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Protein Glycosylation: New Challenges and Opportunities

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Protein glycosylation is the most complex post-translational modification process. More than 50% of proteins in humans are glycosylated, while bacteria such as *E. coli* does not have this modification machinery. Many small-molecule natural products also require glycosylation in order to express their function. Development of effective synthetic tools for use in understanding the effect of glycosylation on the structure and function of biomolecules will lead to the development of new strategies to tackle major problems associated with carbohydrate-mediated biological recognitions.

Of the three major classes of macro-biomolecules—nucleic acids, proteins, and carbohydrates—it is the carbohydrates that are the least studied and understood. Carbohydrates often exist on cell surfaces as glycoprotein or glycolipid conjugates and play important structural and functional roles in numerous biological recognition processes, including, for example, viral and bacterial infection, cancer metastasis, inflammatory response, innate and adaptive immunity, and many other receptor-mediated signaling processes.[1] In addition, a large number of small-molecule natural products require glycosylation in order to express their biological activities. The effect of glycolsylation on the structure and function of glycosylated natural products is, however, not well understood, mainly due to the lack of effective synthesis methods to cover the relatively large structural space in order to address this problem.

Nucleic acids can be synthesized via chemical and biological methods with the aid of the polymerase chain reaction, and protein sequences, which are encoded by DNA, can therefore be easily determined, produced, and manipulated through recombinant DNA technology. In addition, automatic synthesizers are available for the synthesis of these linear polymers using a single protecting group strategy in the iterative process. Saccharides, however, are often branched and made with a diverse set of enzymes. There is no information carrier that encodes a particular saccharide sequence. Traditional synthesis of saccharides requires multiple protection and deprotection steps and stereocontrol in each glycosylation reaction.[2,3] Development of automatic saccharide synthesis has been reported;[3,4] however, the efficiency is not as high as those used in the synthesis of nucleic acids and polypeptides. Creating libraries of saccharides with methods akin to protein mutagenesis is thus impossible.

The lack of convenient, synthetic tools for research in glycobiology has slowed the pace of its development, and the discovery of previously unknown functions associated with carbohydrates has thus been relatively slow when compared to proteins. In addition, synthesis of complex glycoconjugates, especially glycoproteins, is a formidable task, and conventional approaches may not be the solution, especially when considering the practicality of large-scale synthesis and the enormous molecular diversity that can be assembled from the nine common monosaccharides found in humans.[5] A new strategy based on the fusion of chemical and enzymatic methods in a programmable one-pot approach to synthesis has thus been developed in my laboratory to tackle this major problem.[6] Described below are the highlights of our recent development of new synthetic tools and their application to research in glycobiology and carbohydrate-based drug discovery.

Programmable One-Pot Approach to Oligosaccharide Synthesis

In oligosaccharide synthesis, protecting groups, possible participating groups, promoters/catalysts, reaction conditions, and donor leaving groups and acceptors must all be carefully designed in order to generate the correct regio- and stereochemistry for the new glycosidic bond. Each coupling step also requires tedious product isolation and purification protocols, making the synthesis process very time-consuming and expensive. To overcome these difficulties, several strategies, most notably solid-phase synthesis,[3,4] have been developed. The problem of protecting group manipulation in solid-phase synthesis, however, still exists, and the complexity increases with increase of chain length, though the solid-phase approach

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offers its simplicity in process and, to some extent, flexibility in structure.

A new strategy called "programmable one-pot synthesis" has been developed in my laboratory to address the above problems. This strategy is based on the sequential use of thioglycoside building blocks to form glycosidic bonds based on the reactivity difference of the building blocks[5,6] (Figure 1). The activation of the anomeric leaving group can be attenuated through modification of the protecting group strategy and neighboring group participation. This reactivity-based strategy has been applied to one-pot glycosylation reactions where a series of building blocks with identical leaving groups react sequentially in one vessel without laborious intermediate purification steps.[5,6] It provides rapid access to oligosaccharides with a wide-range of molecular diversity.

