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The Chemistry–Glycobiology Frontier

This *JACS* Select issue highlights current and emerging areas of research at the chemistry–glycobiology interface and illustrates how chemical and physical approaches are being used to great advantage to investigate the structures, reactions, and functions of complex glycans and glycoconjugates. While major advances in chemical approaches for synthesis, analysis, and molecular reengineering have fueled our understanding of proteins and nucleic acids, the chemical complexity of naturally occurring glycans and glycoconjugates has proven to be far more challenging. Despite the challenges, the field of glycobiology has become a rich area of research where chemically inspired strategies and technologies are making a major impact.

There are many reasons why the naturally occurring polymers of carbohydrates continue to demand strategic new chemical approaches to help in deciphering the fascinating biology that is concealed within their structures. Unlike nucleic acids and proteins, glycan biopolymers can be linear or branched, and there exists a far greater expansion of structural types due to the potential variety of linkages and linkage stereochemistry. Additionally, the functional group reactivity of individual carbohydrate building blocks is much harder to differentiate, making the development of methods for building glycans *de novo* or for regioselectively labeling glycans and glycoconjugates for biological analysis a daunting task. From a biological perspective, glycans and glycoconjugates are intrinsically far more heterogeneous than proteins and nucleic acids; their biosynthesis is not template driven, and there are no selectivity and editing filters that ensure the biosynthesis of unique products. Thus, samples of glycans and glycoconjugates derived from nature are often complex mixtures of closely related materials that can be very difficult to analyze. Furthermore, navigating through the diversity of glycosyl hydrolase, glycosyl transferase,[1] and glycan-modifying enzymes that ultimately control the structures of naturally occurring glycans can be confounding even with the relatively limited set of building blocks that are commonly observed in eukaryotes. Furthermore, as we start to appreciate the enormous diversity of carbohydrates in prokaryotic glycoconjugates, which far exceeds the basic set of common saccharide building blocks, the difficulties are ever more confounding.

Nevertheless, the biochemistry and biology of carbohydrate-containing macromolecules are fascinating, and so the motivation to develop and apply new chemical approaches is both significant and highly rewarding. This *JACS* Select issue is built around five focus areas that illustrate the ingenuity of the chemists tackling the complex and often arcane field of glycan and glycoconjugate research.

Getting a Grip on Glycoconjugates with Selective Labeling and Imaging. Important initiatives focus on the development of methods for selective labeling and detection of specific glycoconjugates in biological systems. Glycan structures lack intrinsically reactive functional groups and represent challenging targets for molecular recognition, so the development of innovative chemical approaches has been valuable in providing new insight that greatly contributes to the field of glycobiology. Complex glycoconjugates, including glycolipids and glycoproteins, are commonly displayed on cell surfaces, where they play critical roles as mediators in extracellular communication and signaling networks. Since the information contained in carbohydrate polymers is critical in processes ranging from protein localization to cell division and differentiation to organism development, insight into the dynamic changes in glycan and glycoconjugate profiles is very important for understanding the underlying biology. The investigation of glycan modification patterns of cell-surface glycoconjugates also holds great potential in medical diagnostics, as the studies offer insight into cellular pathologies and may provide important molecular markers for disease. Pioneered by Bertozzi and coworkers,[2] the method of glycoconjugate metabolic labeling has proven to be a powerful and versatile approach for detecting and imaging these important macromolecules in cells and in living animals. One of the most important applications is for labeling of cell-surface glycoconjugates. In principle, the methodology involves the use of surrogate carbohydrate building blocks that are armed with chemical handles, such as azide or alkyne functional groups, for bioorthogonal conjugation. Upon uptake into cells, these alternative building blocks are adopted into native glycoconjugate assembly pathways and processed as if they were the native building blocks. When the carbohydrate analogues are efficiently incorporated into cell-surface glycoproteins and glycolipids, the non-native functionality can be chemically conjugated through selective bioorthogonal reactions including Staudinger ligation, "click" chemistry, or strain-promoted cycloaddition. The cell-surface location of targeted glycoconjugates allows considerable flexibility with the labeling reactions, as it is not necessary to deliver reagents into cells. By using carbohydrate metabolic labeling together with bioorthogonal conjugation methods to introduce fluorescent probes for imaging, the dynamic changes in glycoconjugate distributions on the surface of live cells and in living animals can be observed.

