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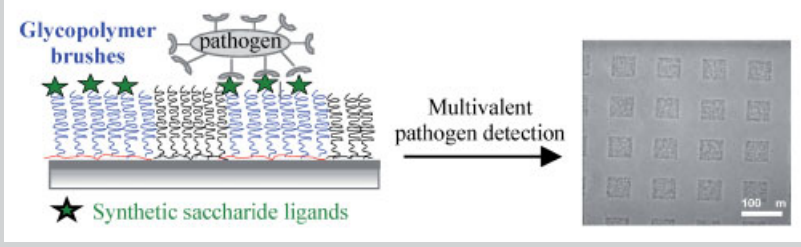
Summary: Oligosaccharides at cell surfaces are known to play a critical role in many biological processes such as biorecognition, interactions between cells and with artificial surfaces, immune response, infection and inflammation. In order to facilitate studies of the role of sugars, an increasing number of novel tools are becoming available. New synthetic strategies now provide much more efficient access to complex carbohydrates or glycoconjugates. Branched carbohydrates and hybrids of carbohydrates conjugated to polymers have been prepared using solution and/or solid-phase synthesis and advanced methods of polymerization. These materials are essential for the development of methodologies to study and map the molecular structure-function relationship at interfaces. This article highlights recent advances in the synthesis of carbohydrates and polymer hybrids mimicking the properties and functionalities of the natural oligosaccharides, as well as selected applications in biology, biotechnology and diagnostics.



Merging Organic and Polymer Chemistries to Create Glycomaterials for Glycomics Applications

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Introduction

Role of Carbohydrates in Biology

Carbohydrates constitute the third class of naturally occurring biopolymers besides nucleic acids and proteins. Sugars are an essential part of every mammalian cell surface forming the so-called glycocalyx. In most cases, carbohydrates are found in the form of glycoconjugates (glycoproteins and glycolipids), where oligosaccharides are attached to proteins or lipids.[1] More than 50% of mammalian proteins are glycosylated.[2] It is now well appreciated that carbohydrates are not merely space filling matrices between proteins of the cell membrane but act to transmit information in a plethora of biological processes. As biological markers, carbohydrates have broad functions in cell-cell recognition events regulating immune response, host-pathogen infection, inflammation and tumor metastasis.[3] All these events are attributed to macromolecular recognition processes occurring at cell surfaces and involving a large number of different proteins with carbohydrate binding domains such as galectins and selectins, enzymes, immune receptors and growth factors.[4] Well documented examples of functional carbohydrates include the terminal oligosaccharide sequences that constitute the major blood group antigens,[5] the cell surface sialic acid residues that serve as attachment sites for the influenza virus[6] or the glycosaminoglycan heparin known for its anticoagulant activity.[7]

Motivation for Research in Glycomics

The diversity of biological functions makes carbohydrates exciting new targets for elucidating crucial pathways in a wide range of diseases and for pharmaceutical applications including antibiotics and vaccines. However, in comparison to the field of nucleic acids and proteins, which has been a

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Peter H. Seeberger received his Vordiplom in 1989 from the Universität Erlangen-Nrnberg, where he studied chemistry as a Bavarian government fellow. In 1990 he moved as a Fulbright scholar to the University of Colorado where he earned his Ph.D. in biochemistry under the guidance of Marvin H. Caruthers in 1995. After a postdoctoral fellowship with Samuel J. Danishefsky at the Sloan-Kettering Institute for Cancer Research in New York City he became Assistant Professor at the Massachusetts Institute of Technology in January 1998 and was promoted to Firmenich Associate Professor of Chemistry with tenure in 2002. In June 2003 he assumed a position as Professor for Organic Chemistry at the Swiss Federal Institute of Technology (ETH) in Zurich, Switzerland and a position as Affiliate Professor at the Burnham Institute in La Jolla, CA where he is currently directing research programs with about 30 coworkers. Professor Seeberger's research has been documented in over 120 articles in peer-reviewed journals, thirteen issued patents and patent applications, more than 70 published abstracts and more than 250 invited lectures. Among other awards he received the Technology Review Top 100 Young Innovator Award (1999), MIT's Edgerton Award (2002), an Arthur C. Cope Young Scholar Award and the Horace B. Isbell Award from the American Chemical Society (2003), the Award of the European Society of Combinatorial Sciences (2005) and the Carbohydrate Research Award (2005). In 2004 he received the Otto-Klung Weberbank Prize for Chemistry. Peter H. Seeberger is the Editor of the Journal of Carbohydrate Chemistry and serves on the editorial advisory boards of nine other journals. He is a founding member of the board of the Tesfa-Ilg "Hope for Africa" Foundation that aims at improving health care in Ethiopia in particular by providing access to malaria vaccines and HIV treatments. He is a consultant and serves on the scientific advisory board of several companies. The research in professor Seeberger's laboratory has resulted in two spin-off companies: Ancora Pharmaceuticals (founded in 2002, Medford, USA) is currently developing a promising malaria vaccine candidate in late preclinical trials as well as several other therapeutics based on carbohydrates. i2chem (founded in 2005, Cambridge, USA) develops integrated microchemical systems based on silicon microreactors Selected Publications (from 130) 1) Plante, O.J.; Palmacci, E.R.; Seeberger, P.H.; Automated Solid-Phase Synthesis of Oligosaccharides; Science 2001, 291, 1523. 2) Schofield, L.; Hewitt, M.C.; Evans, K.; Siomos, M.A.; Seeberger, P.H.; Synthetic GPI as a Candidate Anti-toxic Vaccine in a Model of Malaria; Nature, 2002, 418, 785. 3) Adams, E.W.; Ratner, D.M.; Bokesh, H.R.; McMahon, J.B.; O'Keefe, B.R.; Seeberger, P.H. Oligosaccharide and Glycoprotein Microarrays as Tools in HIV-Glycobiology: Glycan Dependent gp120 / Protein Interactions, Chem Biol. 2004, 11, 875. 4) Disney, M.D.; Seeberger, P.H.; The Use of Carbohydrate Microarrays to Study Carbohydrate-Cell Interactions and to Detect Pathogens; Chem. Biol. 2004, 11, 1701. 5) Ratner, D.M., Murphy, E.R.; Jhunjhunwala, M.; Snyder, D.A; Jensen, K.F.; Seeberger, P.H.; Microreactor-based Reaction Optimization in Organic Chemistry – Glycosylation as a Challenge; Chem. Comm. 2005, 578.