To implement the programmable synthesis, thioglycoside donor reactivity had to be characterized by determining their relative reactivity value (RRV), measured by performing a competition reaction between a given donor and a reference donor with methanol as the acceptor.[6] The RRV database has been used to develop the OptiMer one-pot synthesis program.[6] This program contains information on each thioglycoside building block including its RRV, the position of any unprotected hydroxyls, and the α/β directing nature of the C′2 functionality. OptiMer uses this information to analyze a given oligosaccharide, determines the best combinations of donor−acceptors, and predicts yields. There are currently more than 600 thioglycoside building blocks with defined RRVs ranging from 1 to $>10^6$ available for use in one-pot synthesis.[5]

Several reagents[3,5] can be used as activators for thioglycosides, including dimethylthiosulfonium triflate (DMTST), trimethylsilyl triflate (TMSOTf), methyl triflate, *N*-iodosuccinimide/triflic acid (NIS/TfOH), benzene sulfinylpiperidine/triflic anhydride,[7] and the newly developed *N*-(phenylthio)caprolactam/triflic anhydride.[8] The last two thioglycoside activators are faster, allow for fewer side reactions, and were applied to a wide variety of oligosaccharide and glyconjugate syntheses, including Globo-H,[9] Lewis Y,[10] poly-*N*-acetyllactosamines,[11] and fucosyl GM_1,[12] all commonly found on the surface of cancer cells. Globo-H was identified as an antigen on prostate and breast cancer cells. It is being clinically developed as a therapeutic vaccine for treatment of breast cancer.[13]

Enzymatic Synthesis

Of growing interest as a chemical alternative is the utilization of enzymes in synthesis.[14−17] Enzymatic coupling has several advantages over its chemical counterpart. Enzymatic glycosylation occurs stereo- and regioselectively under mild conditions without protecting group manipulation. Also, even very sterically demanding couplings, such as those involving sialic acid glycosylation, can be performed selectively. Enzymes catalyzing such reactions fall into one of two categories: glycosyl transferases and glycosidases.

Glycosyltransferases, which catalyze the transfer of a monosaccharide from a sugar nucleotide donor to an acceptor, are responsible for the biosynthesis of oligosaccharides. While glycosyltransferase-catalyzed reactions exhibit high stereo- and regiocontrol, both the enzyme and the sugar nucleotide are expensive, and the process can be plagued with feedback inhibition by the generated nucleoside phosphate. Regeneration of the sugar nucleotide substrate from its byproduct nucleoside phosphate eliminates the problem of product inhibition and lowers the cost, allowing for synthesis of oligosaccharides and polysaccharides on large scales[5,14,17] (Figure 2). Two such examples are the enzymatic synthesis of sialyl Lewis x,[18] which is responsible for the binding of neutrophils and leukocytes to the selectins of injured tissues during the inflammation cascade, and hyaluronic acid,[19] which plays a critical role in angiogenesis, hemopoiesis, and adhesion. Both compounds were prepared in one pot with multiple glycosyltransferases and regeneration of each sugar nucleotide. Further improvement of the system has been reported with the use of whole cells or multienzymes on beads.[17]

Glycosidases can be utilized to transfer monosaccharides or oligosaccharides to saccharide acceptors in vitro in a kinetic or thermodynamic mode.[5,15,16] These enzymes are readily available, but they lack the regiocontrol of glycosyl transferases. Some of the issues related to regioselectivity can be overcome, though, with selection of proper enzyme−substrate combinations. Glycosidases also accept a variety of saccharide donors with different leaving groups in a kinetic mode. A new efficient approach based on protein engineering of retaining β-glycosidases has been developed, where the α-face nucleophile is mutated from Asp to Ala. The mutant enzyme, called glycosynthase, was shown to catalyze the formation of β-glycosidic bond using α-glycosyl fluoride as a substrate and no hydrolytic activity was observed.[20−22] The development of glycosynthetases has proven to be useful and several examples have been illustrated, including the synthesis of polysaccharides using cellulase and the creation of a new class of thioglycoligases.

