which galectin-1 promotes muscular regeneration.

**Next-generation tools for the synthesis of bioactive saccharides**

**Maciej Walczak**

*Department of Chemistry, University of Colorado Boulder*

**Abstract:** The Walczak group studies methods to prepare and manipulate complex natural products and biologics such as (oligo)saccharides, peptides, and proteins. Our current interests are focused on the synthesis and chemical biology of mammalian and bacterial glycans that are known to engage in biological recognition and signaling events and show a promising therapeutic and diagnostic potential. While chemical synthesis has enabled a better understanding of the role of saccharides in homeostatic and developmental processes, key obstacles such as suboptimal selectivities in chemical glycosylation reactions await broadly applicable solutions. In this presentation, I will describe the development of metal-catalyzed methods that capitalize on stereoretentive reactions of anomeric nucleophiles suitable for the formation of C(sp3)-C and C(sp3)-heteroatom bonds. This seminar will also feature selected applications of the glycosyl cross-coupling method, mechanistic and computational studies that guided new reaction discovery, and integration with modern automation technologies.

**1,2-*Cis* Selective Glycosylation through the Synergy of Trifluoromethylated Benzyl Protecting Groups and Lewis-Basic Additives**

**Dancan K. Njeri, Erik V. Alvarez, Claude J. Pertuit and Justin R. Ragains**

*Lousiana State University*

**Introduction:** Chemical *O*-glycosylation reactions offer a handle toward accessing carbohydrates and glycoconjugates. This allows in-depth study into their structure-function activities. Carbohydratebased biomolecules are defined, in part, by the stereochemistry at their anomeric centers (*i.e.*, 1,2 *-cis* and 1,2*-trans* glycosidic linkages). Access to the 1,2*-trans* linkage is straightforward as it can be enforced by having acyl-bearing protecting groups at carbon 2. On the other hand, accessing 1,2 *-cis* glycosidic linkage remains a challenge. Many approaches developed so far are laborious and lack universality.

**Methods and Results:** Our lab has recently demonstrated that synergistic effects of non-participating protecting groups and Lewis-basic additives (LB) greatly bolsters 1,2 *-cis* selectivity. In our pilot studies using our donors (*i.e*. 4-(4-methoxyphenyl)-3-butenylthioglycosides (MBTGs) and 4-(4-methoxyphenyl)-4- pentenylthioglycosides (MPTGs)) we demonstrated how halogenated benzyl protecting groups greatly bolster 1,2*-cis* selectivity while using Lewis-basic 1,4 dioxane as solvent. Variable selectivities favoring the 1,2-*cis* stereoisomer were observed. In our recent studies using trichloroacetimidates (TCAs) and *N-*phenyl trifluoroacetimidate (PTFAI) donors, we were able to demonstrate that trifluoromethylated benzyl protecting groups and triphenylphosphine oxide (TPPO) as a Lewis-basic additive work in concert to deliver exceptionally high 1,2 *-cis* selectivity in glycosylations of alcohol substrates with inherently poor stereoselectivity due to their high reactivity. This approach is especially useful when dealing with reactive primary alcohol acceptors which are used as linkers for vaccine development and glycan arrays.

**“Human Milk Oligosaccharides as a Defense Against Group B Streptococcus"**

**Steven D. Townsend**

*Department of Chemistry, Vanderbilt University*

**Introduction:** Carbohydrates are the most abundant organic molecules on earth and are critical to a variety of biological processes. The goal of this project is to characterize how human milk, at the chemistry level, protects infants from infectious diseases. At the outset, our initial hypothesis was that human milk oligosaccharides (HMOs) possess antimicrobial and anti-virulence activities. Previously, we discovered that HMOs do indeed modulate bacterial growth and biofilm production. In light of this discovery, three priorities emerged for the program. The first was to characterize the mode of action for antimicrobial activity. The second was to decipher the functional effects of HMO structural diversity. Finally, we set our sights on using HMOs to treat or prevent non-pediatric illness.

**Methods:** A combination of fractionation techniques, chemical synthesis, and industrial partnerships.

**Results**: We determined the identities of several HMOs with potent antimicrobial activity against the important neonate pathogen Group B *Streptococcus* (Group B Strep; GBS). Additionally, we observed that HMOs are effective adjuvants for intracellular-targeting antibiotics against GBS. This included two antibiotics that GBS has evolved resistance to. At their half maximal inhibitory concentration (IC50), heterogeneous HMOs reduced the minimum inhibitory concentration (MIC) of select antibiotics by up to 32-fold. Similarly, we observed that HMOs potentiate the activity of polymyxin B (Gram-negative-selective antibiotic) against GBS (Gram-positive species). Based on these collective discoveries, we hypothesized that HMOs function by increasing bacterial cell permeability, which would be *a novel mode of action for these molecules*. This hypothesis was validated as HMOs were found to increase membrane permeability by around 30% compared to an untreated control. Moreover, HMOs were shown to perturb central metabolism and the biosynthesis of membrane building blocks

***ABSTRACTS***

**Poster Presentations**

**Poster 1**

**“Potential Modification of Brain Aging by Receptor of Advanced Glycation Endproducts”**

**Brandon G. Ashmore, Cellas A. Hayes, James A. Stewart, Jr., Nicole M. Ashpole**

*Department of BioMolecular Sciences, University of Mississippi*

**Introduction:** Medical and technological advances worldwide have led to increased lifespans, but

individuals in advanced age are at increased risk for numerous diseases and comorbitidies, along with

various physical and cognitive impairments. Previous studies on the *C. elegans* model system have

demonstrated evidence that the accumulation of advanced glycated end products (AGEs) in aging tissue

may play contribute to the hallmark symptoms of aging, yet further studies are needed to determine

direct causality. We hypothesize that systemic knockout of AGE receptors (RAGE) will delay the onset of

cognitive and physical impairments in advanced age, along with the molecular markers of aging by

decreasing AGE accumulation and signaling.

**Methods:** Gene expression of markers of inflammation and senescence were quantified in aged (23-

month-old) RAGE-knock-out mice (RAGE-KO) and age-matched control, as well as young (3-4-month-old)

RAGE-KO mice and controls. For this, RNA was isolated and reverse transcribed and qPCR was

performed to calculate delta-delta Ct. Accumulation of AGE adducts in the hippocampi of aged RAGE-KO

and control mice was visualized with immunohistochemistry and accompanying fluorescence

microscopy. Brain tissue was embedded, cryosectioned, and stained with anti-*N*-carboxy-methyl-lysine

or Conclavanin A, and quantified using Nikon Elements analysis software. Cohorts of RAGE-KO and WT

mice are currently being bred to assess behavioral endpoints in young adulthood and middle age.

**Results:** Aged RAGE-KO mice showed significant reductions in the expression of senescence markers

p16ink4a and p21, indicating reduced senescence in the aged brain. The levels of lectin and CML-tagged

products within the hippocampus were still increased in the RAGE-KO mice, suggesting that the

upstream glycation signaling molecules are still available despite reductions in RAGE. We are currently

assessing glial activation in these same hippocampal tissues to determine whether the reduction in

RAGE signaling while the AGEs are still present alters astrocytic and microglial activation. As our

behavioral cohorts are currently being bred, our future studies include assessing learning and memory in

the radial arm water maze, working memory in the novel object task, circadian rhythms in free running

wheels, and locomotor function in the rotarod. Together, these studies will allow us to better

understand the role of AGE/RAGE signaling in the biological process of aging and the potential of

targeting RAGE signaling in therapeutics.

**Poster 2**

**Measuring Changes in Myxobacterial Metabolic Production Using Lipopolysaccharides of Various Prey Bacteria**

**Andrew Ahearne and Cole Stevens**

*Department of BioMolecular Sciences, University of Mississippi*

**Introduction:** Natural products of microbial origin have long been a promising source of pharmaceutically relevant compounds. Although actinomycetes and cyanobacteria have historically been the preferential source for compound discovery, myxobacteria contain a similar proficiency for producing secondary metabolites.1,2 With large genomes (9-13 Mb), myxobacteria typically have the potential of producing 30-40 secondary metabolites. However, like most bacteria, a large portion of a bacteria’s metabolic pathways remain dormant under standard laboratory growth conditions.2 Due to the predatory nature of myxobacteria, it is hypothesized that myxobacteria will alter their metabolism depending on the species of prey present. Given the diversity of the O-antigen in lipopolysaccharide(LPS) between species3, myxobacteria may utilize this variation as a way of modulating metabolic production. Using the MS spectrum of myxobacterial cultures grown under different conditions, in concert with the tools GNPS and XCMS, we assess the effects of various LPS on myxobacterial metabolism. However, given the test strain’s high relatedness to the well characterized *Corallococcus coralloides*, it is unsurprising that no novel compounds were identified. To increase likelihood of novel compound discovery, isolation and characterization of unique myxobacteria from North American soils is currently being performed.

