ACS chemical biology

Combined Approaches to the Synthesis and Study of Glycoproteins

Gonçalo J. L. Bernardes[†], Bastien Castagner[‡], and Peter H. Seeberger[†,*]

[†]Department of Biomolecular Systems, Max-Planck Institute of Colloids and Interfaces, Research Campus Golm, 14424 Potsdam, Germany, and [‡]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH Zürich), Wolfgang-Pauli-Str. 10, 8093 Zürich, Switzerland

ABSTRACT Carbohydrates are the basis for many therapeutic and diagnostic strategies, yet the full potential of glycans in medicine has not been realized. The study of the precise role of different carbohydrates, bound to either proteins or lipids, is hampered by difficulties in accessing pure, well-defined glycoconjugates. This Review highlights recent advances in glycobiology with a particular emphasis on oligosaccharide synthesis and conjugation techniques for the construction of homogeneous glycoconjugates. New methods for the study of protein−glycan interactions such as carbohydrate arrays and *in vivo* visualization of glycosylation pattern changes will also be addressed. The development of glycotherapeutics is just beginning, and much remains to be understood about the relationship between glycoconjugate structure and function. The emergence of novel tools will certainly facilitate and expand the use of carbohydrates in therapeutics and diagnostics.

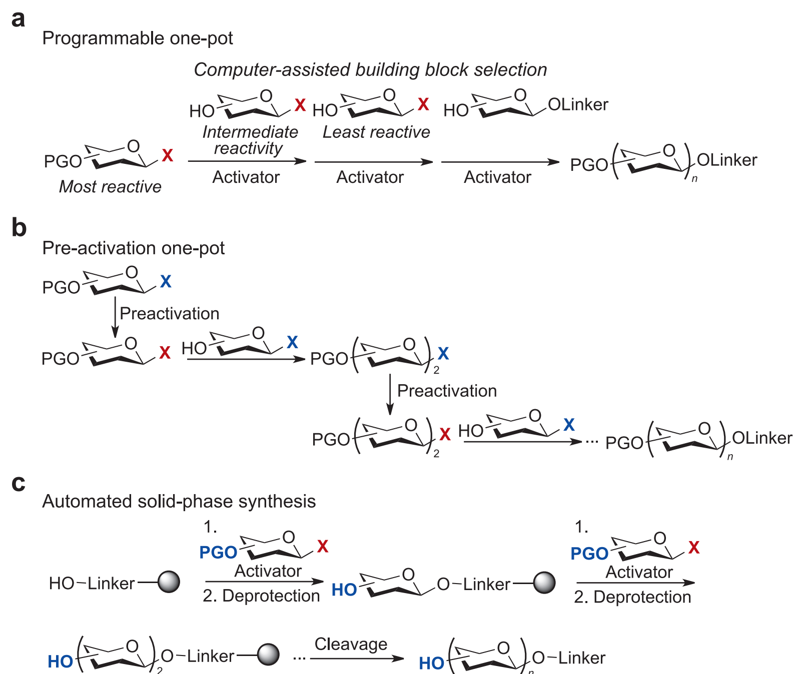
Carbohydrates constitute a large and diverse class of biopolymers. For a long time the primary role of carbohydrates in biology was viewed as a source of energy or as an integral part of cellular structure. However, over recent decades, it has become clear that the expression of complex carbohydrates in human cells is critical in the development and physiology of living systems (*1–4*). The mammalian glycome is thought to be complex, due to the inherent structural diversity of carbohydrate molecules (*5*). Nature uses this abundant repertoire of structures as specific codes in several biological processes such as cellular differentiation (*6*), regulation of cellular signaling (*7*), fertilization (*8*), and immune response (*9*). Unlike proteins and nucleic acids, glycoprotein biosynthesis is not under genetic control, resulting in heterogeneous mixtures−the so-called glycoforms (*2*). Complexity is further increased by competing pathways between different processing enzymes and substrates that result in complex, branched structures. Access to pure samples from natural sources is very challenging, despite some exceptions (*10*). It is also well-established that each component of these glycoforms may have different biological properties (*2*). Recent developments of recombinant glycosylation systems *in vivo* (*11*), oligosaccharide synthesis (*12–15*), site-selective protein glycosylation (*16–18*), glycoarrays (*19, 20*), and *in vivo* targeting of glycans (*21*) are leading to a better understanding of carbohydrate function in nature. However, the field of glycobiology still suffers from the lack of basic tools that fueled advances in genomics and proteomics. This Review focuses on recent advances in the synthesis of complex, biologically relevant carbohydrates, new methods for accessing well-defined glycoproteins and glycolipids, and new tools for analysis of glycosylation patterns and the study of glycan−protein interactions.

*Corresponding author,
peter.seeberger@mpikg.mpg.de.

Received for review January 22, 2009
and accepted March 7, 2009.
Published online March 9, 2009
10.1021/cb900014n CCC: $40.75

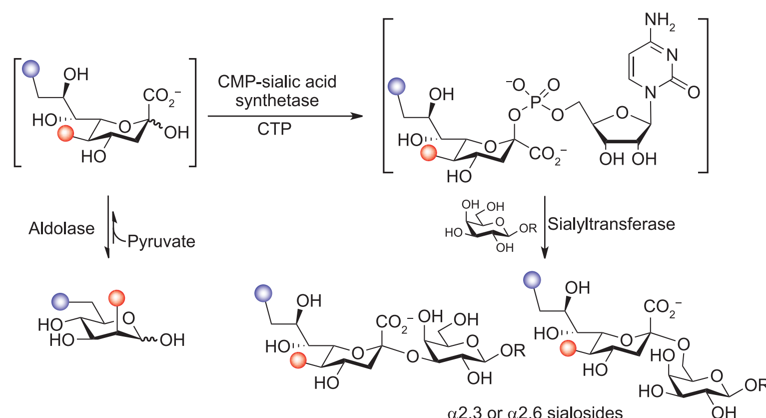
© 2009 American Chemical Society

SCHEME 1. Oligosaccharide synthesis using (a) programmable one-pot synthesis, (b) pre-activation one-pot synthesis, and (c) automated solid-phase synthesis



Oligosaccharide Synthesis. The synthesis of oligosaccharides requires control over the stereochemistry of the glycosidic bond and regioselectivity. Furthermore, the number of glycan structures of interest is very large and thus commands a synthesis method that will allow a large-scale effort to make these glycans available (*22*). Recent advances in the assembly of oligosaccharides offer an unprecedented level of efficiency. Both chemical and chemoenzymatic synthesis have been improved. One-pot chemical synthesis streamlines the assembly of oligosaccharides (Scheme 1, panel a). The programmable one-pot synthesis relies on computer-assisted selection of building blocks with different activity of the anomeric leaving group based on the sugar and its protecting group pattern (*12*). This approach allows for the activation of the building block in the presence of the acceptor, which also possesses an anomeric leaving group. Successive addition of building blocks with decreasing reactivity and finally the reducing-end sugar completes the assembly of the protected oligosaccharide. The one-pot method requires one single purification step and removes the need for temporary protection of the acceptor alcohol. However, disaccharide building blocks are often needed for the assembly of structures above five residues. Protecting group pattern has to be tailored to the specific position of the monosaccharide in the oligosaccharide and increases the required number of building blocks. Preactivation protocols, where the anomeric leaving group is activated irreversibly, prior to the addition of the acceptor have addressed this shortcoming (Scheme 1, panel b) (*23*). The resulting disaccharide can be preactivated prior to the addition of the third sugar. Although preactivation introduces more flexibility in the protecting group scheme, the use of disaccharide buildings blocks is still necessary. The one-pot synthesis of a small library of oligosaccharide has been automated, but solution-phase chemistry will always be challenging to automate (*24*). A different approach to oligosaccharide assembly is based on automated solid-phase synthesis (Scheme 1, panel c) (*13, 25*). The method uses the successive glycosylation of a linker attached to a polymeric support