Saccharides and other glycoconjugates can be further modified by sulfotransferases, which install sulfate esters, to mediate inhibition and binding in a variety of biological pathways. As with other transferases, sulfotransferases are sensitive to feedback inhibition by the product of their sulfonation reaction, 3′-phosphoadenosine-5′-phosphate (PAP). The sulfate donor, 3′-phophoadenosine-5′-phosphosulfate (PAPS), is also too expensive to be used as a stoichiometric reagent. Regeneration of the donor in situ is thus required to make the practical synthesis possible[23,24] (Figure 2). As such, *p*-nitrophenyl sulfate has been successfully used to regenerate the PAPS. Indeed, future discovery and characterization of sulfotransferases should provide useful tools for creating further diversity in oligosaccharide libraries, including heparin sulfates and the complex glycosaminoglycans, and allow for a greater understanding of the roles of sulfonation in Nature. In a similar manner, the coupling system can be used for the high-throughput screening of sulfotransferase inhibitors when a fluorescent substrate is used.

Enzymatic synthesis is also conducive to automation through the use of solid-phase methods.[25,26] Solid-phase enzymatic synthesis of oligosaccharides gives a distinct advantage over either solution-phase synthesis or chemical solid-phase synthesis: facile purification with regio-

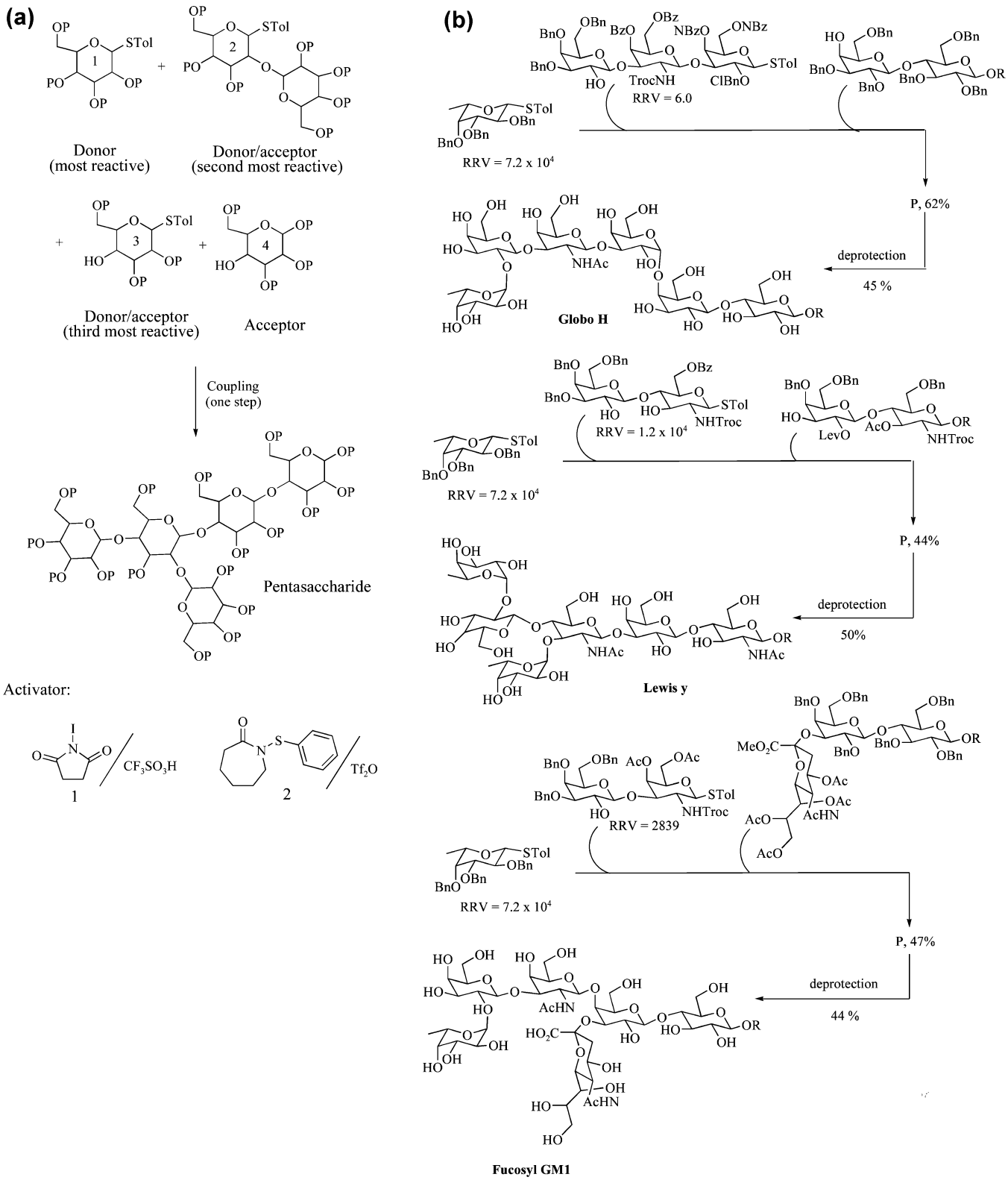

FIGURE 1. (a) Programmable one-pot oligosaccharide synthesis of a branched pentasaccharide depicted as an example. The constituent building blocks are classified into three species: the first sugar at the nonreducing end acts as the donor; the last sugar at the reducing end is the acceptor; all other building blocks that form the inner part of a complex (linear or branched) oligosaccharide are classified as donor/acceptor. Protecting groups determine the reactivity of anomeric centers. Building blocks/promoters are added in the order 1−4 to obtain the pentasaccharide. Activator: *N*-iodosuccinamide/triflic acid (for two couplings) or *N*-(phenylthio)-ϵ-caprolactam (**2**, for more than two couplings). (b) Representative syntheses of cancer antigens Globo H (breast cancer), Lewis Y (colon cancer), and fucosyl GM_1 (lung cancer). P: fully protected product.

