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Perspective

Solid-phase chemical tools for glycobiology

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Abstract—Techniques involving solid supports have played crucial roles in the development of genomics, proteomics, and in molecular biology in general. Similarly, methods for immobilization or attachment to surfaces and resins have become ubiquitous in sequencing, synthesis, analysis, and screening of oligonucleotides, peptides, and proteins. However, solid-phase tools have been employed to a much lesser extent in glycobiology and glycomics. This review provides a comprehensive overview of solid-phase chemical tools for glycobiology including methodologies and applications. We provide a broad perspective of different approaches, including some well-established ones, such as immobilization in microtiter plates and to cross-linked polymers. Emerging areas such as glycan microarrays and glycan sequencing, quantum dots, and gold nanoparticles for nanobioscience applications are also discussed. The applications reviewed here include enzymology, immunology, elucidation of biosynthesis, and systems biology, as well as first steps toward solid-supported sequencing. From these methods and applications emerge a general vision for the use of solid-phase chemical tools in glycobiology.

Keywords: Microarrays; Nanoparticles; Quantum dots; PEGA; Nanobioscience; Glycomics; Microtiter plates; ELISA; ELLA

1. Introduction: glycobiology

Carbohydrate-containing structures are amongst the most complex, heterogeneous, and abundant biomolecules on earth.[1] Glycans are central to many fundamental biological processes including cell–cell recognition, detection, and evasion of immune responses, cell attachment, and detachment, cell fate, development, and morphogenesis. The importance of glycans for health is exemplified by their central roles in cancer metastasis

Abbreviations: α3GalT, α-(1→3)-galactosyltransferase; ADL, *Arundo donax* lectin; Aoa, amino-oxyacetyl; CD, cyclodextrin; CEL, *Cucumaria echinata* lectin; CF, cystic fibrosis; Con A, concanavalin A; DIOS, desorption/ionization on silicon; DTT, dithiotreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; ELLA, enzyme-linked lectin assay; FGF, fibroblast growth factors; FimH, minor type 1 fimbrial subunit, membrane-specific adhesin; FITC, fluorescein isothiocyanate; GAG, glycosaminoglycans; Gal 6, galectin LEC-6; GalAT, α1,4-galacturonosyltransferase; GNP, glyconanoparticle; GnT-V, 5′-diphospho-*N*-acetylglucosamine:α-mannoside β-(1→6)-*N*-acetylglucosaminyltransferase; gp120, HIV envelope glycoprotein; GPA, erythrocyte glycophorin; HEV32, hevein-derived peptide; HRP (HPR), horseradish peroxidase; LCA, *Lens culinaris* (lentil) lectin; NGL, neoglycolipid; NHS, *N*-hydroxysuccinimidyl; OGA, oligogalacturonates; PA, pyridylaminated; PAA, polyacrylamide; PA-IL, *Pseudomonas aeruginosa* agglutinin; PEGA, polyethylene glycol dimethylacrylamide copolymer; PHA, Phaseolus vulgaris (red kidney bean); PNGase F, peptide *N*-glycosidase F; PRV gC, *Pseudorabies* virus glycoprotein C; QD, quantum dots; RANTES, regulated on activation, normal T-cell expressed, and secreted; RCA, *Ricinus communis* agglutinin; SBE, starch branching enzyme; SNA, *Sambucus nigra* agglutinin; SP-D, surfactant protein D; SPE, solid-phase extraction; SPR, surface plasmon resonance; TBS, Tris-buffer saline; TF, Thomsen–Friedenreich; TFA, trifluoroacetic acid; TOPO, trioctylphosphine oxide; VAA, *Viscum album* agglutinin; WGA, wheat germ agglutinin.

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and in mediating immune responses.[1–3] Glycomics entails the comprehensive study of glycans (oligo- and polysaccharides). Carbohydrate structures comprising relatively few components have the capacity to confer vast amounts of information.[2] Variations in sugar composition, and modifications to those sugars, as well as different linkage types and branching patterns mean that even short oligosaccharides can be very information rich. Further layers of complexity are added when carbohydrates are attached, as is frequently the case, to proteins or lipids.

Solid-supported sequencing of peptides, solid-phase synthesis of peptides and oligonucleotides, as well as arrays of DNA, peptides and, to a lesser extent, proteins have been immensely successful methodologies. In contrast to proteins, carbohydrates are not directly encoded but are constructed by the concerted activity of synthesizing and modifying enzymes. This indirect linkage between genome and glycome presents a barrier to unraveling the complex biology of glycans. Many of the milestone technologies that have been so important for understanding the structures and biology of oligonucleotides and proteins—such as rapid sequencing methods, in vitro synthesis, cloning, heterologous expression, and modeling—cannot be readily applied to carbohydrates. Furthermore, micro-heterogeneity in glycan moieties is often observed, thus complicating the analysis and understanding of their biological roles. For these reasons there is a pressing need to develop solid-supported techniques that can be used to analyze the occurrence and interactions of carbohydrates in a high throughput fashion.

Carbohydrate mediated recognition among cells can be envisioned as consisting of several hierarchical levels of molecular information.[4,5] First, the primary oligosaccharide sequence might be recognized by a protein. Second, several oligosaccharides may be presented and recognized along a polypeptide. Third, multiple glycoconjugate molecules, e.g. glycoproteins or glycolipids, may be displayed on the surface; their density, distribution, and relative orientations may contribute to even greater specificity.

The interaction between a carbohydrate and the corresponding individual binding site in a protein (e.g., a lectin) is typically weak and binding strength and specificity is often enhanced through polymeric interactions between carbohydrates and oligomeric carbohydrate-binding proteins.[6] Multivalent display and cooperative recognition are thus very important phenomena in glycobiology. Carbohydrates immobilized on a surface are presented multivalently[7] and solid-supported techniques displaying multiple copies of the same carbohydrate could thus be particularly well suited for studying many carbohydrate–protein interactions. For example, Houseman and Mrksich[7] as well as Corn, Kiessling and co-workers[8] have immobilized carbohydrates onto gold surfaces and used the multivalency in the study of carbohydrate–lectin interactions with surface plasmon resonance (SPR) spectroscopy.

This review provides a unifying perspective on solid-phase chemical tools for glycobiology, that is, on methods and applications for using solid-supported strategies. Here we provide a view across very different approaches, including some well-established ones such as immobilization in microtiter plates and to cross-linked polymers, and also emerging areas such as microarrays and sequencing. This review has a focus on chemical methods for covalent immobilization and their applications. This includes attachment to, for example, cross-linked polymers and microtiter plates as solid supports; we have also included nanoparticles, such as quantum dots and gold nanoparticles for nanobioscience applications. Areas that have already been comprehensively reviewed will be covered in less detail here.

2. An overview of methods for immobilization and anchoring

2.1. Non-covalent adsorption

Non-covalent adsorption is the least technically demanding approach for anchoring carbohydrates to surfaces and relies on the formation of a range of non-covalent bonds between the surface and the arrayed carbohydrates. Surfaces (substrates) used for this type of immobilization include nitrocellulose and polystyrene.[9] Its use includes manufacturing of microarrays on slides and arrays in microtiter plates.

The advantage of arrays based on this strategy is that a very wide range of carbohydrates can be immobilized directly onto the substrate surface. There is no requirement for derivatization of the slide surface, or the need to create reactive groups on the arrayed carbohydrates. Comprehensive carbohydrate microarrays can be created by this method because in addition to saccharides, a wide range of proteoglycans, glycolipids, and other glycoconjugates can be readily immobilized. Importantly, uncharacterized crude extracts from cells or tissues can also be directly immobilized. However, the orientation of the arrayed samples cannot be controlled and some samples may be presented in forms in which their biological meaning is lost by denaturation or steric occlusion.

The widely used binding between streptavidin and biotin has also been used to immobilize carbohydrates.[10,11] Furthermore, polyacrylamide (PAA) glycoconjugates have been used for non-covalent immobilization.[12] As an alternative to PAA glycoconjugates, oligosaccharides have been coupled to a C_{14} hydrocarbon chain that binds non-covalently to the surface of microtiter wells.[13] Wong and co-workers[14] used a Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between an azide and alkyne to attach oligosaccharides to a C_{14} chain by formation of a triazole.[15]

2.2. Covalent immobilization using oriented, chemical linkages

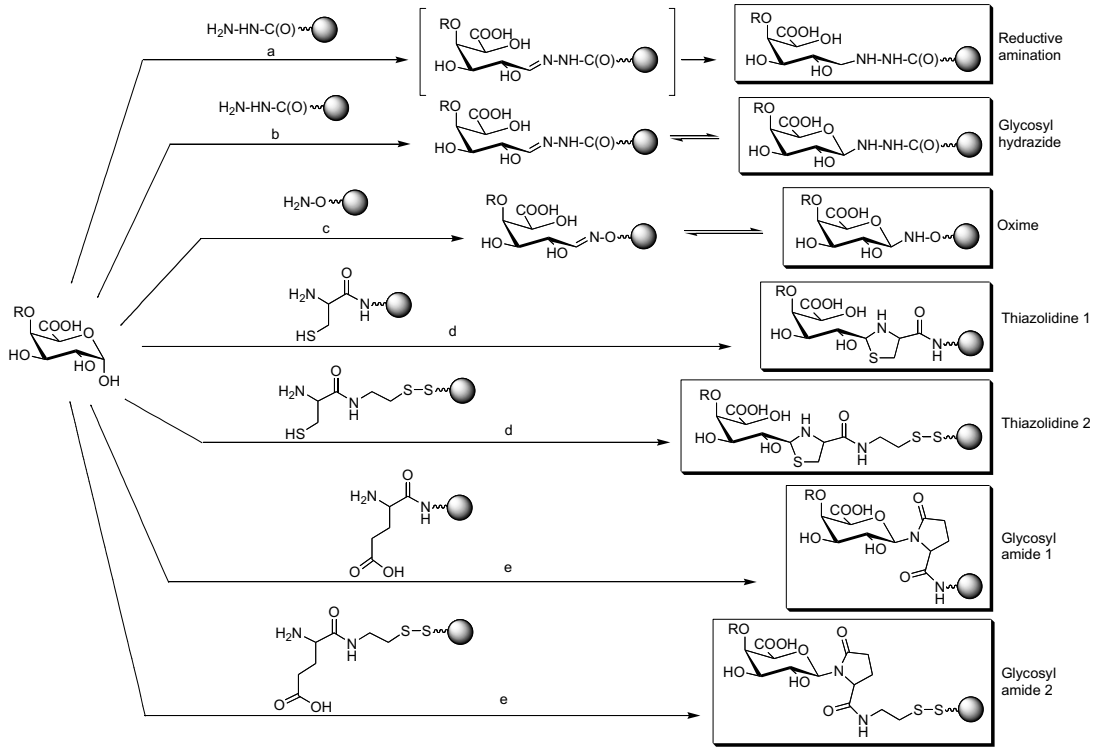
Some of the most promising approaches have used covalent immobilization to surfaces such as glass slides and polymeric supports by the formation of a firm, covalent bond. Covalent attachment is often preferred over other types of interactions, such as hydrophobic, ionic, or biotin-mediated coupling because more stable linkages and more robust functionalized supports are formed, and coupling efficiencies are more readily controlled. Requirements for a covalently fabricated carbohydrate surface include: (i) the spacer between the glycan and the surface should provide optimal presentation of glycans and prevent non-specific binding of, for example, proteins; (ii) careful choice of immobilization method, for example, whether the carbohydrate have to be derivatized prior to immobilization; (iii) suitable surface on the polymeric support or the chip, for example, a functionalized glass slide versus a metallic surface; and for some applications (iv) cleavable linkers for selective release of modified carbohydrates after screening.

2.2.1. Methods for the immobilization of unmodified oligosaccharides. A key issue in solid-phase carbohydrate chemistry and in the development of new advanced tools for glycobiology is the anchoring of oligosaccharides onto a solid matrix. Since the pioneering work by Fréchet and Schuerch[16] on polymer-supported synthesis of oligosaccharides, a wide range of methodologies to covalently immobilize carbohydrates on solid supports has been developed. Often the reducing end serves as the anchoring point, although immobilization of carbohydrates through O-6,[17] O-3,[18] and N-2,[19] as well as random reactions also have been reported. In an effort to focus on general, simple, and efficient tools that can be applied to a range of unprotected and unmodified oligosaccharides, chemoselective immobilization strategies involving the reducing end of carbohydrates will be discussed here. Progress on other linker systems to connect modified or protected saccharides to solid supports has been reviewed elsewhere.[20]

(1) Reductive amination was one of the first immobilization methods to be implemented. It relies on imine formation between the aldehyde of reducing carbohydrates and amino groups of a solid support, followed by selective, sodium cyanoborohydride reduction of the imine to a stable amine. Baues and Gray[21] as well as Matsumoto and co-workers[22] originally applied this strategy to prepare affinity adsorbents for protein purification. For example, lactose, maltose, and melibiose were anchored to amino-terminated Sepharose 4B[22] or aminoethyl-Bio-Gel[21] in a potassium phosphate buffer (pH 9.0) at room temperature. Also, reductive amination has also proved efficient for the immobilization of heparin oligosaccharides to pyrolytic carbon coated graphite.[23] However, this anchoring technique suffers from poor reactivity and long reaction times. These drawbacks can be overcome by substituting amino groups with hydrazide groups, due to a higher reactivity caused by the α-effect.[24]

(2) To improve the immobilization of small ligands to ELISA plates, Satoh and co-workers,[25] coupled acid anhydride groups of methyl vinyl ether–maleic anhydride copolymer on the wells with adipic acid dihydrazide. The resulting hydrazide groups were allowed to react with the reducing end of maltose, lactose, *N*-acetyl-chitooligosaccharides, or heparin by reductive amination. The results showed that immobilization efficiency depended on the oligosaccharide concentration and on the amount of hydrazide reactive groups on the wells. Recently, pectin oligogalacturonides of various sizes were efficiently immobilized onto hydrazide-functionalized PEGA supports through the reducing end by reductive amination.[26] Typically, the solid-phase reactions were performed in DMF–AcOH (99:1) in the presence of $NaBH_3CN$ at room temperature for 24 h (Scheme 1). Coupling efficiencies ranged between 81% and 93%. Solid-phase anchoring of carbohydrates onto hydrazide supports under such conditions leads to a reducing saccharide, which is modified and transformed into an open ring hydrazide.

Also, in the absence of reducing agent, the reaction between hydrazide groups and the reducing end of carbohydrates can be exploited for immobilization purposes. For instance, Lee and Shin[27] have used hydrazide-coated glass slides to immobilize a wide range of carbohydrates including mono-, di-, and oligosaccharides in a simple, efficient, and chemoselective fashion for the construction of carbohydrate microarrays. Optimal immobilization took place in phosphate buffer at pH 5.0, at 50 °C and for 12 h. Another example was reported by Guillaumie et al.[26] for the preparation of pectin oligomer-functionalized supports. Conditions optimized in solution were transferred to solid-phase, using longer reaction times to compensate for slower reaction rates generally encountered with solid supports (Scheme 1, glycosyl hydrazide). The resulting glycosyl hydrazide linkage is very stable under neutral to basic conditions. Overall, this method yields efficient coupling with a minimum number of steps but suffers from poor stability of the linkage at low pH and the fact that excess glycan is generally required to achieve high efficiency. However, this latter disadvantage may be minimized through careful and specific optimization and may be balanced with the numerous advantages offered by a solid-phase approach. The reaction between GalA and a model hydrazide, namely acetic acid hydrazide, generated two acyclic glycosyl hydrazides,[26] whereas Lee and Shin obtained predominantly the cyclic form of a model glycosyl hydrazide in solution.



Scheme 1. Methods for chemoselective solid-phase immobilization of unprotected model pectin oligosaccharides. Reagents and conditions: (a) $NaBH_3CN$, DMF–AcOH (99:1), rt, 24 h; (b) CH_3OH, 65 °C, 20 h; (c) DMF–H_2O (1:1), pH 4.8; 40 °C, 24 h; (d) H_2O–CH_3CN (2:1), pH 4.0, rt, 48 h; (e) (i) imidazole, DMF, 60 °C, 24 h, (ii) PyBOP, imidazole, DMF, rt, 4 h.[26]

(3) Anchoring of the reducing end of carbohydrates to a solid support via an oxime bond has been reported by several groups.[28–31] The main advantages of an oxime linker strategy for solid-phase immobilization of carbohydrates include: (i) ease of formation under mild aqueous conditions and high nucleophilicity via the α-effect; (ii) good stability under a wide range of pH; (iii) versatility in the choice of supports and saccharides. In an ELISA-microtiter plate format, lactose and *N*-acetylchitooligosaccharides have been immobilized through the reducing end via oxime formation.[29] The amino-oxy anchor was installed on the amino-coated plate using Boc-Aoa-OH and *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide as the coupling reagent.

Guillaumie et al. have extended the use of this methodology to successfully immobilize short[26] and long[32,33] anionic oligosaccharides from pectin onto PEGA and Sepharose supports, respectively (Scheme 1, oxime). Amino-oxy groups were installed on the amino-terminated support through standard coupling of Boc-Aoa-OH with PyBOP and HOBt in DMF followed by acidic deprotection of Boc.[26] In an alternative approach, an amino-oxy derivative, synthesized from Boc-Aoa-OH and *S*-(2-pyridylthio)cysteamine hydrochloride, was reacted with the thiols groups of thiopropyl Sepharose to afford an amino-oxy-terminated support functionalized with a disulfide-cleavable linker.[32] In this case, oxime formation usually took place at pH 4.8 at 40 °C in a DMF–H_2O mixture (Scheme 1). The amount of loaded carbohydrates varied depending on the study and their size. Furthermore, this technique gave very efficient coupling in most cases.