**Methods:** Lipopolysaccharides isolated from various prey bacteria, *Escherichia coli* and *salmonella sp.*, were added to the growing myxobacteria culture. The mass spectrum data from the isolates of these cultures were used for analysis using XCMS and GNPS. Using these tools, the changes in metabolite production under LPS exposure were assessed, and searched for induction of new biosynthetic gene clusters. Novel myxobacteria were isolated from soil using *E. coli* baiting techniques, and purified by transfer of fruiting bodies to fresh agar plates. The relatedness of novel species was assessed using total genome sequencing followed by average nucleotide identity(ANI) and digital DNA-DNA hybridization(dDDH).

**Results:** Initial MS based analysis of metabolic changes in the test myxobacteria showed substantial changes in metabolic production under the two LPS stimuli. Notably, there was not much overlap in the changes between *E. coli* and *Salmonella* LPS, suggesting unique reactions from the myxobacteria to each prey. However, unique clusters were not identified on a GNPS network. The use of total genome comparison, rather than 16S homology, for determining homology has resulted in the discovery of 6 novel species of the genera *Corallococcus*, *Myxococcus*, *Pyxidicoccus*, *Stigmatella*, and *Nannocystis.*

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**Poster 3**

**The GLYCORE Imaging Core: Bridging the gap between advanced imaging techniques and the Glycosciences**

**Ruofan Cao, Gregg Roman**

*Department of BioMolecular Sciences, University of Mississippi*

The objective of the GlyCORE Imaging Core is to promote and enhance the growth of glycoscience projects at the University of Mississippi and throughout the mid-south region. The Core brings together new and existing advanced microscopes into a University-wide central platform, offering a wide range of advanced imaging techniques. The Core currently administrates three microscopes, one imaging cytometer, and a computer workstation dedicated to image analysis. The primary microscope is an inverted **Leica SP8 confocal microscope** with a white light laser, piezo encoded stage, resonant scanner, objective inverter, adaptive focus control (AFC), and ultrasensitive HyD detectors. This confocal can handle most fluorescent imaging applications, including Z-stack, time-lapse, spectrum screening, tiling/stitching with robust (AFC), and sensitive and super-fast imaging (up to 28 fps) capabilities. Software modules within the system will assist many advanced imaging techniques, such as Fluorescent Resonant Energy Transfer (FRET) and Fluorescence Recovery After Photo Bleaching (FRAP), and image processing for presentation. The Core also contains a **Zeiss Axio Imager M1 widefield microscope** with epifluorescent, dark-field, phase-contrast, and bright-field imaging functions. This M1 Axio imager is well suited for the general morphometric analysis of tissues. The GlyCORE Imaging Research Core also houses a **Zeiss Discovery v.12 Stereomicroscope** capable of bright field and fluorescent microscopy, with ZEN2.3 software and extended focus technique for increasing depth of field in thick specimens of larger tissues and structures, and the morphometric analysis of these structures. The **FlowCam imaging cytometer** provides a high-throughput screening/analysis platform for suspended particles and cells. Most commonly used image processing software, including Fiji, Photoshop, Zen Lite, and LAS X, can be accessed on our **top-level image workstation**. The GlyCORE Imaging Research Core will also offer many services, including but not limited to new imaging technique development, image processing, and specimen preparation consultation, etc. Amongst the most important will be consulting with the investigators on how the data can be best collected and published. Extra information and services could be found on the core website <https://gic.olemiss.edu/>.

**Poster 4**

**Conformational Analysis of CF2-linked Flavonoid Glycosides**

**Reem A. Alkhodier and David A. Colby**

*Department of BioMolecular Sciences, University of Mississippi*

Many natural products are glycosylated at oxygen atoms. However, they are often chemically and enzymatically labile. CH2-glycosides were developed as stable analogs of Oglycosides. A CH2 group cannot act as a bioisostere of an oxygen atom, due to the lower polarity and the increased conformational flexibility observed with this linker, compromising biological mimicry. The difluoromethylene (CF2) group acts as a nonhydrolyzable and an isopolar oxygen atom bioisostere. For example, CF2-mannopeptides retained activities as selectin inhibitors, and CF2-sialosides were active as sialidase inhibitors. Moreover, conformational bias is introduced by using CF2 as an intersaccharide linker, which may play a role in determining pharmacophoric features needed for the binding of glycosylated molecules.

Flavonoids have been heavily investigated for many health benefits. These compounds are present predominantly as β-O-glycosides, which are hydrolyzed in the body. Herein, we perform conformational analysis using molecular mechanics and molecular dynamics for Oglycosylated flavonoids and their CF2-glycosylated analogs, to investigate conformational changes. Sampling of the φ and ψ dihedral angles along the glycosidic linkage was key in this study.

The compounds were minimized using LigPrep of the Schrodinger package, and conformational analysis and energy calculations were performed using MacroModel in an aqueous solvent. The lowest energy conformer of each molecule was used for geometry optimization and restrained electrostatic potential (RESP) charge calculations using the Gaussian 09 software. The molecules were parameterized, and 100 ns molecular dynamics (MD) simulations were performed in an explicit solvent at 300°K using the General AMBER Force

Field (GAFF2), and AMBER software.

The φ and ψ dihedral angles of crystal structures of all compounds fall within the conformations of O-glycosides sampled in MD simulations, but not the CF2-glycosides. This is expected since all CF2-glycoside analogs impart conformational bias for all flavonoids tested. Each representative compound from heavily populated clusters adopts the chair conformation. For compounds substituted at the 3-position, ψ angles of the CF2-glycosides are restricted to lower values than O-glycosides. The largest change in conformational flexibility is observed with the very restricted CF2-glycosides at the 5-position. CF2-glycosides at the 7-position show changes in the φ angle, and major shifts in ψ angles. This data indicates how different positions of the flavonoid are affected by changing an O-glycoside into a CF2-glycoside, and how conformational bias of the CF2 linking the sugar moiety to the flavonoid ring is observed in all molecules, similar to the behavior of CF2 as an intersaccharide linker.

**Poster 5**

**Mapping Interaction Domains of Cation-Independent Mannose 6-phosphate Receptor and Plasminogen through Fast Photochemical Oxidation of Proteins and High-Resolution Mass Spectrometry**

**Zhi Cheng, Sandeep Misra, Richard N. Bohnsack, Linda J. Olson, Nancy M. Dahms,**

**and Joshua S. Sharp**

*Department of BioMolecular Sciences, University of Mississippi*

The cation-independent mannose 6-phosphate receptor (CI-MPR) is an essential glycoprotein that carries out multiple functions by interacting with other proteins. The most commonly known functions of this protein include lysosomal enzyme recognition, protein trafficking, and cell growth and motility regulation. Plasminogen, a precursor of the glycoprotein plasmin that is synthesized by the liver and extrahepatic cells, plays an important role in cell migration, inflammation, and tissue remodeling. Plasminogen activation can be regulated by many activators, inhibitors, and receptors including CI-MPR. The complex of CI-MPR and plasminogen can further interact with other proteins such as latent transforming growth factor-β (TGF-β) resulting in proteolytic activation. However, the mechanisms of these direct or indirect interactions of CI-MPR on a molecular basis are still not noticeably clear. Therefore, more molecular model studies are needed to identify how plasminogen binds to CI-MPR and facilitate understanding of receptor interactions.