SCHEME 2. Chemoenzymatic oligosaccharide synthesis using a one-pot, three-enzyme system



with different monosaccharide building blocks. After assembly, the oligosaccharide is cleaved from the polymer support, purified, and deprotected. Automated solid-phase synthesis greatly simplifies the assembly of oligosaccharides and has the potential to make the glycan synthesis available for the nonexpert, as is the case for oligonucleotide and peptide synthesis. This method truly will be useful once all of the common glycosidic linkages are accessible. Thus, the focus of automated solid-phase synthesis has recently shifted to the synthesis of difficult glycosidic linkages such as α-galactose (*26*) and β-mannose (*27*). Different supports such as fluorous linkers have been explored (*28*) but have yet to deliver on the promise of automation.

Chemoenzymatic synthesis of oligosaccharides is often used as an alternative to existing chemical methods (*29–31*). The stereo- and regioselective glycosylation of unprotected glycans makes this approach particularly attractive. However, glycosyltransferases are not always available and require the use of expensive nucleotide-activated glycosides. *In vivo* synthesis and multienzyme systems have been used to prepare complex glycans on a large scale (*29, 32–36*). Recently, the discovery of highly active bacterial sialyltransferases has enabled the synthesis of sialosides in a one-pot, three-enzyme system starting from various mannosamines (Scheme 2) (*37, 38*). This method was used to generate a library containing natural and unnatural α2,3- and α2,6-sialosides. The challenge remains to identify other transferases with high activity and broad substrate specificity in order to gain access to unnatural glycans.

Carbohydrate Vaccines. The development of carbohydrate-based vaccines holds great promise for a host of diseases (*39*). Current strategies require conjugation of the low immunogenic carbohydrate antigen through a linker to a protein carrier for good antibody response (Figure 1). The protein carrier induces a potent T-cell response resulting in a cascade of cytokines that aid the antibody response against the protein carrier. In some cases good levels of carbohydrate-specific antibody were raised using conjugate constructs (*40–44*).

Despite these successes, serious challenges remain. Strong adjuvants often are required to induce a good immune response. High antibody titers against protein and linker can result in undesired immune suppression

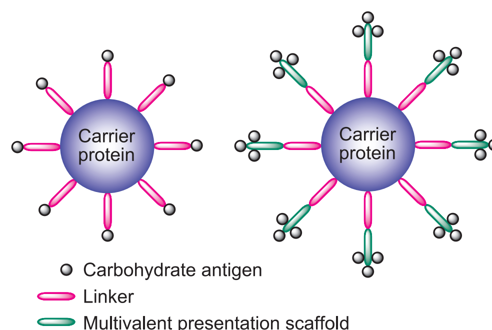


Figure 1. Typical glycoconjugate vaccine constructs.

of the carbohydrate epitope (*45, 46*). While methods for the efficient synthesis of pure antigenic carbohydrates are available, the construction of well-defined glycoconjugates as vaccines is rare. Methods used for the conjugation of the carbohydrate antigen to a protein carrier generally rely on the alkylation of nucleophilic side chains of lysine and cysteine residues (*47*). Lack of chemoselectivity generates highly polydisperse mixtures that are the basis for immunological studies. The efficacy of such constructs in generating antibodies cannot be attributed definitely to a single antigen copy number. Mass spectrometry analysis typically reveals a statistical mixture of carbohydrate antigen copies. Recently, a robust immune response was generated using a *single* copy of a carbohydrate epitope coupled to a synthetic peptide (*48*). This is one of only a few, successful examples of a well-defined glycoconjugate construct used in vaccination (*49–51*). This result encourages the continued investigation of homogeneous constructs in vaccination studies. A coherent strategy that coordinates both carbohydrate synthesis and conjugation methodology allowing for the construction of uniform protein conjugates is highly desirable. Structure–activity relationships and thus glycoconjugate structure and immunogenicity could be correlated.

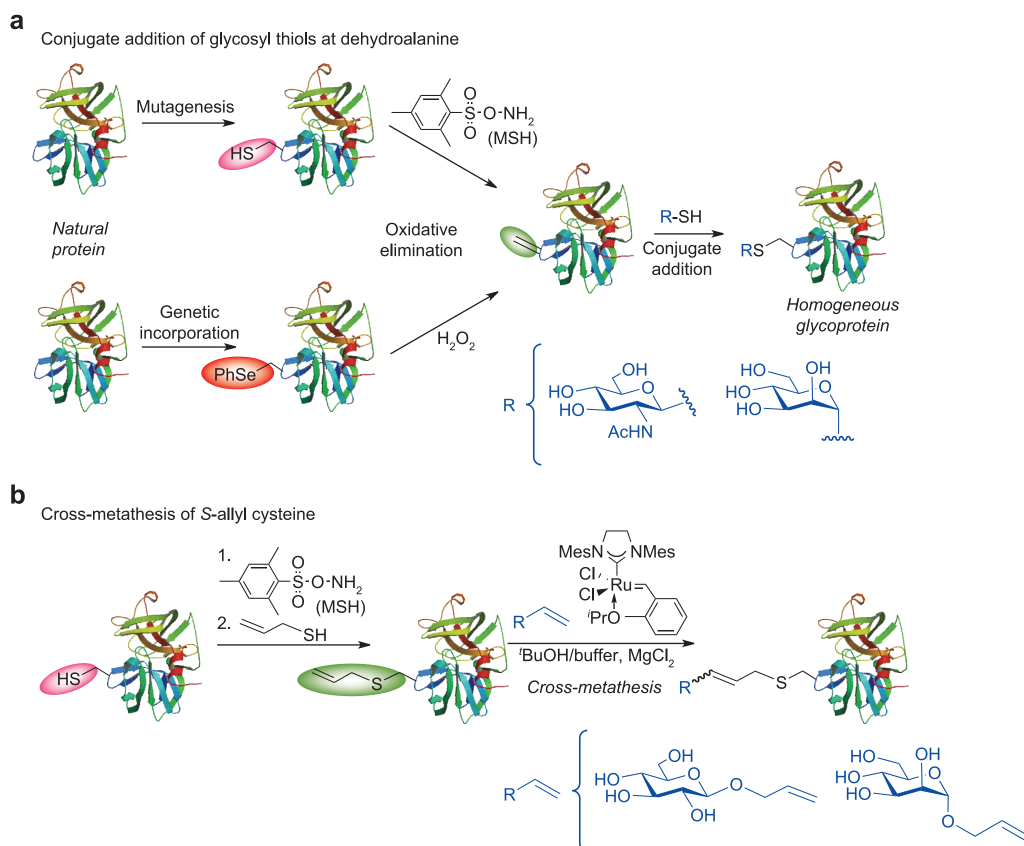
Glycoconjugation Methods. Recent advances in the site-selective modification of proteins enables the creation of pure, well-defined glycoconjugates to study the role of glycans and for applications such as vaccination (*16–18*). One approach that has been explored to avoid selectivity issues is to introduce a non-native amino acid that contains a side chain with a bio-orthogonal functionality. Chemical ligation at that specific site gives access to homogeneous modified proteins. Recently, access to dehydroalanine-containing proteins and its use as a Michael acceptor for glycosyl thiols has been demonstrated (Scheme 3, panel a). A novel, efficient oxidative-elimination reaction of cysteine with *O*-mesitylenesulfonylhydroxylamine (MSH) gives access to dehydroalanine (*52*). Alternatively, incorporation of phenyl selenocysteine into a protein through misacylated tRNAs can be converted to dehydroalanine upon treatment with peroxide (*53*). In both cases, dehydroalanine proved to be an efficient chemical handle to access glycosylated cysteine derivatives. Previous studies on the desulfurization of a disulfide-linked glycoprotein had enabled the first conversion of cysteine to a thioether linked glycoprotein (*54*). Access to glycosylated proteins was also shown to be possible using olefin metathesis (*55*). Allyl sulfides are privileged substrates for aqueous cross-metathesis that are easily installed at dehydroalanine, allowing for the conjugation of carbohydrates to proteins (Scheme 3, panel b) (*55*). This conjugation methodology might prove useful in the development of glycoproteins for vaccination purposes when combined with automated procedures for the synthesis of carbohydrate antigens that often bear a terminal alkene.