One limitation of current metabolic glycan labeling approaches is that they affect many different cell types, relying principally on the efficiency of carbohydrate precursor uptake into cells and on the intracellular biosynthetic pathways. To address this issue, **Chen**[3] has developed a glycan metabolic labeling approach that is cell type specific. This is achieved by encapsulating a synthetic azido carbohydrate analogue, in this case a sialic acid derivative, into liposomes that are surface modified with a ligand that interacts only with certain cell types. In the proof-of-principle studies, selective metabolic labeling followed by fluorophore conjugation and fluorescence-activated cell sorting enabled significant discrimination between two cell types. Therefore, this advance enables selective delivery of the carbohydrate only into targeted cells, which display the cognate receptor for the ligand. These types of studies will enable the

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dynamic analysis of glycans on specific cells, such as cancer cells, in the presence of other cell types.

In some cases, carbohydrate metabolic labeling may not proceed efficiently because of the limitations imposed by the enzymes in the endogenous biosynthetic pathways. As an alternative, **Hsieh-Wilson**[4] has spearheaded the development of chemoenzymatic strategies that complement metabolic glycan labeling and can be used to selectively target specific glycan biomarkers on cell surfaces. The disaccharide motif that is targeted by their current approach is α-D-fucose-(1→2)-galactose, which is found at the nonreducing end of a large family of cell-surface glycans, including important complex structures that are involved in learning and memory, inflammation, and tumorigenesis. Since lectins, which are carbohydrate-binding proteins, show notoriously poor affinity and selectivity for small glycan structures such as disaccharides, this clever approach instead exploits the high selectivity of a bacterial enzyme, BgtA, that acts on the terminal α-D-fucose-(1→2)-galactose disaccharide motif to transfer an azide- or ketone-containing carbohydrate from the corresponding UDP-sugar glycosyl donor. In this way, bioorthogonally reactive functional group handles can be installed uniquely onto target glycan structures and subsequently used, for example, for selective fluorophore labeling for imaging.

Efficient gycan labeling strategies are also important for altering the physical properties of isolated glycoconjugates. In this context, glycoproteins are now widely recognized as essential components of the modern pharmacopeia, and there is an urgent need for selective and practical methods for modifying these complex macromolecules in order to improve their pharmacodynamic and pharmacokinetic properties. Galactose oxidase (GOase) is a copper-dependent alcohol oxidase that selectively oxidizes the C-6 hydroxyl group of free and terminal galactose moieties found at the nonreducing termini of glycans and glycoconjugates. While introduction of the C-6 aldehyde functionality provides a useful site for conjugation with aminooxy alcohols to afford stable oxime products, the high selectivity of the enzyme for galactose can be a limitation. To address this issue, **Turner**, **Flitsch**, and co-workers[5] have applied enzyme engineering approaches introduced by Raetz to drive the GOase to function with other hexose and hexosamine derivatives. The current generations of GOase mutants that are presented oxidize a considerably broader range of carbohydrates, including many of the common hexoses, and will be useful for global labeling strategies. It will be exciting to see if future engineering efforts with this system can provide new tools for the selective labeling of a range of targeted carbohydrates for diagnostic applications.

While many of the recent glycan labeling initiatives feature conjugation strategies based on azide/alkyne click chemistry and oxime formation, there are also excellent opportunities for chemists to engage in the discovery of new bioorthogonal transformations. For example, **Boons** and co-workers[6] have recently reported the development of a new cycloaddition strategy, based on the reaction of a nitrile oxide with a strained alkyne, which is orthogonal to the established azide-based cycloaddition. This chemistry enables sequential introduction of two different labels, which will enable the preparation of multifunctionalized glycoconjugates that feature two different exogenous groups.[6]

Using Glycans To Modulate the Structure and Function. Of the covalent protein modification reactions found in nature, glycosylation is perhaps the most complex and challenging to study. In addition, this modification is widespread and extremely important in human biology, as more than half of all eukaryotic proteins are glycosylated. Glycosylation modulates protein folding and stability, alters solubility and aggregation, influences cellular trafficking and intercellular recognition, and impacts the interactions of proteins with the protein degradation and immune systems. Sometimes the effects of glycosylation are so diverse that it can be challenging to actually parse out the specific effects of individual glycan modifications and their relationship to the structure and function of the protein that they decorate. An additional obstacle for many of these studies is that the native processes of protein glycosylation are so complicated that it is not straightforward to exploit chemoenzymatic approaches to prepare tailored glycoproteins for study. For these reasons, chemists have engaged experimental and computational approaches to help investigate the roles of glycosylation in particular protein targets. Additionally, there are major initiatives that seek to find strategies for site-selectively introducing glycans and glycan-like structures into proteins to exploit the favorable properties shown by native glycans.