Here, we used Fast Photochemical Oxidation of Proteins (FPOP) coupled with mass spectrometry (MS) to determine the oxidation levels of CI-MPR under three different conditions: CI-MPR alone, plasminogen alone, and plasminogen bound to CI-MPR. The result from this experiment will help us understand whether the plasminogen binding interaction causes CI-MPR to change its conformation, and therefore, influence its solvent accessible surface area (SASA). Three groups of protein samples were prepared for the FPOP experiment: free CI-MPR, free plasminogen, and CI-MPR in the presence of plasminogen (n=4). These three groups of FPOP samples contained 2 μM CI-MPR/plasminogen/CI-MPR plasminogen complex, 10 mM Tris (pH 7.5), 150 mM sodium acetate, 0.005% Tween 20, 17 mM glutamine, and 100 mM hydrogen peroxide were illuminated by a COMPex Pro 102 KrF excimer laser and then collected to a quenching solution to eliminate further oxidation. Quenched protein samples were denatured and digested by either trypsin or GluC separately. Digested protein samples were analyzed by Dionex Ultimate 3000 system coupled with an Orbitrap Fusion Tribrid MS. The peptides from tryptic digestion of CI-MPR were identified by Byonic. The oxidation event per peptide was calculated manually by integrating peak areas of the unoxidized and oxidized versions of the same peptide. We found four peptides of CI-MPR (148- 156, 204-214, 260-274, 457-476) show decreased solvent accessibility, and five peptides (102-121, 157- 178, 306-323, 564-586, 699-718) show increased solvent exposure upon interaction with plasminogen. One peptide (475-493) of plasminogen shows increased exposure to the solvent and three peptides (313-324, 409-426, 514-530) show decrease in peptide oxidation, indicating decrease in solvent accessibility upon interaction with CI-MPR. We are in the process of modeling these topographical changes onto the structures of CI-MPR and plasminogen to better understand the interaction of CI-MPR to plasminogen.

**Poster 6**

**Coupling FDM 3D Printing with Hot-Melt Extrusion to Produce Hypertensive Dual Therapy Fixed Dose Combination Tablet**

**Abdullah Alzahrani, Sagar Narala, Dinesh Nyavanandi, Mashan Almutairi, Ahmed Almotairy, Suresh Bandari, Michael Repka**

*Department of Pharmaceutics and Drug Delivery, University of Mississippi*

**Introduction:** Coupling Hot melt extrusion (HME) technology with Fused deposition modeling (FDM) three-dimensional (3D) printing technology as additive manufacturing has huge potential to revolutionize alternative methods of drug delivery system formation. The primary aim of this study was to develop a pharmaceutically suitable core-shell fixed-dose combination product for hypertensive dual therapy using HME paired with FDM 3D printing and direct compression techniques.

**Methods:** The selected drug and polymer combinations were blended, and the obtained physical mixture was

extruded using a co-rotating twin-screw extruder. Filaments used for direct compression tablets (core) were prepared by HME with different drug load of atorvastatin calcium (ATV) using Kollidon® VA 64 as the polymeric carrier and croscarmellose as a super disintegrant. Filaments of amlodipine besylate (AML) with different drug load employing polyvinyl alcohol (PVA) with/without plasticizer were fabricated using HME. The obtained filaments were utilized to produce the shell using FDM 3D printing. The mechanical properties of filaments were assessed using a TA-XT2 texture analyzer and Repka-Zhang test. The tablet shell was designed with 12.5mm diameter and 5.5 mm thickness. The dimensions for the core tablets were 8.0mm diameter and 3.25mm thickness. Thermal stability of both APIs was evaluated using differential scanning

calorimetry (DSC) for all formulations. The interactions between APIs and the polymeric carriers were investigated using Fourier transform infrared spectroscopy (FTIR). The in vitro drug release studies were conducted in 900 mL pH 6.8, 0.05 M phosphate buffer for ATV and pH 2.2, 0.01 N HCl for AML and 0.1% polysorbate 80 in phosphate buffer with pH 6.8 for the shell-core combination.

**Results:** The DSC data showed sharp single melting endothermic peaks of the crystalline ATV and AML

at 164.48 °C and 206 °C, respectively. The characteristic melting endothermic peaks for both APIs were absent in the filaments demonstrating transformation of crystalline API to the amorphous form. Core tablet with high drug load ratio showed better compressibility characteristics and higher dissolution rate than the ATV formulation with low drug load. PVA filaments with 5% w/w of AML showed degradation due to the high HME process temperature at 190°C. The addition of sorbitol with reduced drug load allowed extrusion at low temperature resulting in an acceptable drug content. Extruded filaments of PVA mixtures exhibited good printability with optimum mechanical strength for 3D printing. The hollow shell tablet with low drug load of AML and different infill density showed better printability. ATV-AML shell-core tablets were successfully prepared by coupling HME technology with 3D printing as promising manufacturing technologies for complex personalized oral dosage forms based on the patient's needs.

**Poster 7**

**Dual Responsive Glycopolymeric Nanoparticles for Neoantigen Peptide and Lipophilic Adjuvant**

**Co-delivery**

**Judith U. De Mel1\*, Oluwaseyi Shofolawe-Bakare2, Karan Arora4, John T. Wilson4, Adam E. Smith1,2 and Thomas A. Werfel1, 2, 3**

*1Biomedical Engineering, 2Chemical Engineering, and 3BioMolecular Sciences, University of Mississippi,*

*4Chemical and Biomolecular Engineering, Vanderbilt University*

**Introduction:** Neoantigen peptide vaccines (NPVs) used for boosting immunogenicity in various cancers are prone to aggregation at the site of injection and lack inherent immunogenicity. To address this, immune agonists (*i.e.* adjuvants) capable of provoking a robust immune response to peptide antigens can be co-delivered. Also, the poor cellular uptake and endosomal entrapment of peptides in cancer cells impacting Major Histocompatibility Complex- I (MHC-I) loading and Cytotoxic T Lymphocyte (CTL) activation can be overcome by pH and reduction-responsive release mechanisms facilitating endosomal escape and cytosolic release of both peptide and adjuvant cargo. We address these barriers to NPVs by designing glycopolymeric nanoparticles (NPs) based on stimuli-responsive block copolymers. We utilize a poly[2-(diisopropylamino)ethyl methacrylate] (PDPA) block as the core for loading lipophilic adjuvants and the “pH-responsive switch” to transition from hydrophobic at extracellular pH (7.4) to hydrophilic at pH values of the endolysosomal pathway (below ~6.8). The hydrophilic poly[(pyridyl disulfide ethyl methacrylate)-*co*-(methacrylamidoglucopyranose) (P(PDSMA-*co*-MAG)) corona provides colloidal stability, cryoprotection, and reversible attachment sites for peptide conjugation.

**Methods:** PDPA-*b*-P(PDSMA-*co*-MAG) diblock copolymer was synthesized by RAFT polymerization, characterized by NMR and GPC. NPs were assessed for size, surface charge, stability using DLS, and zeta potential. Nile Red (NR)-loaded NPs in buffer (pH 7.4) and their systematic disassembly was observed in acidic pHs by DLS. Encapsulation efficiency and drug release utilized NR and fluorescence spectroscopy. The conjugation of ovalbumin with PDSMA was confirmed by SDS-PAGE.

**Results and Discussion:** NPs formed from PDPA-*b*-P(PDSMA-*co*-MAG) (PDPA: Mn 20 kDa, Đ 1.07 and PDSMA*co*- MAG: Mn 10 kDa Đ 1.09) in neutral pH (7.4) were 33.5 ± 0.4 nm in diameter with a surface charge of 3.59 ± 0.29 mV. Upon loading NR, NPs expanded to 38.8 ± 0.5 nm with no significant change to the surface potential. We observed that the particles were stable upon rehydration after lyophilization. We hypothesize MAG in the corona adds cryoprotection during the lyophilization process in addition to colloidal stability. Thus, the dual-responsive glycopolymeric NPs developed here represent a promising platform technology that can overcome peptide delivery barriers, enable cytosolic peptide and adjuvant co-delivery, and stability through lyophilization facilitating vaccine transportation.

**Poster 8**

**Synthesis of Difluorinated Benzopyrans**

**Amena Begum, David A. Colby**

*Department of BioMolecular Sciences, University of Mississippi*

Natural antioxidants are widely distributed in food and medicinal plants. The effective extraction and proper assessment of antioxidants from food and medicinal plants are crucial to explore the potential antioxidant sources and promote the application in functional foods, pharmaceuticals and food additives. Of the antioxidants found in nature, anthocyanins are among the most potent. In particular Malvidin-3-glucoside has exhibited exceptional potential as a neuroprotective agent. Malvidin-3-glucoside is a naturally occurring antioxidant and a potent neuroprotective agent. The clinical translation of this natural product is prevented by rapid hydrolysis of glucoside and this process is known as deglycosylation. The resulting malvidin aglycone is unable to cross the blood brain barrier which eliminates its potential use in neurodegenerative disorders. The hydrolytic reaction occurs at the unstable O-glucoside linkage of malvidin-3-glucoside. The goal of this project is to create a model system that replaces the unstable O-glucoside linkage with a stable CF2-glucoside bond. The synthetic strategy will be developed through the production of pentafluoro-gem-diols and conversion to difluoro methyl ketones.

