DOI: 10.1002/cbic.200700788

A Versatile Gold Surface Approach for Fabrication and Interrogation of Glycoarrays

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Glycans attached to proteins and lipids are known to act as regulators of various aspects of cell behaviour. They exhibit high levels of structural diversity, which dictates the functional specificity of their many activities, achieved principally through interactions with proteins. An understanding of the cell's glycome–protein interactions complements data obtained from studies of the proteome and represents an essential facet of post-genome technology development that should underpin the discovery of novel glycotherapeutics and biomarkers. The inherent structural complexity and limited availability of cell-expressed sugars is now driving the development of glycomics approaches for studying bioactive carbohydrates.[1] Major tools in this respect are surface binding-based interrogation techniques including fluorescence microarrays,[2] surface plasmon resonance (SPR) biosensors,[3] and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-ToF-MS).[4] A combination of these interrogation techniques is a very effective tool for elucidating binding patterns, kinetics, affinities, and specificities of these molecular interactions. We reasoned that an appropriately designed terminally functionalised self-assembled monolayer (SAM) on a gold surface could have advantageous features as a generic platform for glycan attachment and binding interrogation by this diverse range of techniques. To achieve this goal, reliable surface chemistries that allow effective covalent attachment of glycans with minimal nonspecific protein binding are central requirements.

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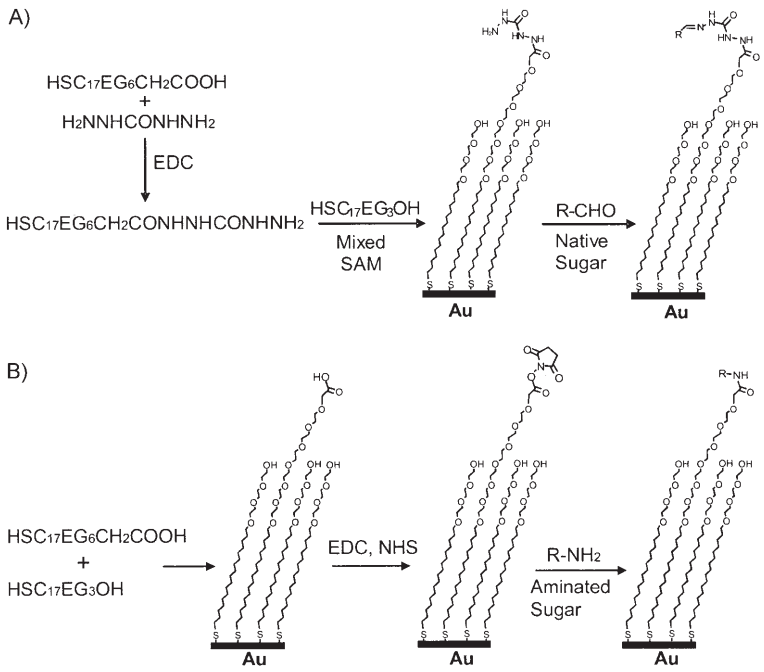
For covalent attachment, three general strategies have been used for attachment of sugars onto gold or glass surfaces, with no single method suited to all types of sugars. The first strategy is based on the synthesis of thiol-terminated sugar derivatives and subsequent formation of a SAM.[5] The second is based on the introduction of sugar moieties onto prefunctionalised surfaces through the use of chemoselective coupling reactions, including thiol addition to a maleimide functional group,[6] Diels–Alder cycloaddition,[7] disulfide exchange,[8] DNA hybridisation[9] and Huisgen 1,3-dipolar cycloaddition.[10] The principle disadvantage of the above two strategies is the requirement for significant derivatisation and purification of each individual probe, complicating platform fabrication and limiting accessibility of the technology to life science researchers.

The third option is based on the direct and chemoselective attachment of nonderivatised glycans to a surface functionalised with nucleophiles such as hydrazide,[11,12] amino-oxy[11c,13] or amino groups,[14,15] through formation of stable Schiff bases with the free reducing end aldehyde functional group. This strategy eliminates the need for sugar prederivatisation, though higher concentrations of spotting sugars[11b,c] are typically needed to drive immobilisation efficiency. Previous studies have demonstrated that mixed SAMs prepared with alkanethiolates incorporating oligo(ethylene glycol) groups display ideal properties in terms of reduced nonspecific protein absorption.[6b,7,16,17] Building on these approaches, here we demonstrate a versatile platform based on a common SAM-gold surface chemistry for displaying both nonderivatised and derivatised natural and synthetic oligosaccharides for diverse protein binding assays in chip formats (Scheme 1).