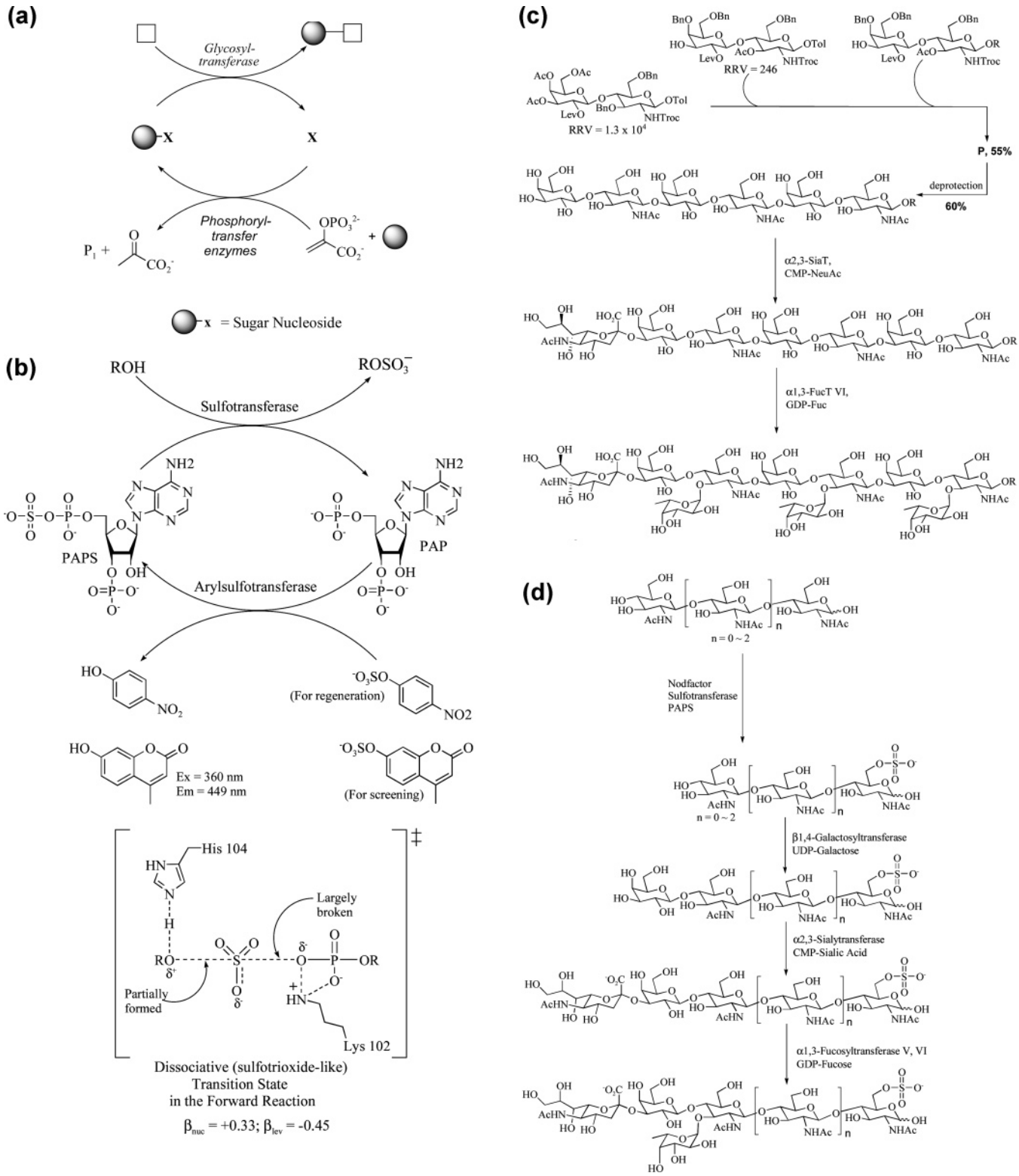

FIGURE 2. (a) Regeneration of sugar nucleotides for the large-scale synthesis of oligosaccharides using glycosyltransferases. (b) Regeneration of phosphoadenosyl-5-phosphosulfate (PAPS) for sulfotransferase-catalyzed sulfation using *o*-nitrophenol sulfate as donor. The sulfotrioxide-like transition state was proposed for the enzymatic reaction. Also shown is a fluorescence-based high-throughput assay used in the discovery of sulfotransferase inhibitors. (c) Synthesis of sialyl trimeric Lewis x using a combined chemical and enzymatic approach (both were carried out in a one-pot manner). P: fully protected product. (d) Synthesis of a heparin-like polysaccharide sulfate using a sulfotransferase and glycosyltransfereases.

and stereocontrol without intermediary protecting group manipulation. One of two methods can be applied: attachment of the acceptor saccharide to solid support or attachment of the enzyme to solid support, though both enzymes and acceptors have been utilized in conjunction on differing supports.