**Poster 9**

**An Immunoblot Assay to Detect Antibodies to the SARS-CoV-2 Spike**

**Glycoprotein Antigen in Serum of COVID-19 Patients**

**Wayne Gray and Shivum Desai**

*Biology Department, University of Mississippi*

**Introduction:** The Coronavirus Disease 19 (COVID-19) pandemic caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has killed more than 3.5 million people worldwide, including nearly 600,000 people in the U.S. An enzyme linked immunosorbent assay (ELISA) is available to diagnose COVID-19 infection by detection of antibodies to the SARS-CoV-2 spike (S) glycoprotein in patient serum. While this COVID-19 ELISA is generally reliable, the assay may have false positive results meaning that a person may not truly have protective antibodies and thus may be susceptible to SARS-CoV-2 infection. In this study, a COVID-19 immunoblot assay was developed to provide a means to confirm the presence of antibodies to the SARS-CoV-2 S glycoprotein in patient serum.

**Methods:** The S1 and Receptor Binding Domain (RBD) components of the SARS-CoV-2 S glycoprotein were expressed in *E. coli*, purified, fractionated by SDS-PAGE gel electrophoresis, and transferred to nylon membranes. Expression of the S1 and RBD proteins was confirmed by immunoblot analysis using rabbit polyclonal antiserum against the SARS-CoV-2 S antigen. Immunoblot analysis was used to detect antibodies to the S1 and RBD antigens in sera derived from COVID-19 positive patients and in uninfected individuals.

**Results:** Antibodies to the SARS-CoV-2 S1 antigen was confirmed in sera derived from multiple COVID-19 positive patients, but not in serum from uninfected patients. Interestingly, to date, antibodies to the RBD portion of the S protein have not been detected in the SARS-CoV-2 patients.

**Conclusion:** The results of this study indicate the feasibility of a SARS-CoV-2 immunoblot assay to detect antibodies in SARS-CoV-2 patients and to confirm that these patients are seropositive for COVID-19.

**Poster 10**

**Chronic protein O-GlcNAcylation induces cardiac hypertrophy**

**Chae-Myeong Ha, Sayan Bakshi, Luke A. Potter, John C. Chatham, Adam R. Wende**

*Department of Pathology,* *University of Alabama at Birmingham*

Cardiovascular disease (CVD) is the leading cause of death in the United States. Lifestyle and metabolic diseases influence severity and pathogenesis of CVD through numerous mechanisms including regulation of gene expression. CVD is associated with cardiac hypertrophy and tightly linked to mortality in diabetes. However, some hypertrophy may be adaptive. A potential link between diabetes and heart failure is hyperglycemia. Chronic high blood glucose levels alter molecular mechanisms through the post-translational modification of *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAcylation). Previous studies reported higher protein *O*-GlcNAcylation in cardiac tissue during heart failure. Dynamic protein *O*-GlcNAcylation is regulated by protein *O*-GlcNAc transferase (OGT) and protein *O*-GlcNAcase (OGA), which in concert sense nutrient status and metabolic flux. In the current study, we hypothesized that protein *O*-GlcNAcylation contributes to cardiac remodeling which may progress to pathophysiological effects when sustained in cardiomyocytes leading to heart failure.

To test this hypothesis, we developed an inducible cardiomyocyte-specific dominantnegative *O*-GlcNAcase (dnOGA) overexpression transgenic mouse model, which leads to 1.8-fold increase in protein *O*-GlcNAcylation. We examined the effects of 2 weeks (acute) and 24 weeks (chronic) overexpression in both male and female mice. Cardiac function (i.e. echocardiography, n>8) and molecular analysis (i.e. mRNA-seq, n=5) of mouse hearts were performed to determine effects of acute and chronic *O-*GlcNAcylation.

Acute *O*-GlcNAcylation in cardiac muscle did not alter heart weight or cardiac function; however, chronic cardiac protein *O*-GlcNAcylation lead to cardiac hypertrophy in both male (1.2-fold) and female (1.4-fold) mice compared to sex-matched controls. Cardiac hypertrophy markers were elevated both on acute and chronic protein *O*-GlcNAcylated heart. Interestingly, chronic *O-*GlcNAcylation produced right ventricular hypertrophy.

Despite this, cardiac function was maintained with preserved ejection fraction. mRNA-seq analysis of acute versus chronic induction showed a remarkably divergent metabolic gene signature including oxidative phosphorylation, branched chain amino acid, fatty acid, and glucose metabolism pathways. In chronic induction, extracellular matrix pathways were also elevated.

Our data indicate that chronic increases in protein *O*-GlcNAcylation induced by overexpression of dnOGA lead to cardiac remodeling with distinctive changes in mRNA expression levels related to cardiac metabolic regulation and cardiac remodeling markers. Specifically producing a functionally compensated right ventricular

hypertrophy is a distinct result compared to other heart failure models, such as pressure-overload or diabetes-associated left ventricular hypertrophy. Furthermore, female mice have more robust hypertrophy compared to male dnOGA mice. Additional mechanistic analysis into the consequences of chronic *O*-GlcNAcylation in

cardiomyocytes are underway to identify specific target proteins and underlying mechanisms of regulation.

**Poster 11**

**Hot Melt Extrusion based Fused Deposition Modeling 3D Printing of Atorvastatin calcium tablets: Impact of infill density on the performance of tablets**

**Preethi Mandati1, Nagireddy Dumpa1, Dinesh Nyavanandi1, Sagar Narala1, Honghe Wang1, Suresh Bandari1, Michael A. Repka1, Sandip Tiwari2, Nigel Langley2**

*1Department of Pharmaceutics and Drug Delivery, University of Mississippi,*

*2BASF Corporation*

**Introduction:** The current research was focused on developing immediate release tablets of

atorvastatin calcium using hot melt extrusion coupled fused deposition modeling technique and

to investigate the effect of shape and infill density (50% and 100%) on drug release profiles.

**Methodology:** Formulations were prepared using different hydrophilic polymers, Kollicoat® IR

as a major polymer and Kollidon® VA64 , Kollidon® 12PF were selected to improve the printability of the filament. All of the filaments were produced with 10% drug load using Kollicoat® IR alone or in combination with 20% of Kollidon® VA64 or Kollidon® 12PF. The physical mixtures were extruded using an 11mm co-rotating twin-screw extruder at 170 ℃ with 75 rpm screw speed. The process was performed by using ThermoFischer standard screw configuration. The filaments and drug excipient interactions were evaluated using differential scanning calorimetry (DSC) and Fourier-transform infrared spectroscopy (FTIR). The filaments obtained were printed using a Prusa i3 FDM 3D printer. Each 3D printed tablet consisted of atorvastatin calcium equivalent to 10.0 mg of atorvastatin. The drug release studies were performed in 900 ml pH 6.8 phosphate buffer at 37± 0.5°C using USP type I dissolution apparatus at 75 rpm. Aliquot of samples (1ml) was collected and evaluated for drug content using UV-visible spectrophotometry at 241 nm with dissolution media as a blank.

**Results:** During the process of FDM 3D printing, filaments of Kollicoat® IR alone has resulted

in separation of layers attributing to the poor adhesive nature of the polymer. Addition of Kollidon® VA64 or Kollidon® 12PF resulted in successful printing of tablets with 50% and 100% infill densities at a nozzle temperature of 190°C. Conversion of the drug to the amorphous form and no drug-excipient interactions was confirmed by DSC and FTIR. Tablets of Kollidon® VA64 and Kollidon® 12PF formulations, with 100% infill density demonstrated incomplete drug release profiles, whereas 50% infill density demonstrated more than 80% drug release in 30 min. Tablet infill densities played a key role in achieving the target release profiles. Thus, HME coupled FDM 3D printing process provides the flexibility of adjusting the release profiles of tablets, which remains as a major limitation of the conventional techniques. Furthermore, the effect of tablet shape on drug release is warranted for the future studies of the 3D printed tablets.