To develop the surface and linker chemistry we first used antibody-based on-chip fluorescence detection of protein binding to immobilised nonderivatised glycans. This fabrication procedure is based on the use of a carbohydrazide-derivatised hexa(ethylene glycol) alkanethiol construct formed into a mixed SAM with a tri(ethylene glycol) terminated C17 alkanethiol (Scheme 1A). For initial optimisation of monolayer structures (hydrazide surface coverage and alkyl chain length), microarrays displaying dilution series of heparin decasaccharides or oligomannose-5 (Man5) were interrogated with protein probes (the heparin-specific protein fibroblast growth factor 2 (FGF2) and the mannose-specific protein concanavalin A (Con A), respectively). Homogenous SAMs made with 100% hydrazide-terminated construct provided strong fluorescence spot signals for the sugars at several different dilutions (Figure 1A), but with elevated background binding similar to that observed with a 16-mercaptohexadecanoic acid (MHDA)-hydra-



Scheme 1. Surface modification chemistries for simple fabrication of gold surface glycoarrays. Optimised mixed C17 thiol SAMs on gold surfaces are prepared with appropriate terminal modifications to permit rapid immobilisation of either nonderivatised or derivatised glycans, and incorporate oligoethyleneglycol groups to minimise nonspecific binding. A) Attachment of nonderivatised glycans by hydrazide chemistry. B) Attachment of derivatised (aminated) glycans by succinimide ester chemistry. These surfaces offer multiple detection options including fluorescence microarrays and label-free biophysical methods such as SPR or MALDI-ToF MS.

zide linker[12] with no hexa(ethylene glycol) group present, demonstrating that the hexa(ethylene glycol) moiety alone is insufficient to reduce nonspecific binding. In contrast, inclusion of shorter tri(ethylene glycol)-terminated C17 thiols as dilutors to produce mixed SAMs permitted adjustment of the hydrazide coverage. Dilution to 60% hydrazide construct relative to total alkanethiolate produced optimal microarray signals, whereas a surface with 40% hydrazide coverage or below resulted in much reduced specific signals (Figures 1A and C).

To promote formation of a densely packed monolayer we introduced a long alkyl chain. We found that C17 thiol-based monolayers provided improved fluorescence signals and lower background and noise than C11-thiols (Figures 1B and D); this suggests differences in their molecular packing into a structurally well defined monolayer. The improved performance of the C17 thiols was also confirmed by contact angle measurements of surface wettability, a useful parameter for evaluation of the quality of the monolayer[18] (see Figure S1A in the Supporting Information). A better ordered monolayer is also beneficial for overcoming fluorescence quenching on gold surfaces by providing improved insulation.[19] We found that the quenching effect was further minimised by application of a commonly used indirect detection format employing primary and labelled secondary antibodies. This "three-layer" approach significantly increases the distance between the gold surface and the fluorescent probes, though we observed that single-layer detection is also possible, but with reduced sensitivity. A comparison revealed that the three-layer format provided much stronger fluorescence signals (Figures 1B and D).

To examine specific and nonspecific protein adsorption onto the gold surfaces further, we utilised SPR to monitor binding of FGF2 to immobilised heparin decasaccharide and a control surface. SPR sensorgrams showed that a SAM with 100% hydrazide surface coverage gave strong binding signals when the sugar was present, but negative control experiments (surface without sugar attachment) demonstrated very strong nonspecific binding (Figure 2A). In contrast, when the tri(ethylene glycol)-terminated C17 thiol was employed as a "dilutor" to adjust the hydrazide surface coverage, nonspecific binding was minimised. A monolayer with 80 or 60% hydrazide surface coverage was found to provide optimal results, giving negligible binding (only "bulk shift" upon introduction of sample; Figure 2A). These data were supported by QCM measurements, which also demonstrated that 60 or 80% surface coverage results in reduced nonspecific binding (see Figures S1B, C). Further control experiments on binding of FGF2 to immobilised heparin decasaccharides in the presence of a 20-fold excess of heparin were carried out on surfaces with 60% hydrazide coverage, and very low nonspecific binding was observed (Figure 2B).

Collectively, these data show that mixed SAMs fabricated with optimised ratios of dilutor can be used to generate binding data for measurement of specific interactions between protein probes and immobilised glycans. Using these conditions we next demonstrated the utility of this approach for interrogation of glycan–protein interactions in a fluorescence microarray format, examining the binding of lectins and heparin-binding proteins to structurally diverse saccharides. Glycoarrays in slide format were fabricated with the aid of a standard pin-type contact arraying robot (~1 nL per spot), and binding of protein probes was detected with a standard fluorescent slide scanner. We first studied the specificity of binding of several lectins to a selection of model glycan ligands (for details of the sugar structures, see Table S1). Con A, either in three-layer (Figure 3A) or in one-layer (Figure 3B) detection formats, showed a strong preference for binding to α1–6 mannose oligosaccharides (6 and 7), as would be expected from its specificity for α-linked mannose residues[20a] (Figures 3A, B and S2). Similarly, wheat germ agglutinin (WGA), which binds to *N*-acetylglucosamine and sialic acid residues,[20b] displayed strong binding to α-2,3-sialylated and α-2,6-sialylated structures (**1** and **2**, respectively, Figure 3E; see also Figure S2). However, WGA showed only weak binding to 6′-sialyl-*N*-acetyllactosamine (**4**), and even weaker binding to LSTc (**3**) and 3′-sialyl-*N*-acetyllactosamine (**5**); this may be due to influences of sugar size and internal structures on the binding. We next also tested two sialic acid-specific binding immunoglobulin-like lectin proteins—human Siglec-7 (hSiglec-7) and murine Siglec-E (mSiglec-E)—using a precomplexed format (anti-Fc antibody precomplexing the Fc fusion component of