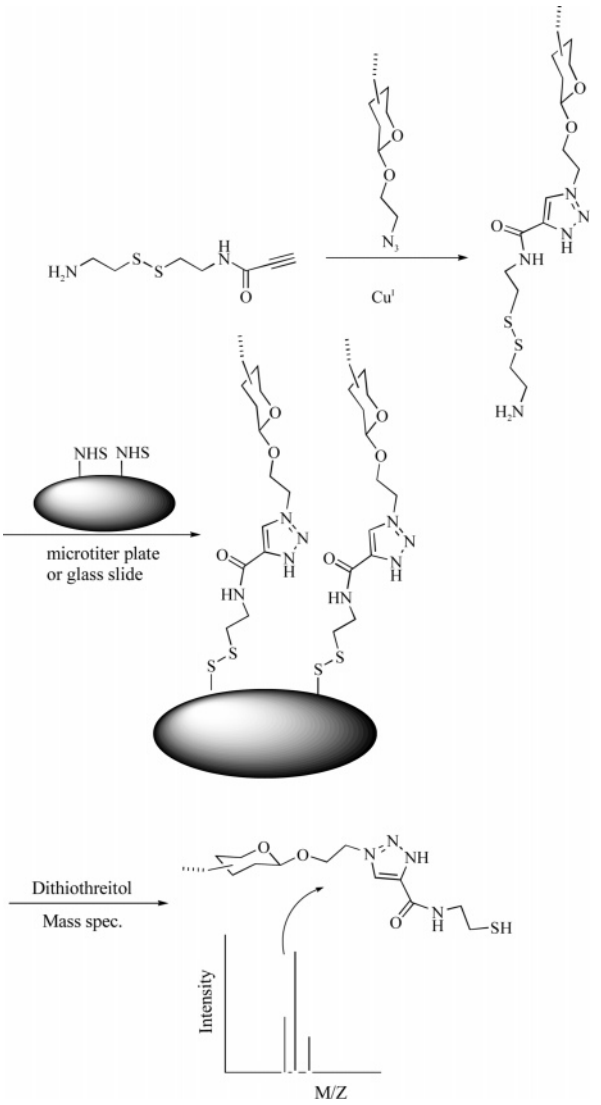

FIGURE 3. Representative synthesis of covalent glycoarrays in microtiter plates or on glass slides. Oligosaccharides containing an azide group were linked to a cleavable linker (e.g., a disulfide-containing or nitrobenzyl group) through a Cu-catalyzed triazole-forming reaction followed by attachment to the succinimide-ester on the surface.

Glycoarrays

A major challenge in cell biology is to define the interaction of oligosaccharides and proteins involved in many biological processes. Conventional approaches to carbohydrate ligand discovery are cumbersome, and there is a need for highly sensitive, high-throughput identification of carbohydrates recognized by various receptors. A technique for attaching oligosaccharides to microplates or glass slides for glycoarrays offers a solution to high-throughput analysis and profiling of such interactions.[27] Both noncovalent and covalent glycoarrays have emerged as a new set of tools to facilitate the study of carbohydrate-protein interactions and identification of optimal sugar ligands and inhibitors[28−36] (Figure 3). Only small amounts of product are required for fabricating microarrays and many saccharides can be screened in parallel in a single operation. For example, a bound *N*-acetylactosamine in microtiter plates was treated with GDP-fucose and a fucosyltransforase to form Le^{x}, which is detected by a fucose-binding lectin.[34] This format was used to identify potent fucosyltransferase inhibitors. Glycoarrays have also found use in the study of RNA−aminoglycoside interactions. As more genetic sequences and their functions are available, RNA has become a new target for drug discovery.[37] The glycoarrays based on aminoglycosides offer a new platform for the high-throughput analysis of aminoglycoside−RNA and other small molecule−RNA interactions. In the future, glycoarrays may be used to profile the glycosylation pattern of tumor cells and their noncancerous counterparts and provide information regarding signaling event, regulation, cellular transport, catalytic activity, targeting, protein fusion and binding, and other biological reactions.[36,37] In addition, glycoarrays can be used to detect the presence of antibodies, T lymphocytes, or other immune cells that recognize antigens associated with cancer and pathogens. One such antigen that is commonly overexpressed in breast cancer is Globo-H, as previously mentioned. Conjugating Globo-H or sections of the hexasaccharide to carrier proteins induces a humoral response in humans to the saccharide, indicating its potential utility as cancer vaccines.[38] Glycoarrays can be used to monitor the level of antibody in the blood after vaccination. Glycoarrays on nanoparticles can also be developed for use in isolation of carbohydrate-binding receptors and for imaging in vivo. The synthesis methods described above should facilitate the development of glycoarrays.

Glycoprotein Synthesis

Protein glycosylation affects a wide range of protein functions, including folding, secretion, targeting, stability in the circulation, and many other intercellular communication processes. Glycoproteins are, however, often produced as a mixture of glycoforms, making it difficult to isolate individual glycoforms for studies of their structure and function.[39] In addition, there is a lack of effective methods available for the synthesis of glycoproteins with a well-defined carbohydrate structure. Recent advances in the field have[5,40−43] provided some new strategies to tackle this formidable problem. Shown in Figures 4 and 5 are several methods for the synthesis of glycoproteins in vitro, including[40] (1) remodeling of recombinant glycoproteins using glycosidases and glycosyltransferases, (2) ligation of synthetic glycopeptides by enzymatic or chemical methods, (3) intein-mediated coupling of glycopeptides to larger proteins expressed as intein-fusion protein, (4) ligation of glycopeptides to larger proteins containing N-terminal cysteine expressed as TEV protease cleavable fusion proteins, (5) in vitro translation, and (6) pathway re-engineering in yeast systems to produce human-type *N*-linked glycoforms.