(4) Another immobilization concept based on the formation of glycosyl amide has been designed by Norberg and co-workers.[34] The first step involves the reaction of a reducing sugar with an amino-functionalized support. The reaction is reversible and a further acylation step using acetic acid is required to stabilize the glycosyl amine. In another report, GalA oligomers were immobilized onto a glutamic acid-functionalized PEGA support, glycosyl amine formation between the carbohydrate reducing end and the glutamic acid amino groups first took place in DMF at 60 °C for 24 h (Scheme 1).[26] Stabilization of the glycosamine linkage was achieved by intramolecular acylation of the secondary amine with PyBOP-activated carboxyl groups on the Glu-side chain[35] (Scheme 1, glycosyl amide). In this work, two different spacers with a terminal Glu-residue were installed on the amino-terminated supports, using either glutamic acid for permanent immobilization or a glutamic acid derivative containing a cleavable linker in the form of a disulfide bridge.

(5) Recently, Guillaumie et al. introduced thiazolidine formation for immobilizing non-protected reducing carbohydrates onto a solid support.[26] Pectin fractions, dissolved in a water–acetonitrile mixture (pH 4), were

anchored onto cysteine-terminated PEGA resins through formation of a thiazolidine linkage (Scheme 1, thiazolidine 1). This methodology provided quantitative coupling of the GalA oligomers in very few steps. Moreover, thiazolidines are expected to be stable over a wide range of conditions. The oligosaccharides could be cleaved off from the support, provided that a disulfide bridge was installed on the resin (Scheme 1, thiazolidine 2). Anchoring of chitosan onto non-porous glass beads through the formation of a 1,3-thiazolidine linker has been achieved by Liu and co-workers.[36] Cysteine moieties were introduced on the glass beads through condensation of L-4-carboxy-3-formyl-2,2′-dimethylthiazolidine with amino groups on the solid support, followed by acidic deprotection. Covalent anchoring with water insoluble chitosan took place in an aqueous acetic acid solution at room temperature for 3 h.

(6) The adsorption of thiol-functionalized carbohydrates to gold surfaces has also been used for covalent chemoselective immobilization.[7,8]

(7) Immobilization of unmodified glycans by random, non-regioselective reactions has also been used. For example, carbohydrates have been immobilized using the bifunctional cross-linking reagent, divinyl sulfone,[37] which randomly links carbohydrates to amino groups on a surface.

2.2.2. Immobilization through a functionalized aglycon. The above immobilization methods use unprotected oligosaccharides, that is, they do not have to be derivatized prior to immobilization. From an operational point of view, this is a very significant advantage, especially when working with complex oligosaccharides isolated from natural sources. However, it limits the functional groups available for immobilization to mainly the reducing end aldehyde moiety. Furthermore, it often leads to an alteration of the reducing end of the oligosaccharide.

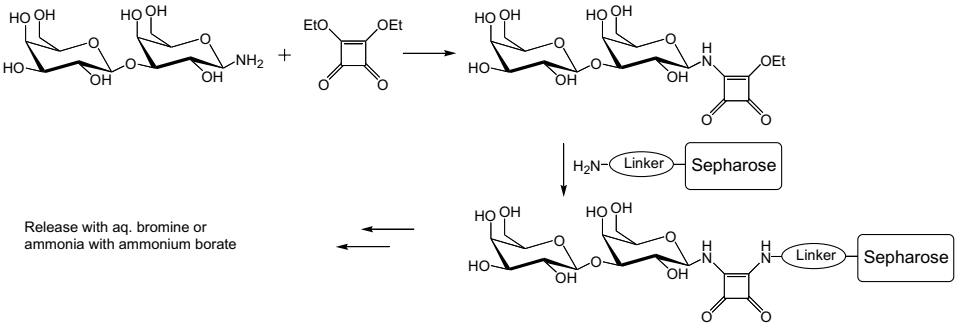
Thus, from a structural perspective, it can be advantageous to first convert carbohydrates to glycosides and then immobilize them through the aglycon. However, this requires chemical manipulation of carbohydrates and the approach is limited to primarily monosaccharides and short oligosaccharides. Reactions used for immobilization have included amide bond formation between amino-functionalized carbohydrates and surfaces functionalized with *N*-hydroxysuccinimidyl (NHS) moieties.[38,39] The Cu(I) catalyzed Huisgen 1,3-dipolar cycloaddition between an alkyne and an azido moiety has also emerged as a promising, chemoselective immobilization technique.[40] The Diels–Alder reaction, using carbohydrates carrying a cyclopentadiene moiety, has been utilized less.[7] Also, any of the reactions used for immobilization of unprotected carbohydrates, for example, oxime formation, can be envisioned for immobilization of glycosides, as long as the aglycon carries a suitable functional group. These reactions will primarily be discussed in the relevant sections.

2.2.3. Immobilization of carbohydrates to functionalized supports. There are a number of 'classical' methods for immobilization of mono- and oligosaccharides to commercially available matrices and supports.[37] These methods have, for example, been used for the preparation of affinity columns using specific ligands. These include amide bond formation, treatment with CNBr, opening of epoxide, and thiol exchange. While some of these methods allow chemoselective immobilization, others will give rise to random attachment to the support.

2.2.4. Methods for anchoring in solid-phase oligosaccharide synthesis. Solid-phase synthesis has been tremendously successful in the field of peptide chemistry due to its inherent simplicity and ease of automation. It has also been implemented in an efficient way for DNA and RNA oligomer synthesis. Despite Fréchet and Schuerch's early pioneering work[16] and significant progress[41–46] since then, considerable developments are still required before solid-phase oligosaccharide synthesis can be put to general use. Crucial to any solid-phase synthesis plan is the efficient and reliable anchoring of the first building block, the stability of the anchoring linkage to the reaction conditions, and upon completion of the synthesis the efficient release under conditions compatible with the structural integrity of the final product. As for any immobilization technique, it is important to differentiate between methods that can anchor unmodified carbohydrates and methods that require specifically functionalized carbohydrate derivatives. In the latter case, the immobilization can either be through a glycosylation or through an often more simple reaction, such as ester or amide formation.

Some of the bonds formed in anchoring of carbohydrates to solid supports have included: esters,[41] silyl ethers,[42] sulfonyl esters,[43] acetals,[44] alkylthiols,[45] ethers,[47] and amides.[19,48] This section provides a short and rather selective view of methods for anchoring the first carbohydrate moiety in solid-phase oligosaccharide synthesis. The selection is based on our estimate of their value for immobilization of carbohydrates in general, and not just for their use in solid-phase oligosaccharide synthesis.

Blixt and Norberg have employed a squarate linker, which can anchor glycosyl amines, while retaining the pyranoside ring-form of the reducing end (Scheme 2).[49] Glycosyl amines can be prepared chemoselectively from unprotected carbohydrates with a free reducing end. They successfully employed this method for enzymatic oligosaccharide synthesis using glycosyltransferases. The same authors have also used a disulfide linker system and determined that the length of the spacer between the support, Sepharose, and the glycosyl

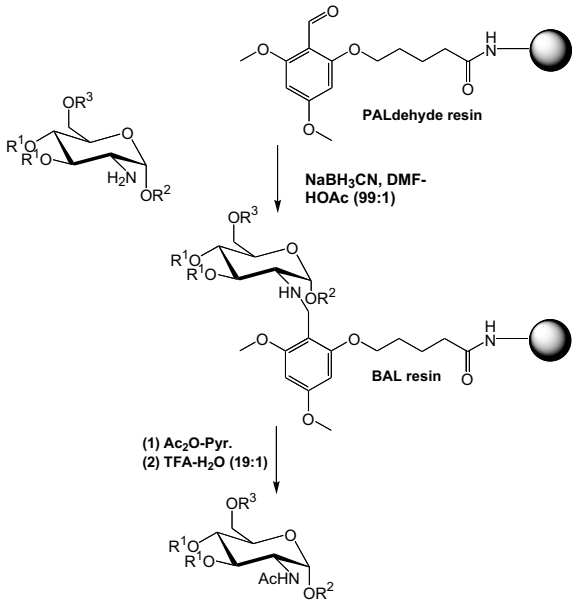


Scheme 2. The 'squarate' linker for anchoring of glycosyl amines.[49]

acceptor is crucial for efficient enzymatic glycosylation (71 atoms in a PEG type spacer backbone gave rise to higher yields than shorter ones).[50]

The Seeberger group has extensively used an octene aglycon linker system, which allows release of final products by olefin metathesis yielding pentenyl glycosides.[51] The first carbohydrate moiety is attached by O-glycosylation; the pentenyl moiety allows further functionalization.

The backbone amide linker (BAL) concept[52] for solid-phase peptide synthesis of C-terminally modified or cyclic peptides has been adapted to oligosaccharide synthesis. Unprotected D-glucosamine as well as glucosamine derivatives were attached to a trialkoxybenzyl handle (linker) on a solid support through a chemoselective reductive alkylation of the 2-amino group. Selectively protected glucosamine derivatives, and also fully unprotected glucosamine, were successfully immobilized. The BAL handle has furthermore been used for anchoring to PEGA supports via the amine in a



Scheme 3. Chemoselective BAL anchoring of D-glucosamine derivatives.[52]

4-aminophenyl aglycon and a backbone amide in a glycopeptide to enable enzymatic glycosylations with glycosynthases.[53] The final products were released by treatment with trifluoroacetic acid (TFA) containing-cocktails at room temperature (Scheme 3).

3. Methods for determination of immobilization efficiency

In parallel with the development of anchoring conditions, analytical techniques have been advanced to follow the progress of reactions and for quantification purposes. The monitoring of immobilization reactions relied initially on the quantitative cleavage of the carbohydrate from the support followed by traditional analysis such as NMR spectroscopy, HPLC, TLC, or by the use of specific assays. Alternatively, non-destructive 'on-bead' methods have been available for a few years, although they may require more advanced equipment. In this section, we will outline a few methods for the quantification of solid-support reactions. Recent tools for on-bead analysis in solid-phase oligosaccharide synthesis have been reviewed elsewhere.[20]

Oligosaccharides immobilized on microtiter (ELISA) plates have been detected by a horseradish peroxidase (HPR)-based colorimetric assay.[29] First, the oligosaccharides were bound to biotinylated or HPR-conjugated lectins in Tris-buffer saline (TBS, pH 7.5). Diluted HPR-conjugated biotin–streptavidin complex with TBS was then added. After incubation with HPR conjugates, the wells were washed and developed by the addition of 0.04% *o*-phenylenediamine and 0.01% H_2O_2 in citrate-phosphate buffer. Absorbance at 490 nm allowed quantitation of immobilized carbohydrates. This method, which provides the amount of available ligands, is convenient, highly sensitive and requires only small amounts of samples. However, a prerequisite is the availability of specific lectins for each individual application.

Guillaumie et al.[26] have determined immobilization efficiencies on PEGA supports by the anthrone assay.[54] This colorimetric method was chosen as, (i) it is a general and well-established method for the quantification

of neutral and charged carbohydrates, and (ii) it is performed at low pH and at high temperatures. Such conditions allow cleavage of the glycosidic bonds along the oligosaccharide chain, liberating single sugar units. The harsh conditions dissolve the resin; however, the spectroscopic contribution from this can easily be deducted. Typically, the solid support prior to and after immobilization, respectively, was dried and treated with 0.2% anthrone in concentrated H_2SO_4. The difference in optical density (at 570 nm) between the pre- and the post-immobilization resins was used as a measure of the amount of immobilized carbohydrates. For those anchoring methodologies, which gave fixed open ring structures at the reducing end, the contribution of the carbohydrate unit connected to the solid matrix had to be subtracted. In some cases, the anthrone assay is not the preferred choice and alternative assays have been developed to circumvent problems such as a too high absorbance of the resin before immobilization. In such cases, the Ellman test[55] has been applied to the quantitation of galacturonides immobilized onto Cys-terminated PEGA supports.[26] This colorimetric method is typically used for the quantification of free thiols. Pre- and post-immobilization resins were dried and treated with a 0.01 M solution of 5,5′-dithiobis(2-nitrobenzoic acid) in aqueous sodium phosphate buffer (pH 8.5). The amount of free thiol groups was detected by reverse-phase HPLC. Negative tests were indicative of quantitative coupling of GalA oligomers. Another assay available for the quantitation of immobilized carbohydrates has been reported by Liu and co-workers.[36] Chitosan-modified glass beads were treated with 4 M hydrochloric acid at 100 °C for 16 h, allowing the hydrolysis of the polymer into glucosamine units. The amount of released glucosamine was subsequently quantified by a modified Svennerholm method.[56]

It is also possible to determine the amount of incorporated saccharides onto a solid support in an 'indirect' fashion. For instance, reactive amino groups on a Sepharose support have been quantified by the ninhydrin test before and after their reaction with a functionalized lactose.[49] Assuming a 'clean' derivatization reaction, the difference between the amino group content before and after solid-phase coupling corresponded to the amount of immobilized oligosaccharide.

4. Carbohydrate–protein interactions in microtiter plates

Early work on carbohydrate arrays used conventional microtiter plates, which are simple to use but do not offer the advantages of miniaturization and the possibility for advanced methods for 'reading' the chip, beyond using photometric readers. These plates have been used in a variety of biological applications by binding an analyte of interest to the wall of the microtiter plate and examining the interaction with reactants in the surrounding assay mixture. The enzyme-linked immunosorbent assay (ELISA) builds on the observation that either antibodies or antigens adsorbed to a solid phase retain their ability to participate in specific binding reactions. Carbohydrates arrayed in microtiter plates have been used in a range of chemical and biological transformations, such as ELISA or solid-phase assays of enzyme recognition and action. In 1999, Gervay and McReynolds published a review covering ELISA technology used in the measurement of biological activities of carbohydrates relevant in certain disease states.[57] The following will therefore focus on protein–carbohydrate interactions such as in enzyme-linked lectin assay (ELLA),[58] where lectins are used as markers instead of antibodies.

Most microtiter plates are made of polystyrene, which poses a challenge, as carbohydrates do not adsorb well to polystyrene due to few hydrophobic interactions. Accordingly, covalent bonding to the well is often the only solution. A number of modifications have been made to allow for covalent linkage to the polystyrene surface. These include commercial surfaces with short spacers with a terminal amine attached.[59] Other alternatives are terminal maleimide, hydrazine, or *N*-oxysuccinimide groups (discussed in Section 2.2). Another possibility is to couple one part of the analyte to a conjugate, such as polyacrylamide or a protein, which will subsequently interact with the wells of the microtiter plate.