Three-dimensional structural investigations of glycoproteins have been hampered by the heterogeneous nature of the available samples. Recently, a new NMR-based method for the study of glycoproteins has been demonstrated (*56*). Sequential labeling and *in vitro* *N*-glycosylation was used to access a nonlabeled glycan attached to a uniformly labeled protein in milligram amounts. The glycosylation acceptor site as well as the glycan structure of *Campylobacter jejuni* glycoprotein was fully characterized using this strategy.

Chemoenzymatic methods have been successfully employed for the construction of glycoproteins (*57*). This approach involves preparation of a homogeneous glycoprotein acceptor by either selective deglycosylation of natural or recombinant proteins or by modern chemical protein synthesis techniques. Subsequent stereo- and regiospecific enzyme-catalyzed transglycosylation gives access to complex, well-defined glycoproteins (*57*). Incorporation of a ketone handle into a protein followed by reaction with a *N*-acetylglucosamine (GlcNAc) derivative containing a hydroxylamine reactive group results in a GlcNAc oxime-linked glycoprotein (*58*). Sequence-specific and differential enzymatic elaboration at the previously installed GlcNAc site was shown (*58*). More recently, endo A-catalyzed transglycosylation (*59, 60*) has been applied to the synthesis of complex glycoproteins (*61, 62*). Importantly, assembly of the native core *N*-pentasaccharide ($Man_3GlcNAc_2$) and further elaboration is possible using this method (Scheme 4) (*62*). Combined yeast expression and *in vitro* chemoenzymatic glycosylation was used to assemble human IgG1-Fc (*63*). These examples illustrate the power of combined chemical and enzymatic methods.

Many proteins are attached to cell surfaces *via* a glycosyl phosphatidylinositol (GPI)-anchor. Native chemical ligation (*64*) (NCL) was employed to ligate a synthetic, cysteine-tagged GPI anchor with recombinant prion protein (PrP) bearing a C-terminal thioester in the

SCHEME 3. Chemical site-selective protein glycosylation



first example for homogeneous GPI-anchored PrP (Figure 2) (*65*). Access to this important class of post-translationally modified proteins provides the basis for a detailed analysis of the influence of GPI anchors on protein structure and function. NCL has often been used for the construction of well-defined glycoproteins (*66*). For example, the glycoprotein ribonuclease C has been accessed using this strategy (*67, 68*). Importantly, the semisynthetic enzyme displayed activity comparable to that of natural enzyme.

Novel Tools for Carbohydrate Research. Carbohydrates are abundantly displayed on the surface of cells with implications in various physiological and pathological processes (*4*). The surface of malignant cells displays a characteristic aberrant glycosylation pattern (*69*). The ability to explore the biological information content of carbohydrates, attached to either proteins or lipids, has become a primary focus of glycomics research.

Probing Glycosylation. Real-time visualization of changes in glycosylation patterns in cells and living animals is now possible (*21, 70*). Metabolic incorporation of a non-native carbohydrate bearing a bio-orthogonal functional group that acts as a chemical reporter into the cell biosynthetic machinery initiates the process. The modified glycan

KEYWORDS

Automated synthesis of oligosaccharides: Programmed synthesis of oligosaccharide structures by a machine.

Bio-orthogonal: Non-native chemical handle that allows chemical modification without compromising the protein solution or living cell.

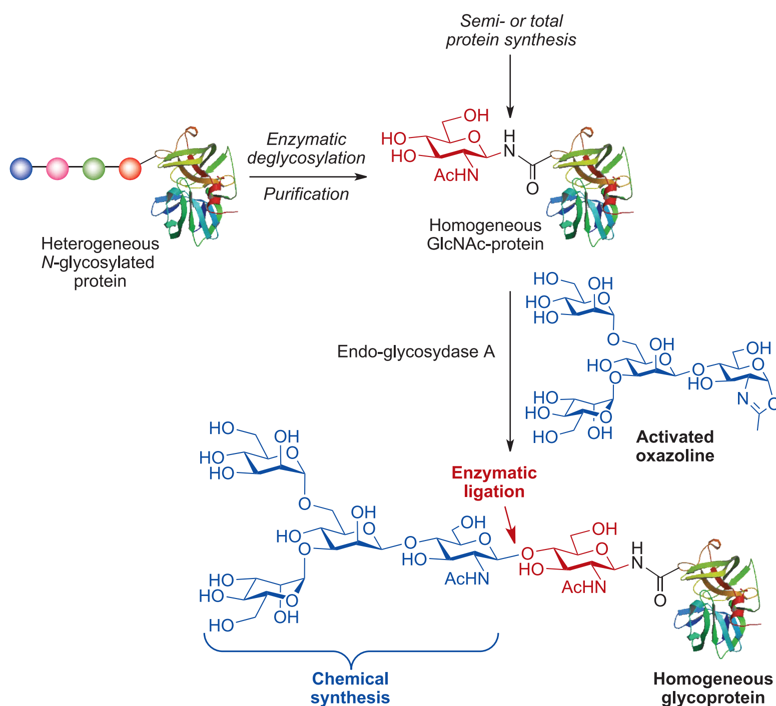
Chemoenzymatic: Combined chemical and enzymatic approach toward the synthesis of biomolecules.

Glycome: Entirety of glycans associated within an organism.

Glycoarrays: Glycans attached to a surface in a spatially defined and miniaturized fashion.

Glycoform: Glycosylated proteins that possess the same peptide backbone but different nature and site of glycosylation.