Marcus Textor has been a professor of biologically oriented surface science at the Swiss Federal Institute of Technology (ETH) Zurich since 1994. His research and teaching interests cover the modification and characterization of surfaces and interfaces, quantitative techniques to sense in situ interfacial reactions and the application of functional surfaces in the biomaterials, biosensor and drug delivery field. Textor graduated in chemistry at the University of Zurich, followed by a postdoctoral two years at the University of Sussex in catalysis on single crystal surfaces. From 1978 to 1994 he worked for the company Alusuisse in the development of new materials and fabrication technologies for automotive and packaging applications. He is a member of the editor board of several journals including Biomaterials, co-editor of the book "Titanium in Medicine" and a member of the Executive Committee of the AVS Biomaterials Interface Division. Textor can be reached by e-mail at marcus.textor@mat.ethz.ch.

major focus of biomolecular research (genomics and proteomics), glycomics is still in its infancy. Automated synthesis, efficient methods of sequencing and microarrays, which are of prime importance for elucidating protein and gene function, have only recently begun to emerge in the field of glycobiology. A primary reason is the extreme complexity and variability of carbohydrates, compared to genes and proteins, which makes the sugars difficult to synthesize, purify and analyze. The complexity derives from the large number of existing monosaccharide building blocks, the linkage diversities and the structural complexity associated with highly branched systems (Appendix: diversity of carbohydrates). Although feasible, the isolation of carbohydrates from natural sources is difficult and yields only limited quantities, which are moreover often heterogeneous in composition. Therefore there is a clear need to develop tools and sensitive techniques as platforms to investigate and learn on a molecular level how biochemical information is encoded by carbohydrates and translates into biochemical events. Synthetic approaches have been developed recently to access sufficient quantities of pure, structurally and chemically defined monosaccharides, oligosaccharides and polysaccharides, a prerequisite for being able to profile their structure-binding activities. Synthetic mimics of cell surface carbohydrates can indeed be used to modulate cellular interactions and thus open up new avenues to the development of more efficient therapeutic agents, anti-inflammatory and anti-cancer drugs and vaccines. Relevant approaches include the development of methods for enzymatic synthesis, automated solid-phase synthesis and the production of functional glycopolymers. At the same time, these advances provide glycomaterials as a new tool kit to sense, understand and make use of carbohydrate binding events.

Content

This feature article covers recent progress in the design and synthesis of multivalent ligands and tools that are being developed for biomedical applications of carbohydrates. First, we will illustrate some approaches based on both glycosylation and polymer chemistries to prepare pure and structurally defined carbohydrate analogs. While the former allows for the synthesis of oligosaccharides with well defined sequences of monosaccharide building blocks and linkage chemistry, the latter is particularly attractive in providing good control over multivalency. It will be shown how the two strategies can be combined to prepare new glycomaterials that will be useful in biotechnology for applications ranging from diagnostics to pharmacy and drug development. The advantages and the limitations of the different approaches will be discussed in the context of the applications.

Synthetic Routes to Glycomaterials

The most difficult challenge in glycomics remains the generation of carbohydrates through either isolation from natural resources or by synthesis. However, the last decade has seen a number of innovative approaches to synthesize, with much higher efficiency, carbohydrates and glycomimetics with good control over critical parameters such as their composition and structure including topology, linkages and functionality. One aim of these synthetic approaches is to develop libraries of oligosaccharides, glycoconjugates and analogs as inhibitors or upregulators of carbohydrate-mediated biological processes and exploit them for medical applications. Another driving force is to understand and probe the effect of the multivalent binding character of the synthetic ligands. Below, different strategies towards functional glycomaterials are highlighted, based on organic and polymer chemistries and, in the context of generating a broad new class of architecturally defined synthetic carbohydrates, oligosaccharides and polysaccharide mimics.

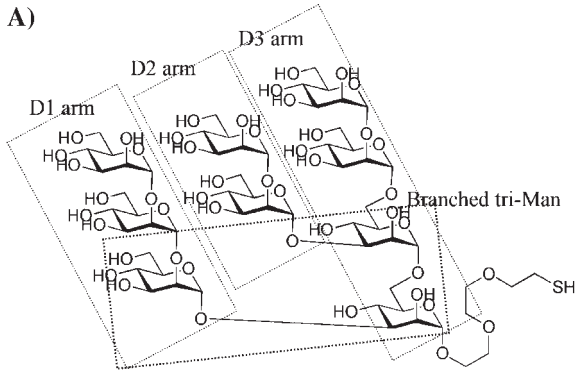
Automated Solid Phase Synthesis of Oligosaccharides

Studying the biological role of sugars obtained from natural sources is tedious because they are difficult to extract in sufficient quantities, require extensive characterization and purification, and the products are generally heterogeneous in composition and structure. Investigation of mixtures of different oligosaccharides or glycoconjugates makes it difficult to determine their specific structure-function relationship. This problem can be circumvented through the chemical synthesis of complex carbohydrates and carbohydrate-containing macromolecules in the laboratory. However, carbohydrate preparation is also challenging since the conventional method, i.e., the single phase, solution-based biosynthesis of complex oligosaccharides, is extremely time consuming and difficult. Nevertheless, synthetic glycosylation strategies have been employed to overcome these difficulties and access reasonably large quantities of pure and chemically well defined oligosaccharides. Complementary approaches include the development of methods for chemical synthesis,[8] enzymatic synthesis[9] and automated solid-phase synthesis.[10] The chemical approaches typically involve a large number of organic reactions and require very specialized organic chemistry expertise toward the assembly of carbohydrate building blocks. The enzymic approaches using glycosyltransferases can be used to prepare complex carbohydrates in aqueous media with regio and stereo-control, often with high efficiency. However, since specific enzymes are needed for specific bonds, the isolatation and purification of various enzymes is necessary in order to synthesize diverse structures. The recent development of solid-phase techniques based on the successive addition of carbohydrate building blocks to an insoluble polymer substrate (Merrifield's resin) has been an important breakthrough in this field.[11] Purification after each step is no longer required; instead successive washing steps are used to remove excess reagents. Automated synthesis reduces the carbohydrate production time by a factor of 50–500, when

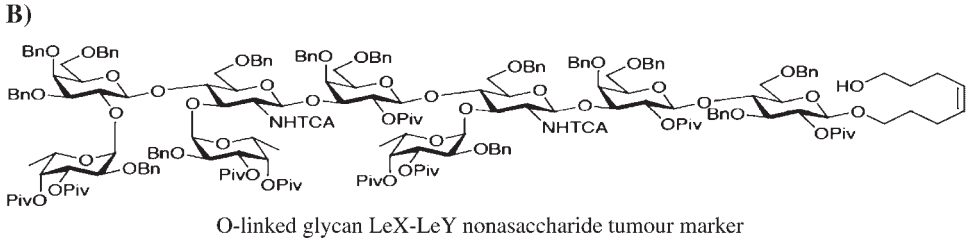
compared to the traditional methods. Seeberger and co-workers have demonstrated the advantage of this approach based on a modified peptide-synthesizer for the synthesis of both linear and branched oligosaccharide structures.[10a] Various biologically important complex carbohydrates of differing lengths and architectural complexity are now available and ready for conjugation with proteins to design vaccines or with surfaces for the production of carbohydrate microarrays. Examples include the branched tetrasaccharide expressed on the cell surface of Leishmania parasites, malarial toxin glycolipids and the *N*-glycoside branched high-mannose oligosaccharide present on the human immunodeficient virus (HIV) envelope gp120 glycoprotein and Ebola virus. The O-glycosyl sialyl Lewis X antigen (Le^x-Le^y) marker of tumor cells and the core structure of the glycosaminoglycan heparin are further examples.[7b,1] A selection of these chemical structures is presented in Figure 1.