Among the first examples using microtiter plates for rapid screening of lectin specificity was by Shao[10,11] in 1992. It relied on the fact that streptavidin adsorbs strongly and irreversibly to the wells of titer plates. A series of biotinylated glycans was first coated on the streptavidin treated plates and then six lectins (concanavalin A (Con A), wheat germ agglutinin (WGA), red kidney bean erythroagglutinin (PHA), lentil agglutinin (LCA), *Datura stramonium* agglutinin or elderberry bark agglutinin) coupled to horseradish peroxidase were added. After incubation and washing, only the lectin bound to a complementary glycan remained and could be detected via the peroxidase reaction. It was established that the lectins retained their oligosaccharide-binding specificities after coupling to the peroxidase. Other examples of the biotin/avidin-mediated microtiter plate lectin assay are from Wu and co-workers.[60,61] Here, human erythrocyte glycophorin A (GPA) was adsorbed onto the wells of the microtiter plate.[62] GPA contains multiple O-linked tetrasaccharides (α-Neu*p*Ac-(2→3)-β-Gal*p*-(1→3)[α-Neu*p*Ac-(2→6)]-α-Gal*p*NAc-1→Ser/Thr).[63] Desialylation of GPA by mild acid hydrolysis gave the β-Gal*p*-(1→3)-α-Gal*p*NAc-1→Ser/Thr (Thomsen–Friedenreich (TF)) receptor, and subsequent degalactosylation by Smith degradation gave the α-Gal*p*NAc-1→Ser/Thr antigen (Tn antigen). It was

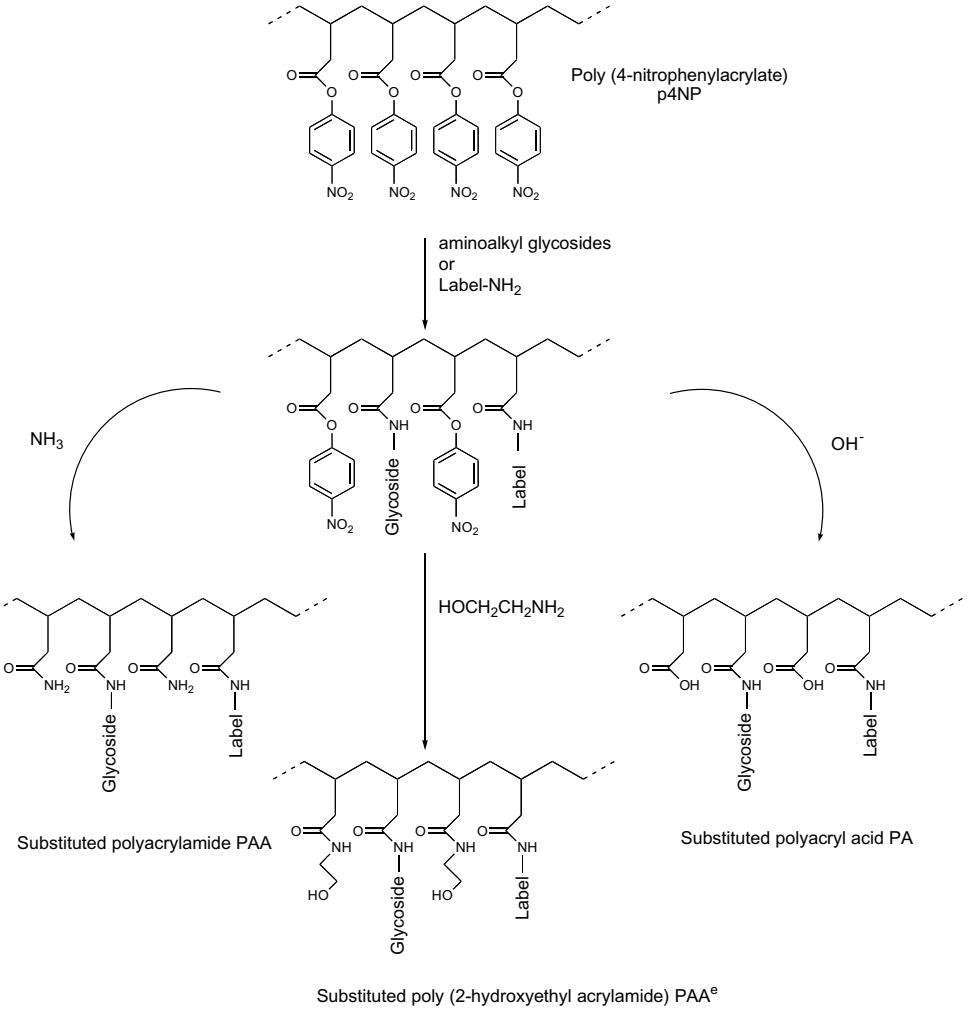
shown that the lectin binding assay in combination with use of chemically degraded glycoproteins offers the possibility of fast and sensitive characterization of lectin specificity with a great number of low- and high-molecular-weight ligands. Wu et al. also investigated the binding site of the galactose-specific agglutinin PA-IL from *Pseudomonas aeruginosa*.[61] PA-IL had the strongest affinity to two glycoproteins containing α-Gal*p*-(1→4)-Gal*p* determinants and a human blood group ABO precursor equivalent group. Inhibitory studies with disaccharide fragments showed that PA-IL has a preference for the α-anomer in decreasing order as follows: α-Gal*p*-(1→6) > α-Gal*p*-(1→4) > α-Gal*p*-(1→3).

The methodology for the synthesis of polyacrylamide glycoconjugates (reviewed by Bovin[12]) enables optimization of ligand presentation on the surface of polyacrylamide (PAA) conjugates, thus providing the type of conjugate having the most suitable signal-to-background ratio the principle is outlined in Scheme 4.

The maximum amount of saccharide introduced depends on the size of the saccharide; for bulky tri- and tetrasaccharides the range is 30–35 mol % at 40 °C, whereas with monosaccharides, the incorporation can be as high as 80%. With bulky lysogangliosides only 15% is incorporated. Spacers can be incorporated, but due to the flexibility of the polyacrylamide chain, a short aminopropyl spacer often gives enough flexibility for interaction studies of carbohydrate-binding proteins, such as lectins, antibodies, or glycosyltransferases. PAA-derivatives are obtained by the action of ammonia, other examples such as substituted polyacrylic acid (PA) or poly(2-hydroxyethyl acrylamide) (PAA^e) are also possible modifications (Scheme 4).

A PAA glycoconjugate was used by Khraltsova et al., to study the action of the α-(1→3)-galactosyltransferase (α3GalT), thought to be responsible for the synthesis of the xenoantigen, α-Gal*p*-(1→3)-β-Gal*p*-(1→4)-Glc*p*NAc (Fig. 1).[64]

Formation of the product by enzymatic extension of the glycan chain was detected by the biotinylated plant lectin *Viscum album* agglutinin (VAA) and was found to be proportional to enzyme activity and the concentra-



Scheme 4. Synthesis of soluble polyacrylamide-derived conjugates for non-covalent immobilization in microtiter plates. Redrawn from Bovin and co-workers.[12]

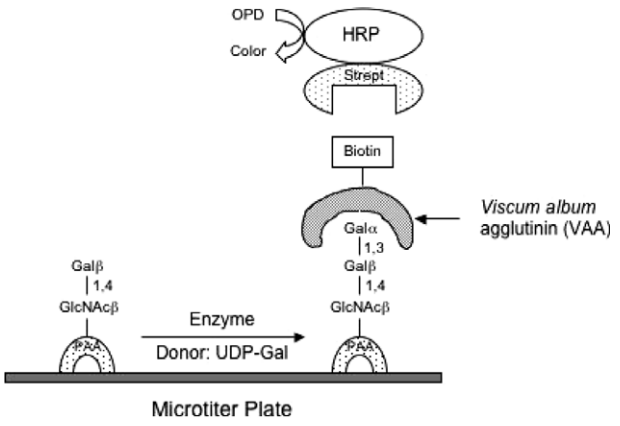


Figure 1. Microtiter plate assay of α-(1→3)-galactosyltransferase. Redrawn from Khraltsova.[64]

tion of the acceptor and the glycosyl donor, UDP-Gal. The method affords high sensitivity (better than 1 pmol/well), substrate specificity and linearity. Application of neoglycoconjugates as substrates afford additional possibilities to attain optimal results by taking any structural fragments into account: (i) optimization of substrate by variation of carbohydrate ligand density on the matrix, (ii) variation of spacer length, (iii) variation of ligand structure including mimetics with enhanced affinity.[64]

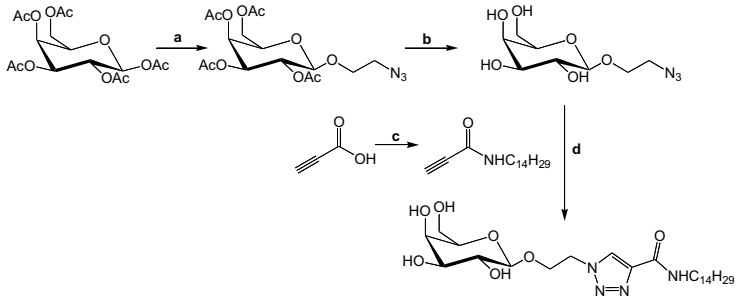
Another study using PAA neoglycoconjugates investigated the 6-sulfo-sialyl-Le^x epitope as a receptor for different strains of *P. aeruginosa*.[65] *P. aeruginosa*, the main pathogen in the airways of patients suffering from cystic fibrosis (CF), recognizes a set of neutral and acidic carbohydrate epitopes found at the periphery of respiratory mucins, especially sialyl-Le^x. The study was conducted with the bacterial strain 1244-NP and four mucoid strains isolated from CF patients. Affinity measurements conducted for all strains indicated that the 6-sulfo-sialyl-Le^x epitope bound all strains, except for one, just as well as the sialyl-Le^x determinant. This together with the equal adherence of bacteria strain 1244-NP to glycoconjugates bearing 6-sulfo-sialyl-Le^x and sialyl-Le^x epitopes indicated that the two epitopes are among the best receptors for *P. aeruginosa*. 6-Sulfo-sialyl-Le^x is also known to bind selectins such as L-Selectin, which is constitutively expressed on leukocytes. By binding leukocytes and bacteria, mucins with 6-sulfo-sialyl-Le^x and sialyl-Le^x determinants might thus limit the defense properties of leukocytes against *P. aeruginosa*.

An alternative to PAA glycoconjugates is the addition of oligosaccharides to a C_{14} hydrocarbon chain that also binds non-covalently to the surface of microtiter wells.[13] Wong and co-workers[14] used a Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between an azide and alkyne to attach oligosaccharides to a C_{14} chain by formation of a triazole[15] (Scheme 5).

The advantage of the Cu(I)-catalyzed reaction is that the triazoles are formed at room temperature and thus when carried out in CH_3OH in a 100 μL microtiter well, the crude product can be purified in situ, allowing the reaction to be carried out at micromolar scale in high yields (92%) (Fig. 2).

The glycolipids thus non-covalently linked are stable under repeated aqueous washings. In a test of the assay functionality, Wong and co-workers devised a biological screen in which ranges of oligosaccharides were displayed in microtiter plate wells in concentrations corresponding to densities of 4.1–7.6 μmol/cm^2. Seven of these, displayed various types and linkages of terminal sialic acid known to be ligands for lectins from *Sambucus nigra* (SNA). SNA was able to bind all sialic acid containing carbohydrates with preference to α-(2→6) linked over α-(2→3) linked sialic acids. The authors were also able to conduct enzymatic transformations of oligosaccharides attached to the microtiter plate. In a competitive assay, human α-(1→3)-fucosyltransferase [EC 2.4.1.65] fucosylated the trisaccharide α-Neu*p*Ac-(2→3)-β-Gal*p*-(1→4)-Glc*p*NAc over the regioisomer α-Neu*p*Ac-(2→3)-β-Gal*p*-(1→3)-Glc*p*NAc and produced sialyl Le^a, which was recognized by a fucose specific lectin from *Tetragonolobus purpureas*.

Another non-covalent glycoarray created by mixing isocyanate-containing C_{14}-hydrocarbon and amine-containing carbohydrates thus exploiting a reagent-free urea formation between isocyanates and amines has also been reported.[66]



Scheme 5. Reagent and conditions: (a) 2-azidoethanol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, rt, overnight; (b) 0.3 M $NaOCH_3$ in CH_3OH; (c) $C_{14}H_{29}NH_2$, DCC, CH_2Cl_2, 0 °C to rt, 2 h; (d) DIPEA, CuI, CH_3OH, rt, 8 h. From Fazio et al.[14]

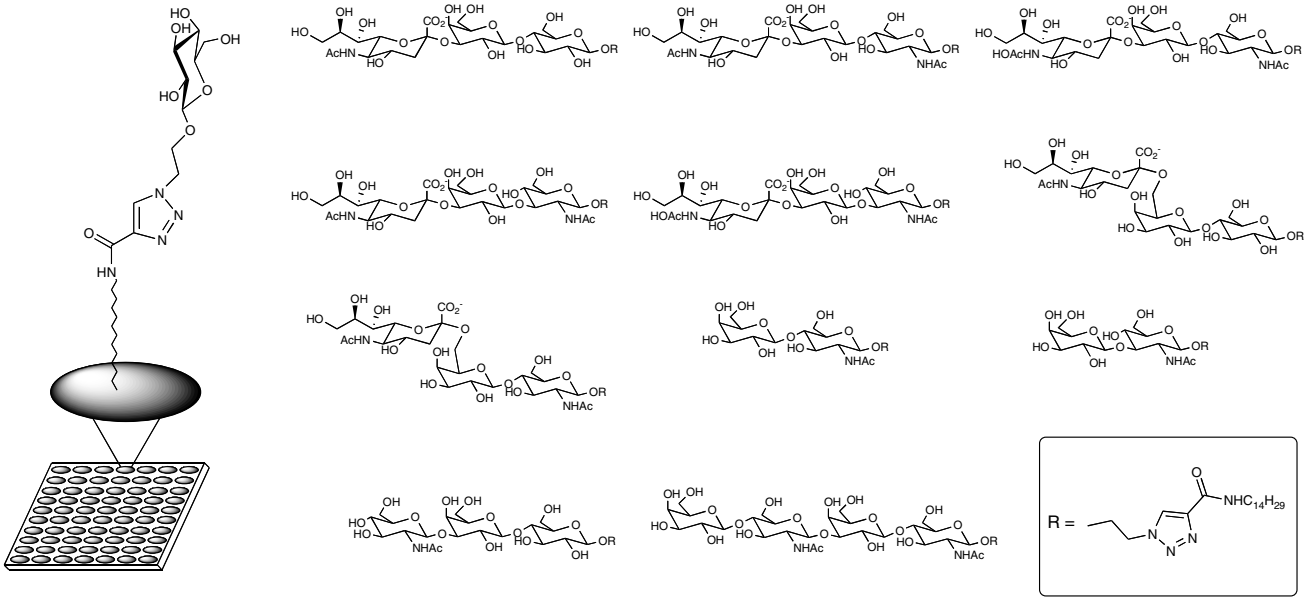
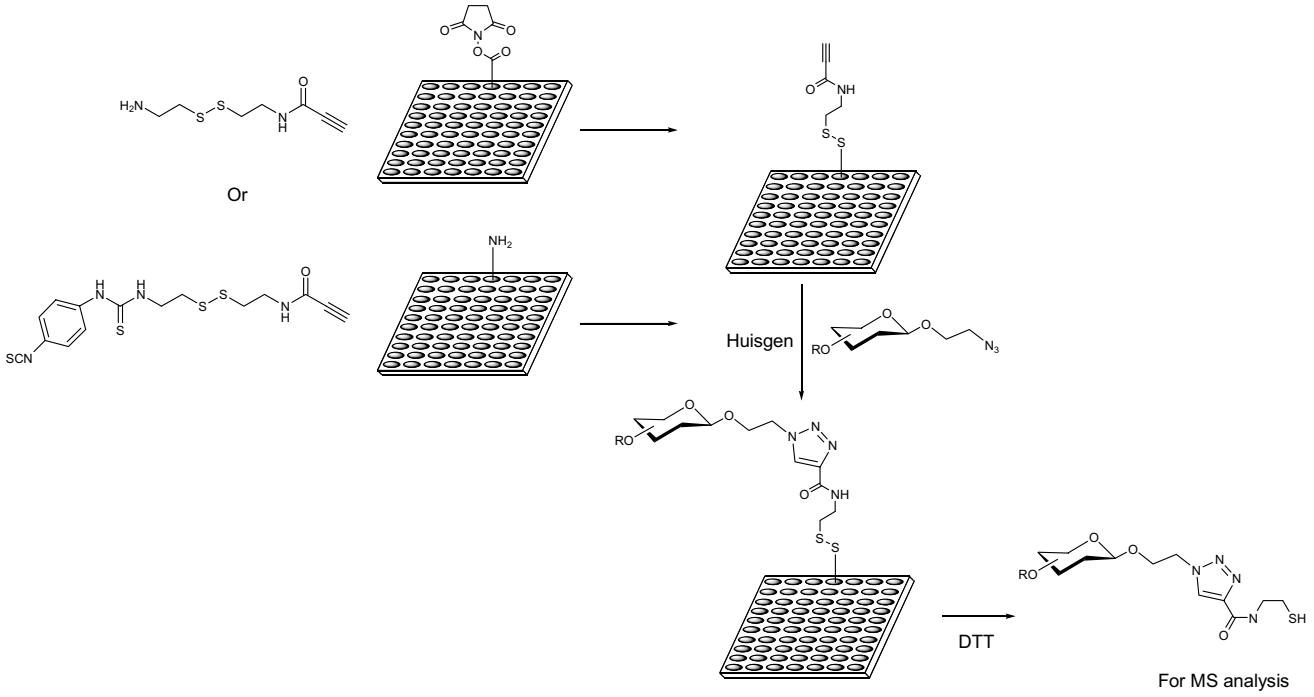


Figure 2. Oligosaccharides displayed in the microtiter plate via a Huisgen 1,3-dipolar cycloaddition. From Fazio et al.[14]

Wong and co-workers[40] improved in 2004 their concept of oligosaccharide arrays avoiding the difficult handling of glycolipids used for non-covalent attachment in microtiter plate format. The sugars were covalently attached to either amine or NHS-functionalized microtiter surfaces (Scheme 6).

A new feature was the cleavable linker in the form a disulfide bond, which is stable in many biological applications, but is easily cleavable with dithiothreitol (DTT). The linker facilitated the analysis of the covalent array by NMR or mass spectrometry. Using this concept, an array of 23 mono- and oligosaccharides was prepared and tested against lectin and antibody binding.