Scheme 4. Combined chemical and enzymatic approach for the synthesis of homogeneous *N*-glycoproteins



is then processed and incorporated on the cell surface. Subsequent reaction with a detectable probe equipped with a complementary bio-orthogonal functional group enables detection of the incorporated non-native glycan. The chemical reporter is metabolically stable and inert in biological settings and selectively reacts with phosphines to generate an aza-ylide intermediate that, upon hydrolysis, gives an amine and phosphine oxide−Staudinger ligation (*71*) and alkynes in a [3 + 2] Cu(I)-promoted cycloaddition resulting in a triazole

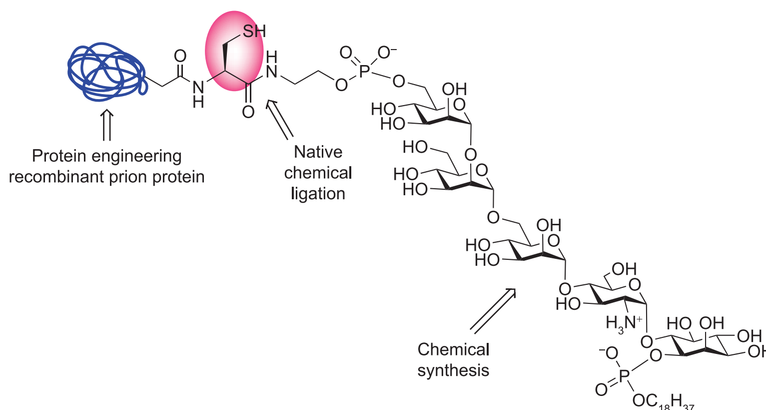


Figure 2. Synthesis of a semisynthetic, homogeneous GPI prion protein (PrP).

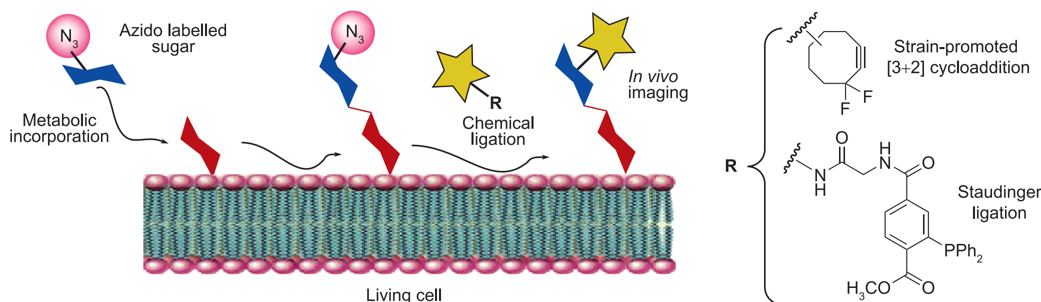


Figure 3. Schematic view of the use of azido-labeled sugars for carbohydrate imaging in living systems.

(*72, 73*). These two reactions are now standard tools in chemical biology, with the Staudinger ligation finding extensive use to probe *in vivo* glycosylation in cells (*74, 75*), allowing, for example, identification of *O*-GlcNAc proteins (*76*). More recently, a strain-promoted [3 + 2] cycloaddition between azides and cyclooctyne that does not require cytotoxic copper has been disclosed (Figure 3) (*77, 78*). This methodology was successfully used for *in vivo* imaging of membrane-associated glycans in developing zebrafish embrios (*77*). 4-Dibenzocyclooctinols are also efficient reagents in copper-free Huisgen cycloaddition reactions allowing for real-time monitoring of glycan trafficking in living cells (*79*).

Carbohydrate Arrays. System-wide analysis techniques such as DNA and protein arrays have benefited genomics and proteomics. Glycobiology lacked routine tools for glycan profiling until carbohydrate arrays emerged as the high-throughput analytical technique of choice for the study of glycan–protein interactions, starting in 2003 (*80, 81*). Chip-based glycan arrays enable screening of several thousand binding events in a single read-out. Importantly, this technique requires very little glycan sample. The presence of a linker between the glass surface and the sugar ensures presentation of the glycan to the binding partner. The diverse display of glycans on a single chip mimics glycan presentation on cells allowing multivalent interactions of relatively weak protein–glycan interactions. These interactions are generally detected by the use of fluorophore-labeled proteins or by antibody incubation (*19, 20*). Surface plasmon resonance (SPR) allows for label-free analysis of protein–carbohydrate interactions on microarrays (*82*).

Carbohydrate arrays rapidly became the tool of choice for the study of glycans in systems biology (*83*). Carbohydrate arrays have found most use in the efficient screening of carbohydrate-binding proteins (*19, 20*). Focused carbohydrate microarrays complement the Consortium for Functional Glycomics (USA) general carbohydrate array. These include, for instance, host specificity of avian and human influenza strains (*84*) or epitope mapping of HIV- and tumor-associated antibodies (*85–87*). Heparin microarrays aided the identification of specific sequences recognized by different fibroblast growth factors (*88*), and serologic search for autoimmune disease antibodies led to the discovery of novel Crohn's disease markers (*89*). Immobilization of sulfated chondroitin tetrasaccharides led to the identification of novel tumor necrosis factor-α antagonist (*90*). Enzymatic elaboration of immobilized glycans is also possible (*91*). Additionally, kinetic constants can be calculated from binding intensities at different dilutions (*92, 93*). More recently, covalently immobilized synthetic GPI glycans were used to assess the level and specificity of anti-GPI antibodies in individuals exposed to malaria disease and naïve individuals (Figure 4) (*94*). Additionally, phosphatidylinositol mannosides (PIMs)—essential precursors of more complex mycobacterial cell wall glycolipids—have been synthesized and immobilized in

KEYWORDS

Glycotools: Toolkit for the synthesis, conjugation, and analysis of carbohydrates in biological systems.

Carbohydrate-based vaccines: Carbohydrate antigen based vaccines; typically the carbohydrate antigen is conjugated to a protein carrier.

GPI anchored protein: Protein modified posttranslationally by glycosylation and lipidation; glycosyl phosphatidylinositol (GPI) anchor is used for the correct attachment of proteins to cell surfaces.

Native chemical ligation (NCL): widespread method for the total synthesis of proteins based on seminal ligations developed by Wieland and co-workers in 1953 (*98*); involves the reaction of two unprotected peptide moieties bearing a C-terminal thioester and a N-terminal cysteine.