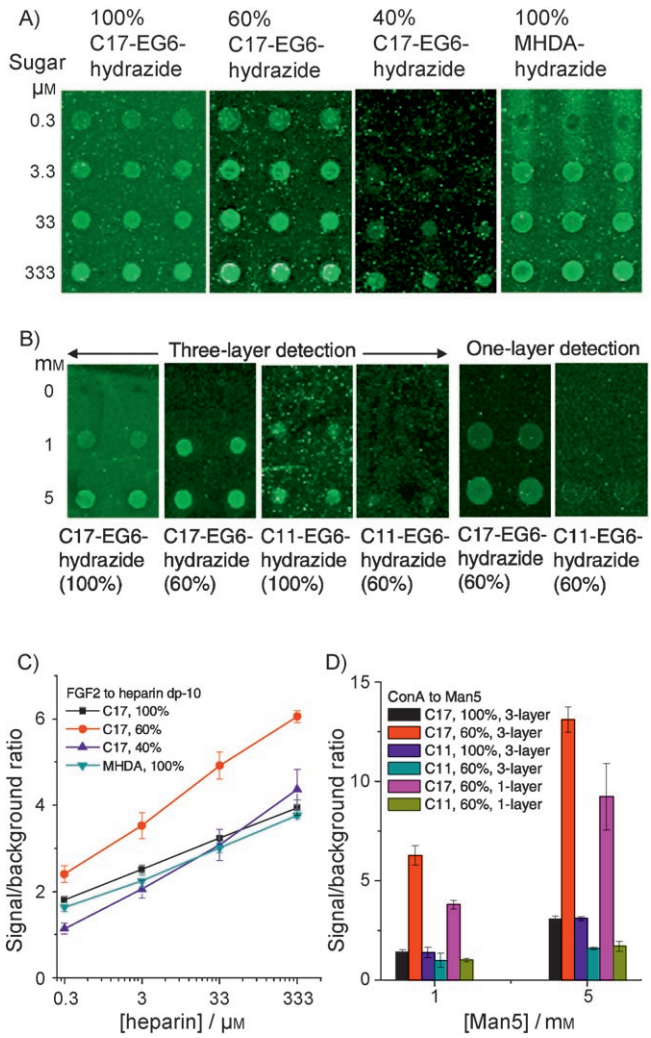


Figure 1. Optimisation of gold platform for fluorescence glycoarray fabrication. A) Binding of FGF2 to immobilised sugars (heparin dp-10, dp: degree of polymerisation) on hydrazide-terminated SAMs on gold as shown in Scheme 1 A. B) Comparison of one-layer (direct) and three-layer (indirect) detection formats for Con A to immobilised Man 5 on hydrazide-terminated SAM. FGF2: 100 nM, Con A: 50 nM (three-layer format), 200 nM (one-layer format). The slide with C11-EG6-hydrazide was noisy due to poor wettability of the surface. C) and D) Quantitative data analysis of the data in A and B. Note that false-colour images are shown for fluorescence microarrays.

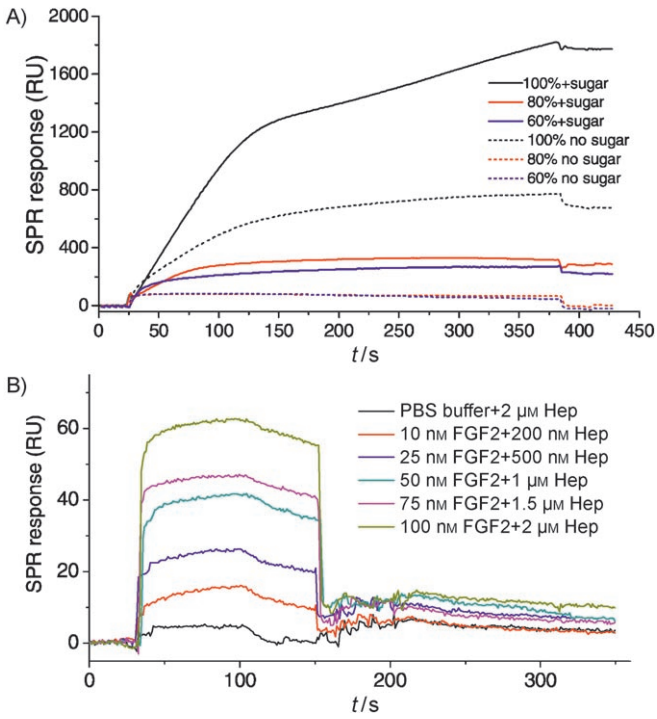


Figure 2. SPR characterisation of the hydrazide surface. Specific and nonspecific binding of FGF2 to an immobilised heparin saccharide (dp-10) was examined by SPR. A) Effect of hydrazide surface coverage on protein binding to surfaces with and without immobilised heparin (33 μM in 1 M betaine; 30 μL of 100 nM FGF2 was injected at 5 μL min^{-1}). B) Nonspecific binding of heparin-saturated FGF2 to the immobilised heparin (40 μL of 1–100 nM FGF2 saturated with a 20-fold excess of heparin dp–10 was injected at 20 μL min^{-1}).