Synthesis of Carbohydrate-Polymer Hybrids

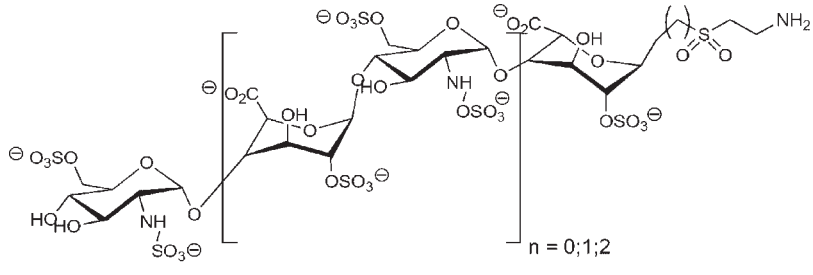
Even though much progress has been achieved in the synthesis of branched and linear oligosaccharides over the last few years, the synthesis of large libraries of target molecules remains a difficult and time consuming task. Therefore, substantial efforts have been directed toward the synthesis of biologically active carbohydrate-polymer hybrids, neoglycopolymers, that do not necessarily have to be assembled by traditional glycosylation reactions. This concept is interesting since it enables variation in chemical structure (chain length, blocks), and the three-dimensional architecture (branching) as well as the type, position and density of the sugar functional groups at the macromolecular level. At the same time, the biological properties of the natural counterpart are preserved or even surpassed.[13] The interaction between a protein and a monosaccharide is weak



N-linked glycan high-oligomannose structure key to gp120



O-linked glycan LeX-LeY nonasaccharide tumour marker



Glycosaminoglycan heparin

Figure 1. Examples of oligosaccharides assembled by an automated solid phase synthesizer: (A) high mannose oligosaccharide from the HIV surface envelope gp120 protein;[12f] (B) sialyl Lewis X observed in various types of cancer;[12e] (C) heparin oligosaccharide antithrombotic drug.[7b]

with dissociation constants, K_d, typically in the range of 10^{-4}–10^{-6} M compared with 10^{-6}–10^{-9} M for antigen-antibody interactions. Multivalent presentation strongly enhances the binding to receptors on the cognate cells via polyvalent interactions, forming oligomeric structures of carbohydrate-protein complexes.[14] Collectively, the interactions are strong and specific due to what is called ''the cluster glycoside effect'' or ''multivalent effect''.[15] The best characterized example of affinity enhancement achieved through multivalency and the cluster effect described first by Lee et al.[16] is the binding of the hepatic asialoglycoprotein to its Gal/GalNAc receptors. On the intact hepatocyte surface, the site affinity of a lectin for Gal is 1×10^{-3} M, whereas its affinity to a synthetic trivalent oligosaccharide with appropriately spaced and oriented galactose residues is 5×10^{-9} M, nearly 6 orders of magnitude greater, while the number of galactose residues per mol of ligands increased only 3-fold. Such multivalent ligands have been exploited to probe the effect of the multivalency in protein-sugar interactions or for therapeutic applications to prevent viral or bacterial infections. Whitesides et al.[17] reported polyacrylamide-based polyvalent molecules presenting sialic acid as an inhibitor of adhesion between hemagglutinin molecules of the influenza virus with sialic acid residues of erythrocytes. While the interaction could be inhibited by monovalent α-methyl sialic acid in solution in the $\times 10^{-3}$ M range, the neoglycopolymers with increased valency showed an IC_{50} value 1 000-fold lower. However, while the increased binding affinity is a consequence of the number of residues per cluster, the branching mode and spacing between adjacent residues also plays a critical role. In most cases, the exact structures of receptors for biologically active carbohydrates are unknown and tight polyvalent bindings have been created based on a highly empirical endeavor. Multivalent ligands tailored to receptor structures may offer the best activity gains. Based on crystallographic studies, Bundle and co-workers[18] have designed a pentavalent star-like glycoligand, based on a carbohydrate core molecule as a scaffold, whose structure fitted into the binding pocket of the Shiga-like bacterial AB_5 toxin (*E. coli* O157:H7). Binding simultaneously to 5 subunits of the toxin overcomes the low affinity of every single interaction, and induces by far the highest molar activity for toxin anti-adhesive therapeutics at the nanomolar level relatively to the millimolar concentrations needed for monovalent ligands. To be able to probe the effects of multivalency in protein-sugar interactions it is therefore essential to have access to materials and surfaces that present high and controlled ligand surface densities with suitable accessibility/orientation of the oligosaccharides. The architecture of the scaffold, determined by the size, shape and flexibility of the macromolecular structures and morphologies, influences significantly the formation of the macromolecular receptor complexes. Multivalent ligands generated from different scaffolds function by different mechanisms.[19] Polymer chemistry methods provide opportunities to synthesize well defined carbohydrate ligands. Particularly promising are the ''living/controlled'' polymerization techniques, which proceed in the absence of termination steps and chain transfer, such as living atom transfer radical polymerization (ATRP)[20] involving the addition of vinyl monomers (i.e., methacrylate, acrylate, styrene) to growing polymers with a reactive radical chain end as well as ring-opening metathesis polymerization (ROMP) of olefins such as norbornene by organometallic initiators (i.e., Grubbs' catalyst ruthenium-based carbenes)[21] (Figure 2). Each technique tolerates multiple unprotected functionalities, such as carbohydrate hydroxys, and can produce polymers with a precise degree of polymerization (DP) (variation in the monomer-to-initiator ratio) and narrow polydispersity indices (PDIs ≈ 1.2) both in organic or aqueous media. Well defined comb-polymers and blocks are also accessible through those methods allowing for variations in structure and function. Kiessling and co-workers first exploited ROMP to synthesize multivalent arrays of sugar epitopes (i.e., mannosyl, galactosyl, sialic acid).[22] The polymers were assessed usually regarding their functional affinity and lengths and whether they can cluster and thereby precipitate lectins.[23] For example, α-*D*-mannose conjugated linear oligomers of well defined lengths (10 to 143-mers) have been found to act as selective inhibitors of carbohydrate binding proteins such as ConA, with an inhibitory potency that increased exponentially in agglutination inhibition assays up to a degree of polymerization of 52.[22c] This is in agreement with modeling that showed that about 35-mers are enough to bridge two ConA binding sites that are 65 Å apart. ATRP has also been employed to achieve the synthesis of glycopolymers, although even less examples have been reported in the literature.[24] For example, Gupta and co-workers[24f] have designed polymers with glucoside moieties by ATRP for conjugation with nanoparticles that showed high binding affinities in hemagglutination assays with the plant lectin Concanavalin A (ConA) used as a model system. These approaches also permit the attachment of sugar moieties at the chain terminus through the initiating or the terminating units by using functionalized initiators in ATRP.[24b] This concept was further extended to produce galactose-coated block copolymer micelles (35–40 nm),[24d] and to produce mannose-decorated crosslinked nanoparticles (7–20 nm).[24e] Agglutination inhibition assays with lectin (RCA120, ConA) and red blood cells supported semi-quantitatively the surface availability of the galactose or mannose chain ends indicating the possibility to design polyvalent nanoscaffolds. Both ATRP and ROMP methods tolerate the presence of polar groups and may be conducted using unprotected sugars in aqueous solution,[24c,25] although living conditions take place when hydroxyl groups are protected, i.e., acetyl groups. Sulfated glycopolymers have been generated by ROMP as inhibitors of protein-carbohy-