State-of-the-art physical methods including neutron diffraction are being used to investigate specific aspects of the unique β-glycosylamide linkage that is common to all *N*-linked glycoproteins. This has been a challenging initiative because of the difficulties associated with getting suitable quality crystals for analysis. A multinational team including **Loganathan**, **Mason**, **Pérez**, and **Imberty**[7] has now derived very accurate physical data that will be extremely valuable for parametrizing molecular mechanics force fields, which will in turn contribute to future experimental and theoretical studies.

Glycosylation improves the properties of therapeutic glycoproteins by enhancing solubility and protecting the protein from aggregation, proteolysis, and degradation, which enhances circulation times and improves efficacy. However, these advantages come at a significant cost because of the more complex expression systems that are needed to produce glycoproteins with glycosylation patterns that are compatible with human administration. While alternative strategies such as PEGylation (covalent modification with polyethylene glycol polymers) have been exploited, there are opportunities for site-specific introduction of more native modifications that might better mimic natural glycosylation. To address this issue, **Palmer** and **Wang**[8] present reagents for the cysteine thiol-specific labeling of proteins with specific homooligomers of β-maltose ranging from 500 to 10,000 Da. The approach is illustrated with the modification and biophysical analysis of hemoglobin, which is of interest due to the potential importance of semisynthetic hemoglobins for artificial red blood cell systems.

Since protein glycosylation plays such an important role in defining protein structure and function, methods for the sensitive and rapid analysis of very small amounts of glycoproteins are in high demand. Such methods are not only essential for the assessment of recombinant therapeutic glycoproteins but also of considerable interest in medical diagnostics, where aberrant glycosylation is an important marker for diseases including cancer and autoimmune disorders. This need has prompted **Herr** and co-workers[9] to develop sensitive new analytical methods that use a microfluidic platform together with a combination of size-based and lectin-based separations to enable rapid analysis of aberrant glycoproteins.

Synthetic systems with pendant glycan structures also show interesting and unexpected properties when integrated into higher order supramolecular structures. For example, **Sen Gupta** and **Ambade**[10] have used ring-opening polymerization to prepare glycopolypeptides that can then be conjugated to different hydrophobic dendrons using click chemistry. The variation of different components of the design affords a remarkable variety of supramolecular structures.

Assembling Tailored Glycans and Glycoconjugates with a Little Help from Nature. While the synthesis and semisynthesis of defined glycans is a Herculean task, it represents one of the important research areas where chemists can contribute in a unique way to glycobiology. The preparation of pure samples of chemically defined structures is the surest way of establishing critical structure−function relationships and unambiguously confirming the identity of glycans and glycoconjugates from complex biological samples. In recent years, major strides have been made toward the automated synthesis of oligosaccharides; however, particular carbohydrate building blocks and certain glycosidic linkages can be quite recalcitrant, thus complicating total synthesis efforts. In these cases, help from nature's glycan assembly catalysts and the application of chemoenzymatic and biomimetic approaches can make a major difference. For example, **Flitsch**[11] has developed a versatile semisynthesis of glycopeptides that include the α-linked mannosyl threonine glycoamino acid. The mannosyl threonine is first incorporated into mono-mannosyl peptide using standard solid-phase peptide synthesis, avoiding the challenging enzymatic step of mannose transfer to peptide. The assembled peptide is then elaborated to the α-D-NeuNAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→2)-α-D-Man tetrasaccharide using three glycosyl transferases that have been selected for logistical reasons from diverse organisms. The glycopeptides that are assembled using this chimeric approach will help to shed light on the role of protein *O*-mannosylation and its function in regulating the activity of α-dystroglycan, which is dysregulated in congenital muscular dystrophy.