the siglec constructs). The data showed that whereas mSiglec-E displayed more relaxed specificity, binding to 1, 2, and 4 (Figures 3 C and H), hSiglec-7 bound strongly to the branched α-2,6-sialylated structure (2), but more weakly to one of the unbranched α-2,6-sialylated glycans (4, Figures 3 D and G). The specificity data for these siglecs are consistent with those reported previously, with hSiglec-7 binding strongly to α-2,6-sialylated glycans,[21] but more weakly to α-2,3-sialylated structures.[21b,22] Our data on mSiglec-E are also consistent with previous findings that indicated similar binding to hSiglec-7,[22] but with a broader preference for binding α-2,3- and α-2,6-sialylated structures.[23] We noted that WGA, hSiglec-7, and mSiglec-E all showed some cross-reactivity to heparin decasaccharide at the higher spotting concentrations (~33–333 μM, Figures 3 C–E); it is feasible that *N*-acetylglucosamine residues, and also anionic sulfate groups,[23] may contribute to generation of significant affinity of these lectins for large heparin saccharides.

For microarrays with immobilised heparin saccharides we observed strong specific binding for known heparin-binding proteins such as the growth factors FGF1, FGF2 and glial cell line-derived neurotrophic factor (GDNF), with no binding to a negative control protein, Con A (Figure 3 F). Note that much lower spotting concentrations (~3–33 μM) are required, reflecting the higher affinity of heparin–protein interactions relative to *N*-linked glycans; heparin is known to bind specifically to these growth factors with low nanomolar affinities.[24] We have found that binding of protein targets such as FGF2 to heparin saccharides as small as tetrasaccharides can be examined with this microarray format (see Figure S3). Overall, this facile glycoarray approach should be particularly advantageous for rapid multiplex screening of large libraries of natural glycans against protein probes; such applications can readily be envisaged in the developing field of glycomics.

MALDI-ToF MS analysis of SAMs on gold surfaces[7,25–27] has recently emerged as an alternative, label-free method for probing bound molecules. In order to extend the versatility of our gold platform we also examined its application for MALDI-ToF MS analysis of immobilised glycans. For this application we utilised a second variant of the surface by functionalising it with activated succinimide esters in order to capture aminated sugars (Scheme 1 B). Aminated sugars are common products of