**Poster 12**

**Altered Mucin Sialylation Results in Delayed Mucociliary Transport in Cystic Fibrosis**

**Elex Harris, E Scott Helton, Marina Mazur, Stefanie Krick, Steven M. Rowe, Jarrod W. Barnes**

*Gregory Fleming James Cystic Fibrosis Research Center, Univ. of Alabama at*

*Birmingham*

**Introduction:** Mucus stasis is a pathologic hallmark of Cystic Fibrosis (CF). Many of the gel-forming

properties of mucus are provided by mucins, a primary constituent of mucus. These mucins are extensively O-linked glycosylated and terminal sialylation of these glycans contributes to their negative charge state and regulation of the ionic microenvironment. Changes in O-linked sialylation of gel-forming mucins would therefore be expected to alter its physiochemical characteristics. Early evidence shows that defective CFTR can affect mucin glycosylation; however, the consequences of altered sialylation on mucus transport and physiology have not been determined. In this study, we investigated the changes in sialyltransferase gene expression in CF human bronchial epithelial cells (HBECs) and the direct effects of sialyltransferase inhibition on mucus physiology *in vitro* and *in vivo*.

M**ethods:** Well-differentiated HBECs were treated with vehicle or 200μM sialyltransferase inhibitor (STI, 3Fax-Peracetyl Neu5Ac) to the basolateral compartment; mucus physiology was assessed at 24 and 48 hours after treatment. Glycogene expression was also analyzed by a qPCR array of predetermined glycogenes using RNA isolated from non-CF and CF HBECs. In vivo, vehicle or 500μM STI was instilled intratracheally into WT rats daily for 7 days, and then tracheas were excised to assess mucus physiology. Mucus physiology was assessed by micro-Optical Coherence Tomography (μOCT) to measure air surface liquid (ASL) and periciliary liquid (PCL) depths, mucociliary transport (MCT), and (ciliary beat frequency) CBF.

**Results:** To determine the glycosyltransferases involved in mucin biosynthesis that may be affected by STI, we performed a targeted qPCR glycogene array in non-CF and CF HBECs. Notably, 5 out of 7 of the targeted sialyltransferase genes were downregulated, suggesting that sialylation may be decreased in CF HBECs. To measure the functional impact, we investigated the effects of decreased mucin sialylation on mucus transport. Following removal of pre-existing mucus, treated cells were subjected to STI to prevent sialylation of nascent mucins. non-CF HBECs had significantly decreased MCT after 24 and 48 hours without substantial effects on

PCL depth. Similarly, STI administration to WT rats for 7 days significantly reduced MCT in ex vivo tracheas, without meaningful changes in ASL or PCL depth or CBF.

**Conclusion:** Basal mRNA levels of multiple sialyltranferases potentially involved in mucin biogenesis are reduced in CF HBECs compared to controls. Inhibiting sialylation in non-CF HBECs and rat trachea impairs MCT Sialyation warrants further investigation as a mechanism of CF mucus stasis and may be a potential therapeutic target.

**Poster 13**

**Computational Chemistry and Bioinformatics Research Core**

**Sushil K. Mishra, Priyanka Samanta, and Robert J. Doerksen**

*Department of BioMolecular Sciences, University of Mississippi*

Glycoscience continues to emerge as a high-value information-rich field providing medical insight in the post-genomic era. Computational approaches to study protein‒carbohydrate interactions are essential for understanding their direct contribution and role in biological function, including adhesion, recognition, differentiation, metastasis, pathogenesis, and immunological recognition [1-2]. The Computational Chemistry and Bioinformatics Research Core (CCBRC), one of the three research cores of the Glycoscience Center of Research Excellence, supports performing computations on a wide range of glycoscience topics. In this poster, we will provide an overview of the capabilities of CCBRC in terms of computations that we can support and/or perform, and the training we provide. Notably, we show our hardware resources that encompass several standalone workstations, servers, and a list of available software. We also present how CCBRC can assist in identifying computational approaches that can foster and encourage glycoscience research in the Mid-South region of the USA.

**Reference:**

[1] Karlsson. Trends Pharmacol. Sci. 12 (1991) 265–272

[2] Gabius. Sci. 40 (2015) 341

**Poster 14**

**A novel octasaccharide isolated from mild acid hydrolysis of the Isostichopus badionotus sulfated fucan**

**Seon Beom Kim, Rohini Dwivedi, Maggie C. Taylor, Pavel Kucheryavy, Vitor H. Pomin**

*Department of BioMolecular Sciences, University of Mississippi*

**Introduction:** Sea cucumber has traditionally been consumed as a tonic food in East Asia countries. The

*Isostichopus badionotus* from the Stichopodidae family is widely spread in the warm Atlantic found from North Carolina in the United States. Multiple biological activities of *I. badionotus*derived sulfated glycans have been reported including anticoagulant, antithrombotic, angiogenic modulation and metastasis inhibition. Effects in coagulation and against viral infections have attracted considerable attention in the community. The sulfated fucan from *I. badionotus* presents a linear tetrasaccharide repeating structure composed of the following sequence [→3Fuc(2S,4S)- (α1→3)-Fuc(2S)-(α1→3)-Fuc(2S)-(α1→3)-Fuc-(α1→]n.

**Method:** The crude polysaccharide from the dried *I. badionotus* was extracted by papain digestion and partially purified by ethanol precipitation. The crude polysaccharides were subjected to ionexchange chromatography (DEAE Cellulose column, 2.5 x 20 cm, 2.5 mL/20min/fraction) to isolate the sulfated fucan. The Sephadex G-15 (1.5 x 45 cm, 2 mL/10 min/fraction) was employed for desalting of fractions. Mild acid hydrolysis was performed by dissolving 30.0 mg of the purified sulfated fucan in 3.0 mL of 0.05 M H2SO4 during 10 hours. The pH of the solution was then adjusted to 7.0 with the addition of 3.0 mL of 0.05 M NaOH. The hydrolyzed sulfated fucan was subjected to size-exclusion chromatography (Bio-Gel P-10 column, 1.5 x 160 cm, 1 mL/15

min/fraction) eluted with aqueous 10 % EtOH in 1.0 M NaCl resulting into eight fractions. Fractions were desalted using Sephadex G-15 column. The molecular weight of an octasaccharide was confirmed by mass spectrometry, negative mode, using a three-charged molecular ion. The obtained octasaccharide (830 μg) was dissolved in 150 μL of D2O (99.8%) in a Shigemi tube and subjected to 1D; 1H and 2D; COSY, TOCSY, HSQC, NOESY NMR experiments at 25 oC in 600 MHz Bruker spectrometer.

**Results:** In our research, we report for the first time, a stereospecific 2-desulfation reaction during the mild acid hydrolysis of the *I. badionotus* sulfated fucan. In addition, a novel octasaccharide has been isolated from a controlled mild acid hydrolysis and structurally characterized by NMR, resulting the following sequence [→3Fuc(4S)-(α1→3)-Fuc(2S)-(α1→3)-Fuc(2S)-(α1→3)-Fuc-(α1→]2. The 2-desulfation reaction that occurs at the fucose residue between 2- and 4-sulfated units during the mild acid hydrolysis was confirmed via cross-peak assignments in the 1H-13C HSQC NMR spectrum. This novel and chemically defined octasaccharide will be subjected to NMR experiments for assessment of its conformation in solution.

**Poster 15**

**GlyCORE: Analytical and Biophysical Chemistry Research Core**

**Sandeep K. Misra, Anter Sami, Joshua S. Sharp**

*Department of BioMolecular Sciences, University of Mississippi*

The University of Mississippi received center of biological research excellence (COBRE) grant from NIH in 2020 to create the Glycoscience Center of Research Excellence (GlyCORE). The Analytical & Biophysical Chemistry Research Core is one of the three dedicated research cores created in GlyCORE to support the glycoscience work of researchers at the University of Mississippi and throughout the Mid-South region. We currently operate a high resolution Orbitrap Exploris 240 system coupled to Dionex Ultimate 3000 nano-UHPLC system for LCMS/ MS analyses. We are collaborating with the researchers of Ole Miss in a wide variety of research projects encompassing proteomics, structural analyses, metabolomics and glycomics

utilizing these instruments. A few of the representative projects include the identification of protein oxidation, untargeted metabolomics and identification of glycosylation patterns on isolated glycoproteins. We are also working with researchers in the Department of BioMolecular Sciences to separate glycosides and other compounds of interest using chiral chromatography, supporting researchers doing unique synthesis reactions. These chiral columns offer excellent resolution of racemates as well as rapid and easy method development.

We are in the process of installing a UHPLC system with a Corona Veo RS Charged Aerosol Detector. It can be used for the universal detection of analytes with poor optical properties, such as carbohydrates and polymers. Using this detector, concentrations of the oligosaccharides and other analytes of interest can be measured against any known standard because response is near uniform and independent of chemical structure. We are happy to consult researchers within Ole Miss and the Mid-South region to discuss their

needs and how we can help in advancing their research projects.