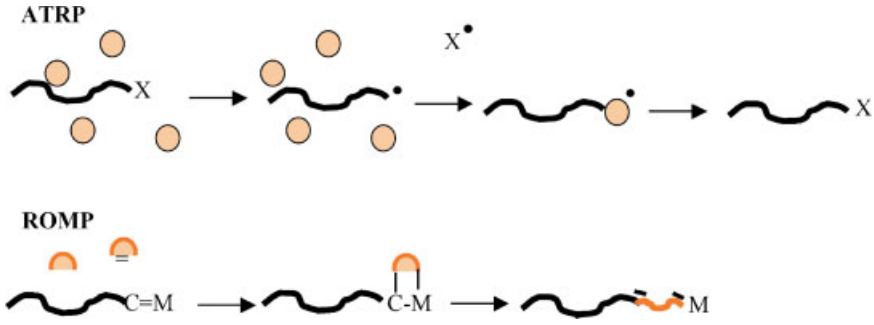


Figure 2. Schematic representation of strategies to synthesize multivalent neoglycopolymers using ATRP and ROMP. In ATRP, the polymer chain is stabilized by a halogen end-group (X) that rapidly associates and dissociates allowing monomer addition. In ROMP, a transition metal catalyst breaks the double bond of an alkene glycomonomer yielding a complex that reacts with another monomer.

drate interactions involved in cell adhesion or to promote specific cellular responses.[22e,26] Heparan sulfate chains of proteoglycans (polysaccharide heparin) mediate important physiological processes including binding to growth factors important in tumor angiogenesis or to cell adhesion molecules (*L*- and *P*-selectin) involved in the inflammatory response and facilitating the attachment of leukocytes from the bloodstream to sites of tissue damage.[7] Generating heparin analogs is still a significant challenge and has only been achieved so far by ROMP as a living polymerization method. It was found that oligomers (15-mers sulfated saccharide units) could be highly effective inhibitors of the binding of an *L*-selectin with an 80-fold potency increase when compared with similar monovalent compounds (sLe^X) and served as anti-inflammatory agents.[26] Also, a neoglycopolymer with galactose-3,6-disulfate displayed *P*-selectin inhibitory activity which was found to be 500-fold higher when compared to natural LeX.[22d] The application of solid-phase methods to living polymerization methods is feasible and could also be used for the ROMP synthesis of libraries of neoglycopolymers.[22g,27] Alternatively, carbohydrates can be conjugated to preformed activated polymers, as pioneered by Bovin and Whitesides.[17,28] Both ATRP and ROMP can be used to synthesize well defined polymers that bear reactive side chains (e.g., *N*-hydroxysuccinimide esters) to further introduce the sugars.[29] The shape of the macromolecule has been recognized to be important for controlling carbohydrate interactions. A multivalent display can also be achieved with monodisperse dendritic sugars of a few nanometers in size.[30] Dendrimers show great promise for applications in therapeutic processes since they can adopt a protein-like structure and thus mimic the branched carbohydrates of natural glycoproteins.[31] Such glycosylation techniques, performed by step growth condensation strategies, may help to overcome problems associated with heterogenous glycoproteins synthesis.[32] A variety of scaffolds are available, e.g., commercial polyamidoamine PAMAM, which can be used to synthesize multivalent ligands with carbohydrate density increasing with the generation.[33]

Coupling Chemistries for Glycomaterial Synthesis

At the heart of chemical glycobiology is the preparation of glycomaterials that bridge the approaches based on organic and polymer chemistries. Platforms are being developed with the aim of creating glycomaterials to perform mechanistic studies of carbohydrate interactions as well as for medical applications (diagnostics, drugs). Two categories of glycomaterials, based on surface immobilized and soluble glycomimetics and dedicated to the study and exploitation of molecular recognition in thin films and in solution can be distinguished. Various devices and particles presenting bioavailable monosaccharides or oligosaccharides are becoming accessible,[34] for applications such as imaging (quantum dot conjugates), carbohydrate-affinity screening for the chromatographic separation of proteins from crude mixtures (latex beads), drug delivery systems (neoglycoproteins, micelles and dendrimers) and chips for high throughput screening (carbohydrate microarrays) (Figure 3). Applications of the last type to diagnostics will be described in the next section. Often the creation of glycotools requires the use of various coupling chemistries and linkers, depending on the source of the carbohydrate probes that are either isolated from natural products or are synthetic. Various immobilization strategies to present carbohydrate on solid surfaces for multivalent display have been developed for studying high throughput carbohydrate-lectin interactions and sugar arrays. For example, Wang et al.[35] immobilized non-covalently by physical adsorption naturally occurring oligosaccharides and polysaccharides, glycoproteins and glycolipids (chemically unconjugated) via hydrophobic interactions to unmodified nitrocellulose-coated glass slides and nitrocellulose membranes for the fabrication of microarrays. The advantage of the method is that carbohydrates are not modified before immobilization. The

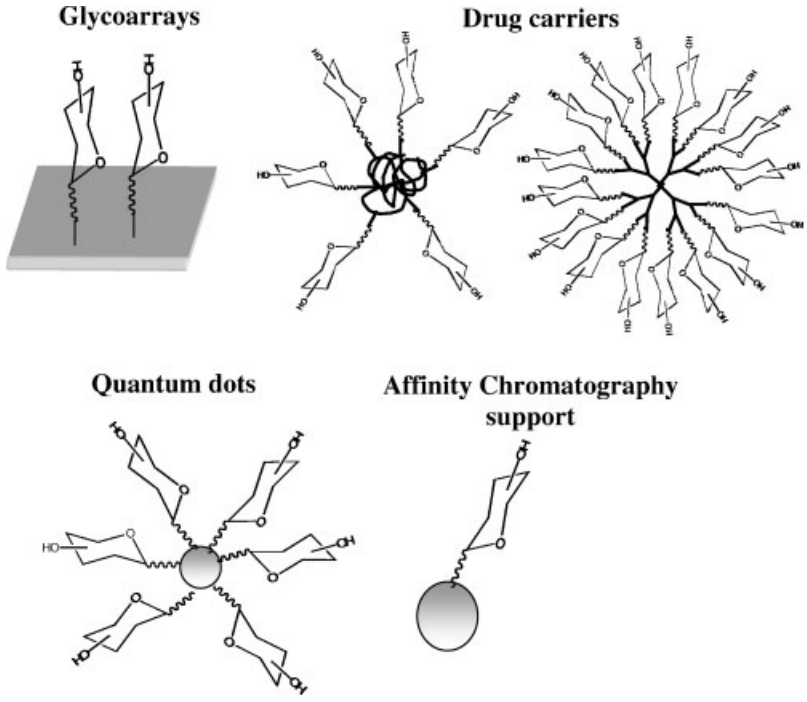


Figure 3. Examples of glycomaterials for glycobiology: modified surfaces for carbohydrate arrays, neoglycoproteins and dendrimer drug carriers, latex microspheres for affinity chromatography columns and multivalent quantum dots for cell biology.