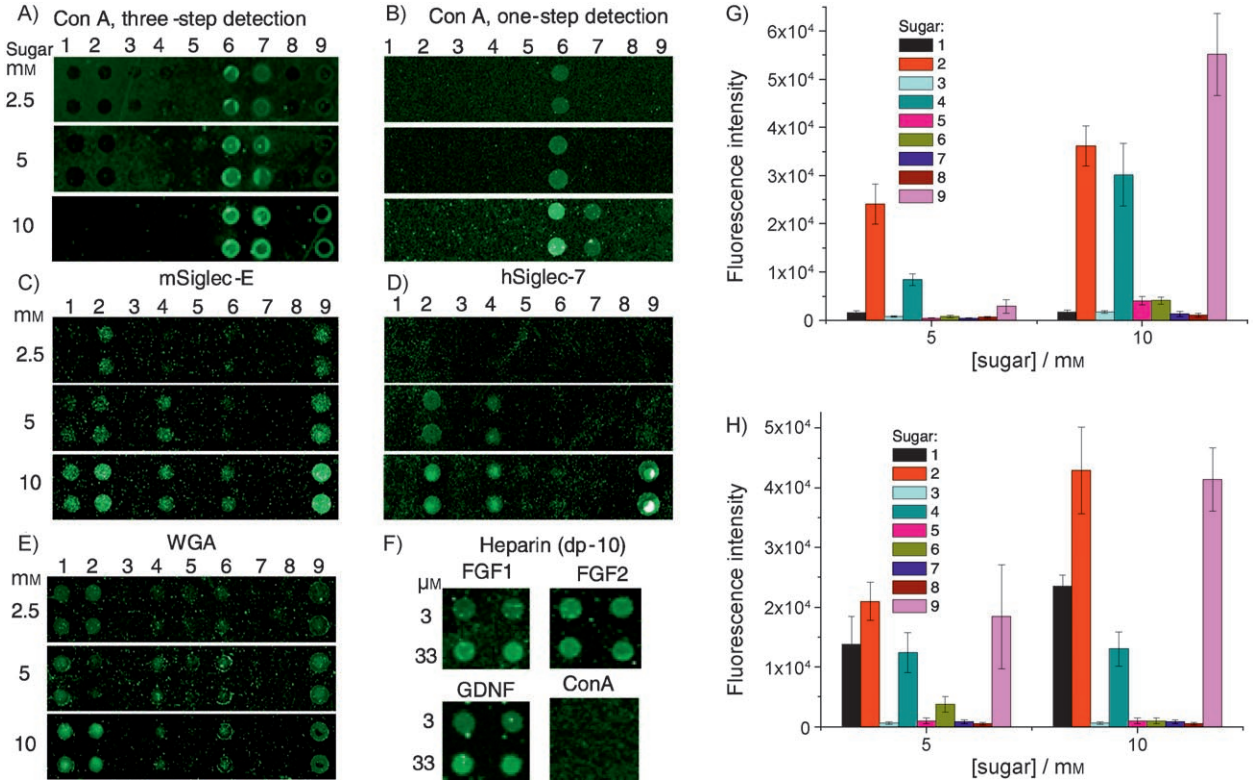


Figure 3. Interrogation of protein binding to immobilised glycans in fluorescence array format. Microarrays displaying immobilised *N*-linked glycans and a heparin decasaccharide were fabricated on a 60% hydrazide mixed SAM gold surface, and interrogated for binding of selected protein targets. A) and B) Con A; C) mSiglec-E; D) hSiglec-7; E) WGA; F) heparin-binding growth factors FGF1, FGF2, and GDNF (with Con A as negative control). G) and H) Quantitative data for detection of binding of hSiglec-7 and mSiglec-E to glycan ligands, respectively. Quantitative data for Con A and WGA are shown in Figure S2. Bar graphs of the data corresponding to the means of five spots ± SD are shown. Assays using the primary and secondary antibodies were also tested as the negative control to exclude the possibility of antibodies binding directly to the attached sugars. Glycans were printed in three twofold dilutions at the concentrations shown: 1) LSTa; 2) LSTb; 3) LSTc; 4) 6′-sialyl-*N*-acetyllactosamine; 5) 3′-sialyl-*N*-acetyllactosamine; 6) oligomannose-5; 7) α1-6-mannobiose; 8) α1-3-mannobiose; 9) heparin dp-10. Note that heparin was printed at 3, 33, and 333 μM. Only duplicate spot data for each glycan are given for clarity.

carbohydrate synthetic schemes and are readily available for array fabrication. 2-Aminoethyl glycosides were synthesised by known procedures.[28] Briefly, 2-*N*-benzyloxycarbonylethanolamine was glycosylated with *O*-acetylated glycosyl donors (acetate, trichloroacetimidate, or bromide) under standard conditions and purified by flash chromatography. Acetyl protecting groups were removed under Zemplen conditions (MeONa/MeOH), followed by *N*-Cbz cleavage by hydrogenolysis to afford the desired glycosides suitable for immobilisation. Initial experiments were carried out with the β-D-GlcNAc derivative. Immobilisation was performed by coating clean gold surfaces with a mixture of C17 alkanethiolates presenting either a carboxylic acid (suitable for functionalisation) or a tri(ethylene glycol) group (dilutor), in an optimal molar ratio of 50:50 for further enzymatic transformation.[24b] Activation of the carboxylic acid with a dimethylformamide (DMF) solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS)[29] allowed for immobilisation of the sugar through amide bond formation.

MALDI-ToF MS analysis of the resulting monolayer (Figure 4) indicated efficient immobilisation, the main signal detected being the mixed disulfide formed by the sugar-terminated alkanethiol and the tri(ethylene glycol) alkanethiol (*m/z* 1298; see also Figure 5E). Reproducibility was assessed by spotting the sugar in triplicate on the same gold-coated slide, and in all cases a strong signal at *m/z* 1298 was detected (data not shown).

Nine different sugars were then spotted in triplicate in an array format on the same SAM gold surface, with the aid of a modified Tecan liquid-handling robot (Figures 5A, B; Table S1).

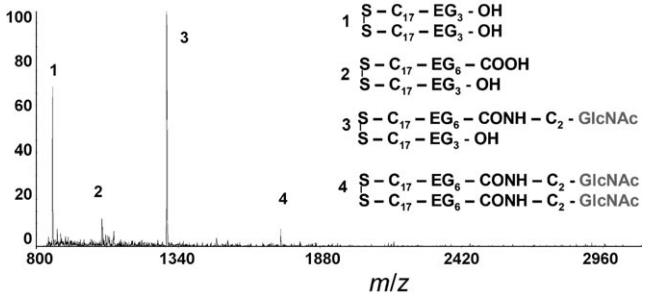


Figure 4. MALDI-ToF MS analysis of glycans immobilised on gold surfaces. Direct MALDI-ToF analysis of immobilised 2-aminoethyl 2-acetamido-2-deoxy-β-D-glucopyranoside. Structures of the detected species are indicated: 1) *m/z* 861, 2) *m/z* 1051, 3) *m/z* 1298, 4) *m/z* 1734 for the sodium adducts of the species 1, 2, 3, and 4, respectively.