**Poster 16**

**Polysaccharide as Colon Drug Delivery Carrier: Hot-melt Extrusion**

**Sagar Narala\*, Dinesh Nyavanandi, Abdullah Alzahrani, Preethi Mandati,**

**Suresh Bandari, Michael A. Repka**

*Department of Pharmaceutics and Drug Delivery, University of Mississippi*

**Introduction:** Colon-targeted drug delivery has drawn the researcher's attention due to the local and systemic therapy, and for the enhanced oral delivery of drugs susceptible to acidic degradation in the stomach. This study was aimed to develop the colon drug delivery system containing ketoprofen with AFFINISOL™ HPMC HME 4M and pectin using hot-melt extrusion.

**Methodology:** The physical mixtures of ketoprofen and polymer (20:80 % W/W) were prepared in different pectin and HPMC ratios and processed through a co-rotating twin-screw extruder (Process 11, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 110° C and 50 rpm screw speed. The extruded filaments were characterized using DSC. Extruded filaments were cut into pellets manually and used for dissolution studies. A three-stage dissolution was conducted for all the formulations using the USP type II apparatus. Initially, dissolution was performed in 750 mL 0.1 N HCl (pH = 1.2) for two hours, followed by adjusting the pH of the medium to 6.8 with 250 mL of 0.2 M sodium phosphate tribasic buffer, and continued for four hours. Finally, the

dissolution medium pH was adjusted to 7.4 with 0.1 N NaOH, and the dissolution continued for 18 h. Samples were collected at regular intervals and analyzed using a UV spectrophotometer at max of 256 nm. Dissolution was performed with and without the enzyme (Pectinex Ultra SP-L).

**Results:** The DSC data revealed that drug was converted into an amorphous form. In the dissolution study without Pectinex Ultra SP-L, a relatively similar drug release was observed within all formulations at 2, 6, and 24 h. A similar release profile was observed with all formulations at 2 and 6 h in dissolution with Pectinex Ultra SP-L. However, all the formulations showed a substantial improvement in % drug release at 24 h except the formulation with only HPMC. Formulation with high pectin concentration (40:60 % w/w pectin:HPMC) showed the highest % drug release (97%) at 24 h over other formulations. The presence of Pectinex in dissolution media, increased breakdown of the pectin chains resulting in increased erosion rate of the formulations causes enhanced drug release. Colon-targeted drug delivery was successfully achieved with the developed formulations consisting of pectin and HPMC. The pectin concentration mainly influenced drug release in the prepared formulations.

**Poster 17**

**Carbohydrate structures shared between the *Streptococcus pneumoniae* capsule**

**and common food**

Moon H. Nahm and Feroze Ganaie

*Department of Medicine, University of Alabama at Birmingham*

**Introduction:** Since birth, we are exposed to many glycans from bacteria and food plants. Food antigens can influence the development of our immune system and may even induce tolerance. If a bacterium mimics a food antigen, then the host immune system can fail to recognize the bacterial antigen as foreign. Polysaccharide (PS) is an essential component of food and is used by *S. pneumoniae* as a capsule to protect the bacterial

cell wall and/or other critical surface structures, thereby shielding the bacteria from the host immune system. Since the capsule PS is an important virulence factor for pneumococci and is used as a vaccine, the impact of food consumption on vaccine responses requires investigation.

**Methods:** We studied 14 different plant foods for cross-reactivity in our multiplex bead array assay with monoclonal antibodies (MAbs) against 26 pneumococcal serotypes, which commonly cause infections and are included in pneumococcal vaccines. To directly show that βGal(1-6) is the epitope shared between RG-I and 10A PS, we created a 10A variant lacking *wcrG* gene and examined it with the two Mabs (Hyp10AG1 and CCRC M7). Urinary capsule test, a sandwich immunoassay, was used to investigate the pectin-mediated immunological interference. Next, *in vitro* opsonization assay was performed to determine if RG-I pectin could inhibit the opsonic capacity of a human serum pool (007sp) containing 10A antibodies.

**Results:** We discovered that capsule type 10A specific monoclonal antibody (Mab) cross-reacts with many natural and processed plant foods. A similar cross-reaction was noted between capsule type 15B specific Mab and fruit peels. Further investigation of the L:\Feroze\4 Conferences\9. Mid-South Glycoscience meeting\Abstract\_v4.docx 4/21/2021 capsule type 10A revealed that the cross-reactive epitope is 1,6-β-galactosidase [β Gal(1-6)], present in the rhamnogalacturonan I (RG-I) domain of pectin found in almost

all plant material. Besides, the structure is found among the capsules produced by oral streptococci as well.

**Conclusion:** Immune response to 10A is comparable to that seen with other capsule types, and pectin ingestion may have little impact on vaccine responses. However, antibody to pectin can kill serotype 10A pneumococci, and this shared epitope may be considered in pneumococcal vaccine designs. Moreover, the epitope is widely present among food items and microorganisms; thus, it may interfere with pneumococcal serotyping assays.

**Poster 18**

**NMR-based structural biology in marine medicinal glycomics**

**Vitor H. Pomin1,2,\*, Seon Beom Kim1, Rohini Dwivedi1**

1Department of BioMolecular Sciences, University of Mississippi, 2Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, \* email: vpomin@olemiss.edu

**Introduction**: Nuclear Magnetic Resonance (NMR) spectroscopy plays an essential role in structural biology. From this analytical technique, unknown structures can be revealed, reactions can be followed, conformational and dynamical properties of molecules can be studied in solution, either free or bound to proteins, and biding sites and/or atoms involved in intermolecular complexes can be mapped. Here, we employ NMR in multiple investigations regarding the structural biology of marine sulfated glycans endowed with biomedical actions.

Attention is given on the (i) characterization of the 3D structure of the tetrasaccharide building block of the sea urchin *Lytechinus variegatus*-derived sulfated fucan (LvSF), (ii) stereospecific 2 desulfation of the sulfated fucan from the sea cucumber *Isostichopus badionotus* (IbSF), and (iii) structural elucidation of the new fucosylated chondroitin sulfate from the sea cucumber *Pentacta pygmaea* (PpFucCS).

**Methods:** The 3D structure of LvSF tetrasaccharide was achieved by a combination of measurements from 3*J*H-H scalar coupling and nuclear Overhauser effect (NOE)-derived internuclear distances using 85:15% H2O:acetone‑*d*6 solution at 0 °C. For accurate NOEderived conformational restraints, calibration of NOE cross-peaks was performed by analysis of NOE build-up curves acquired at multiple mixing times (0, 4, 8, 12, 16, 20, 24, 28, 32 ms). The selective 2-desulfation reaction of IbSF was followed by 1D 1H NMR spectral analysis on a series of oligosaccharide fractions obtained after size-exclusion chromatography (Bio-Gel P-

10 column, 1.5 x 160 cm, 1 mL/15 min/fraction eluted with aqueous 10 % EtOH in 1.0 M NaCl) of a sample submitted to mild acid hydrolysis (0.05 M H2SO4 for 10 hours). The pH of the solution was adjusted at the end of the reaction to 7.0 with the addition of same volume of 0.05 M NaOH. Fractions were desalted using Sephadex G-15 column and analysed by 1D 1H NMR in a Bruker 400 MHz magnet at 25 oC. The structure of PpFucCS was revealed by spin assignments of multiple 2D NMR methods (COSY, TOCSY, NOESY and HSQC spectra) acquired in a Bruker 400 MHz magnet at 25 oC.

**Results:** The solution 3D structure of LvSF pentasulfated tetrasaccharide was generated and deposited at PDB (7KS6). The 2-desulfation of IbSF was identified to occur selectively at the disulfated fucosyl unit of its tetrasaccharide-repeating unit. The structure of PpFucCS was characterized as {→3)-*β*-GalNAcX-(1→4)-*β*-GlcA-[(3→1)Y]-(1→}, where X = 4S (80%) or 6S (10%), Y = *α*-Fuc2,4S (40%), *α*-Fuc2,4S-(1→4)-*α*-Fuc (30%), or *α*-Fuc4S (30%), and S = SO3-.