Recently, in vivo suppressor tRNA technology[43] has been exploited for the recombinant production of neoglycoproteins and glycoproteins. Successful in vivo incorporation of unnatural amino acids in *E. coli* has been achieved systematically by (1) evolving an orthogonal tRNA synthetase-tRNA pair from *Methanococcus jannaschii* that is capable of accepting and charging an unnatural amino acid onto Amber-suppressing $tRNA_{CUA}$ and (2) introducing permissible Amber stop codons (TAG) into a protein of interest that serve to site-specifically

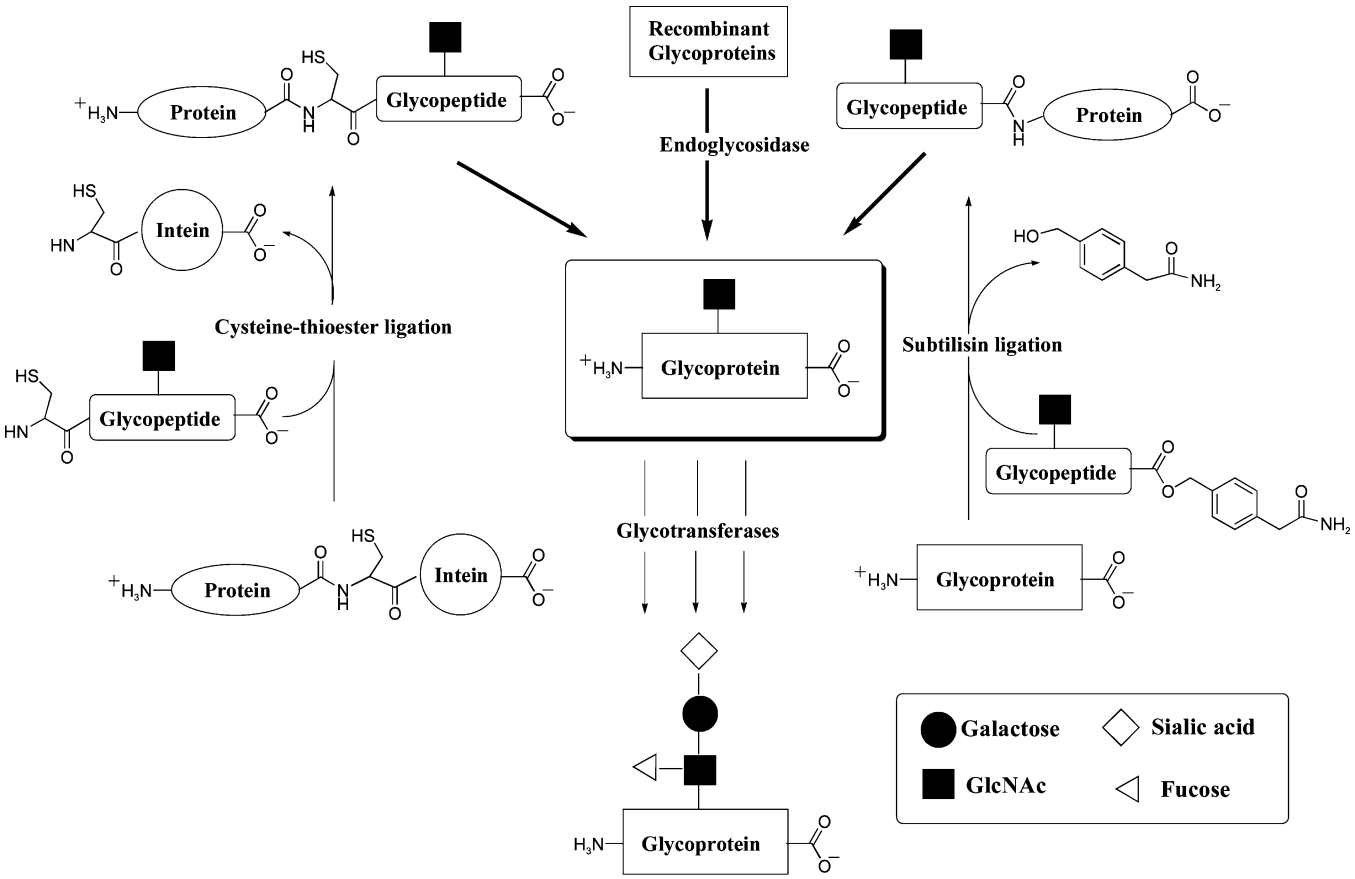

FIGURE 4. Strategies for glycoprotein synthesis in vitro. Center, a recombinant glycoprotein was treated with an endoglycosidase to remove some sugars followed by incorporation of additional sugars with glycosyltransferase. Left, an intein fusion protein overexpressed in *E. coli* is isolated and reacted with a synthetic glycopeptide with N-terminal cysteine to form a glycoprotein. Right, a synthetic glycopeptide benzyl ester is ligated with another polypeptide (prepared in *E. coli* via TEV protease cleavage). (b) Intein-mediated or subtilisin-mediated glycopeptide ligation. TEV protease: tobacco etch virus NIa protease.

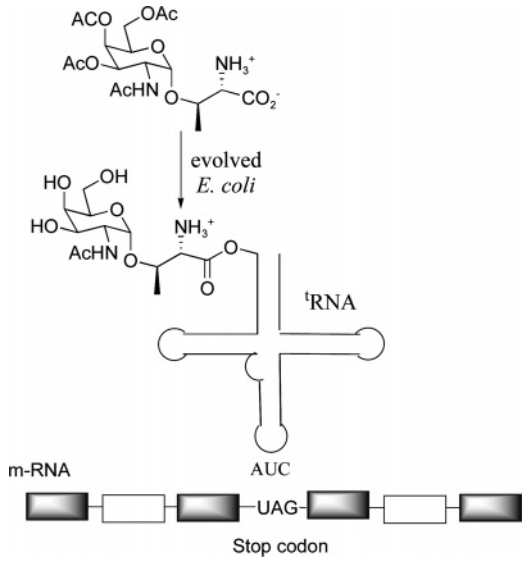

FIGURE 5. Glycoprotein synthesis in vivo in *E. coli* to produce homogeneous glycoproteins. Directed evolution of an orthogonal tRNA synthetase and tRNA pair from *Methanococcus jannaschii* to accept and charge a glycosylamino acid onto the $tRNA_{CUA}$ in response to the stop codon TAG for specific incorporation of the glycosylamino acid.