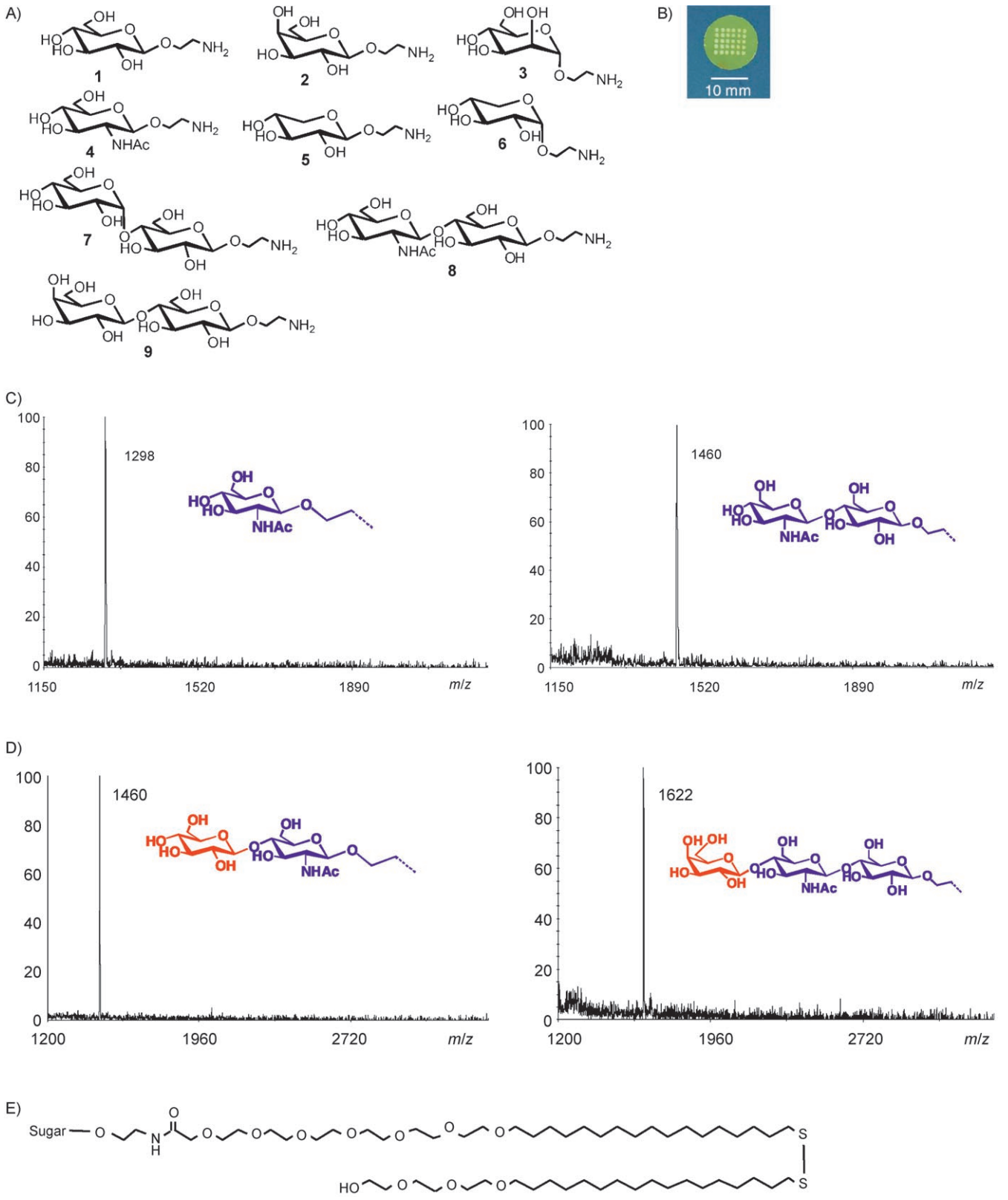


Figure 5. MALDI-ToF MS analysis of immobilised carbohydrates and detection of enzymatic transformations. A) Structures of the sugar derivatives (see also Table S1). B) Representative 6×5 array (*picture taken after spotting of the matrix*). C) MALDI-ToF MS analysis of the βGlcNAc **4** (left) and βGlcNAc(1,4)βGlc **8** (right). MALDI-ToF MS mass data for the complete glycoarray are given in Table S1, and the corresponding MALDI spectra are shown in Figure S4. D) MALDI-ToF MS of spots 4 (top) and 8 (bottom) after enzymatic transformation with β1,4-GalT in the presence of UDP-Gal. Masses shown correspond to the mass of the sodium adduct of the mixed disulfide formed by the sugar-terminated alkanethiol and the tri(ethylene glycol) alkanethiol. No mass shifts were observed for the other glycans in the array (see Table S1). E) Molecular structure of the sugar-terminated mixed disulfide of alkanethiol and tri(ethylene glycol) alkanethiol.

Again, MALDI-ToF MS analysis confirmed immobilisation and detection of all sugars (see Figure S4 and Table S1 for mass data). Despite increasing development of glycoarrays, only a few examples of enzymatic glycosylation on solid surfaces, mostly based on the use of fluorescent lectins to probe successful transformation,[6d,7,30] have been reported. MALDI-ToF MS analysis has recently been employed to probe the activities and specificities of enzymes, though not in array format.[7,25,27] To test the potential of our SAM gold surface for detecting enzymatic transformations in arrays, we assayed the activity of bovine β1,4-GalT by covering the surface of the nine-sugar array with a mixture containing the enzyme, UDP-Gal and $MnCl_2$, and incubating overnight at 37 °C in a wet chamber to prevent evaporation. MALDI-ToF MS of the resulting array indicated full conversion of **4** (β-D-GlcNAc) and **8** (β-D-GlcNAc-(1,4)β-D-Glc) (Figures 5C, D), in agreement with the known substrate specificity of this enzyme.[31] The masses of all other sugars remained unchanged, as would be expected from the known enzyme specificity (see Table S1). These results demonstrate the effectiveness of this approach for examining enzymatic transformations of immobilised glycans. Potential applications include multiplex studies on the substrate specificities of glycan biosynthetic enzymes and monitoring of on-chip glycan synthesis. We have also exploited this surface for MALDI-MS investigation of enzymatic glycosylation of peptides.[32]