One limitation when repurposing naturally occurring glycan-modifying enzymes is their high specificity and limited substrate scope. However, the ever-expanding genome sequence data provides a treasure trove of new information for bioinformatics analyses to identify enzyme targets with modified specificities. In this context, endoglycosidases such as EndoH and EndoA natively hydrolyze off the glycans that are linked to β-GlcNAc-Asn in *N*-linked glycoproteins. Recently, there has been considerable progress in remodeling *N*-linked glycan structures using the Endo enzymes as catalysts to exchange one glycan structure for another; however, the specificity of EndoH and EndoA limits this approach, as neither enzyme tolerates the presence of core fucosylation on the β-GlcNAc-Asn. Therefore, **Davis** and **Scanlan**[12] have surveyed all the known and putative *ndo* genes and identified a new endoglycosidase (EndoS), which shows greater tolerance for *N*-linked glycans with core fucosylation. EndoS shows great potential for the remodeling of *N*-linked glycoproteins, in particular for human therapeutic proteins, where core fucosylation is a prevalent feature of the *N*-linked glycans. The challenge and prominence of core fucosylation have also been addressed by **Reichardt**,[13] who has implemented *N*-glycan arrays for screening the substrate specificities of α-1,6- and α-1,3-fucosyl transferases. The results from these studies are significant, as they provide approaches for the assembly of new glycan ligands for investigating the biological roles of core fucosylation. Furthermore, the late-stage introduction of fucose into preassembled *N*-linked glycan structures is extremely useful, as it enables the study of uniquely fucosylated *N*-linked glycan structures without the redesign of existing synthetic routes to these complex targets.

Xyloglucans are complex polysaccharides from plants that have attracted attention due to their highly branched structures and interesting physicochemical properties. However, progress toward understanding these materials has been hampered by difficulties associated with obtaining samples of the uniquely branched glycans from natural sources. To address this challenge, **Brumer**[14] has exploited the glycosynthase technology first introduced by Withers in 1998.[15] Glycosynthases are repurposed β-glycosidases that have been modified by mutation of a key active-site residue to make them hydrolytically inactive but capable of accepting α-glycosyl fluoride donors for efficient transglycosylation reactions. The power of the glycosynthase approach is remarkable and beautifully illustrated in the development of an efficient broad specificity xyloglucan synthase that can be used for the production of high-molecular-weight (60,000) uniformly galactosylated xyloglucans. The polydispersed xyloglucans, possessing defined branching patterns, can be further elaborated with a xyloglucan-specific fucosyl transferase to create synthetic samples of the complex plant polysaccharides for further study.

Synthetic fluorosugars have also found an interesting application in the **Kiessling** laboratory,[16] where they have been used as chain terminators for investigating the sequence specificity of carbohydrate polymerases that are involved in the biosynthesis of the galactan cell wall of *Mycobacterium tuberculosis*. In this mechanism-driven concept, fluorinated UDP-galactofuran analogues, having fluorine substitution in place of a hydroxyl group near the site of chain elongation, effect chain termination by deactivating subsequent polymerization steps, which offers insight into the fidelity of galactan biosynthesis.

Exploring Glycan Recognition and Processing Enzymes through Chemical and Physical Approaches. Although the mechanisms of glycosidase enzymes have been studied for decades, there continue to be new surprises. For example, in studies on the unsaturated glucuronyl hydrolases, **Withers**[17] has provided evidence for a novel mechanism involving vinyl ether hydration leading to substrate-assisted glycan breakdown, rather than direct hydrolase action at the glycosidic linkage. These enzymes are of interest because they are unique to bacteria and they are involved in the breakdown of glycosaminoglycans in the extracellular matrix of eukaryotic cells, which ultimately contributes to infection. Novel physical methods have also afforded new insight into the function of T4 lysozyme, a muramidase, which catalyzes hydrolysis of glycosidic bonds between the alternating *N*-acetyl glucosamine and *N*-acetyl muramic acid building blocks of the peptidoglycan of bacterial cell walls. One look at the complex cross-linked architecture of native peptidoglycan might suggest that it represents an impenetrable barrier, and indeed mechanistic studies have focused principally on studying the linear glycan chains that make up just part of the peptidoglycan. Undeterred, **Weiss**, **Collins**, and co-workers[18] exploit single-molecule studies on both linear and cross-linked substrates, which provide support for a fascinating view of lysozymes wherein the enzyme rapidly and processively degrades linear substrates with very little lost time. In contrast, in the face of the cross-linked substrate, the enzyme appears to literally lose time in

nonproductive motions as it sidesteps the roadblocks presented by peptide cross-links.