In 1988, Macher and co-workers reported an ELISA to monitor glycosyltransferase and glycosylhydrolase activities.[67] The paper described the identification and quantification of reaction products from the actions of β-galactosyltransferase [EC 2.4.1.22] on β-Glc*p*NAc-



Scheme 6. Triazole formation and cleavage of disulfide linker by DTT for MS analysis. From Bryan et al.[40]

(1→3)-β-Gal*p*-(1→4)-β-Glc*p*-(1→1)Cer and of α-galactosidase [EC 3.2.1.22] on α-Gal*p*-(1→3)-β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→4)-β-Glc*p*-(1→1)Cer (IV3αGal-*n*Lc$_4$Cer). The method takes advantage of carbohydrate sequence-specific monoclonal antibodies and an ELISA technique that follows substrate disappearance and product formation. This eliminates the need for treatment with specific *exo*-glycosidases and methylation analysis, which is the traditional method of analysis. Another advantage of the method is the omission of detergents for the enzyme to be functional, whereas most glycosphingolipid glycosylhydrolases require a detergent for activity. In the solid-phase system used, the glycosphingolipids are immobilized in the well, presumably with their carbohydrate moiety oriented away from the plastic surface, thus, no detergent was necessary to provide the proper orientation of the glycosphingolipids. Also, enzyme characteristics such as K_m, V_{max}, divalent cation requirement, and pH optimum were investigated using this method. Stults and Macher reported the use of an ELISA-based assay for the study of β-galactosyltransferase [EC 2.4.1.38][68] and for the study of β-(1→3)-*N*-acetylglucosaminyltransferase in human leukocytes [EC 2.4.1.149].[69]

Other uses of ELISAs for the investigation of enzymatic transformations include the work carried out by Hindsgaul and Palcic.[70] This assays the enzymatic modification of the synthetic substrate β-Glc*p*NAc-(1→2)-α-Man*p*-(1→6)-β-Man*p*-R (acceptor for uridine 5′-diphospho-*N*-acetylglucosamine:αmannoside β-(1→6)-*N*-acetylglucosaminyltransferase (GnT-V)) into the product β-Glc*p*NAc-(1→2)-[β-Glc*p*NAc-(1→6)]α-Man*p*-(1→6)-β-Man*p*-R when these structures are covalently attached to bovine serum albumin (the R-group), which was coated to microtiter wells. The conversion was followed using purified polyclonal antiserum raised against the product. Further exploitation of glycosyltransferase assays in ELISA format have been reported[71–74] and reviewed by Palcic and Sujino in 2001.[75] Baek and Roy[76] have described the synthesis of water-soluble glycopolymers bearing the TF-antigen for use in solid-phase screening in an ELISA plate format. The glycopolymers were immobilized in ELISA plates and increasing the lipophilicity by adjusting the length of an alkyl chain of the glycopolymer proved advantageous (up to M_w 42 kDa). The system was tested with a monoclonal mouse antibody and with a glycosyltransferase.

Hatakeyama[77] developed a simple microtiter plate assay for lectins, which utilized carbohydrates immobilized in the wells of the microtiter plate functionalized with primary amino groups at their surface. The carbohydrates were immobilized using the bifunctional cross-linking reagent, divinyl sulfone,[37] which randomly linked the carbohydrates to the amino groups on the plate. The binding of *Ricinus communis* agglutinin (RCA), Con A, and WGA was measured using microtiter plates coated with lactose, mannose, or *N*-acetylglucosamine, demonstrating that the lectins bound in accordance with their known specificities. As an application of the assay, the carbohydrate-binding activity of the proteins from the marine invertebrate *Cucumaria echinata* was examined. Different carbohydrate-binding activities were observed with different elution profiles, which suggested that the assay indeed could be applied to the simultaneous detection of lectins from various sources, when it was combined with a competitive binding assay to confirm the specific binding of the protein.

5. Carbohydrate microarrays

Since 2001, several short reviews have provided an overview of the emerging field of carbohydrate microarrays.[78–83] A very recent review describes the importance of surface selection and immobilization chemistry.[84] This section will provide an overview and place the topic in relation to immobilization methods and other solid-phase techniques.

5.1. Non-covalent immobilization

Microarrays based on non-covalent coupling (adsorption) have several advantages: a very wide range of carbohydrates can be immobilized directly onto the substrate surface; there is no requirement for derivatization of the slide surface, nor the need to create reactive groups on the arrayed carbohydrates; microarrays of saccharides, proteoglycans, glycolipids, and other glycoconjugates can be readily immobilized. Importantly, uncharacterized crude extracts from cells or tissues can also be directly immobilized. However, the simplicity of adsorption arrays comes at a price. The orientation of the arrayed samples cannot be controlled and structures may be 'forced' out of native configurations by the formation of numerous attachment points between sample and substrate. Therefore, some samples may be presented in forms in which their biological meaning is lost by denaturation or steric occlusion—a potentially worrying prospect for carbohydrate–protein interaction analysis.[85] Furthermore, the stability and spatial organization of arrayed samples is dependent on the structural and physical properties of each sample. The high potential for variability is likely to be problematic for direct comparative analysis between samples and for quantitative studies. Nevertheless, despite these shortcomings, adsorption arrays have been successfully used for a number of applications involving carbohydrate–protein interactions.

One of the first demonstrations of this approach was by Wang and co-workers.[86] A variety of carbohydrates

were spotted at high density onto glass slides coated with nitrocellulose (FastSlides[87]). A wide range of microbial antigen samples was used in this study including polysaccharides, glycosaminoglycans, glycoproteins, and semi-synthetic conjugates. The arrays were used to detect the presence of antibodies in human and murine serum, and using this system, antibody repertoires could be analyzed using as little as 1 μL of serum. The work clearly demonstrated the enormous potential of carbohydrate arrays for the high throughput analysis of carbohydrates using levels of sample that would be limiting for most other types of assays. It is well known that one limitation of nitrocellulose when used in membrane format is that the efficiency of immobilization is size dependent, with smaller molecules being less effectively attached. Wang et al., examined this potential limitation of the FastSlides by arraying fluorescently labeled (α1–6) dextrans ranging in size from 20 to 2000 kDa. Although all the samples were immobilized to some extent, the smaller samples were significantly less efficiently immobilized.[86]

Willats et al.,[88] have produced carbohydrate microarrays based on a modified polystyrene surface known as 'MaxiSorp'.[89] The MaxiSorp modified polymer substrate has been widely used in the production of microtiter plates and assay dishes for applications including antibody binding and carbohydrate–protein interaction studies. Microarray slides of standard dimensions were produced by injection molding of black polystyrene. The MaxiSorp surface modification was subsequently generated by oxidation of the polymer slide surface. This is a treatment that chemically alters the polystyrene surface, but is not an applied coating. This is important because it is extremely difficult to produce coated surfaces that are sufficiently intra- and inter-slide consistent. Binding is established by passive adsorption mediated by hydrogen bonding, ionic bonding and van de Waals hydrophobic interactions. Hydrogen and ionic interactions are primarily involved in capturing polar parts of molecules and securing a tight junction between the surface and the molecule. Immobilization is further stabilized by the removal of water molecules between the relatively hydrophobic surface and the immobilized molecule. Water removal is driven by a lowering of the free energy in the system by exchanging the weak water bonds with hydrophobic bonds. Willats et al.[88] tested the suitability of this surface for generating carbohydrate microarrays by immobilizing a range of glycan structures (Fig. 3).

All the samples were derived from, or occurred in, plant cell walls, which contain some of the most complex glycans found in nature. The use of black polymer was important in reducing background fluorescence; white and transparent MaxiSorp slides were also tested and in both cases the signal to noise ratios were inferior compared to the black slides.

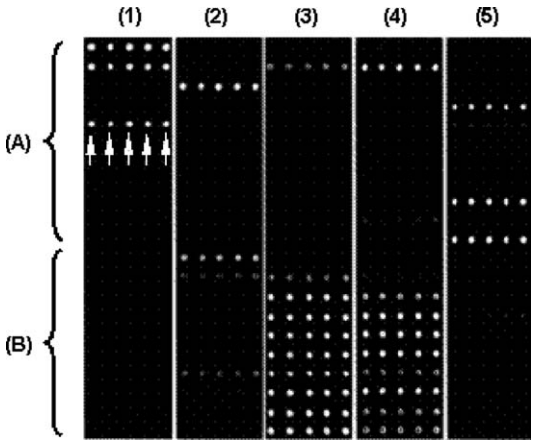


Figure 3. Plant cell wall glycan microarrays. A series of polysaccharides, proteoglycans, neoglycoproteins and plant cell extracts (A) and pectin samples (B) were arrayed onto MaxiSorp polystyrene slides. Each array (1–5) contained the same range of samples and five replicates are shown (as indicated by arrows on array number 1). The different profiles result from the fact that each array was probed with a different monoclonal antibody: (1) anti-arabinogalactan protein; (2) anti-homogalacturonan (non-methyl-esterified); (3) anti-homogalacturonan (high degree of methyl-esterification); (4) anti-homogalacturonan (low degree of methyl-esterification); (5) anti-galactan.[88]

The two examples above illustrate the simplest adsorption arrays, where samples are simply spotted directly onto the chosen substrate without any modification at all. Several groups have also developed a second class of adsorption array where the arrayed molecules are modified prior to spotting, but immobilization itself is still non-covalent. This was exemplified by Fukui et al. who used neoglycolipid (NGL) technology to generate carbohydrate probes from small oligosaccharides.[90] An important additional aspect of this work is that a deconvolution approach, utilizing TLC, antibody binding, and mass spectrometry was used to further characterize the arrayed samples. This kind of parallel analysis is an important concept for the future of carbohydrate microarrays. Because carbohydrates cannot the readily sequenced, synthesized or cloned, it is highly unlikely that really comprehensive arrays of pre-defined carbohydrates will be generated in the near future. However, the extraction and fractionation of tissue samples, followed by spotting with parallel deconvolution offers a means of greatly extending the scope of carbohydrate microarrays.

Galanina et al. have compared non-covalent immobilization of oligosaccharides in standard 96-well polystyrene ELISA plates with quasi-covalent multi-stage anchoring of derivatized oligosaccharides to the XNAonGold microarray platform.[91] The latter involved self-assembled monolayers, where some alkylthiols were functionalized with biotin, followed by multi-stage binding. This study highlighted a number of problems, including low loading. A non-covalent microarray has

also been used by Wong et al., to screen for possible inhibitors to fucosyltransferases.[92]

5.2. Covalent immobilization using oriented, chemical linkages

The chemical details of the covalent immobilization methods employed in this section have been described in Section 2.2. As previously mentioned, from an operational point of view it is important to differentiate between methods that can use unmodified carbohydrates and those that require specifically modified carbohydrates.

In 2002, Houseman and Mrksich reported the preparation of a carbohydrate microarray by Diels–Alder reaction immobilization of 10 different carbohydrate–cyclopentadiene conjugates to self-assembled monolayers (gold-coated glass slides) that presented benzoquinone and penta(ethylene glycol) moieties. Binding of several lectins was detected by SPR. This was also used to characterize substrate specificity of a galactosyltransferase; detection was by rhodamine-labeled lectins.[7]

Park and Shin have described microarrays displaying mono- and disaccharides that are covalently coupled via a linker to glass slides.[93] Maleimide-linked sugars were attached via thioether linkages to slides coated with thiol groups. Microarrays of 12,000 features were created by spotting of the maleimide-linked carbohydrate probes GlcNAc, lactose, cellobiose, and maltose. After a 5 or 10 h coupling reaction, unreacted thiols were capped by incubation with *N*-ethylmaleimide. The binding of FITC-conjugated lectins demonstrated that the samples had been effectively and reproducibly immobilized. Spotting of the maleimide-linked carbohydrate probes created microarrays from four different mono- and disaccharides using various linkers. Lectins bound more effectively to samples that were attached with longer linkers, demonstrating the importance of correct presentation of the arrayed carbohydrates on the slide surface. FITC-Con A (an α-Man*p* and α-Glc*p*-binding lectin) bound strongly to maltose (α-Glc*p*-(1→4)-Glc*p*) but weakly to cellobiose (β-Glc*p*-(1→4)-Glc*p*). This work was extended in 2004.[94]

Blixt et al., have recently reported a carbohydrate microarray with 200 oligosaccharides on a glass slide representing glycan structures from major glycoproteins and glycolipids. The immobilization was achieved by standard amide bond formation between amino-functionalized carbohydrates and NHS-activated commercially available glass slides. This very interesting approach is, however, limited to the detection of carbohydrate-binding events, which can be detected by fluorescence.[95]

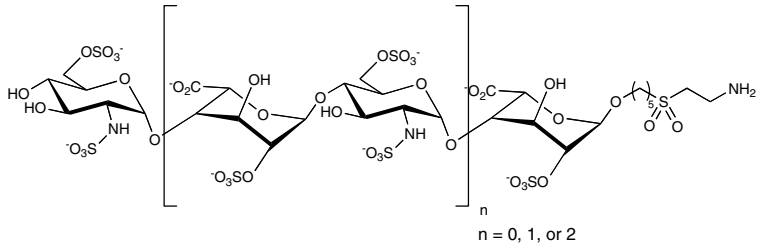
Disney and Seeberger have reported a microarray assembled with five amino-functionalized (ethanolamine spacer) monosaccharides, which were covalently anchored to glass slides that had been derivatized with homobifunctional disuccinimidyl carbonate or disuccinimidyl tetrapolyethyleneglycol linkers. This very simple setup was used to test several types of glass slides and concentrations of monosaccharide. They screened with *E. coli* (ORN178) for adhesion (Mannose preferred). This limited study showed a potential for development of anti-adhesion therapeutics.[38] The Seeberger group has also described a microarray based on seven thiol-functionalized oligosaccharides to study the binding profiles of five gp120 proteins, with a potential for HIV vaccine development.[39] They also described an aminoglycoside microarray for the study of antibiotic resistance.[96]

Seeberger, Walt and co-workers in 2003 reported a carbohydrate microarray based on a fiber-optic random microsphere array using encoding with fluorescent dyes. This format differs from the above approaches in using encoding and a random ordered microsphere array.[97]

Seeberger and co-workers[98] reported very recently the construction of a carbohydrate microarray based on a series of di-, tetra-, and hexasaccharides containing the $GlcNSO_3$(6-OSO_3)-IdoA(2-OSO_3) repeating unit of the major sequence of heparin (Scheme 7).

The potential of the microarray was demonstrated using two heparin-binding proteins, fibroblast growth factors (FGF-1 and FGF-2), which could differentiate between heparin structures and monosaccharides known not to be substrates of FGF-1 and FGF-2.

A few papers have suggested combining carbohydrate microarrays with mass spectrometric analysis. Wong et al.,[40] prepared an array in conventional microtiter plates with a cleavable disulfide linker, which allowed release for ESI-MS analysis. Mrksich et al.,[99] reported



Scheme 7. Synthetic heparin oligosaccharides with amine linker used to construct a heparin microarray.[98]

use of mass spectrometry (MALDI-TOF) to characterize self-assembled monolayers presenting carbohydrates. However, this interesting work was *not* applied to microarrays.

Shen et al., have very recently reported a specialized surface-based mass spectrometric technique, desorption/ionization on silicon (DIOS), for monitoring enzyme activity and inhibition. This appealing approach uses no matrix, in contrast to MALDI-TOF. However, a limitation is that in its present format it is not fully compatible with a proper microarray format.[100]

In summary, a few aspects should be highlighted. First, it is obviously crucial to avoid non-specific interactions; using PEG-based spacers can be beneficial. Secondly, non-covalent immobilization is very convenient from an operational perspective, especially for complex oligosaccharides from natural sources. However, it leads to randomly oriented ligands and the ligand density is often low, as physisorption depends on the nature of glycan or glycoconjugate. 'Reading' of the arrays has been limited mainly to fluorescence detection. Third, covalent immobilization can lead to robust arrays with an oriented display of carbohydrate ligands, although there are also methods, which will give random immobilization. While non-covalent immobilization has a higher efficiency for larger glycans than for short oligosaccharides, covalent immobilization in general is more efficient for shorter oligosaccharides. These arrays, although limited in size, have been used in the areas of glycomics, drug discovery, and diagnosis. There is currently no generally accepted format for carbohydrate microarrays. However, the 'Consortium for Functional Glycomics' has developed promising glycan microarrays using *N*-hydroxysuccinimide activated glass slides. The microarrays are used within the consortium and may constitute a step toward standardized formats.

6. Solid-supported peptides, glycopeptides, and oligosaccharides as biological probes

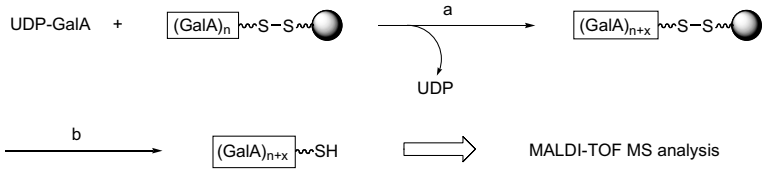
The ability to immobilize peptides and oligonucleotides on solid supports has enabled a tremendous gain in our knowledge of these biomolecules and their applications. Currently, there are no fully equivalent methods for oligosaccharides, but substantial efforts have been put into the development and use of oligosaccharide-functionalized solid supports. Such tools can for instance serve as starting materials for the synthesis of glycoconjugates and as substrates for biochemical investigations. This section will provide a few examples of these applications.

Sepharose supports, containing a disulfide-cleavable linker and functionalized with unprotected pectin oligogalacturonides of defined sizes, have been employed in solid-phase enzymatic reactions with the pectic biosynthetic enzyme, α-(1→4)-galacturonosyltransferase (GalAT) and the glycosyl donor, uridine diphosphate GalA (UDP-GalA).[32] Solid-supported biosynthesis was followed by cleavage of the immobilized OGAs with DTT and direct analysis of the products released into the liquid phase by MALDI-TOF mass spectroscopy (Scheme 8).

Long oligosaccharides up to the 16-mer were synthesized, starting from immobilized $(GalA)_{13}$. Furthermore, this strategy allowed complete elucidation of the mode of action of GalAT and of the mechanism of homogalacturonan biosynthesis. Indeed, the results showed that elongation of the oligogalacturonan chain proceeded via the transfer of single GalA residues from UDP-GalA in the presence of GalAT, that is, in a non-processive manner.