disadvantage is that the molecular weight of the polysaccharides has to be high enough to guarantee sufficient immobilization (e.g., the surface loading was found to be higher for dextran with a molecular mass of 2 000 000 $g \cdot mol^{-1}$ than for 20 000 $g \cdot mol^{-1}$); oligosaccharides cannot be densely and stably immobilized at the surface unless they are first modified by coupling to another larger moiety such as bovine serum albumin (BSA). Washing steps and type of detergents used to reduce non-specific interactions are critical factors in this approach. Another non-covalent immobilization strategy is based on the use of lipid linkers, as demonstrated by the Feizi group,[36] who immobilized various *O*-linked and *N*-linked glycans as well as various glycosaminoglycan fragments (containing 2–20 monomers) also on nitrocellulose coated glass slides. The immobilization efficiency was found to be high even for small carbohydrates. The drawback is that the neoglycolipids, synthetic or natural carbohydrates conjugated to lipids, having an open unit on the reducing end of the sugar are not all compatible with this approach and only oligosaccharides that do not require the reducing end for binding should be studied in this way. To develop new materials and study biological systems, bioconjugation and covalent linkages are often preferred. The methods require modified surfaces and chemical-conjugated carbohydrates. The covalent coupling must be high yielding, few or no by-products should be formed and extensive modification of the carbohydrates is not desired. Those criteria are fulfilled by various organic reactions that have been used in the preparation of glycoarrays on glass slides or gold coated surfaces: e.g., aminoglycosides react with activated esters (succinimide)[37] or carboxylic acid groups, while thiol-containing glycomimetics can be coupled through maleimide groups (on the derivatized protein BSA)[38] and vice versa.[39] In a recent report[40] the reaction between amino-terminated sugars and succinimides was also used for their conjugation to fluorescent polymers (poly(*p*-phenylene ethynylene) (number average molecular weight 128 000 $g \cdot mol^{-1}$; PDI = 1.53; 25% of the reactive sites were functionalized with glycosides). The specific chemical approach depends also on the linker added at the reducing end (anomeric center) of the carbohydrate which must be compatible with the synthesis protocol. Tethers of proper lengths are usually inserted to reduce steric hindrance during the protein binding. In particular, the use of 2-[2-(2-mercaptoethoxy)ethoxyl]ethanol has proven to be successful in view of the ease of temporarily masking the thiol with a protecting group.[41] Other examples of powerful and versatile coupling reactions for the production of glycomaterials include Diels-Alder chemistry exploiting the cycloaddition of an alkene (e.g., cyclopentadiene) to a dienophile (e.g., benzoquinone) and the copper (I) catalyzed azide-alkyne 1,3-cycloaddition (the "click" chemistry).[42] These reactions have also been successful for the preparation of glycoarrays. Houseman and Mrksich used the Diels-Alder chemistry to immobilize cyclopentadiene monosaccharides on benzoquinone-derivatized gold surfaces.[43] This reaction was found to be highly

selective (high yield) for the immobilization of the carbohydrates on surfaces. Specific monosaccharide binding was observed for five lectins. Also, the GlcNAc monosaccharide array was probed with a glycosyltransferase, β-1,4-galactosyltransferase, and showed that enzymatic glycosylation of immobilized monosaccharide is possible on surfaces. Wong and co-workers[44] used the click chemistry to immobilize azide containing mono- and oligosaccharides to alkylynated lipids (14 carbon lipid) although immobilized non-covalently on polystyrene microtiter plates. The required azide tagged sugars are facilely synthesized by solid-phase synthesis. The disadvantage of the approach is the low retention of the lipid-bearing sugars when washed with buffers or solvents. Although developed for chemoselective ligation of biomolecules to surfaces, the click chemistry is also applicable to the build-up of macromolecules and attachment of a variety of different carbohydrates to macromolecular systems, e.g., for producing mannose-tagged glycodendrimers.[45] This might be a useful future approach to prepare a wide range of macromolecular and nano-objects mimicking glycoproteins.

Applications in Diagnostics

Multivalent glycomimetics with controlled structural features are important materials to decipher the information coded by carbohydrates in diagnostic assays (such as carbohydrate arrays).

Carbohydrate Functionalized Polymers to Detect Pathogens

Pathogens, such as viruses, bacteria and other microbes, use cell surface carbohydrates to invade host organisms and to deliver toxins.[46] Multivalent protein-sugar interactions generate adhesive forces.[14] Therefore, linear polymers tagged with multiple copies of carbohydrates provide a potential means of stimulating those binding events and neutralizing the pathogens in liquid media,[47] or can be used for their specific detection. Seeberger et al.[40] reported the use of a water-soluble π-conjugated polymer (poly-(para-phenyleneethynylene) (PPE)) presenting galactosides or mannosides, for the detection of *Escherichia coli* (*E. coli*) bacteria (Figure 4). The type 1 pili of *E. coli* express mannose-specific adhesin FimH at the end of the bacterial fimbria, that specifically recognize d-mannose.[48] Using confocal microscopy, they showed that the mannose binding plant lectin Concanavalin A (ConA) was found to interact with mannose displayed on the polymer. Mannosylated polymers showed a strong fluorescence when binding *E. coli*. while a mutated strain was unable to bind

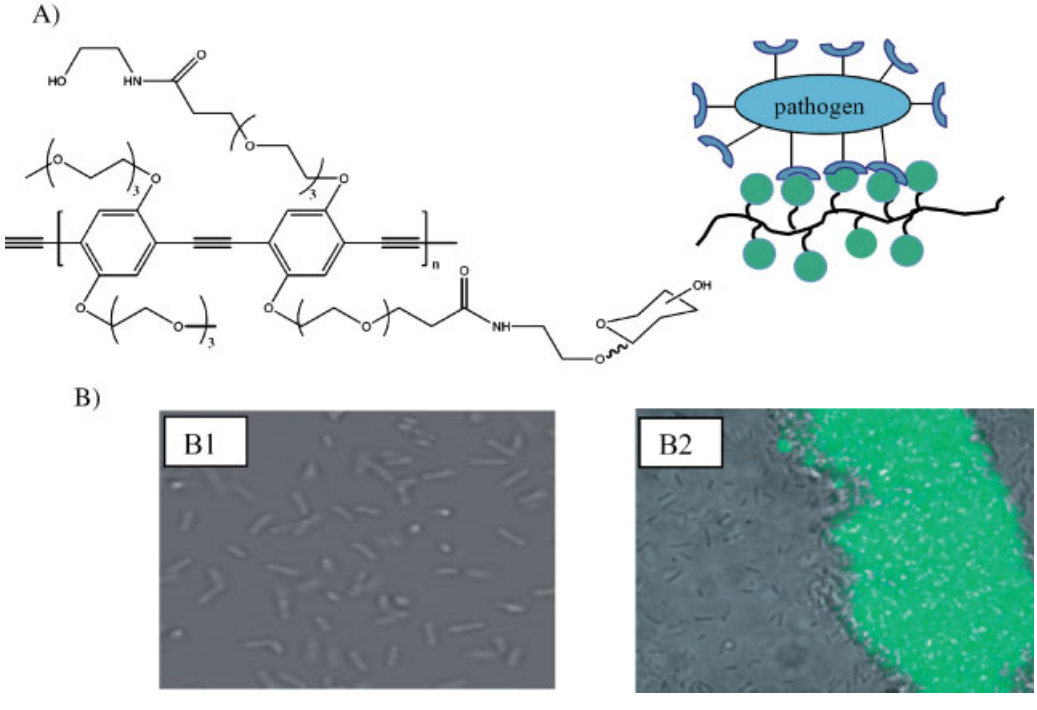


Figure 4. (A) Schematic of multivalent interactions between a pathogen to clustered display of saccharides on a polymer scaffold mimicking host cell-pathogen interaction; chemical structure of carbohydrate-functionalized fluorescent poly(phenylene ethynylene). (B) Exploiting multivalent mannose-tagged polymer for the clearance of *E. coli* (strain ORN178) in a complex mixture; the image obtained by laser scanning confocal microscopy shows (B1) individual cells of a mutant that does not bind and (B2) a large bacterial polymer cluster (Images in Figure 4(B) are reprinted with permission from ref.[40]).

mannose (Figure 4(A)), the mannose-binding bacteria formed extensive cell clusters (Figure 4(B)). A concentration of 10×10^{-3} M of d-mannose was needed to inhibit binding to the polymer which is indicative of strong multivalent interactions. The clusters contained tens to thousands of cells and the detection limit was found to be between 10^3–10^4 cells in the bacterial suspension. This functional polymers approach proved to be rapid and efficient to detect pathogens.