direct the incorporation of the unnatural amino acid. Using this method, *p*-acetylphenylalanine was incorporated into proteins and subsequently derivatized with aminooxy saccharides to produce homogeneous neoglycoproteins.[44] Further, a naturally occurring homogeneous glycoprotein population was produced in *E. coli* for the first time via the direct incorporation of the core glycosylamino acids *N*-acetylglucosamine-β-serine[45] and *N*-acetylgalactosamine-α-threonine[46] (Figure 5). The glycoproteins were easily isolated and the sugar chains further elongated using glycosyltransferase in vitro. Though the current production level is relatively low, ~4 mg/L, this new method may eventually lead to the development of fermentation methods for the large-scale production of glycoproteins with well-defined carbohydrates at genetically controlled positions.

Conclusion and Outlook

Development of simple and convenient methods for oligosaccharide synthesis is important as the biological impact of oligosaccharides and glycoconjugates has become increasingly apparent. It is hoped that automatic methods will be developed to synthesize complex oligosaccharides as individual entities or as arrays to address biological problems associated with carbohydrate-mediated biological recognition, glycomics, and glycoproteomics. Enzymes have facilitated the large-scale synthesis of saccharides for chemical studies, and complex molecules such as glycoproteins, with well-defined car-

bohydrate structures can be prepared in vitro and in vivo through directed evolution. These advances together with the development of iminocyclitols and other small molecules for inhibition of carbohydrate biosynthesis should provide a new set of tools for glycobiology research and for the production of glycoproteins and other carbohydrate-related substances as a new class of molecules for therapeutic investigation.

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