Fluorescently labeled polysaccharides (pullulan, laminarin, xylan, chondroitin sulfate, alginic acid) have been anchored to epoxy-activated agarose beads and used to investigate the activities and structural specificities of *endo*-acting extracellular enzymes (i.e., enzymes that cleave a polymer mid-chain) in complex media (e.g., seawater and homogenized sediments).[101] Hydrolysis rates were measured by means of the fluorescently labeled substrates, released into the media. In contrast to most investigations on enzyme activities and specificities that rely on the use of purified material, this solid-phase approach does not require prior purification of the enzymes.

Toward the elucidation of the interactions between proteins and glycosaminoglycans (GAGs), Vivès et al.,[102] have designed a strategy for identifying protein residues involved in the binding to heparin. Commercially available heparin beads were first incubated with EDC/NHS to activate the polysaccharide carboxylic groups, which were allowed to react with functional groups in proteins (e.g., RANTES-(9-68), PRV gC, or

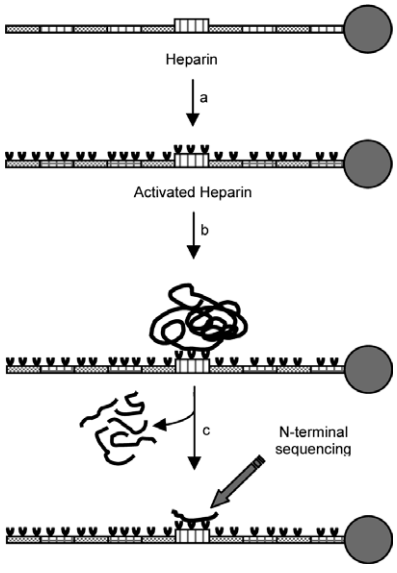


Scheme 8. Solid-supported biosynthesis followed by analysis of oligogalacturonide products. n is the initial degree of polymerization (DP) and $(n + x)$ represents the DP of biosynthetic products. Reagents and conditions: (a) 5 mM HEPES (pH 7.8), 20 mM sucrose, 0.005% BSA, 2.5 mM KCl, 350 μM $MnCl_2$, GalAT, 30 °C, 45 min; (b) DTT, 50 mM Tris (pH 8.0), rt, 3 h.

laminin-5 α3LG4/5). This cross-linking step used to stabilize the heparin–protein complex was mainly aimed at avoiding undesirable protein–protein interactions. Then, the heparin–protein conjugate was subjected to proteolytic digestion. Finally, the peptide fragments retained by the covalent linkages with heparin were identified by Edman type N-terminal sequencing (Scheme 9).

Yao et al., have described the use of high resolution magic angle spinning (HR-MAS) NMR to characterize the conformation of vancomycin anchored to PEGA or TentaGel resin.[103] By comparing directly the NMR spectra of free vancomycin in DMSO and of immobilized vancomycin, they demonstrated that the conformation of the on-bead antibiotic glycopeptide was identical to that of the free drug. Furthermore, when a vancomycin-functionalized PEGA support was swollen in the presence of Ac_2-L-Lys-D-Ala-D-Ala, a small peptide component of bacteria cell wall, they observed similar peak broadening and chemical shifts of the same protons as for vancomycin- Ac_2-L-Lys-D-Ala-D-Ala complex in solution. This study, albeit limited to one example, validates the use of on-bead screening of combinatorial libraries.

Chemically well-defined surfaces coated with selected glycan targets have been generated for SPR studies.[28] For instance, a model glycopeptide based on penta-*N*-acetylglucosamine $(GlcNAc)_5$-Aoa-GFAKKG was immobilized through an oxime linkage in one of the flow channels of a CM5 sensor chip. In this case, the N-terminus of the peptide served as anchoring point to the solid matrix, thereby granting good accessibility of the sugar-moiety. The active surface was then subjected to solutions of WGA or of a hevein-derived peptide (HEV32) at various concentrations, and the carbohydrate–lectin interactions were monitored by recording of sensorgrams. The recorded interactions ranged from a strong affinity of $(GlcNAc)_5$ with lectin ($K_A \approx 10^9\ M^{-1}$) to a weak binding of the same oligosaccharide with a hevein domain ($K_A \approx 10^4\ M^{-1}$). Importantly, the peptide linker did not interfere during the recognition event. Overall, this method may constitute a general tool for the investigation of protein–carbohydrate interactions.



Scheme 9. Protein-capture on immobilized heparin, followed by proteolytic digestion for defining critical amino acid residues (dashed zone) involved in protein/GAG interactions. Reagents and conditions: (a) (i) EDC, NHS, PBS buffer, rt, 15 min, (ii) β-mercaptoethanol; (b) protein, PBS, rt, 2 h; (c) (i) 2 M urea, PBS, 60 °C, 15 min, (ii) thermolysin, PBS, 50 °C, 16 h.[102]

Covalent coupling has been used to create protein and nucleotide microarrays[85,104] and this approach has the advantage that the arrayed molecules can be presented in a controlled orientation. The orientation of glycans is crucial for many carbohydrate interactions. For receptor–ligand or antibody binding assays, binding sites, and epitopes must be presented in a biologically meaningful fashion. Similarly, to be useful for enzyme characterization, cleavage sites must be assessable.

7. Glycoblotting

Glycoblotting, a concept introduced by Nishimura and co-workers, is an approach for the purification of small amounts of carbohydrates in biological samples. The method consists of the capture of free saccharides by chemoselective ligation to amino-oxy functionalized polymers followed by release of the purified saccharides under acidic conditions.[105,106] Subsequent analysis by, for example, MALDI-TOF/TOF mass spectrometry facilitates the assignment of complex oligosaccharides to the high level of sensitivity needed for this purpose (see Fig. 4). The applied polymers are preferably nanoparticles of 200–300 nm in diameter prepared by UV photo-polymerization of liposomal mixtures of diacetylene-containing amino-oxy-lipids and diacetylene-containing phospholipids. The size of the glycoblotting nanoparticles makes them easily separable from the crude digested biological sample by centrifugation.

As a proof of concept, the glycoblotting approach has been applied to the analysis of the saccharide composition of the glycoprotein ovalbumin. Ovalbumin (1 mg) was digested by two proteases, pronase and trypsin, and de-glycosylated with peptide N-glycosidase F (PNGase F). The glycoblotting nanoparticles were added to the crude mixture, and after an incubation period and subsequent washing, the saccharides were released by addition of a sulfonic acid resin. The glycoblotting nanoparticles and the resin were removed by centrifugation, and the filtrate was analyzed by MALDI-TOF mass spectrometry. Oligosaccharide compositions could be assigned to all major peaks observed in the MALDI-TOF mass spectrum, and further assignments could be made by tandem mass fragmentation analysis.

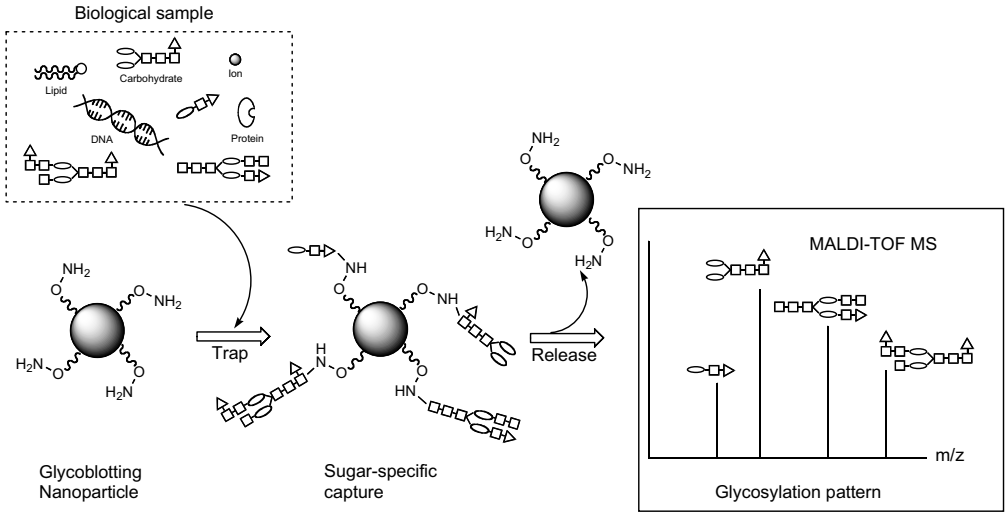


Figure 4. The glycoblotting concept based on trap and release of carbohydrates from complex biological samples and subsequent glycopattern analysis. Redrawn from Niikura et al.[106]

The glycoblotting approach has further been explored for the identification of glycosylation sites of glycoproteins in a model study of a glycopeptide. Enzymatic oxidation of a peptide-bound β-Gal*p*-(1→4)-Glc*p*NAc saccharide to generate a terminal aldehyde group at the C-6 position of the galactose unit followed by glycoblotting showed the versatility of sensitivity enhancement in the subsequent sequencing analysis of the peptide.

The glycoblotting model studies indicate that this approach coupled with advances in carbohydrate sequencing may become a powerful tool for mapping of glycoproteins and glycolipids.

8. Glyconanoparticles

Glyconanoparticles (GNPs) have lately attracted attention as probes for the study of carbohydrate–protein and carbohydrate–carbohydrate interactions. These metal and semiconductor nanoparticles linked with monolayers of carbohydrates offer some unique advantages over multivalent carbohydrate-functionalized dendrimers, polymers and liposomes in the combined properties of the clustered multivalent presentation of the carbohydrates on the surface, the facile tracing via electron microscopy or fluorescence emission, the small size that allows the study of in vivo bio-imaging, and the high degree of solubility and stability in biological and buffered aqueous media. The three-dimensional presentation on the spherical metal or semiconductor nanoparticles results in a flexible globular shape that can be thought of as a model for the glycocalyx of cell surfaces.[107] Additionally, the clustering of the carbohydrates at the GNPs has been found to result in a very high enzymatic stability that makes GNPs well-suited for interactions with biological material.[108]

The preparation of gold glyconanoparticles (Au-GNPs) has mainly been performed by a modification of the procedure of Brust et al.,[109] in which tetrachloroauric acid, $HAuCl_4$, is reduced by sodium borohydride in the presence of a thiol- or disulfide-linked saccharide in methanol, resulting in mean gold core sizes in the range of 1–20 nm. The length and nature of the linker between the reducing end of the saccharide and the mercapto group(s) not only affects the ligand density but may also play a role in the organization of the monolayer and on the solubility and biocompatibility of the resulting nanoparticles. Manipulation of the ratio of tetrachloroauric acid to organic ligand allows control over the size and polyvalency of the GNPs. This approach for the preparation of Au-GNPs was introduced by Penadés and co-workers[108] to present glucose, lactose, maltose, and the Lewis^x trisaccharide on gold nanoparticles attached via ω-mercapto-oligo(ethylene glycol) and/or straight-chain alkyl sulfide linkers (Fig. 5).[107,108] The

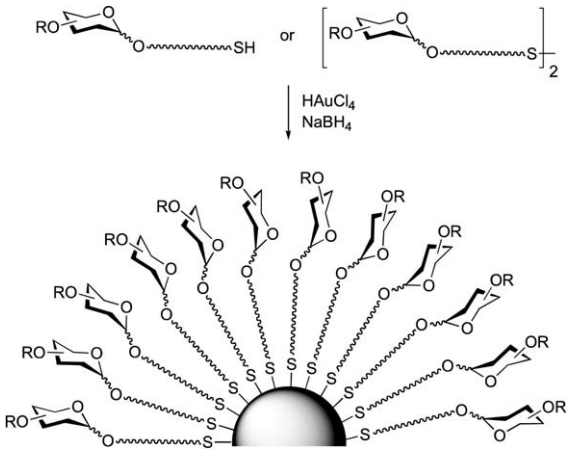


Figure 5. Preparation of Au-GNPs. Redrawn from Rojo et al.[114]

Au-GNPs can be purified by centrifugation or centrifugal filtration and can be easily handled as powders with high storage stability. They can be analyzed directly by NMR and possess a characteristic surface plasmon band centered at 520 nm that can be used for tracing. Au-GNPs incorporating a variety of complex oligosaccharides such as the Lewis^y tetrasaccharide α-Fuc*p*-(1→2)-β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)]-Glc*p*NAc, a colon and liver tumor antigen;[110] β-Glc*p*NAc3S-(1→3)-Fuc*p*, a sulfated disaccharide from marine sponge cells;[111] the TF disaccharide β-Gal*p*-(1→3)-Gal*p*NAc, a tumor antigen present in 90% of carcinomas;[112] and the P^k trisaccharide α-Gal*p*(1→4)-β-Gal*p*-(1→4)-Glc*p*, a cell differentiation antigen[113] have all been prepared in a similar manner.

An alternative approach for the preparation of gold glyconanoparticles consists of an initial preparation of citrate stabilized gold nanoparticles, in which sodium citrate is added to an aqueous solution of $HAuCl_4$ and functions as both reducing and capping agent.[115,116] The introduction of thiol-linked saccharides is subsequently performed by ligand displacement. One advantage of this method is that Au-GNPs having identical gold cores but displaying different carbohydrates can be easily prepared, a task that is difficult with the Brust reaction due to the influence of the carbohydrate ligand nature on particle growth.[117]

The preparation of carbohydrate-functionalized semiconductor quantum dots (QD-GNPs) was first reported for CdSe/ZnS core–shell quantum dots having a surface coating of a mannose-containing phosphine oxide.[118] Recently, maltose and Lewis^x functionalized CdS QD-GNPs were prepared by addition of sodium sulfide to an aqueous solution of cadmium nitrate in the presence of the thiol- or disulfide-linked saccharides.[119] The QD-GNPs showed emission in the green region under an ultraviolet lamp, however the fluorescence spectra showed multiple and broad emission maxima, thus limiting the application of these QD-GNPs.

QD-GNPs having sharp and symmetrical emission maxima can be obtained by the more classical approach of quantum dot ligand displacement, and this has recently been performed in the preparation of *N*-acetylglucosamine functionalized QD-GNPs.[120] High-quality TOPO (trioctylphosphine oxide) capped CdSe/ZnS core–shells were prepared by a one-pot procedure, and ligand exchange was effected by pyridine capping followed by addition of the disulfide of 11-mercapto-undecyl-β-*N*-acetylglucosaminoside under reducing ($NaBH_4$) conditions. The intermediate pyridine capping was used to attain a more efficient ligand displacement by the thiol-functionalized saccharide.

8.1. Lectin binding studies

The proteins (lectins) involved in specific carbohydrate–protein binding are typically present in the native form as oligomeric aggregated structures, thus presenting multiple carbohydrate recognition sites. Traditional efforts to study structure-binding relationships of mono- and oligosaccharides have faced the challenge of measuring the weak interactions, in the range of millimolar affinity, of monovalent carbohydrates with lectins. Due to the multivalent nature of GNPs, they seem ideally suited for the study of carbohydrate-binding affinity to lectins, lectin purification, identification of unknown lectins and for lectin binding-epitope mapping.

One of the first reports on glyconanoparticle binding to lectins is that of Kataoka and co-workers,[121] who studied the binding of lactose/mannose functionalized gold nanoparticles to the galactose-binding lectin *R. communis* agglutinin (RCA_{120}). The nanoparticles were prepared by Brust reaction with α-diethylacetal-ω-mercapto-PEG_{3090}, followed by unmasking of the aldehyde and reductive amination with mixtures of *p*-aminophenyl-β-D-lactoside and mannoside by borane–dimethylamine complex to install the carbohydrates. Binding to RCA_{120} was monitored visually by a color change from pinkish-red to purple and quantitatively by a broadening and red shift in the assigned gold nanoparticle surface plasmon band in the optical spectra. A linear relationship in optical absorbance with lectin concentration was established and absorbance was fully restored by addition of D-galactose, thus these glyconanoparticles can be used as a simple lectin assay with a high sensitivity and stability in physiological buffers. This study was extended in 2005.[122] A similar approach was taken by Russell and co-workers for the study of binding of mannose functionalized gold nanoparticles to Con A.[117] Here, the use of a short linker, 2-mercaptoethanol, was thought to induce a better-defined change in the optical spectra to provide a more sensitive assay. In fact, it was later shown by comparison of similar mannose functionalized gold nanoparticles, having 2-mercaptoethanol versus *N*-(2-hydroxyethyl)thioctic amide linker, that sensitivity was improved and non-specific binding reduced with the latter linker. This is presumably due to greater organization of the monolayer to provide a more optimal ligand presentation with the longer linker.[123]

An alternative method for quantification of lectin affinity has been demonstrated by Lin and co-workers in which GNP binding to Con A was quantified by a SPR competition binding assay.[124] Mannose and glucose functionalized gold nanoparticles competed with the mannose functionalized SPR chip and Con A-GNP aggregation was measured as inhibition of the Con A SPR response. Various GNPs were prepared to evaluate the influence of average particle size (6 nm vs 20 nm), linker (5-mercaptopentanol vs α-hydroxy-ω-(3-mercaptopropyl)-tetra(ethylene glycol)), and monosaccharide (mannose vs glucose vs galactose). The GNPs were prepared by glycosylation of the alkene-precursors

of the linkers with glycosyl bromides followed by installation of the mercapto group and subsequent formation of the nanoparticles by the Brust reaction. Inhibition constants of the mannose GNPs were in the range of five orders of magnitude lower than that of α-methyl mannopyranoside, whereas a Glc*p*-GNP was four orders of magnitude lower, clearly showing an increase in binding affinity as a consequence of the cluster effect. Relative to the averaged number of mannose units for each individual nanoparticle, this corresponds to an increase in binding affinity per mannose unit of one to two orders of magnitude. The increase in particle size from 6 to 20 nm caused a 10-fold increase in relative binding affinity, whereas the penta(ethylene glycol) linked GNPs showed a 2-fold lower relative binding affinity compared with the 5-mercaptopenatanol linked GNPs. The latter result was speculated to be due to orientation and rigidity effects of the linkers. A similar study has been reported by Chen and co-workers.[125]

A broadening of the scope of glyconanoparticles has been introduced by Kamerling and co-workers in the application of a reductive amination protocol with tritylated cysteamine (2-mercaptoethylamine) for the facile thiol-functionalization of free saccharides, which obviates the need for protecting group manipulations and glycosylation chemistry[126] (see Section 2.2). In this procedure, the reducing end saccharide unit is converted into an open-chain secondary amine, which can be used as a handle for SPE purification. A variety of different mannose and glucose containing GNPs were prepared in this fashion and lectin binding affinity was measured by SPR studies of chip-immobilized Con A. Qualitatively, mannose containing GNPs gave stronger SPR responses compared to glucose (mannose-free) GNPs. Unfortunately, the monosaccharide containing GNPs prepared from glucose and mannose showed poor stability in aqueous media resulting in partial precipitation and both were excluded from the binding studies. Thus, it remains questionable whether the open-chain configuration of the attached reducing end saccharide units using this protocol are in fact able to bind to their lectin carbohydrate-binding domains.