Catching Bacteria with Glycoarrays of Monosaccharides

Carbohydrate arrays in ''chip'' format presenting synthetic sugars on surfaces for high throughput screening assays have become an important tool to study interactions of cells, viruses and bacteria with specific sugar epitopes and facilitate discoveries in the emerging field of glycomics. Sugars presented in clusters on surfaces facilitate multivalent interactions and are ideal for the measurement of binding specificities.[49] Thanks to assay miniaturization, only small amounts of ligands (a few picomoles per array) are required. The platforms available to date have been recently reviewed.[50] Considering the structural characteristics of the carbohydrates displayed at surfaces, one can distinguish chips presenting monosaccharides,[39,43] oligosaccharides[36] or macromolecules such as natural or semi-synthetic polysaccharides and glycoconjugates. [35,51] As reported in an earlier section, methods have been developed for the non-covalent immobilization of carbohydrates on nitrocellulose or polystyrene microtiter plates (polysaccharides and neoglycolipids)[35,36,44a,52] and for covalent immobilization of mono- and oligosaccharides on gold or glass surfaces.[37,38b,39,43] Standard printing technologies based on arraying robots can be used to immobilize sugar probes onto various substrate surfaces including plastic, gold or glass. Single spots on glass slides are highly suitable for binding detection. The density of different carbohydrates on a single slide is higher than 10 000.[53] Carbohydrate microarrays have been successfully used to investigate carbohydrate-binding specificity of intact bacterial cells, as shown by Disney and Seeberger.[54] As a proof of the concept, five monosaccharides (mannose, glucose, *N*-acetyl-glucosamine, galactose, fucose) were immobilized on succinimide-derivatized glass surfaces via an ethanolamine linker (Figure 5(A)). The cells were stained with fluorescent dyes for detection by fluorescence microscopy. From homogeneous and complex mixtures, *E. coli* (strain ORN 178) were shown to bind selectively to domains containing d-mannose (spots of ≈200 μm in diameter) (Figure 5(B)). Bacteria did not adhere outside the spots thanks to a background that resisted non-specific adsorption. Furthermore, differences in carbohydrate binding affinities were observed as a function of the mannose surface density as well as with a mutant strain (ORN209) that gave a lower signal (Figure 5(C)). Importantly, this study highlighted the ability to specifically detect bacteria from heterogeneous mixtures containing sheep erythrocytes, other pathogens and serum. This proved to be difficult with the more conventional approach of using functional polymers in solution: cross-reactivity tests with *Salmonella enterica* were found to also form aggregates with the mannose-tagged polymer.[54] The limit of detection was 10^5–10^6 cells, similar to ELISA or PCR based analyses.[55] The selection of the carbohydrates was restricted to monosaccharides, but demonstrated the potential of the method to investigate the role of carbohydrates in pathogenesis. Arrays of more complex oligosaccharides are expected to provide efficient ways to investigate the specificity of their interactions with various pathogens and are a potential platform to test anti-adhesion therapeutics.

Glycoarrays to Screen Interactions of Highly Mannosylated Ligands

First established to identify and classify novel carbohydrate-binding proteins, the glycoarrays now allow one to address diverse biological problems as well, such as the antigenicity of carbohydrates, and provide a basis for the design of new vaccines, e.g., those intended to interfere with HIV or malarial toxin virus entry, or to test new antimicrobial aminoglycoside antibiotics. Carbohydrate arrays have been used, for example, to characterize carbohydrates that bind with high affinity, potent inactivating proteins (Cyanovirin-N (CVN), Scytovirin-N, DC-SIGN, and the human antibody 2G12)[56] to the *N*-glycans $(Man)_9$-$(GlcAc)_2$ (Figure 1(A)) of the viral envelope glycoprotein of the HIV virus.[38b] Arrays of seven oligomannoside structural determinants of the $(Man)_9$-$(GlcAc)_2$ (Man, Man3 (linear (D1) and branched), Man4 (D3), Man6, Man9) with variable lengths and stereochemical linkages on maleimide-activated glass surfaces were tested. The oligosaccharides were tagged with the thiol containing linker 2-[2-(2-mercaptoethoxy)ethoxyl]ethanol and immobilized, with and without protein carrier (BSA), at different densities.[51] Testing for differences in carbohydrate binding affinities as a function of structural motifs was possible. CVN was binding to fragments containing terminal Manα1-2, while Scytovirin-N additionally required the presence of α1-6 trimannoside for efficient binding. Furthermore, the amount of lectin CVN depended strongly on the surface density of the immobilized saccharide.[38b]

Multifunctional Glycopolymer Brushes at Surfaces

The detection of specific interactions of carbohydrates with cells and proteins depends on multivalent interactions induced by carbohydrates immobilized on macromolecular scaffolds or surfaces. However, typical surface density might not always be sufficient for the detection of weak