**Poster 19**

**Effects of Alginate Exposure on Metabolite Production in Novel *Corallococcus* Species**

**Kayleigh Phillips, Shukria Akbar, Andrew Ahearne, and D. Cole Stevens**

*Department of BioMolecular Sciences, University of Mississippi*

Predation by myxobacteria often provides insight into the diversity of microbial communities because of their capability to produce secondary metabolites and hydrolytic enzymes to lyse prey organisms. The *Corallococcus* genus of myxobacteria is found abundantly in the soil and is one of the most isolated genera for myxobacteria. Two newly isolated, novel *Corallococcus* spp. Of myxobacteria were utilized to determine the susceptibility of a *Pseudomonas putida* phenotype recalcitrant to myxobacterial predation. This predation-resistant phenotype has previously been observed to overproduce alginate as well as phenazine-1-carboxylic acid both of which impede myxobacterial swarming and predation*.* Previously determined resistance to myxobacterial predation was done using *Cystobacter ferrugineus* using traditional predation assays coupled

with comparative transcriptomics and metabolomics. Due to the *Corallococcus* genus having a diverse prey consumption, an identified phenazine degradation pathway, and numerous alginate or poly(beta-D-mannuronate) lyases present in their sequenced genomes, there is potential for *Corallococcus* spp. to consume the predation-resistant phenotype. *Pseudomonas putida* survivor phenotype and wild type were co-cultured with *Corallococcus* spp*.* by creating a bacterial suspension of prey spotted on nutrient-limited agar introduced with *Corallococcus* on the edge of the prey spot. *Corallococcus* spp. was also cultured on VY/2 agar for 7 days and supplemented with a 10 μM alginate solution around the perimeter of the swarming biomass to determine whether alginate has any effect on predator swarming. Organic phase extraction of

*Corallococcus* spp. exposed to exogenous alginate was also performed to observe any potential shift in metabolic profile due to perception of glycans associated with mucoid prey. With these methods, we can further evaluate whether alginate produced by the survivor phenotype of *P. putida* contributes to predation-resistant phenotypes and what myxobacterial adaptations overcome these resistances.

**Poster 20**

**Synthesis of ROS-Responsive Mannose Glycopolymers for Macrophage-Targeted Drug Delivery**

**Oluwaseyi T. Shofolawe-Bakare1\*, Judith U. De Mel2, Adam E. Smith1, 2, Thomas A. Werfel1, 2, 3**

1Chemical Engineering, University of Mississippi, 2Biomedical Engineering, University of Mississippi,

3BioMolecular Sciences, University of Mississippi,

**Introduction:** Macrophages play an essential role in the pathophysiology of a variety of disorders, including cancer, inflammation, atherosclerosis, and others. Moreover, although therapeutic strategies targeting macrophages hold promise for treatment of these diseases, these therapies often suffer from toxicity associated with systemic delivery of the therapeutics. For this reason, it is desirable to develop macrophage-specific drug delivery vehicles. In this work, we synthesize nanoparticles (NPs) that target macrophages via polymeric mannose ligands on the nanoparticle surface and preferentially release drug in response to reactive oxygen species (ROS), which is elevated inside macrophages. This dual-targeting strategy is expected to improve the selectivity of delivery to macrophages over mannose-targeting or ROS-responsiveness alone and improve the therapeutic window of macrophage-specific therapeutic agents.

**Materials and Methods:** The high-mannose decorated-nanoparticles will consist of triblock copolymers of poly

(propylene sulfide) (PPS) for hydrophobic drug loading and release, polymethacrylamidoglucopyranose PMAG) for nanoparticle stability, and polymethacrylomidomannose (PMAM) for targeting to M2-polarized macrophages. The methacrylate-modified carbohydrates, MAG and MAM, were synthesized by addition of methacryloyl chloride to amino-modified glucose and mannose sugars. PPS was synthesized by anionic ring-opening polymerization (ROP), followed by transformation into a RAFT macroCTA (via conjugation to 4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT)). The PPS-ECT macroCTA was then chain extended with MAG to form PPS-*b*-PMAG, and PPS-*b*-PMAG was chain extended further with MAM to yield PPS-*b*-PMAG-*b*-PMAM triblock copolymers. The resulting polymers were characterized by 1H NMR and GPC, and NPs formed form these polymers were characterized using DLS.

**Results:** PPS-*b*-PMAG diblock copolymers were successfully synthesized by a combination of anionic and RAFT polymerizations, respectively. The successful synthesis of a hydroxyl-terminated PPS polymer was confirmed through 1H NMR and the molecular weight was measured to be 3.6 kDa (Mn) using GPC. Conjugation of ECT was subsequently confirmed using 1H NMR spectra. Chain extension of PPS-ECT was confirmed by observing a reduction in the elution time for the diblock copolymer compared to the homopolymer. The molecular weight of diblock copolymers was determined to be 6 kDa (Mn). The PPS-*b*-PMAG deblock copolymer formed micelles of 98nm with a polydispersity of 0.184. The micelles also exhibited a slightly negative zeta potential of -13.3mV.

**Poster 21**

**Anti-SARS-CoV-2 and binding properties of fucosylated chondroitin sulfates**

**Priyanka Samanta1, Rohini Dwivedi1, Poonam Sharma2, Fuming Zhang3, Sushil K. Mishra1, Pavel Kucheryavy1, Seonbeom Kim1, AyoOluwa O. Aderibigbe1, Robert J. Linhardt3, Ritesh Tandon2, Vitor H. Pomin1,4, Robert J. Doerksen1,4**

*1Department of BioMolecular Sciences, University of Mississippi, 2Department of Microbiology and Immunology, University of Mississippi Medical Center, 3Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 4Research Institute of Pharmaceutical Sciences, University of Mississippi,*

*Priyanka Samanta: psamanta@go.olemiss.edu, Robert J. Doerksen: rjd@olemiss.edu*

**INTRODUCTION:** The outbreak of severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) has created an ongoing global pandemic. The entry of SARS-CoV-2 inside the host cell is mediated by spike glycoprotein (SGP). Several glycosaminoglycans (GAGs) have been found to bind to SGP. The emergence of new SARS**-**CoV**-**2 variants with a key N501Y mutation in the SGP receptor binding domain (RBD) has enabled tighter binding to angiotensin converting enzyme 2 (ACE2) receptor, causing higher infection and transmission rates. In the current work, we have studied the structural characteristics of novel fucosylated chondroitin sulfates (fCS) extracted from *Pentacta pygmaea* and *Isostichopus badionotus*, and investigated their anti-SARSCoV- 2 and binding properties with wild type (WT) and mutant RBD of SARS-CoV-2 SGP. The isolated marine fCS could be further developed for their prophylactic and therapeutic benefits.

**METHODS:** We used molecular dynamics (MD) simulations and molecular docking to study free and protein-bound fCS. Models of the WT and N501Y RBD SGP were built using the published X-ray crystal structure (PDB ID: 6M0J). All-atom MD simulations of the isolated fCS and of fCS– protein interactions were performed using Glycam06 and Amberff14SB forcefields for glycans and proteins, respectively. The Amber20 package was used for MD simulations. Docking was performed using Autodock Vina. Surface Plasmon Resonance (SPR) was used to study the molecular interactions of the sulfated glycans and SGP.

**RESULTS:** Using MD simulations, we investigated the dynamic structural conformations of the fCS molecules in solution. We observed that the sulfation pattern of the extracted fCS played an important role in the intramolecular interaction between neighboring carbohydrate moieties. The molecular structures of the GAGs were then used for studying SGP–fCS complexes. The energetically minimized structures of fCS were docked into the RBD of SGP. A potential GAG binding site was identified by docking a heparin disaccharide unit and the fCS to WT and N501Y SGP. Several key interactions between the fCS and SGP were identified. The dynamics of the SGP–fCS complexes were further studied using MD simulations. Conformational analyses were compared for the isolated fCS and the protein-bound fCS. Molecular docking studies were in excellent agreement with SPR measurements of SGP WT and N501Y binding to surface heparin. Our results provide insights into potential anti-SARS-CoV-2 benefits of the marine sulfated glycans.