In summary, here we report a versatile gold-surface-based glycoarray platform for presenting multiple oligosaccharides to protein targets. The approach is based on the utilisation of self-assembled monolayers of functionalised PEGylated C17-alkyl-thiolate linkers on a gold chip surface for direct chemoselective attachment of glycans either in natural (nonderivatised) form (to hydrazide-functionalised surfaces) or as prederivatised glycoconjugates. The latter case is exemplified here by succinimide ester coupling of aminated glycans, though alternative chemistries to extend the applications of this surface platform can readily be envisaged. Diverse natural and synthetic oligosaccharide probes can be rapidly immobilised on gold chip surfaces, and their interactions with target proteins can be assessed for binding and specificity by use of microarrays with fluorescence detection, label-free interrogation techniques including SPR and MALDI-MS, and on-chip enzymatic modifications. Importantly, the functional group density on the monolayer can be adjusted by use of a "dilutor" (thiol-alkyl-EG3-OH) to form mixed SAMs. This minimises nonspecific protein adsorption, provides optimal signal to noise ratios, and abrogates the need for blocking agents (the latter being critical for effective SPR and MALDI-MS). This diversified, generic surface platform is facile, does not require specialist chemical expertise, and can be applied by use of widely available equipment. It is readily accessible to standard life science labs and could provide the basis for development of integrated methods with potential for exploitation as tools for decoding the protein interactions of the glycome. Applications of this platform with extended libraries of chemically and structurally diverse glycans should prove valuable, as has already been convincingly demonstrated with other glycoarray platforms.[23,35]

Experimental Section

Materials: All chemical and biochemical products were of analytical grade. PEGylated thiol linkers—HS-$(CH_2)_{17}$-EG_6-CH_2-COOH (C17 thiol) and HS-$(CH_2)_{11}$-EG_6-CH_2COOH (C11 thiol)—and dilutors—HS-$(CH_2)_{17}$-EG_3OH and HS-$(CH_2)_{11}$-EG_3-OH—were purchased from ProChimia Surfaces (Poland). All thiol structures were verified by ^{1}H NMR and mass spectrometry. Carbohydrazide and MHDA were purchased from Aldrich. EDC was purchased from Fluka. Gold-coated microarray slides (coating: 100 nm Au, 5 nm Ti; size: 1″× 3″×0.04″) were obtained from EMF (New York, USA). Alexa Fluor 546 rabbit anti-goat IgG (H + L) conjugate was obtained from Invitrogen-Molecular Probes. Goat anti-human IgG Fc, FGF1, FGF2, GDNF and their corresponding antibodies were obtained from R & D Systems (Minneapolis, USA). Con A, anti-Con A antibody, rhodamine-labelled Con A, WGA, and anti-WGA antibody were purchased from Vector Labs (Burlingame, USA). hSiglec-7 and mSiglec-E were examined as recombinant soluble IgG Fc chimeras in culture supernatants. These consist of the extracellular Ig-like domains 1–3 of hSiglec-7, and mSiglec-E, stably secreted by transfected Chinese hamster ovary cells harvested in X-VIVO-10 serum-free medium, and quantified by immunoassays (ELISA). Oligomannose-5 (Man5), α1–6-mannobiose, α1–3-mannobiose, sialyllacto-*N*-tetraose a (LSTa), sialyllacto-*N*-tetraose b (LSTb), sialyllacto-*N*-tetraose c (LSTc), 6′-sialyl-*N*-acetyllactosamine, and 3′-sialyl-*N*-acetyllactosamine were from Dextra Lab. Heparin decasaccharides (dp-10) were prepared by gel filtration chromatography.[33]

Hydrazide derivatisation of thiol linkers: Carboxylic acid-terminated C17 or C11 thiols (2 mg) and carbohydrazide (35 mg) were mixed in dimethyl sulfoxide (DMSO, 10 mL), and sonicated to dissolve the mixture completely. EDC (30 mg) was then added, and the reaction mixture was stirred at ambient temperature for 6 h; the reaction product was divided into aliquots (200 μL) and freeze-dried. The molecular structures of the prepared C17 and C11 thiol-carbohydrazide constructs were verified by MALDI-ToF and electrospray ionisation (ESI)-MS analysis.

Fabrication of hydrazide-functionalised gold surfaces: Gold-coated glass slides were cleaned by sonication in ethanol for 10 min. Slides were coated with a monolayer of the thiol-carbohydrazide construct by overlaying for 24 h in a solution of thiol (0.1 mg mL^{-1}) dissolved in 50% methanol (400 μL). This solvent was chosen after extensive testing, and provided reduced evaporation and diffusion on the gold surface (alternative polar solvents include 50% or 100% ethanol, isobutyl alcohol, or acetonitrile). The SAM-covered slides were washed and sonicated for 10 min in ethanol and dried with nitrogen. For the formation of the mixed monolayer on gold, a precalculated amount of the "dilutor" was added to the thiol-carbohydrazide construct solution.