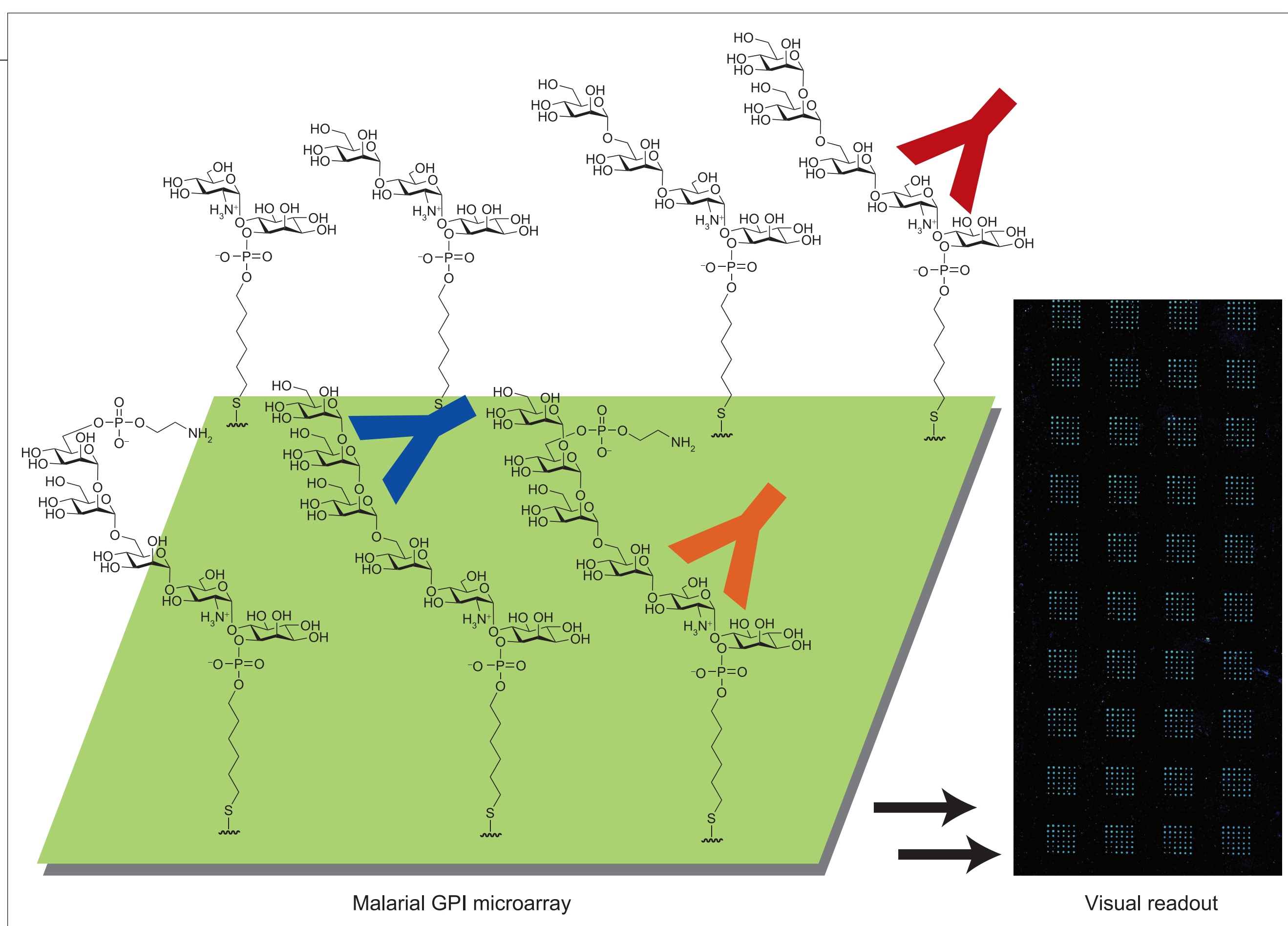


Figure 4. Malarial GPI microarray to probe anti-GPI antibody levels and specificity. Use of covalently bound chemically synthesized GPI on a glass surface to specifically analyze anti-GPI malarial antibodies of noninfected and infected serum.

microarray slides to elucidate differences in binding to the dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN) receptor (*95*). Immobilized lectin arrays have been used to identify the presence of certain glycan structures in a glycoprotein sample (*96*). This methodology was further applied in the analysis of a dynamic bacterial glycome (*97*).

Carbohydrate array technology is an important tool that will play a critical role in the correlation of the genome and proteome with the glycome of an organism.

Conclusions. Glycan-based therapies hold great promise. However, realizing the full potential of glycans in medicine has been hampered by difficult access to glycoconjugates and the lack of tools for the study of the biological role of these biopolymers. While recent advances in oligosaccharide synthesis, glycoconjugation methodologies, and array and biochemical techniques for glycan profiling have reduced barriers in glycobiology, the glycotools reviewed here are still far from methods available for proteomic and genomic research. Glycomics will certainly benefit from continued efforts in developing glycan technologies. These tools will not only bring deeper insights into the molecular mechanisms but also result in greater advances into their use in therapeutics and diagnostics. We anticipate that automated synthesis in combination with novel glycoconjugation techniques will allow for the construction of well-defined glycoproteins for vaccination, with the design of such vaccines greatly benefiting from the use of glycoarrays for epitope profiling. For three recent publications of considerable relevance to this topic, see refs 99–101.

Acknowledgment: We gratefully acknowledge an EU Marie-Curie Fellowship to G.J.L.B.