Carbohydrate-functionalized quantum dots have been applied in the study of β-*N*-acetylglucosamine adhesion to WGA.[120] Pyridine-capped CdSe/ZnS core–shells were prepared and ligand exchange was effected by addition of the disulfide of 11-mercapto-undecyl-β-*N*-acetylglucosaminoside under reducing (sodium borohydride) conditions. Binding of the GNPs to WGA was monitored by partial (up to 50%) fluorescence quenching of the quantum dot upon addition of WGA, an effect ascribed to electron transfer from the quantum dot to amino acid residues on WGA. The binding constant of the QD-GNPs was three to four orders of magnitude higher than that of the monomeric *N*-acetylglucosamine.

The use of GNPs as probes for the study of lectin binding has been extended by Lin and co-workers to combine the probing ability with mass spectrometry for the study of carbohydrate–protein recognition and binding-epitope mapping.[113] The target lectins were affinity captured on the GNPs and analyzed on-probe by MALDI-TOF mass spectrometry. Careful on-probe protein digestion followed by removal of unbound peptides allowed direct mapping of the peptide sequences involved in the carbohydrate–protein interactions of the target lectin. This approach was demonstrated by analysis of the galactose-binding lectin PA-IL by the use of galactose and P^k antigen (the trisaccharide α-Gal*p*-(1→4)-β-Gal*p*-(1→4)-Glc*p*) functionalized gold GNPs. While still being bound to the glyconanoparticle, the lectin, having a mass of nearly 13 kDa, showed a single peak in MALDI-TOF MS. Dilution experiments showed that the lectin was readily detectable down to femtomolar concentrations. The glyconanoparticle itself did not give any detectable background signal. By subsequent digestion of the GNP-bound lectin by chymotrypsin, without denaturation of the native protein structure, followed by centrifugation, the remaining GNP-bound peptides could be analyzed by MALDI-TOF MS. A comparison with the peptide mass fingerprinting map of chymotryptic digestion of PA-IL revealed that 5–7 peptide fragments, several being discontinuous, out of 17 were still bound to the GNPs after digestion. The galactose and P^k antigen GNPs showed an identical major peptide fragment, a 23-mer peptide, as well as several other similar peptide fragments suggesting that PA-IL interacts with the P^k antigen through the terminal galactose unit. The results correlated well with a recent crystal structure determination confirming the applicability of this approach for the uncovering of carbohydrate–protein recognition and interactions.

8.2. Carbohydrate–carbohydrate interaction studies

Apart from protein–protein and protein–carbohydrate interactions, carbohydrate–carbohydrate interactions between cell surface glycolipids have been suggested as an important mechanism of cell-adhesion and cell-recognition processes. Recent model studies of this mechanism using suitably functionalized GNPs have started to address the inherent challenges of the great complexity and low affinity of carbohydrate–carbohydrate interactions.

In one such study, mimicking cell adhesion through interactions of glycosphingolipid clusters, Au-GNPs were used to study the calcium dependent self-recognition of the trisaccharide $Lewis^x$ antigen, β-Gal*p*-(1→4)-[α-Fuc*p*-(1→3)]-Glc*p*NAc.[108,127] The trisaccharide was attached to the nanoparticles in the β-configuration via a ω-mercapto-tetra(ethylene glycol) linker by the Brust

reaction.[107] Nanoparticle aggregation upon addition of calcium chloride, as observed by transmission electron microscopy, was used as a qualitative measure of carbohydrate–carbohydrate interactions of the Lewis^x GNPs. The calcium dependency was proved by complete dispersion of the aggregates upon addition of EDTA. The self-aggregation was further evidenced by atomic force microscopy[128] and SPR[129] measurements of the interaction between GNPs and two-dimensional self-assembled monolayers displaying identical carbohydrates, as well as by isothermal titration calorimetry.[130] Analogous lactose functionalized GNPs showed minimal aggregation[130,131] and maltose GNPs did not show aggregation at all. The poor ability of the lactose GNPs to undergo calcium-induced self-aggregation has recently been contradicted by a very similar study of lactose Au-GNPs, having larger mean core diameters (16 nm vs 2 nm) and oligo(ethylene glycol) linkers instead of a straight-chain alkyl linker.[132] Here, a high degree of calcium-induced aggregation was apparent with all lactose containing GNPs, which was speculated to be due mainly to the increased hydrophilicity of the linker, facilitating an important hydration of calcium in the binding conformation. These results emphasize the importance of the linker and/or core design in mediating the activity of the surface-bound carbohydrates.

A recent model study on the carbohydrate mediated self-recognition of marine sponge cells[133] clearly underscores the applicability of glyconanoparticles in the understanding of carbohydrate–carbohydrate interactions of complex glycans at the molecular level. Gold nanoparticles were functionalized with β-Glc*p*NAc3S-(1→3)-Fuc*p*, a disaccharide identified as a highly repetitive epitope of a 200 kDa acidic glycan, constituting a repetitive element of a 2×10^4 kDa proteoglycan-like aggregation factor of red-beard marine sponge cells. The disaccharide was attached in the α- and β-Fuc configuration through a propyl-thio-hexylthiol linker. Additionally, analogous GNPs were prepared with each of the monosaccharide units of the disaccharide, as well as with three disaccharides incorporating minor modifications.[111] As in the study of Lewis^x interactions, self-recognition of β-Glc*p*NAc3S-(1→3)-Fuc*p* was monitored by aggregation of the GNPs upon addition of calcium chloride. The results showed a striking importance not only of the presence of each of the monosaccharide units, but also of the C6 methyl group of the fucose unit, the sulfate group and the acetamide group of the glucosamine unit, and the anomeric configuration of the fucose unit, highlighting the extremely high degree of selectivity in carbohydrate–carbohydrate interactions. The aggregation was specific for and dependent on calcium ions, as witnessed by a lack of aggregation upon addition of magnesium chloride and complete dispersion of the calcium-induced aggregates upon addition of EDTA.

These studies show the great potential of glyconanoparticles in the probing and understanding of the weak interactions between carbohydrates.

8.3. Bio-imaging

Despite the number of publications on GNPs, very few are dealing with bio-imaging and interactions with biological systems. One bio-imaging study using Au-GNPs functionalized with mannose has been performed for the visualization of mannose binding domains, the so-called FimH adhesins, on type 1 pili in *E. coli*.[134] The type 1 pili are fibrous organelles on the surface of the bacteria involved in cell adhesion and invasion. The bacterial strains were incubated with the mannose functionalized GNPs and examined by TEM. The cell surface-bound nanoparticles were localized in patterns on the pili in accordance with previous reports on FimH expression patterns along type 1 pili. Displacement of the GNPs by free mannose required concentrations in excess of 2000-fold of the GNP concentration.

A recent bio-imaging study of mice, pig, and sea-urchin sperm using glucosamine and mannose functionalized quantum dot GNPs has focused on the distribution of complimentary binding proteins on the surface of sperm

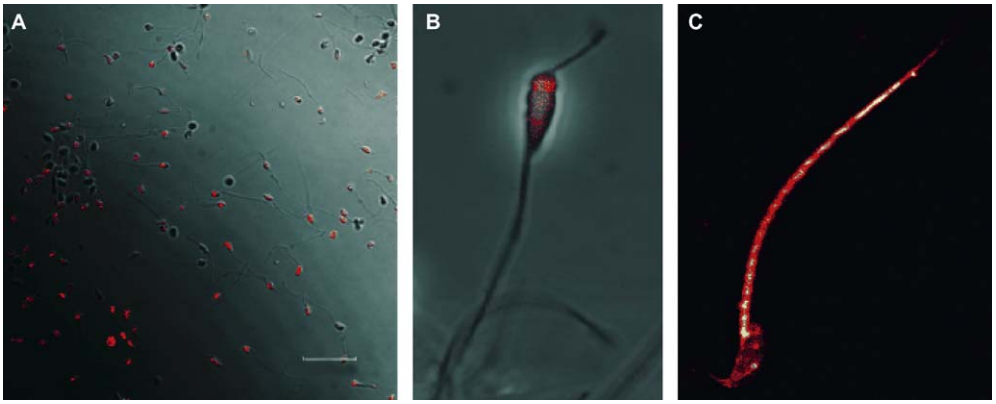


Figure 6. Bio-imaging of (A) and (B) sea-urchin sperm by glucosamine QD-GNPs and (C) mouse sperm by mannose QD-GNPs. Reproduced from Ref. 120.

from mice, pigs, and sea-urchins.[120] Confocal microscopy revealed high concentrations of glucosamine GNPs on the sperm heads, whereas the mannose GNPs were spread over the entire sperm body indicating different distributions of glucosamine and mannose receptors on the sperm surface (see Fig. 6). The binding capability of the GNPs with live sperm was proven by flow cytometry.

Glyconanoparticles have also been studied as a potential therapeutic intervention. Lactose functionalized Au-GNPs have been applied as an anti-adhesive therapy in a remarkable exploratory study of lung metastasis in mice[114] in which the adhesion of the B16F10 melanoma cell line to endothelial cells was interfered by lactose Au-GNPs in cellular assays as well as in vivo. The B16F10 cell line is known to aggressively induce lung metastasis in mice upon intravenous inoculation, however lung metastasis could be reduced by up to 70%, as measured by ex vivo analysis, by pre-treatment of the B16F10 cells with Au-GNPs before inoculation. This effect has been speculated to be due to carbohydrate–protein and/or carbohydrate–carbohydrate interactions between GNPs and surface glycoconjugates of endothelial cells and/or tumor cells.

Undoubtedly, bio-imaging using glyconanoparticles for direct visualization of target carbohydrate receptors in biological environment will provide new insights into glycobiology that may facilitate new approaches in clinical diagnostics and therapy.

9. Affinity separation and chromatography

In the most common approach to affinity chromatography, a ligand immobilized to a support specifically binds to a molecule, for example, a protein, in solution, thus enabling its highly selective purification. The ligand can either be a small organic molecule, a carbohydrate, a peptide, or a protein. The target protein is recovered by changing the conditions over the matrix to favor desorption. The technique is often so specific that no further purification is required. Ligands used for mono-specific affinity chromatography can be selected to be very specific of the sample to be analyzed or purified. One implementation of the affinity-catch principle relies on specific binding of lectins to carbohydrate ligands. Lectins are carbohydrate-binding proteins,[135] which lack enzymatic activity (nor are they antibodies). Lectins are thought to be involved in biological recognition processes but not associated with any catalytic activity within the same polypeptide; this has been reviewed in part by Berteau and Stenutz,[136] and Boraston.[137] Lectins are often classified according to their carbohydrate-binding specificity,[138,139] but from the standpoint of modern biology, lectins should be understood as a protein family.[140]

One study using sequential lectin-affinity chromatography is the analysis of asparagine-linked oligosaccharides.[141] Structures of asparagine-linked oligosaccharides fall into three classes,[142] which all share the common core structure α-Man*p*-(1→3)-[α-Man*p*-(1→6)]-β-Man*p*-(1→4)-β-Glc*p*NAc-(1→4)-Glc*p*NAc-Asn. High-mannose-type oligosaccharides have two to six additional α-mannose residues added to the core structure. Complex-type oligosaccharides contain two to four branches with a sialyllactosamine sequence. Finally, the hybrid-type structures have high-mannose-type and complex-type oligosaccharide antennas (Fig. 7).

Using the experimental setup outlined in Scheme 10, Yamamoto and co-workers[141] were able to fractionate a complex mixture of asparagine-linked oligosaccharides into distinct fractions.

The *glyco-catch method*, as reviewed by Hirabayashi and Kasai,[143] is one method where affinity chromatography has been used to acquire information about different sets of glycans produced by different organism, that is glycomics. The basic procedure is outlined in Figure 8. Here groups of target glycopeptides are captured by means of lectin-affinity chromatography after protease digestion.

From the glyco-catch method, four essential attributes are adopted to characterize each glycopeptide: (i) cosmid identification number (ID), (ii) molecular weight (M_r), (iii) retention (R_s) of pyridylaminated (PA) oligosaccharides, and (iv) dissociation constants (K_d's) of PA-oligosaccharides for a set of lectins. A recent application of the glyco-catch method has resulted in the gene assignment of soluble glycoproteins produced by the nematode *Caenorhabditis elegans*.[144] Using Con A and galectin LEC-6 (Gal6) columns, 44 (Con A) and 23 (Gal6) glycopeptides were captured and successfully analyzed and assigned to 32 and 16 corresponding genes, respectively. For these glycopeptides, 49 N-glycosylations sites were experimentally confirmed, whereas 21 remained as potential sites. However, the authors

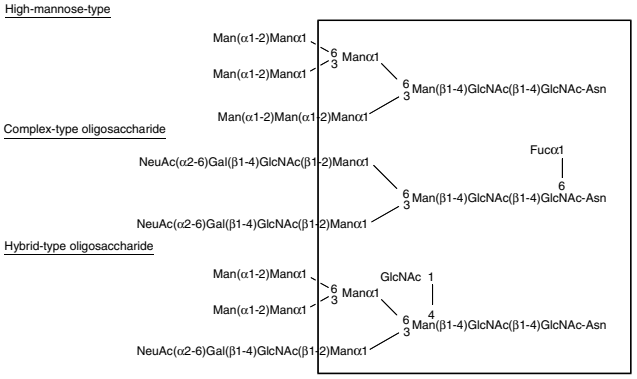
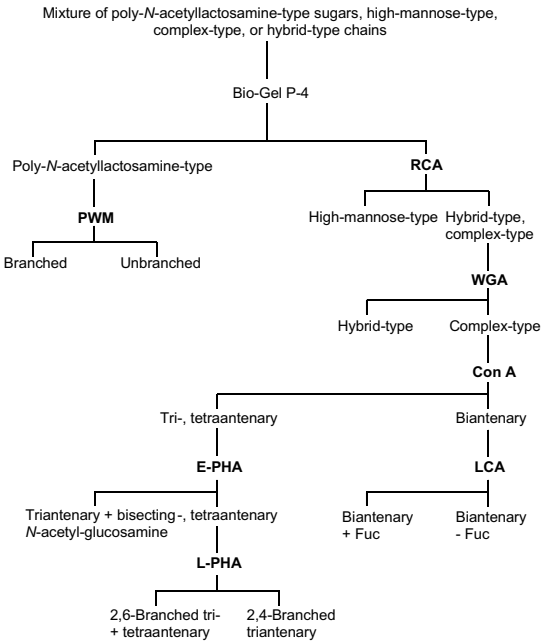


Figure 7. Structures of the three major classes of asparagine-linked oligosaccharides. The boxed area is the common structure of all asparagine-linked oligosaccharides. Redrawn from Yamamoto et al.[141]



Scheme 10. Flow-chart for fractionation of Asn-linked oligosaccharides. Used lectins are marked in bold. Redrawn from Yamamoto et al.[141]

have pointed to some limitations of their strategy.[145] One disadvantage of the glyco-catch method is the inapplicability to O-glycosides, due to the lack of a universal glycosidase to liberate all kinds of *O*-glycans. Another limitation is the biased collection of glycopeptides that the method produces, using a limited number of lectins in the capture of the glycopeptides.

Using a Con A Sepharose-derived matrix, a lectin specific for mannose/glucose was isolated and purified from mulberry seeds.[146] The lectin agglutinated rat red blood cells and in a hapten inhibition test, D-mannose and D-glucose were found to be inhibitors. A lectin from the rhizomes of *Arundo donax* was isolated by Singh and co-workers[147] using affinity chromatography with *N*-acetyl-D-glucosamine linked to epoxy-activated sepharose-6B. The *A. donax* lectin (ADL) agglutinated rabbit erythrocytes and the agglutination was inhibited by *N*-acetyl-D-glucosamine and its di- and trimer (N,N''-diacetylchitobiose and N,N',N''-triacetylchitotriose). Thirty-two other sugars and three glycoproteins were tested but showed no inhibitory effect.