Determining the structural principles of protein−carbohydrate interactions is an important focus in glycobiology that has been greatly hampered by the limited availability of suitable materials for analysis. The improved availability of powerful biochemical and chemical approaches for glycan synthesis are now remedying this situation. **Laguri** and co-workers[19] have developed biochemical methods for preparing $^{13}C/^{15}N$-labeled octasaccharides of defined sequence that are uniquely sulfated. These important glycans can be used for NMR analysis to define the molecular interactions that drive complex formation between the heparan sulfate components of the extracellular matrix and chemokines. More recently, **Nifantiev**[20] has applied chemical synthesis for the preparation of oligosaccharides related to the human natural killer (HNK) cell carbohydrate HNK-1, which is an important epitope that is critical for regulating nervous system function but also the proposed target of autoantibodies that lead to serious neuropathies. Physical studies on the complexes formed between the synthetic HNK-1 oligosaccharides and proteins that bind them can now promote a deeper understanding of the normal and pathological responses to these important cell-surface glycans.

Structures of the binding domains of receptors such as the human asialoglycoprotein receptor, which binds to simple monovalent carbohydrates such as *N*-acetyl galactosamine, are also valuable since they explain not only why these simple ligands bind with very poor affinity but also how glycomimetic ligands might be designed that might bind more avidly. Using principles from the asialoglycoprotein receptor structure, **Finn**, **Mascitti**, and co-workers[21] show that small-molecule ligands with low or even sub-micromolar affinity for the receptor can be developed by exploring substitution around the pyranose ring starting from synthetic azide- and alkyne-modified galactosamine precursors, which can be readily subjected to click chemistry. These types of studies serve as a valuable paradigm for researchers seeking to find useful glycomimetic ligands for diverse carbohydrate-binding proteins.

Strength in Numbers and the Recurring Paradigm of Multivalency. Protein−carbohydrate interactions are central to numerous biological processes, but because the affinity of simple glycans for their protein partners is often weak and relatively nonspecific, multivalent interactions often prevail. An important area of research at the glycobiology interface centers on understanding and exploiting multivalency, and here, chemical approaches for the design, assembly, and evaluation of tailored systems are providing valuable tools and new insight. **Seeberger**[22] has led the development of a series of hexameric scaffolds that are based on functionalized tris(2,2′-bipyridine) ruthenium(II) complexes, which are fluorescent. These scaffolds support the supramolecular assembly of defined and highly multivalent complexes, which present carbohydrates with unique spatial orientation. The fluorescent complexes are sensitive fluorescent reporters of bacterial strains that display receptors for specific carbohydrates. The modular nature of this glycan presentation system represents a promising approach for the assembly of new sensing scaffolds with diverse multivalent carbohydrate epitopes.

The multivalent interactions of proteins with cell-surface glycoconjugates are important for activating cellular signaling processes. However, because cell surfaces are covered with a huge variety of different glycoconjugates, the details of these processes are very challenging to study, particularly in live cells. Now, **Bertozzi** and co-workers[23] have developed a targeted approach for studying the function of galectins, a family of galactose-binding lectins, which are important for clustering cell-surface glycoproteins and glycolipids. The method is based on the assembly of fluorescently labeled glycopolymers, which include fluorescence resonance energy transfer donor or acceptor fluorophores and integrate into cell-surface membranes. Engineered cells displaying these conjugates are used for the real-time analysis of galectin-mediated cross-linking, forming a "galectin lattice", which is monitored by fluorescence imaging. This approach is versatile and can, in principle, be used to investigate a variety of other processes that involve ligand-mediated clustering of glycoconjugates on cell surfaces. Cell-surface clustering also influences the activity of enzymes that act at the membrane interface to modify the structures of glycolipid conjugates. For example, **Flitsch**, **Webb**, and co-workers[24] show that, when *N*-acetylglucosaminolipids are modified with a fluorous tag that forms a microdomain in a fluid phospholipid bilayer, enzyme-catalyzed galactosylation is significantly enhanced relative to that of the corresponding dispersed glycolipids.

The contributions that are highlighted in this *JACS* Select issue reveal important themes in current research at the chemistry−glycobiology interface. Many of the challenges that are faced in connecting the chemistry to the biology are compounded by the fact that glycans and glycoconjugates frequently interact and function at membrane interfaces and in higher order multivalent complexes. These publications demonstrate how chemists are contributing broadly and creatively in the development of new strategies and defined materials that are critical for studies on the structures, functions, and interactions of glycans and glycoconjugates and how the community, undaunted by the complexity, is making major contributions toward understanding the fascinating biology that is concealed within these complex structures.

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Notes

Views expressed in this editorial are those of the author and not necessarily the views of the ACS.

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