REFERENCES

1. Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct, *Glycobiology 3*, 97–130.
2. Dwek, R. A. (1996) Glycobiology: toward understanding the function of sugars, *Chem. Rev. 96*, 683–720.
3. Bertozzi, C. R., and Kiessling, L. L. (2001) Chemical glycobiology, *Science 291*, 2357–2364.
4. Ohtsubo, K., and Marth, J. D. (2006) Glycosylation in cellular mechanisms of health and disease, *Cell 126*, 855–867.
5. Hirabayashi, J., Arata, Y., and Ken-ichi, K. (2001) Glycome project: concept, strategy and preliminary application to *Caenorhabditis elegans*, *Proteomics 1*, 295–303.
6. Nishiwaki, K., Kubota, Y., Chigira, Y., Roy, S. K., Suzuki, M., Schvarzstein, M., Jigami, Y., Hisamoto, N., and Matsumoto, K. (2004) An NDPase links ADAM protease glycosylation with organ morphogenesis in *C. elegans*, *Nat. Cell Biol. 6*, 31–37.
7. Helenius, A., and Aebi, M. (2001) Intracellular functions of *N*-linked glycans, *Science 291*, 2364–2369.
8. Talbot, P., Shur, B. D., and Myles, D. G. (2003) Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion, *Biol. Reprod. 68*, 1–9.
9. Lowe, J. B. (2001) Glycosylation, immunity, and autoimmunity, *Cell 104*, 809–812.
10. Rudd, P. M., Joao, H. C., Coghill, E., Fiten, P., Saunders, M. R., Opdenakker, G., and Dwek, R. A. (1994) Glycoforms modify the dynamic stability and functional activity of an enzyme, *Biochemistry 33*, 17–22.
11. Gerngross, T. U. (2004) Advances in the production of human therapeutic proteins in yeasts and filamentous fungi, *Nat. Biotechnol. 22*, 1409–1414.
12. Zhang, Z., Ollmann, I. R., Ye, X. S., Wischnat, R., Baasov, T., and Wong, C.-H. (1999) Programmable one-pot oligosaccharide synthesis, *J. Am. Chem. Soc. 121*, 734–753.
13. Plante, O. J., Palmacci, E. R., and Seeberger, P. H. (2001) Automated solid-phase synthesis of oligosaccharides, *Science 291*, 1523–1527.
14. Seeberger, P. H. (2008) Automated oligosaccharide synthesis, *Chem. Soc. Rev. 37*, 19–28.
15. Koeller, K. M., and Wong, C.-H. (2001) Enzymes for chemical synthesis, *Nature (London) 409*, 232–240.
16. Gamblin, D. P., Scanlan, E. M., and Davis, B. G. (2009) Glycoprotein synthesis: an update, *Chem. Rev. 109*, 131–163.
17. Foley, T. L., and Burkart, M. D. (2007) Site-specific protein modification: advances and applications, *Curr. Opin. Chem. Biol. 11*, 12–19.
18. Chalker, J. M., Bernardes, G. J. L., Lin, Y. A., and Davis, B. G. (2009) Chemical modification of proteins at cysteine: opportunities in chemistry and biology, *Chem. Asian. J.* DOI: 10.1002/asia200800427.
19. Horlacher, T., and Seeberger, P. H. (2008) Carbohydrate arrays as tools for research and diagnostics, *Chem. Soc. Rev. 37*, 1414–1422.
20. Park, S., Lee, M.-R., and Shin, I. (2008) Chemical tools for functional studies of glycans, *Chem. Soc. Rev. 37*, 1579–1591.
21. Prescher, J. A., and Bertozzi, C. R. (2006) Chemical technologies for probing glycans, *Cell 126*, 851–854.
22. Werz, D. B., Ranzinger, R., Herget, S., Adibekian, A., von der Lieth, C.-W., and Seeberger, P. H. (2007) Exploring the structural diversity of mammalian carbohydrates ("glycospace") by statistical databank analysis, *ACS Chem. Biol. 2*, 685–691.
23. Huang, X., Huang, L., Wang, H., and Ye, X. S. (2004) Iterative one-pot synthesis of oligosaccharides, *Angew. Chem., Int. Ed. 43*, 5221–5224.
24. Tanaka, H., Matoba, N., Tsukamoto, H., Takimoto, H., Yamada, H., and Takahashi, T. (2005) Automated parallel synthesis of a protected oligosaccharide library based upon the structure of dimeric Lewis X by one-pot sequential glycosylation, *Synlett* 824–828.
25. Castagner, B., and Seeberger, P. H. (2007) Automated solid phase oligosaccharide synthesis, *Top. Curr. Chem. 278*, 289–309.
26. Werz, D. B., Castagner, B., and Seeberger, P. H. (2007) Automated synthesis of the tumor-associated carbohydrate antigens Gb-3 and Globo-H: incorporation of α-galactosidic linkages, *J. Am. Chem. Soc. 129*, 2770–2771.
27. Codee, J. D. C., Krock, L., Castagner, B., and Seeberger, P. H. (2008) Automated solid-phase synthesis of protected oligosaccharides containing β-mannosidic linkages, *Chem.–Eur. J. 14*, 3987–3994.
28. Jaipuri, F. A., and Pohl, N. L. (2008) Toward solution-phase automated iterative synthesis: fluorous-tag assisted solution-phase synthesis of linear and branched mannose oligomers, *Org. Biomol. Chem. 6*, 2686–2691.
29. Koizumi, S., Endo, T., Tabata, K., and Ozaki, A. (1998) Large-scale production of UDP-galactose and globotriose by coupling metabolically engineered bacteria, *Nat. Biotechnol. 16*, 847–850.
30. Sears, P., and Wong, C.-H. (2001) Toward automated synthesis of oligosaccharides and glycoproteins, *Science 291*, 2344–2350.
31. Nishimoto, M., and Kitaoka, M. (2007) Practical preparation of Lacto-*N*-biose I, a candidate for the bifidus factor in human milk, *Biosci. Biotechnol. Biochem. 71*, 2101–2104.
32. Wong, C. -H., Haynie, S. L., and Whitesides, G. M. (1982) Enzyme-catalyzed synthesis of *N*-acetyllactosamine with *in situ* regeneration of uridine 5′-diphosphate glucose and uridine 5′-diphosphate galactose, *J. Org. Chem. 47*, 5416–5418.
33. Hokke, C. H., Zervosen, A., Elling, L., Joziasse, D. H., and van den Eijnden, D. H. (1996) One-pot enzymatic synthesis of the Galalfa1-3Galbeta1-4GlcNAc sequence with *in situ* UDP-Gal regeneration, *Glycoconjugate J. 13*, 687–692.
34. Gilbert, M., Bayer, R., Cunningham, A. M., DeFrees, S., Gao, Y., Watson, D. C., Young, N. M., and Wakarchuk, W. W. (1998) The synthesis of sialylated oligosaccharides using a CMP-Neu5Ac synthetase/sialyltransferase fusion, *Nat. Biotechnol. 16*, 769–772.
35. Antoine, T., Priem, B., Heyraud, A., Greffe, L., Gilbert, M., Wakarchuk, W. W., Lam, J. S., and Samain, E. (2003) Large-scale *in vivo* synthesis of the carbohydrate moieties of gangliosides GM1 and GM2 by metabolically engineered *Escherichia coli*, *ChemBioChem 4*, 406–412.
36. Zhang, J., Kowal, P., Chen, X., and Wang, P. G. (2003) Large-scale synthesis of globotriose derivatives through recombinant *E. coli*, *Org. Biomol. Chem. 1*, 3048–3053.
37. Yu, H., Chokhawala, H., Karpel, R., Yu, H., Wu, B., Zhang, J., Zhang, Y., Jia, Q., and Chen, X. (2005) A multifunctional *Pasteurella multocida* sialyltransferase: a powerful tool for the synthesis of sialoside libraries, *J. Am. Chem. Soc. 127*, 17618–17619.
38. Yu, H., Huang, S., Chokhawala, H., Sun, M., Zheng, H., and Chen, X. (2006) Highly efficient chemoenzymatic synthesis of naturally occurring and non-natural α-2,6-linked sialosides: a *P. damsela* α-2,6-sialyltransferase with extremely flexible donor-substrate specificity, *Angew. Chem., Int. Ed. 45*, 3938–3944.
39. Danishefsky, S. J., and Allen, J. R. (2000) From the laboratory to the clinic: a retrospective on fully synthetic carbohydrate-based anticancer vaccines, *Angew. Chem., Int. Ed. 39*, 836–863.
40. Schofield, L., Hewitt, M. C., Evans, K., Siomos, M. A., and Seeberger, P. H. (2002) Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria, *Nature (London) 418*, 785–789.
41. Verez-Bencomo, V. (2004) A synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type b, *Science 305*, 522–525.