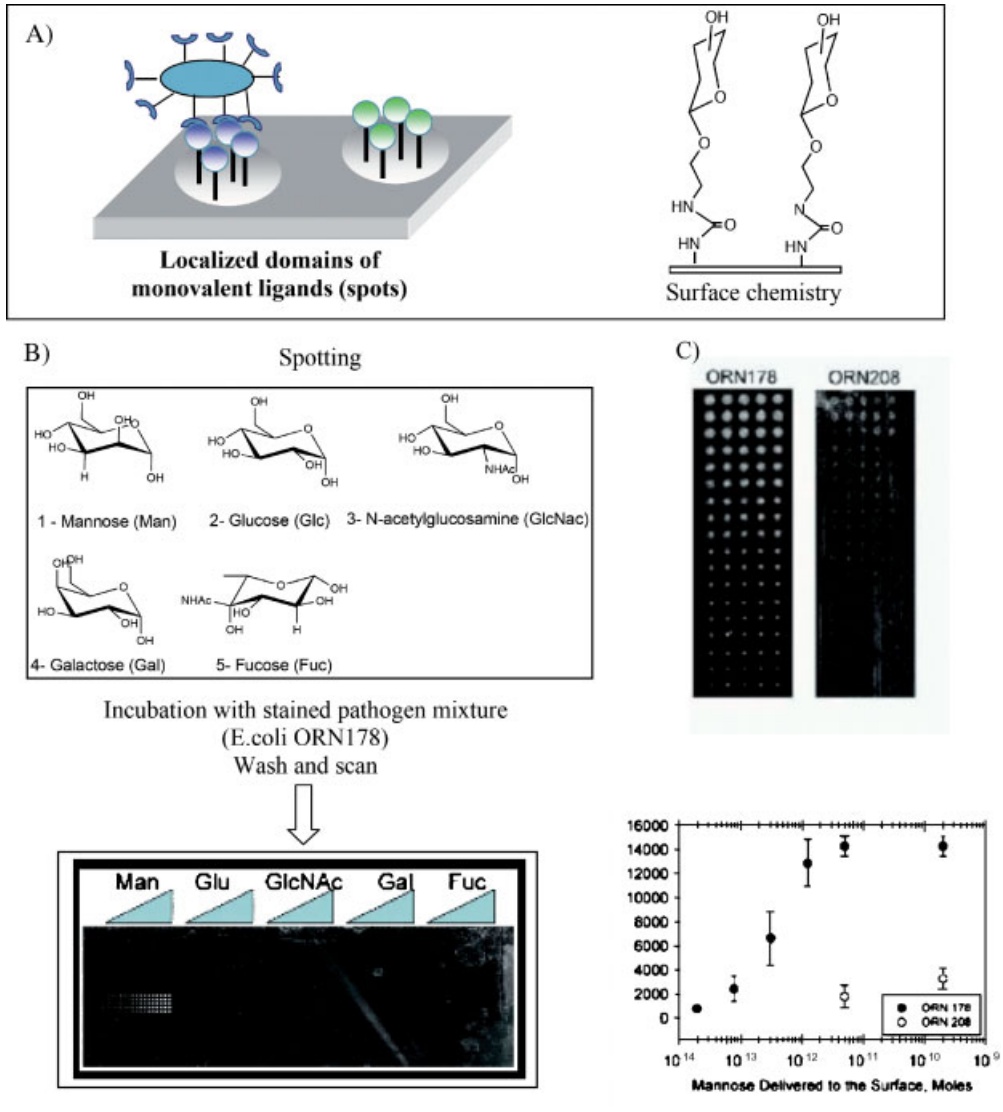


Figure 5. (A) Array to screen binding affinities of *E. coli* (ORN178) to localized microdomains of monovalent ligands (monosaccharides). (B) The image of 5 monosaccharides containing array after incubation with stained *E. coli* shows the interaction with d-mannose. (C) Fluorescence quantitative analysis shows differences in binding affinities after hybridization of ORN178 and a mutant ORN208 to various mannose surface densities (Images in Figure 5(B) and 5(C) are reprinted with permission from ref.[54]).

interactions. A possibility for the investigation of such weak interactions is to immobilize multivalent glycopolymer probes on the surface in order to enhance binding affinity. Secondly, current efforts are directed at the development of surface chips compatible with efficient biosensing methods. Surface plasmon resonance spectroscopy (SPR) has proven to be suitable to profile kinetic and affinity various carbohydrate-receptors interactions, e.g., carbohydrate-antibody and carbohydrate-lectin interactions.[57] SPR imaging studies have been used, for example to determine the strength of the interactions of plant lectins Concanavalin A (ConA) and Jacalin with mannose and galactose immobilized on thiol-self-assembled monolayers (SAMs), respectively. Adsorption coefficients (K_{ADS}) of 5.5×10^6 M^{-1} and 2.2×10^7 M^{-1} were obtained, respectively. The results of competitive binding experiments showed that ConA binds more tightly to mannose than jacalin to galactose with respective solution equilibrium dissociation constants (K_D) of 200×10^{-6} M and 16×10^{-6} M.[58] Also, SPR studies have highlighted the utility of changing ligand surface densities to study both binding selectivity and multivalency of the interactions. Ratner et al. have also studied the interaction of cyanovirin to SAMs of linear trimannosides at different surface densities and tested the inhibition of the association by the SPR technique. It was found that a higher fraction of proteins remained tighly

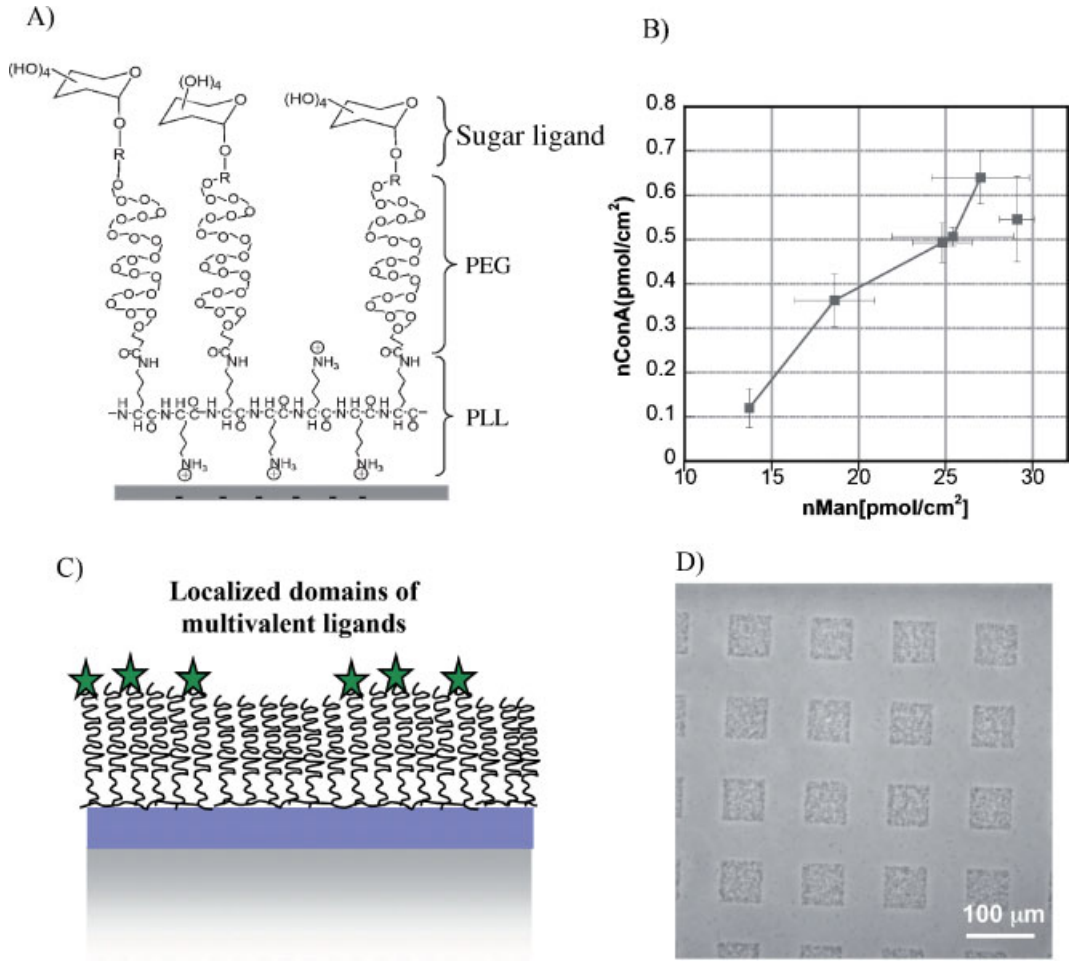


Figure 6. Multifunctional glycopolymer brushes, PLL-*g*-PEG/PEG-sugar, to detect proteins and bacteria. (A) Schematic of the PLL-*g*-PEG/PEG-sugar chemical structure adsorbed on negatively charged metal oxide substrates. (B) The mannose surface density tunes the amount of adsorbed ConA. (C) Schematic of localized domains of multivalent ligands on patterned surfaces. (D) Patterned surface ($60 \times 60\ \mu m^2$) after incubation with *E. coli* (strain K12).

bound at higher densities of carbohydrates reflecting the divalent binding mode of the lectin CNV.[38b] SPR is a technique that allows the binding events to be monitored in situ and in real time, without requiring the use of fluorescently labeled proteins. However, SPR is not the technique of choice to characterize protein-carbohydrate interactions in a high throughput manner. Furthermore, SPR is typically limited to gold or silver coated surfaces.