In another example, the porcine surfactant protein D (SP-D) was purified using maltose affinity chromatography.[148] SP-D, a collectin, takes part in the innate immunity by binding to complex glycoconjugates on a wide range of pathogenic organisms involved in respiratory infection, thus initiating different biological functions, such as antimicrobial activity, regulation of surfactant lipid homeostasis, and macrophage function. After purification of porcine SP-D by maltose affinity chromatography eluting with an $MnCl_2$ gradient, contaminations from IgM were removed by IgM affinity chromatography providing pure SP-D with a molecular mass of 53 kDa according to SDS-PAGE analysis.

Hatakeyama et al.[149] purified the *N*-acetyl-D-galactosamine-specific C-type lectin (CEL-I) from *C. echinata* (sea cucumber) using lactosyl-Cellulofine, GalNAc-Cellulofine, and Sephadex G-75. Subsequent analysis of CEL-I carbohydrate-binding activity, using a

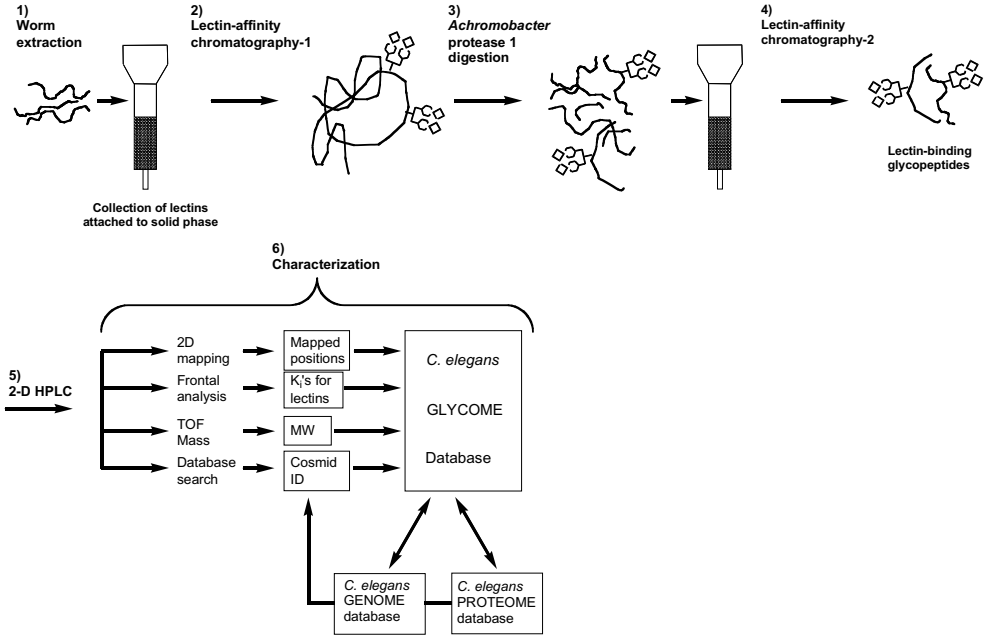


Figure 8. The glyco-catch procedure: (1) extraction of glycoprotein; (2) lectin affinity-1; (3) protease digestion; (4) lectin affinity-2; (5) purification by 2-D HPLC; (6) characterization of released isolated glycoproteins. Redrawn from Yamamoto et al.[2]

solid-phase microtiter plate assay, revealed that CEL-I has a higher affinity for *N*-acetyl-D-galactosamine compared to other galactose-related carbohydrates.

Other recent examples of lectin derived glycoaffinity chromatography and biological recognition have been reported by Ujita et al.,[150] Mann et al.,[151] and Caron et al.[152]

A different application of carbohydrate–protein interaction for purification is the isolation of starch branching enzyme I (SBE-I) [EC 2.4.1.18] from potato.[153] Here columns were prepared with agarose gel beads (divinyl sulfone activated agarose) substituted γ-cyclodextrin (γ-CD). CDs do not serve as substrates or are very poor substrates for most starch-metabolizing enzymes. However, strong binding of CDs may occur in the active site of the enzyme or at specific binding domains. The results from the study showed that potato SBE-I binds very strongly to the γ-CD matrix, whereas amylases and other hydrolytic enzymes was removed from the column by the washing step. This resulted in a 296-fold overall purification of the SBE-I.

Rosenfeld et al.[135] have discussed the properties of different solid supports used in affinity chromatography and how attachment of lectins influences surface properties. The supports (either tailor-made or commercially available) studied were analyzed regarding immobilization kinetics and isotherms. The conclusions were that the tailored supports based on silica or cellulose are better than commercially available polymeric supports (Toyopearl AF-Tresyl and Eupergit C). However, if non-specific adsorption of the lectin has to be at a minimum, Toyopearl appears to be the better option. Using voluminous lectins such as Con A (M_r 108 kDa), spacer molecules between the solid support and the lectin proved to be helpful.

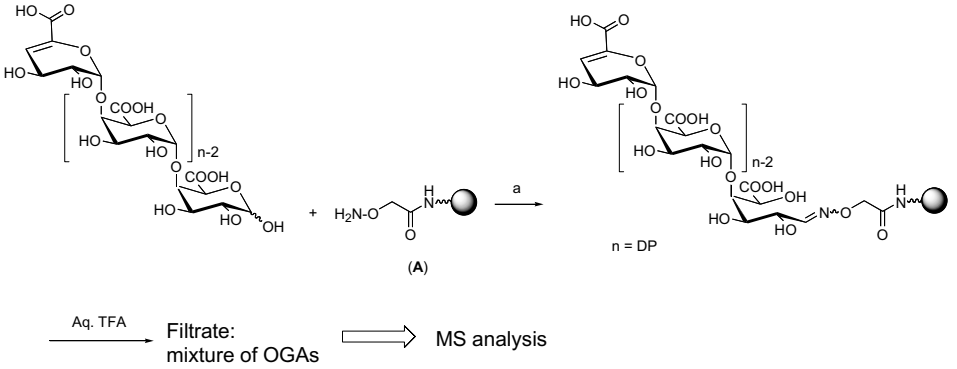
10. Toward solid-supported sequencing

The development of automated sequencing techniques for proteins and nucleic acids has very markedly contributed to the advancement of biochemistry. For instance, solid-phase methodologies routinely provide direct information on protein sequences. However, there is no equivalent to the peptide sequencing by the Edman degradation for poly- and oligosaccharides. Important among possible reasons for this are the higher structural complexity and diversity of polysaccharides compared to proteins and the different nature of linkage between monomers. A natural step toward the development of new tools for the sequencing of carbohydrates would be the use of a solid-phase approach combined with suitable conditions for the partial and repetitive degradation of an immobilized carbohydrate. Initial steps toward this were made by Guillaumie et al., using pectin oligogalacturonides as model substrates.[33] GalA oligomers of defined sizes were first chemoselectively immobilized onto amino-oxy-terminated PEGA supports through the reducing end, resulting in the formation of an oxime linkage. The OGA-functionalized supports were subsequently treated with aq. TFA at 40 or 60 °C, in an attempt to partially cleave the immobilized OGA and to release smaller hydrolysis fragments in solution (Scheme 11).

The use of a solid-phase approach facilitated isolation of these by filtration and the filtrate composition was analyzed by ESI-MS. The results showed that the applied conditions allowed for partial cleavage of the oligosaccharide into shorter products. Despite the lack of perfect stability of the oxime linkage under the hydrolysis conditions, a control experiment in solution confirmed that smaller oligosaccharides resulted primarily from the chemical degradation of immobilized substrate. These data illustrate the basic principles underlying a strategy that may prove important toward the sequencing of oligosaccharides.

11. Overview and conclusions

Solid-phase tools offer the prospect for fast, parallel analysis in glycobiology. As carbohydrates occurring



Scheme 11. General strategy for the preparation and use of pectin oligogalacturonide-functionalized solid-supports toward sequence analysis. Reagents and conditions: (a) Water–DMF (1:1), pH 4.8, 40 °C, 48 h.

in nature are presented multivalently, solid-supported techniques displaying multiple copies of the same carbohydrate could thus mimic this and be particularly well suited for studying many carbohydrate–protein interactions.

Solid-phase tools for glycobiology include classical affinity column matrices, oligosaccharides immobilized in microtiter plates, as well as oligosaccharides immobilized to supports (such as PEGA) or to nanoparticles, and carbohydrate arrays. Central to this is the nature of the solid phase and the nature of linkage to the support. While non-covalent immobilization methods can be convenient from an operational point of view and can be compatible with complex oligosaccharides and conjugates from natural sources, their use is limited due to the non-oriented immobilization and the lability of the anchoring. Some methods for covalent anchoring will give random immobilization but most methods enable chemoselective anchoring and oriented display of the carbohydrate. Among the chemoselective methods, amide and oxime formation, Huisgen 1,3-dipolar cycloaddition, and thiazolidine formation are especially useful.

Carbohydrate arrays offer the opportunity for parallel and fast analysis as well as the prospect of miniaturization with reduction of sample and reagent volumes. Microtiter plates allow the use of standard equipment but are not compatible with true miniaturization. Carbohydrate microarrays are very promising but are still early in their development. Carbohydrates immobilized to specialized matrices, such as Sepharose and PEGA, are also very useful tools for a range of applications. Gold nanoparticles and quantum dots offer unique opportunities, maybe especially for in vivo applications.

In most applications it is crucial that the displayed carbohydrate can interact with biomacromolecules, especially proteins. Thus, the interaction with the biomacromolecule should not be hampered by proximity to the solid support. A suitable linker, such as PEG spacers, can provide distance and flexibility. Some matrices, such as the PEG-based cross-linked PEGA support, have the flexibility of the spacer moiety built-in.

Applications have ranged from affinity chromatography, monitoring of glycosyltransferase and glycosylhydrolase activity, screening of lectin binding, studies on bacterial adhesion, studies on carbohydrate-binding activity of proteins, chemo-enzymatic synthesis of oligosaccharides and glycopeptides, purification of small amounts of oligosaccharides (glycoblotting), studies on carbohydrate–carbohydrate recognition, bio-imaging, and initial steps toward solid-supported sequencing. For future applications, it will be very important to not just monitor by binding, for example, in binding of a lectin to a carbohydrate, but also to analyze the processed oligosaccharide directly. Furthermore, methods for label-free detection will be useful.

Solid-phase tools are thus emerging as powerful tools for glycobiology. However, in contrast to oligonucleotides, carbohydrates can still not be synthesized in situ on the array. This, and a general lack of pure oligosaccharides isolated from natural sources, limits the chemical diversity of current carbohydrate arrays.

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References

1. Koeller, K. M.; Wong, C. H. *Nat. Biotechnol.* **2000**, *18*, 835–841.
2. Hirabayashi, J.; Arata, Y.; Kasai, K. *Proteomics* **2001**, *1*, 295–303.
3. Feizi, T. *Glycoconjugate J.* **2000**, *17*, 553–565.
4. Yarema, K. J.; Bertozzi, C. R. *Curr. Opin. Chem. Biol.* **1998**, *2*, 49–61.
5. Varki, A. *Glycobiology* **1993**, *3*, 97–130.
6. Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754–2794.
7. Houseman, B. T.; Mrksich, M. *Chem. Biol.* **2002**, *9*, 443–454.
8. Smith, E. A.; Thomas, W. D.; Kiessling, L. L.; Corn, R. M. *J. Am. Chem. Soc.* **2003**, *125*, 6140–6148.
9. Willats, W. G. T. Microarrays for the analysis of carbohydrate–protein interactions. In *Protein Microarrays*; Schena, M., Ed.; Jones and Bartlett, 2004; pp 57–70.
10. Shao, M. C. *Anal. Biochem.* **1992**, *205*, 77–82.
11. Shao, M. C.; Chin, C. C. Q. *Anal. Biochem.* **1992**, *207*, 100–105.
12. Bovin, N. V. *Glycoconjugate J.* **1998**, *15*, 431–446.
13. Bryan, M. C.; Plettenburg, O.; Sears, P.; Rabuka, D.; Wacowich-Sgarbi, S.; Wong, C.-H. *Chem. Biol.* **2002**, *9*, 713–720.
14. Fazio, F.; Bryan, M. C.; Blixt, O.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **2002**, *124*, 14397–14402.
15. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2021.
16. Fréchet, J. M.; Schuerch, C. *J. Am. Chem. Soc.* **1971**, *93*, 492–496.
17. Roberge, J. Y.; Beebe, X.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1998**, *120*, 3915–3927.
18. Di Fabio, G.; De Capua, A.; De Napoli, L.; Montesarchio, D.; Piccialli, G.; Rossi, F.; Benedetti, E. *Synlett* **2001**, *3*, 341–344.
19. Tolborg, J. F.; Jensen, K. J. *Chem. Commun.* **2000**, 147–148.
20. Haase, W.-C.; Seeberger, P.-H. *Curr. Org. Chem.* **2000**, *4*, 481–511.
21. Baues, R. J.; Gray, G. R. *J. Biol. Chem.* **1977**, *252*, 57–60.
22. Matsumoto, I.; Ito, Y.; Seno, N. *J. Chromatogr., A* **1982**, *239*, 747–754.
23. Yuan, S. M.; Cai, W. Z.; Szakalasgratzl, G.; Kottkemarchant, K.; Tweden, K.; Marchant, R. E. *J. Appl. Biomater.* **1995**, *6*, 259–266.
24. Seppällä, I.; Mäkelä, O. *J. Immunol.* **1989**, *143*, 1259–1264.