42. Ragupathi, G., Koide, F., Livingston, P. O., Cho, Y. S., Endo, A., Wan, Q., Spassova, M. K., Keding, S. J., Allen, J., Ouerfelli, O., Wilson, R. M., and Danishefsky, S. J. (2006) Preparation and evaluation of unimolecular pentavalent and hexavalent antigenic constructs targeting prostate and breast cancer: a synthetic route to anticancer vaccine candidates, *J. Am. Chem. Soc. 128*, 2715–2725.
43. Wu, X., Lipinski, T., Carrel, F. R., Bailey, J. J., and Bundle, D. R. (2007) Synthesis and immunochemical studies on a *Candida albicans* cluster glycoconjugate vaccine, *Org. Biomol. Chem. 5*, 3477–3485.
44. Joyce, J. G., Krauss, I. J., Song, H. C., Opalka, D. W., Grimm, K. M., Nahas, D. D., Esser, M. T., Hrin, R., Feng, M., Dudkin, V. Y., Chastain, M., Shiver, J. W., and Danishefsky, S. J. (2008) An oligosaccharide-based HIV-1 2G12 mimotope vaccine induces carbohydrate-specific antibodies that fail to neutralize HIV-1 virions, *Proc. Natl. Acad. Sci. U.S.A. 105*, 15684–15689.
45. Musselli, C., Livingston, P. O., and Ragupathi, G. (2001) Keyhole limpet hemocyanin conjugate vaccines against cancer: the Memorial Sloan Kettering experience, *J. Can. Res. Clin. Oncol. 127*, R20−R26.
46. Buskas, T., Li, Y., and Boons, G.-J. (2004) The immunogenicity of the tumor-associated antigen Lewisy may be suppressed by a bifunctional cross-linker required for coupling to a carrier protein, *Chem.–Eur. J. 10*, 3517–3524.
47. Hermanson, G. T. 2008*Bioconjugation Techniques*, 2nd ed., Academic Press, Inc., New York.
48. Ingale, S., Wolfert, M. A., Gaekwad, J., Buskas, T., and Boons, G.-J. (2007) Robust immune responses elicited by a fully synthetic three-component vaccine, *Nat. Chem. Biol. 3*, 663–667.
49. Kudryashov, V., Glunz, P. W., Williams, L. J., Hintermann, S., Danishefsky, S. J., and Lloyd, K. O. (2001) Toward optimized carbohydrate-based anticancer vaccines: epitope clustering, carrier structure, and adjuvant all influence antibody responses to Lewisy conjugates in mice, *Proc. Natl. Acad. Sci. U.S A. 98*, 3264–3269.
50. Xin, H., Dziadek, S., Bundle, D. R., and Cutler, J. E. (2008) Synthetic glycopeptide vaccines combining β-mannan and peptide epitopes induce protection against candidiasis, *Proc. Natl. Acad. Sci. U.S.A. 105*, 13526–13531.
51. Bettahi, I., Dasgupta, G., Renaudet, O., Chentoufi, A. A., Zhang, X., Carpenter, D., Yoon, S., Dumy, P., and Benmohamed, L. (2009) Antitumor activity of a self-adjuvanting glyco-lipopeptide vaccine bearing B cell, CD4(+) and CD8 (+) T cell epitopes, *Cancer Immunol. Immunother. 58*, 187–200.
52. Bernardes, G. J. L., Chalker, J. M., Errey, J. C., and Davis, B. G. (2008) Facile conversion of cysteine and alkyl cysteines to dehydroalanine on protein surfaces: versatile and switchable access to functionalized proteins, *J. Am. Chem. Soc. 130*, 5052–5053.
53. Wang, J., Schiller, S. M., and Schultz, P. G. (2007) A biosynthetic route to dehydroalanine-containing proteins, *Angew. Chem., Int. Ed. 46*, 6849–6851.
54. Bernardes, G. J. L., Grayson, E. J., Thompson, S., Chalker, J. M., Errey, J. C., Oualid, F. E., Claridge, T. D. W., and Davis, B. G. (2008) From disulfide- to thioether-linked glycoproteins, *Angew. Chem., Int. Ed. 47*, 2244–2247.
55. Lin, Y. A., Chalker, J. M., Floyd, N., Bernardes, G. J. L., and Davis, B. G. (2008) Allyl sulfides are privileged substrates in aqueous cross-metathesis: application to site-selective protein modification, *J. Am. Chem. Soc. 130*, 9642–9643.
56. Slynko, V., Schubert, M., Numao, S., Kowarik, M., Aebi, M., and Allain, F. H. (2009) NMR structure determination of a segmentally labeled glycoprotein using *in vitro* glycosylation, *J. Am. Chem. Soc. 131*, 1274–1281.
57. Bennett, C. S., and Wong, C.-H. (2007) Chemoenzymatic approaches to glycoprotein synthesis, *Chem. Soc. Rev. 36*, 1227–1238.
58. Liu, H., Wang, L., Brock, A., Wong, C. -H., and Schultz, P. G. (2003) A method for the generation of glycoprotein mimetics, *J. Am. Chem. Soc. 125*, 1702–1703.
59. Rising, T. W. D. F., Heidecke, C. D., Moir, J. W. B., Ling, Z., and Fairbanks, A. J. (2008) Endohexosaminidase-catalysed glycosylation with oxazoline donors: Fine tuning of catalytic efficiency and reversibility, *Chem.–Eur. J. 14*, 6444–6464 and references therein.
60. Huang, W., Ochiai, H., Zhang, X., and Wang, L.-X. (2008) Introducing *N*-glycans into natural products through a chemoenzymatic approach, *Carbohydr. Res. 343*, 2903–2913.
61. Li, B., Song, H., Hauser, S., and Wang, L.-X. (2006) A highly efficient chemoenzymatic approach toward glycoprotein synthesis, *Org. Lett. 8*, 3081–3084.
62. Ochiai, H., Huang, W., and Wang, L.-X. (2008) Expeditious chemoenzymatic synthesis of homogeneous *N*-glycoproteins carrying defined oligosaccharide ligands, *J. Am. Chem. Soc. 130*, 13790–13803.
63. Wei, Y., Li, C., Huang, W., Li, B., Strome, S., and Wang, L. X. (2008) Glycoengineering of human IgG1-Fc through combined yeast expression and *in vitro* chemoenzymatic glycosylation, *Biochemistry 47*, 10294–10304.
64. Dawson, P. E., Muir, T. W., Clark-Lewis, I., and Kent, S. B. (1994) Synthesis of proteins by native chemical ligation, *Science 266*, 776–779.
65. Becker, C. F. W., Liu, X., Olschewski, D., Castelli, R., Seidel, R., and Seeberger, P. H. (2008) Semisynthesis of a glycosylphosphati dylinositol-anchored prion protein, *Angew. Chem., Int. Ed. 47*, 8215–8219.
66. Muir, T. W. (2003) Semisynthesis of proteins by expressed protein ligation, *Annu. Rev. Biochem. 72*, 249–289.
67. Piontek, C., Ring, P., Harjes, O., Heinlein, C., Mezzato, S., Lombana, N., Pöhner, C., Püttner, M., Silva, D. V., Martin, A., Schmid, F. X., and Unverzagt, C. (2009) Semisynthesis of a homogeneous glycoprotein enzyme: Ribonuclease C: Part 1, *Angew. Chem., Int. Ed. 48*, 1936–1940.
68. Piontek, C., Silva, D. V., Heinlein, C., Pöhner, C., Mezzato, S., Ring, P., Martin, A., Schmid, F. X., and Unverzagt, C. (2009) Semisynthesis of a homogeneous glycoprotein enzyme: Ribonuclease C: Part 2, *Angew. Chem., Int. Ed. 48*, 1941–1945.
69. Dube, D. H., and Bertozzi, C. R. (2005) Glycans in cancer and inflammation−potential for therapeutics and diagnostics, *Nat. Rev. Drug Discovery 4*, 477–488.
70. Keppler, O. T., Horstkorte, R., Pawlita, M., Schmidt, C., and Reutter, W. (2001) Biochemical engineering of the *N*-acyl side chain of sialic acid: biological implications, *Glycobiology 11*, 11R–18R.
71. Staudinger, J. M. H. (1919) Über neue organische phosphorverbindungen III. Phosphinmethylenderivate und phosphinimine, *Helv. Chim. Acta 2*, 635–646.
72. Tornoe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides, *J. Org. Chem. 67*, 3057–3064.
73. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective ligation of azides and terminal alkynes, *Angew. Chem., Int. Ed. 41*, 2596–2599.
74. Saxon, E., and Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction, *Science 287*, 2007–2010.
75. Köhn, M., and Breinbauer, R. (2004) The Staudinger ligation−a gift to chemical biology, *Angew. Chem., Int. Ed. 43*, 3106–3116.
76. Nandi, A., Sprung, R., Barma, D. K., Zhao, Y., Kim, S. C., Falck, J. R., and Zhao, Y. (2006) Global identification of *O*-GlcNAc-modified proteins, *Anal. Chem. 78*, 452–458.
77. Laughlin, S. T., Baskin, J. M., Amacher, S. L., and Bertozzi, C. R. (2008) *In vivo* imaging of membrane-associated glycans in developing zebrafish, *Science 320*, 664–667.