Evanescent-field based sensing techniques are particularly attractive as they allow for the sensing of interfacial reactions quantitatively and with high sensitivity, providing information that is limited to the penetration depth of the evanescent field into the analyte solution, typically 100–200 nm. Apart from lectin microarrays,[59] recent applications of this technique by Kuno et al.[60] cover the profiling of complex glycan structures expressed on glycoproteins by 39 lectins. There is good evidence that in comparison to ELISA and PCR-based techniques, comparable or higher detection sensitivity can be reached. A version of a microarray reader using grating coupler chips and an evanescent-field excited fluorescence readout is commercially available today,[61] allowing one to analyze samples with an increased signal-to-background ratio (typically by a factor of 60–100 in comparison to conventional fluorescence read-out) and detection sensitivity of the order of 1 zeptomole. The probes are arrayed on glass slides coated with high refractive index metal oxides such as tantalum oxide. An example of dedicated surface functionalization of such chips for the glycoengineering field is based on the spontaneous adsorption of sugar-tagged comb copolymers from aqueous solution and the formation of a monolayer with film thicknesses preferentially below 10 nm, thus taking full advantage of the high evanescent field intensity close to the chip surface. Polycationic poly(*L*-lysine)-*graft*-poly(ethylene glycol) based copolymer (PLL-*g*-PEG) with part of the PEG side chains end functionalized with

mono- or oligosaccharides (Figure 6(A)) was assembled on negatively charged metal oxide coated waveguide chips and successfully tested for their ability to sense specifically lectins and bacteria while keeping non-specific adsorption very low thanks to a dense PEG brush background. Using optical waveguide lightmode spectroscopy (OWLS), specific interactions with and the affinity to ConA was measured as a function of the mono-mannose surface loading[62] (Figure 6(B)). A particular advantage of this self-assembly system is the feasibility to vary and quantitatively control the sugar surface density through either the percentage of saccharide-functionalized PEG side chains in the polymer or by co-assembly from mixed solutions of non-functionalized and sugar-functionalized PLL-*g*-PEG present at different molar ratios. Using a lithography and self-assembly based patterning method called molecular assembly patterning by lift-off (MAPL), chemical patterns of different shapes and dimensions presenting mono-mannose ligands at a controlled density were produced and incubated with *E. coli* bacteria. Bacterial adhesion took place exclusively on the mannosylated patterns, demonstrating the functionality of the presented mannose ligands as well as the excellent resistance of the background to protein adsorption and bacterial attachment. In a next step, this technique will be exploited towards the production of arrays presenting multiple mono- and oligosaccharides to efficiently study biospecific interactions with protein, bacteria and other cells.

Conclusion and Outlook

Carbohydrates are important macromolecular constituents of the surface of cells, bacteria and viruses. Research in glycomics aimed at defining their biological functions depend strongly on the availability of pure, natural or synthetic ligands. Advanced automated synthetic approaches and coupling chemistries provide individual building blocks for glycomaterials. By merging organic and polymer chemistry, a platform of defined oligosaccharides and analogs of controlled composition and multivalency is now becoming accessible. One promising application is the production and use of carbohydrate arrays that permit rapid screening of the fingerprints and specificity of carbohydrate binding proteins and bacteria. To overcome problems linked to weak affinities and to develop systems compatible with sensing methods, glycopolymers are particularly attractive, exploiting strong multivalent interactions. Glycopolymers are expected to further provide powerful tools for the design of new glycomaterials, thus bringing materials science and biotechnology to the ''Glyco-world''. In combination with bio-nanotechnology, new molecular insight into the mechanisms and dynamics of carbohydrate-steered processes can be expected in the near future. The time is considered appropriate for an increased, concerted effort to combine forces from the different disciplines involved and to develop novel strategies for fundamental studies and future applications in the life sciences.

Appendix

Carbohydrates carry biological information in their structures. Compared to nucleic acids and proteins, carbohydrates offer the highest capacity because they have the greatest potential for structural variety. The nine common monosaccharides found in mammalian cells may combine in a variety of ways having multiple sites of attachment to the next sugar moiety (hydroxyl and amino groups), e.g., 1–2, 1–3, 1–4, 1–6 for two pyranoses, which allows branching and a large number of possible polysaccharide structures. In addition, each glycosidic linkage connecting two sugar rings can take the two possible isomeric forms (α or β) at the anomeric carbon. A comparison of all the possible permutations in hexamer formation illustrates well

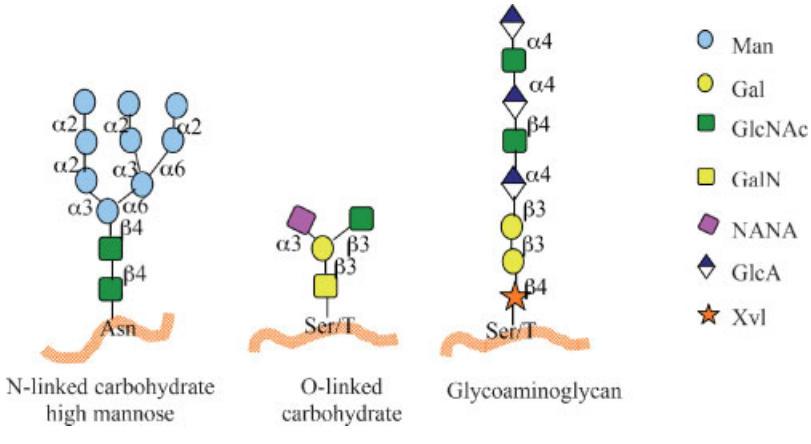


Figure 7. Chemical diversity of the carbohydrates.

the diversity of the resulting chemical structures. Whereas hexanucleotides and hexaaminopeptides may construct 4 096 and 6.4×10^7 variations, carbohydrates have access to 1.05×10^{12} different possible hexasaccharides.[63] The great variety of possible combinations defines the ''language'' of the carbohydrates named as the ''glycocode''. Some proteins, the lectins, are able to recognize those small variations of the oligosaccharide structures and decipher this carbohydrate-encoded information.[64] Based on their backbone chemical structures, the carbohydrates can be classified broadly as linear and branched sugars as illustrated below (Figure 7). The branched *N*-linked and *O*-linked sugars are present on glycoproteins and glycolipids.[65] The *N*-linked glycoproteins (*N*-glycans) are the most abundant in nature. The most predominant forms are the high mannose glycans linked to the protein via an asparagine aminoacid. They all contain a common pentasaccharide core structure $(\text{mannose})_3(N\text{-acetylglucosamine})_2(\text{Man}_3\text{GlcNAc}_2)$. The *O*-linked glycosylation is the second major type of protein glycosylation. The predominant form is the mucin-type, which is characterized by an initial *N*-acetylgalactosamine (GalNAc) residue α-linked to the hydroxy groups of Thr or Ser protein side chains.[66] The majority of the linear sugars are the glycosaminoglycans[67] that contain the polymers of sulfated disaccharides as repeating units like heparin. The chain lengths of the carbohydrates can vary widely from oligosaccharides with 20 units up to several thousand building blocks in the case of polysaccharides.

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