**Poster 22**

**N262-Associated Glycan Microdomain of HIV-1 Env gp120 Exerts a Ripple Effect on Env Glycan-Shield**

**Density, Function, and Antibody Recognition**

**Qing Wei,1 Audra A. Hargett,2 Barbora Knoppova,1 Alexandra Duverger,4 Reda Rawi,5 Chen-Hsiang Shen,5 S. Katie Farney,5 Stacy Hall,1 Rhubell Brown,1 Brandon F. Keele,6 Sonya L. Heath,4 Michael S Saag,4 Olaf Kutsch,4 Gwo-Yu Chuang,5 Peter D. Kwong,5 Zina Moldoveanu,1 Milan Raska,3**

**Matthew B. *Renfrow,2 Jan Novak,1***

*1Department of Microbiology, University of Alabama at Birmingham,*

*2Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham,*

*3Department of Immunology, Palacky University Olomouc, Olomouc, Czech Republic*

*4Depatment of Medicine, University of Alabama at Birmingham,*

*5Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health,*

*6AIDS and Cancer Virus Program, Frederick National Laboratory for Cancer Research*

**Introduction:** The densely glycosylated HIV-1 envelope (Env) glycoprotein is involved in virus-cell entry and immune evasion. Single-genome-amplification of viral RNA has identified nucleotide sequence of *env* genes from viruses that established the productive infection as well as from viruses isolated during subsequent chronic infection. Sequence analyses revealed that mutations in *env* of chronic-stage viruses result in loss of some existing potential *N*-glycosylation sites (NGS) and appearance of new potential NGS. These changes can account for viral escape from neutralizing antibodies (nAbs), although some may affect viral infectivity. The impact of NGS changes on the structure and function of the Env glycan shield is not well understood.

**Methods:** Potential NGS in HIV-1 Env variants were identified by using multiple-sequence alignment and NetNglyc software. Two Env variants with seven differences in potential NGS were selected, WEAU-d16 from an early virus and WEAU-d391 from a late virus. Modifications of N262 potential NGS by site-directed mutagenesis generated WEAU-d16-N264S (adding N262) and WEAU-d391-S264N (deleting N262). Env-pseudotyped virions were produced in Free-Style 293F cells (293F), viral infectivity and neutralization by nAbs were assessed in reporter cells, TZM-bl. Recombinant gp120 with His tag was expressed in 293F cells and isolated by affinity chromatography. The glycosylation heterogeneity of individual NGS of rgp120 was determined quantitatively by a validated LC-MS-based glycomics workflow. A comprehensive Env structure modeling was performed using molecular dynamics simulations (MDS) with follow-up analyses of glycan betweenness centrality and the positioning of the center of mass (COM) of glycans.

**Results:** The presence of N262 NGS in WEAU-d16-N264S and WEAU-d391 viruses resulted in significantly higher infectivity compared to WEAU-d16 and WEAU-d391-S264N (lacking N262). Although these N262 NGS-lacking viruses exhibit reduced binding of Env trimers to CD4 receptor, WEAU-d16 and WEAU-d391-S264N were sensitive to some broadly nAbs. Glycomic profiling revealed that N262 NGS exerted a ripple effect on glycan processing throughout rgp120, altering site-specific glycosylation heterogeneity of some other glycans. MDS revealed that N262 glycans affected movements of other glycans within a glycan microdomain, a cluster of several NGS spatially close to N262. Determination of COM for these NGS tracked the movements of specific glycans in MDS. Betweenness centrality heatmap indicated weaker glycan-glycan interaction on Env

glycoforms without N262. Together, our results demonstrate that N262 glycans interact with several other

glycans in a glycan microdomain and impact processing of other glycans and, thus, have a functional role in

Env glycan shield.

**Poster 23**

**Hydroxyl Radical Carbohydrate Footprinting for Probing Protein-Binding Components of**

**Oligosaccharides**

**Hao Liu, Lianyan Xu, Steven D. Townsend and Joshua S. Sharp**

*Department of BioMolecular Sciences, University of Mississippi*

**Introduction:** The study of protein-carbohydrate interactions is very challenging. This is mainly due to the

structural complexity of carbohydrates, the dynamic nature of many carbohydrate conformations, and

often low binding affinity of the interactions. In order to study and characterize the carbohydrateprotein

interaction, we are developing a new approach using mass spectrometry-based hydroxyl radical

carbohydrate footprinting (HRCF). This method characterizes changes in solvent accessibility of different

regions of an oligosaccharide by exposing the carbohydrate to hydroxyl radicals generated in situ. By

monitoring the extent of oxidation of different oligosaccharides using LC-MS/MS, we can measure

changes in the amount of oxidation of the oligosaccharide, and infer changes in solvent accessibility, as

well as localize which portion(s) of the oligosaccharide are oxidized to determine regions involved in the

binding interface.

**Methods:** A glycan mixture was generated consisting of equal amounts of five trisaccharides: NAG3,

isomaltotriose, 1-kestose, raffinose and melezitose was generated. Four model proteins tested in this

study were ubiquitin, lysozyme, Griffonia siplicifolia lectin, and myoglobin. 50 uM of each protein in 10

mM sodium phosphate buffer along with 17 mM glutamine as scavenger and 1 mM adenine as radical

dosimeter were mixed with 25 uM of glycan mixture for FPOC. After 1h incubation, FPOC labeling of

each protein was performed at 150 mM hydrogen peroxide by a 248 nm COMPexPro 102 high pulse

energy excimer laser, then immediately quenched. The FPOC samples were analyzed by HILIC LC-MS on

a Thermo Orbitrap Fusion Tribrid coupled with a Dionex UltiMate 3000 liquid chromatography.

**Results:** Non-binding trisaccharide isomers showed no significant change in oxidation in the presence or

absence of any protein. Oxidation of NAG3 shows no change in the presence of ubiquitin or myoglobin.

Oxidation of NAG3 decreases in the presence of two proteins that binds NAG3: lysozyme and Gs lectin,

demonstrating capability of FPOC to detect oligosaccharide binding to protein. MS/MS analysis localizes

the major site of oxidation of NAG3 to be the reducing end of the oligosaccharide. Lysozyme binds NAG3

at the reducing end; in contrast, Gs lectin binds NAG3 at the non-reducing end. Comparing the amount

of oxidation of NAG3 in the presence of the two binding proteins, there is a much larger decrease in

oxidation in the presence of lysozyme. These data support that FPOC could differentiate which part of

the glycan is involved in the protein-binding interface. Experiments examining more complex mixtures of

human milk oligosaccharides are ongoing.

**Poster 24**

**An evaluation of the Glycoscience Center of Research Excellence (GlyCORE)**

**Shannon R. Sharp, Sarah Mason, Anna T. Beavers, Olivia Melvin**

*Center for Research Evaluation, University of Mississippi*

**Introduction:** The Center for Research Evaluation (CERE) has partnered with the Glycoscience Center of

Research Excellence (GlyCORE) at the University of Mississippi (UM) to evaluate and help maximize the program’s impact. GlyCORE includes four research Cores providing unique glycolscience-related services to researchers throughout the mid-south and supports Junior Investigators (JIs) and Pilot Project Program awardees in an effort to further encourage glycoscience-related research and funding acquisition.

**Methods:** To evaluate GlyCORE, CERE applied a mixed-methods design, utilizing surveys, interviews and

secondary data. Specifically, CERE administered an annual survey of GlyCORE users; conducted Zoom interviews with each of the GlyCORE JIs, PIs and Core Directors; and conducted monthly altmetrics and social media analyses. CERE developed protocols for these evaluation tools with a focus on measuring progress toward GlyCORE’s aims during its first year of programming.

**Results:** At a high level, responses indicate JIs benefit from their affiliation with GlyCORE and its provision

of funding, professional development, devoted research time, external funding acquisition assistance, science career advancement, and access to more experienced and expert faculty. All JIs reportedly meet or otherwise communicate with their mentors regularly and 75% report their mentors provide beneficial support related to proposal and/or manuscript-writing and career development. Interview and survey responses indicate most JIs are making good progress on their research, manuscript-writing and grant-proposal development. Interviews with PIs and Core Directors revealed that, despite COVID-19-related challenges, GlyCORE leaders felt they made good progress toward supporting the development of UM faculty in glycoscience, establishing state-of-the-art research Core facilities, mentoring JIs, recruiting Pilot Project Program awardees, increasing demand and interest in the Cores and glycoscience, and hiring exceptional employees. Seventy-five percent of PI and Core Director interview respondents indicate GlyCORE influences and/or reaches UM faculty, in general, by helping them run experiments in a new way. Survey responses indicate GlyCORE reaches glycoscience researchers *outside* UM through social media, while other target groups heard about GlyCORE primarily through colleagues. Social media data shows engagement is highest on Twitter, increasing in March with the employment of a Social Media Specialist, and the number of tweets per month seems to positively influence Twitter engagement. Overall, findings indicate that, while GlyCORE is still working to expand its reach

beyond UM, current users and affiliates are highly satisfied with the services and supports GlyCORE provides; mean satisfaction and experience scores from Core users ranged from 4.36 to 5.00 on a 5-point scale.