78. Codelli, J. A., Baskin, J. M., Agard, N. J., and Bertozzi, C. R. (2008) Second-generation difluorinated cyclooctynes for copper-free click chemistry, *J. Am. Chem. Soc. 130*, 11486–11493.
79. Ning, X., Guo, J., Wolfert, M. A., and Boons, G. J. (2008) Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast Huisgen cycloadditions, *Angew. Chem., Int. Ed. 47*, 2253–2255.
80. Wang, D., Liu, S., Trummer, B. J., Deng, C., and Wang, A. (2002) Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells, *Nat. Biotechnol. 20*, 275–281.
81. Fukui, S., Feizi, T., Galustian, C., Lawson, A. M., and Chai, W. (2002) Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions, *Nat. Biotechnol. 20*, 1011–1017.
82. Karamanska, R., Clarke, J., Blixt, O., MacRae, J., Zhang, J., Crocker, P., Laurent, N., Wright, A., Flitsch, S., Russell, D., and Field, R. (2008) Surface plasmon resonance imaging for real-time, label-free analysis of protein interactions with carbohydrate microarrays, *Glycoconjugate J. 25*, 69–74.
83. Paulson, J. C., Blixt, O., and Collins, B. E. (2006) Sweet spots in functional glycomics, *Nat. Chem. Biol. 2*, 238–248.
84. Stevens, J., Blixt, O., Paulson, J. C., and Wilson, I. A. (2006) Glycan microarray technologies: tools to survey host specificity of influenza viruses, *Nat. Rev. Microbiol. 4*, 857–864.
85. Adams, E. W., Ratner, D. M., Bokesch, H. R., McMahon, J. B., O'Keefe, B. R., and Seeberger, P. H. (2004) Oligosaccharide and glycoprotein microarrays as tools in HIV glycobiology: glycan-dependent gp120/protein interactions, *Chem. Biol. 11*, 875–881.
86. Huang, C.-Y., Thayer, D. A., Chang, A. Y., Best, M. D., Hoffmann, J., Head, S., and Wong, C.-H. (2006) Carbohydrate microarray for profiling the antibodies interacting with Globo H tumor antigen, *Proc. Natl. Acad. Sci. U.S.A. 103*, 15–20.
87. Manimala, J. C., Roach, T. A., Li, Z., and Gildersleeve, J. C. (2007) High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems, *Glycobiology 17*, 17C–23.
88. Noti, C., Paz, J. L. d., Polito, L., and Seeberger, P. H. (2006) Preparation and use of microarrays containing synthetic heparin oligosaccharides for the rapid analysis of heparin-protein interactions, *Chem.–Eur. J. 12*, 8664–8686.
89. Iris, D., Sigal, F., Yaara, D., Mikael, S., Amir, K., Aaron, L., Oori, W., Larissa, S., Avi, S., Rom, T. A., Nir, D., and Zamir, H. (2006) Antibodies against laminaribioside and chitobioside are novel serologic markers in Crohn's disease, *Gastroenterology 131*, 366–378.
90. Tully, S. E., Rawat, M., and Hsieh-Wilson, L. C. (2006) Discovery of a TNF-α antagonist using chondroitin sulfate microarrays, *J. Am. Chem. Soc. 128*, 7740–7741.
91. Park, S., Lee, M.-r., Pyo, S.-J., and Shin, I. (2004) Carbohydrate chips for studying high-throughput carbohydrate-protein interactions, *J. Am. Chem. Soc. 126*, 4812–4819.
92. Liang, P.-H., Wang, S.-K., and Wong, C.-H. (2007) Quantitative analysis of carbohydrate-protein interactions using glycan microarrays: determination of surface and solution dissociation constants, *J. Am. Chem. Soc. 129*, 11177–11184.
93. Park, S., and Shin, I. (2007) Carbohydrate microarrays for assaying galactosyltransferase activity, *Org. Lett. 9*, 1675–1678.
94. Kamena, F., Tamborrini, M., Liu, X., Kwon, Y.-U., Thompson, F., Pluschke, G., and Seeberger, P. H. (2008) Synthetic GPI array to study antitoxic malaria response, *Nat. Chem. Biol. 4*, 238–240.
95. Boonyarattanakalin, S., Liu, X., Michieletti, M., Lepenies, B., and Seeberger, P. H. (2008) Chemical synthesis of all phosphatidylinositol mannoside (PIM) glycans from *Mycobacterium tuberculosis*, *J. Am. Chem. Soc. 130*, 16791–16799.
96. Pilobello, K. T., Krishnamoorthy, L., Slawek, D., and Mahal, L. K. (2005) Development of a lectin microarray for the rapid analysis of protein glycopatterns, *ChemBioChem 6*, 985–989.
97. Hsu, K.-L., Pilobello, K. T., and Mahal, L. K. (2006) Analyzing the dynamic bacterial glycome with a lectin microarray approach, *Nat. Chem. Biol. 2*, 153–157.
98. Wieland, V. T., Bokelmann, E., Bauer, L., Lang, H. U., and Lau, H. (1953) Über Peptidsynthesen. 8. Mitteilung Bildung von S-haltigen Peptiden durch Intramolekulare Wanderung von Aminoacylresten, *Liebigs Ann. Chem. 583*, 129–149.
99. Rich, J. R., and Withers, S. G. (2009) Emerging methods for the production of homogeneous human glycoproteins, *Nat. Chem. Biol. 5*, 206–215.
100. Huang, W., Li, C., Li, B., Umekawa, M., Yamamoto, K., Zhang, X., and Wang, L.-X. (2009) Glycosynthases enable a highly efficient chemoenzymatic synthesis of N-glycoproteins carrying intact natural N-glycans, *J. Am. Chem. Soc. 131*, 2214–2223.
101. Krishnamoorthy, L., Bess, J. W., Preston, A. B., Nagashima, K., and Mahal, L. K. (2009) HIV-1 and microvesicles from T cells share a common glycome, arguing for a common origin, *Nat. Chem. Biol. 5*, 244–250.