25. Satoh, A.; Kojima, K.; Koyama, T.; Ogawa, H.; Matsumoto, I. *Anal. Biochem.* **1998**, *260*, 96–102.
26. Guillaumie, F.; Thomas, O. R. T.; Jensen, K. J. *Bioconjugate Chem.* **2002**, *13*, 285–294.
27. Lee, M.; Shin, I. *Org. Lett.* **2005**, *7*, 4269–4272.
28. Vila-Perelló, M.; Gallego, R. G.; Andreu, D. *ChemBioChem* **2005**, *6*, 1831–1838.
29. Satoh, A.; Fukui, E.; Yoshino, S.; Shinoda, M.; Kojima, K.; Matsumoto, I. *Anal. Biochem.* **1999**, *275*, 231–235.
30. Peri, F.; Dumy, P.; Mutter, M. *Tetrahedron* **1998**, *54*, 12269–12278.
31. Langenhan, J. M.; Griffith, B. R.; Thorson, J. S. *J. Nat. Prod.* **2005**, *68*, 1696–1711.
32. Guillaumie, F.; Sterling, J. D.; Jensen, K. J.; Thomas, O. R. T.; Mohnen, D. *Carbohydr. Res.* **2003**, *338*, 1951–1960.
33. Guillaumie, F.; Justesen, S. F. L.; Mutenda, K. E.; Roepstorff, P.; Jensen, K. J.; Thomas, O. R. T. *Carbohydr. Res.* **2006**, *341*, 118–129.
34. Blomberg, L.; Wieslander, J.; Norberg, T. *J. Carbohydr. Chem.* **1993**, *12*, 265–276.
35. Quétard, C.; Bourgerie, S.; Normand-Sdiqui, N.; Mayer, R.; Strecker, G.; Midoux, P.; Roche, A.-C.; Monsigny, M. *Bioconjugate Chem.* **1998**, *9*, 268–276.
36. Liu, X. D.; Tokura, S.; Nishi, N.; Sakairi, N. *Polymer* **2003**, *44*, 1021–1026.
37. Porath, J. General methods and coupling procedures. In *Methods in Enzymology*; Jakoby, W. B., Wilchek, M., Eds.; Academic Press: New York, 1974; Vol. 34, pp 13–30.
38. Disney, M. D.; Seeberger, P. H. *Chem. Biol.* **2004**, *11*, 1701–1707.
39. Adams, E. W.; Ratner, D. M.; Bokesch, H. R.; McMahon, J. B.; O'Keefe, B. R.; Seeberger, P. H. *Chem. Biol.* **2004**, *11*, 875–881.
40. Bryan, M. C.; Fazio, F.; Lee, H.-K.; Huang, C.-Y.; Chang, A.; Best, M. D.; Calarese, D. A.; Blixt, O.; Paulson, J. C.; Burton, D.; Wilson, I. A.; Wong, C.-H. *J. Am. Chem. Soc.* **2004**, *126*, 8640–8641.
41. Adinolfi, M.; Barone, G.; De Napoli, L.; Iadonisi, A.; Piccialli, G. *Tetrahedron Lett.* **1998**, *39*, 1953–1956.
42. Zheng, C. S.; Seeberger, P. H.; Danishefsky, S. J. *J. Org. Chem.* **1998**, *63*, 1126–1130.
43. Hunt, J. A.; Roush, W. R. *J. Am. Chem. Soc.* **1996**, *118*, 9998–9999.
44. Ito, Y.; Ogawa, T. *J. Am. Chem. Soc.* **1997**, *119*, 5562–5566.
45. Rademann, J.; Schmidt, R. R. *J. Org. Chem.* **1997**, *62*, 3650–3653.
46. Douglas, S. P.; Whitfield, D. M.; Krepinsky, J. J. *J. Am. Chem. Soc.* **1995**, *117*, 2116–2117.
47. Hodosi, G.; Krepinsky, J. J. *Synlett* **1996**, 159–162.
48. Petersen, L.; Jensen, K. J. *J. Chem. Soc., Perkin Trans. 1* **2001**, 2175–2182.
49. Blixt, O.; Norberg, T. *Carbohydr. Res.* **1999**, *319*, 80–91.
50. Blixt, O.; Norberg, T. *J. Org. Chem.* **1998**, *63*, 2705–2710.
51. Plante, O. J.; Palmacci, E. R.; Seeberger, P. H. *Science* **2001**, *291*, 1523–1527.
52. Jensen, K. J.; Alsina, J.; Songster, M. F.; Vágner, J.; Albericio, F.; Barany, G. *J. Am. Chem. Soc.* **1998**, *120*, 5441–5452.
53. Tolborg, J. F.; Petersen, L.; Jensen, K. J.; Mayer, C.; Jakeman, D. L.; Warren, R. A. J.; Withers, S. G. *J. Org. Chem.* **2002**, *67*, 4143–4149.
54. Dische, Z. *Methods Carbohydr. Chem.* **1964**, *1*, 478–481.
55. Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
56. Svennerholm, L. *J. Neurochem.* **1956**, *1*, 42–53.
57. Gervay, J.; McReynolds, K. D. *Curr. Med. Chem.* **1999**, *6*, 129–153.
58. Patil, A. R.; Misquith, S.; Dam, T. K.; Sharma, V.; Kapoor, M.; Surolia, A. *Methods Enzymol.* **2003**, *362*, 567–583.
59. Supplied by Nunc or Corning.
60. Duk, M.; Lisowska, E.; Wu, J. H.; Wu, A. M. *Anal. Biochem.* **1994**, *221*, 266–272.
61. Chen, C. P.; Song, S.-C.; Gilboa-Garber, N.; Chang, K. S. S.; Wu, A. M. *Glycobiology* **1998**, *8*, 7–16.
62. Maxisorp from Nunc.
63. Lisowska, E. In *Molecular Immunology of Complex Carbohydrates*; Plenum: New York/London, 1988.
64. Khraltsova, L. S.; Sablina, M. A.; Melikhova, T. D.; Joziasse, D. H.; Kaltner, H.; Gabius, H. J.; Bovin, N. V. *Anal. Biochem.* **2000**, *280*, 250–257.
65. Scharfman, A.; Delmotte, P.; Beau, J.; Lamblin, G.; Roussel, P.; Mazurier, J. *Glycoconjugate J.* **2000**, *17*, 735–740.
66. Fazio, F.; Bryan, M. C.; Lee, H. K.; Chang, A.; Wong, C. H. *Tetrahedron Lett.* **2004**, *45*, 2689–2692.
67. Stults, C. L. M.; Wilbur, B. J.; Macher, B. A. *Anal. Biochem.* **1988**, *174*, 151–156.
68. Stults, C. L. M.; Macher, B. A. *Arch. Biochem. Biophys.* **1990**, *280*, 20–26.
69. Stults, C. L. M.; Macher, B. A. *Arch. Biochem. Biophys.* **1993**, *303*, 125–133.
70. Crawley, S. C.; Hindsgaul, O.; Alton, G.; Pierce, M.; Palcic, M. M. *Anal. Biochem.* **1990**, *185*, 112–117.
71. Yan, L. Y.; Smith, D. F.; Cummings, R. D. *Anal. Biochem.* **1994**, *223*, 111–118.
72. Nogrady, N.; Pocsi, I.; Paffard, S. M.; Katona, E.; Miles, R. J.; Balazs, A.; Sandor, E.; Fachet, J.; Price, R. G.; Szentirmai, A. *J. Biotechnol.* **1998**, *60*, 15–22.
73. Oubihi, M.; Kitajima, K.; Kobayashi, K.; Adachi, T.; Aoki, N.; Matsuda, T. *Anal. Biochem.* **1998**, *257*, 169–175.
74. Han, N. S.; Tao, B. Y. *Enzyme Microb. Technol.* **1999**, *24*, 35–40.
75. Palcic, M. M.; Sujino, K. *Trends Glycosci. Glycotechnol.* **2001**, *13*, 361–370.
76. Baek, M.-G.; Roy, R. *Biomacromolecules* **2000**, *1*, 768–770.
77. Hatakeyama, T.; Murakami, K.; Miyamoto, Y.; Yamasaki, N. *Anal. Biochem.* **1996**, *237*, 188–192.
78. Dove, A. *Nat. Biotechnol.* **2001**, *19*, 913–917.
79. Shin, I.; Park, S.; Lee, M. R. *Chem. Eur. J.* **2005**, *11*, 2894–2901.
80. Mellet, C. O.; Fernandez, J. M. G. *ChemBioChem* **2002**, *3*, 819–822.
81. Feizi, T.; Fazio, F.; Chai, W. C.; Wong, C. H. *Curr. Opin. Struct. Biol.* **2003**, *13*, 637–645.
82. Love, K. R.; Seeberger, P. H. *Angew. Chem., Int. Ed.* **2002**, *41*, 3583–3586.
83. Raman, R.; Raguram, S.; Venkataraman, G.; Paulson, J. C.; Sasisekharan, R. *Nat. Methods* **2005**, *2*, 817–824.
84. Dyukova, V. I.; Shilova, N. V.; Galanina, O. E.; Rubina, A. Y.; Bovin, N. V. *Biochim. Biophys. Acta* **2006**, *1760*, 603–609.
85. Wilson, D. S.; Nock, S. *Curr. Opin. Chem. Biol.* **2001**, *6*, 81–85.
86. Wang, D.; Liu, S.; Trummer, B. J.; Deng, C.; Wang, A. *Nat. Biotechnol.* **2002**, *20*, 234–235.
87. 'FastSlides', www.schleicher-schuell.com.

88. Willats, W. G. T.; Rasmussen, S. V.; Kristensen, T.; Mikkelsen, J. D.; Knox, J. P. *Proteomics* **2002**, *2*, 1666–1671.
89. www.nuncbrand.com.
90. Fukui, S.; Feizi, T.; Galustian, C.; Lawson, A. M.; Chai, W. *Nat. Biotechnol.* **2002**, *20*, 1011–1017.
91. Galanina, O. E.; Mecklenburg, M.; Nifantiev, N. E.; Pazynina, G. V.; Bovin, N. V. *Lab Chip* **2003**, *3*, 260–265.
92. Bryan, M. C.; Lee, L. V.; Wong, C.-H. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3185–3188.
93. Park, S.; Shin, I. *Angew. Chem., Int. Ed.* **2002**, *41*, 3180–3181.
94. Park, S.; Lee, M.; Pyo, S.-J.; Shin, I. *J. Am. Chem. Soc.* **2004**, *126*, 4812–4819.
95. Blixt, O.; Head, S.; Mondala, T.; Scanlan, C.; Huflejt, M. E.; Alvarez, R.; Bryan, M. C.; Fazio, F.; Calarese, D.; Stevens, J.; Razi, N.; Stevens, D. J.; Skehel, J. J.; van Die, I.; Burton, D. R.; Wilson, I. A.; Cummings, R.; Bovin, N.; Wong, C.-H.; Paulson, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17033–17038.
96. Disney, M. D.; Magnet, S.; Blanchard, J. S.; Seeberger, P. H. *Angew. Chem., Int. Ed.* **2004**, *43*, 1591–1594.
97. Adams, E. W.; Ueberfeld, J.; Ratner, D. M.; O'Keefe, B. R.; Walt, D. R.; Seeberger, P. H. *Angew. Chem., Int. Ed.* **2003**, *42*, 5317–5320.
98. de Paz, J.-L.; Noti, C.; Seeberger, P. H. *J. Am. Chem. Soc.* **2006**.
99. Su, J.; Mrksich, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 4715–4718.
100. Shen, Z.; Go, E. P.; Gamez, A.; Apon, J. V.; Fokin, V.; Greig, M.; Ventura, M.; Crowell, J. E.; Blixt, O.; Paulson, J. C.; Stevens, R. C.; Finn, M. G.; Siuzdak, G. *ChemBioChem* **2004**, *5*, 921–927.
101. Arnosti, C. *J. Chromatogr., B.* **2003**, *793*, 181–191.
102. Vivès, R. R.; Crublet, E.; Andrieu, J.-P.; Gagnon, J.; Rousselle, P.; Lortat-Jacob, H. *J. Biol. Chem.* **2004**, *279*, 54327–54333.
103. Yao, N.-H.; He, W.-Y.; Lam, K. S.; Liu, G. *J. Comb. Chem.* **2005**, *7*, 123–129.
104. Benters, R.; Niemeyer, C. M.; Wohrle, D. *ChemBioChem* **2001**, *2*, 686–694.
105. Nishimura, S.-I.; Niikura, K.; Kurogochi, M.; Matsushita, T.; Fumoto, M.; Hinou, H.; Kamitani, R.; Nakagawa, H.; Deguchi, K.; Miura, N.; Monde, K.; Kondo, H. *Angew. Chem., Int. Ed.* **2005**, *44*, 91–96.
106. Niikura, K.; Kamitani, R.; Kurogochi, M.; Uematsu, R.; Shinohara, Y.; Nakagawa, H.; Deguchi, K.; Monde, K.; Kondo, H.; Nishimura, S.-I. *Chem. Eur. J.* **2005**, *11*, 3825–3834.
107. Barrientos, Á. G.; de la Fuente, J. M.; Rojas, T. C.; Fernández, A.; Penadés, S. *Chem. Eur. J.* **2003**, *9*, 1909–1921.
108. de la Fuente, J. M.; Barrientos, Á. G.; Rojas, T. C.; Rojo, J.; Cañada, J.; Fernández, A.; Penadés, S. *Angew. Chem., Int. Ed.* **2001**, *40*, 2258–2261.
109. Brust, M.; Walker, M.; Bethell, D.; Schiffrin, D. J.; Whyman, R. *J. Chem. Soc., Chem. Commun.* **1994**, 801–802.
110. de Paz, J.-L.; Ojeda, R.; Barrientos, Á. G.; Penadés, S.; Martín-Lomas, M. *Tetrahedron: Asymmetry* **2005**, *16*, 149–158.
111. de Souza, A. C.; Halkes, K. M.; Meeldijk, J. D.; Verkleij, A. J.; Vliegenthart, J. F. G.; Kamerling, J. P. *Eur. J. Org. Chem.* **2004**, 4323–4339.
112. Svarovsky, S. A.; Szekely, Z.; Barchi, J. J. *Tetrahedron: Asymmetry* **2005**, *16*, 587–598.
113. Chen, Y.-J.; Chen, S.-H.; Chien, Y.-Y.; Chang, Y.-W.; Liao, H.-K.; Chang, C.-Y.; Jan, M.-D.; Wang, K.-T.; Lin, C.-C. *ChemBioChem* **2005**, *6*, 1169–1173.
114. Rojo, J.; Díaz, V.; de la Fuente, J. M.; Segura, I.; Barrientos, Á. G.; Riese, H. H.; Bernade, A.; Penadés, S. *ChemBioChem* **2004**, *5*, 291–297.
115. Enüstün, B. V.; Turkevich, J. *J. Am. Chem. Soc.* **1963**, *85*, 3317–3328.
116. Turkevich, J.; Stevenson, P. C.; Hillier, J. *J. Phys. Chem.* **1953**, *57*, 670–673.
117. Hone, D. C.; Haines, A. H.; Russell, D. A. *Langmuir* **2003**, *19*, 7141–7144.
118. Tamura, J.; Fukuda, M.; Tanaka, J.; Kawa, M. *J. Carbohydr. Chem.* **2002**, *21*, 445–449.
119. de la Fuente, J. D. M.; Penadés, S. *Tetrahedron: Asymmetry* **2005**, *16*, 387–391.
120. Robinson, A.; Fang, J.-M.; Chou, P.-T.; Liao, K.-W.; Chu, R.-M.; Lee, S.-J. *ChemBioChem* **2005**, *6*, 1899–1905.
121. Otsuka, H.; Akiyama, Y.; Nagasaki, Y.; Kataoka, K. *J. Am. Chem. Soc.* **2001**, *123*, 8226–8230.
122. Takae, S.; Akiyama, Y.; Otsuka, H.; Nakamura, T.; Nagasaki, Y. K. K. *Biomacromolecules* **2005**, *6*, 818–824.
123. Karamanska, R.; Mukhopadhyay, B.; Russell, D. A.; Field, R. A. *Chem. Commun.* **2005**, 3334–3336.
124. Lin, C.-C.; Yeh, Y.-C.; Yang, C.-Y.; Chen, G.-F.; Chen, Y.-C.; Wu, Y.-C.; Chen, C.-C. *Chem. Commun.* **2003**, 2920–2921.
125. Tsai, C.-S.; Yu, T.-B.; Chen, C.-T. *Chem. Commun.* **2005**, *34*, 4273–4275.
126. Halkes, K. M.; de Souza, A. C.; Maljaars, C. E. P.; Gerwig, G. J.; Kamerling, J. P. *Eur. J. Org. Chem.* **2005**, 3650–3659.
127. Rojas, T. C.; de la Fuente, J. M.; Barrientos, Á. G.; Penadés, S.; Ponsonnet, L.; Fernández, A. *Adv. Mater. (Weinheim, Germany)* **2002**, *14*, 585–588.
128. Tromas, C.; Rojo, J.; de la Fuente, J. M.; Barrientos, Á. G.; García, R.; Penadés, S. *Angew. Chem., Int. Ed.* **2001**, *40*, 3052–3055.
129. Hernáiz, M. J.; de la Fuente, J. M.; Barrientos, Á. G.; Penadés, S. *Angew. Chem., Int. Ed.* **2002**, *41*, 1554–1557.
130. de la Fuente, J. M.; Eaton, P.; Barrientos, Á. G.; Menéndez, M.; Penadés, S. *J. Am. Chem. Soc.* **2005**, *127*, 6192–6197.
131. de la Fuente, J. M.; Penadés, S. *Glycoconjugate J.* **2004**, *21*, 149–163.
132. Reynolds, A. J.; Haines, A. H.; Russell, D. A. *Langmuir* **2006**, *22*, 1156–1163.
133. de Souza, A. C.; Halkes, K. M.; Meeldijk, J. D.; Verkleij, A. J.; Vliegenthart, J. F. G.; Kamerling, J. P. *ChemBioChem* **2005**, *6*, 828–831.
134. Lin, C.-C.; Yeh, Y.-C.; Yang, C.-Y.; Chen, C.-L.; Chen, G.-F.; Chen, C.-C.; Wu, Y.-C. *J. Am. Chem. Soc.* **2002**, *124*, 3508–3509.
135. Rosenfeld, H.; Aniulyte, J.; Helmholz, H.; Liesiene, J.; Thiesen, P.; Niemeyer, B.; Prange, A. *J. Chromatogr., A* **2005**, *1092*, 76–88.
136. Berteau, O.; Stenutz, R. *Carbohydr. Res.* **2004**, *339*, 929–936.
137. Boraston, A. B.; Bolam, D. N.; Gilbert, H. J.; Davies, G. J. *Biochem. J.* **2004**, *382*, 769–781.
138. Kennedy, J. F.; Palva, P. M. G.; Corella, M. T. S.; Cavalcanti, M. S. M.; Coelho, L. *Carbohydr. Polym.* **1995**, *26*, 219–230.
139. Gabius, H. J.; Siebert, H. C.; Andre, S.; Jimenez-Barbero, J.; Rudiger, H. *ChemBioChem* **2004**, *5*, 741–764.
140. Hirabayashi, J. *Trends Glycosci. Glycotech.* **2004**, *16*, 63–85.

141. Yamamoto, K.; Tsuji, T.; Osawa, T. *Mol. Biotechnol.* **1995**, *3*, 25–36.
142. Kornfeld, R.; Kornfeld, S. *Annu. Rev. Biochem.* **1985**, *54*, 631–664.
143. Hirabayashi, J.; Kasai, K. *J. Chromatogr., B* **2002**, *771*, 67–87.
144. Hirabayashi, J.; Hayama, K.; Kaji, H.; Isobe, T.; Kasai, K. *J. Biochem.* **2002**, *132*, 103–114.
145. Hirabayashi, J. *Glycoconjugate J.* **2004**, *21*, 35–40.
146. Absar, N.; Yeasmin, T.; Raza, M. S.; Sarkar, S. K.; Arisaka, F. *Protein J.* **2005**, *24*, 369–377.
147. Kaur, A.; Singh, J.; Kamboj, S. S.; Sexana, A. K.; Pandita, R. M.; Shamnugavel, M. *Phytochemistry* **2005**, *66*, 1933–1940.
148. Soerensen, C. M.; Nielsen, O. L.; Willis, A.; Heegaard, P. M. H.; Holmskov, U. *Immunology* **2005**, *114*, 72–82.
149. Hatakeyama, T.; Matsuo, N.; Shiba, K.; Nishinohara, S.; Yamasaki, N.; Sugawara, H.; Aoyagi, H. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 157–163.
150. Ujita, M.; Sakai, K.; Hamazaki, K.; Yoneda, M.; Isomura, S.; Hara, A. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 2402–2407.
151. Mann, K.; Weiss, I. M.; Andre, S.; Gabius, H. J.; Fritz, M. *Eur. J. Biochem.* **2000**, *267*, 5257–5264.
152. Caron, M.; Seve, A. P.; Bladier, D.; Joubert-Caron, R. *J. Chromatogr., B* **1998**, *715*, 153–161.
153. Viksø-Nielsen, A.; Blennow, A. *J. Chromatogr., A* **1998**, *800*, 382–385.