Fabrication of NHS-functionalised gold surfaces: Microscope glass coverslips (13 mm diameter, no. 2 thickness; Agar Scientific) were cleaned in "Piranha" solution (5:1 H_2SO_4/H_2O_2, ***CAUTION!*** very reactive oxidising agent) for 20 min, rinsed with distilled water, and dried under nitrogen. An adhesion layer of 5 nm of chromium and a subsequent 100 nm of gold were sputtered onto the glass coverslips with a Denton Vacuum Desk III sputter coater. Gold-coated glass slides were cleaned in "Piranha" solution, rinsed with deionised water and ethanol, and dried under a stream of nitrogen. The substrates were then immersed overnight in a DMSO solution of carboxylic acid-terminated alkanethiols and tri(ethylene glycol) alkanethiols (final concentration 0.1 mg mL^{-1}, molar ratio 50:50) and rinsed and dried as above. Activation of the carboxylic acid was performed by dipping the substrates into a solution of EDC and

NHS (final concentrations 0.2 M and 0.05 M, respectively, in dry DMF) for 2 h, followed by washing and drying as above. For immobilisation of glycans on these surfaces, a solution of 2-aminoethyl glycosides (100 mM in PBS pH 8.0, approximately 50 nL) were spotted onto the freshly activated monolayers with the aid of a modified Tecan liquid handling robot and allowed to react overnight, then rinsed and dried as above.

MALDI-ToF MS analysis: After spotting of the matrix (2,4,6-trihydroxyacetophenone, 10 mg mL^{-1} in acetone), the gold substrates were attached to a modified MALDI-ToF sample holder and loaded on a Voyager-DE STR Biospectrometry mass spectrometer (PerSeptive Biosystems) operating with a 337 nm nitrogen laser. Spectra were acquired with reflector mode for positive ions with use of an accelerating voltage of 20 kV and an extraction delay of 200 ns.

On-chip enzymatic glycosylation with bovine β1,4GalT: Slides were incubated overnight at 37 °C with a mixture of β1,4GalT[34] (1.1 mU, 1 unit corresponded to the transfer of 1 μmol of Gal from UDP-Gal to D-GlcNAc per min at 37 °C at pH 8.0), UDP-Gal (final concentration 2 mM), and $MnCl_2$ (final concentration 10 mM) in Tris-HCl buffer (40 mM, pH 8.0). After rinses with deionised water and ethanol they were dried under a stream of nitrogen.

Microarray printing for fluorescence detection: Oligosaccharide printing solutions were prepared in betaine (1 M, pH 5.5) and spotted onto hydrazide-derivatised gold-coated glass slides with the aid of a MicroGrid II compact pin-type contact arrayer and MicroSpot 2500 split pins (Genomic Solutions, UK) in 65% relative humidity. Betaine (1 M) was added in the samples to prevent water evaporation from the droplets. The oligosaccharides were typically arrayed as ten- or twofold dilution series with starting pickup solution of heparin (333 μM) and *N*-linked sugars (10 mM) for 10 replicate spots (with ~1 nL per spot delivered by contact of the pins with the surface). The distance between the centers of adjacent spots (spot size: ca. 150 μm) was 400 μm. The printed slides were incubated overnight at 18 °C in a closed environment (a plastic dish sealed with Parafilm). Unbound saccharides were removed by washing and sonicating the slides twice in distilled water, and slides were stable to storage for at least several weeks at 4 °C in a sealed container.

Protein binding on microarrays: The microarray-bound specific proteins were probed with appropriate cognate antibodies, followed by a fluorescence-labelled (Alexa Fluor 546) secondary antibody. Con A and WGA were applied on the slides (5 μg mL^{-1}, 50 nM in DPBS buffer (Invitrogen) supplemented with 1 mM Mn^{2+} and 0.05% Tween 20). Rhodamine-labelled Con A (20 μg mL^{-1}, 200 nM) was also used to probe the binding to mannose sugars by the single-layer binding format. hSiglec-7 and mSiglec-E in PBS buffer (Oxoid) were precomplexed with goat anti-human-IgG Fc (Vector) 1:3 *w/w* ratio for 1 h at ambient temperature. For hSiglec-7, the precomplexed proteins were applied onto the slides at a final concentration of 2 μg mL^{-1}. In the experiments in which mSiglec-E was used, the precomplexed proteins were applied onto the slides at a final concentration of 10 μg mL^{-1}. The binding was detected by use of Alexa Fluor 546-labelled secondary antibody (2 μg mL^{-1} in PBS-T, 0.05% Tween 20). For each binding step, an incubating step of 40 min was used. HybriWells (Grace Biolabs) were used to cover the slides during the incubation to prevent evaporation. The slide was washed gently between binding steps with a magnetically stirred wash bath (in 10 mM Tris-HCl plus 0.01% Tween 20, pH 7.4), and finally rinsed with water.

Scanning and evaluation: Fluorescence glycoarrays were read by use of a Genepix 4000 A laser microarray scanner (Molecular Devices, UK) with PMT voltage set at 800 V and laser power at 100%; signals of the ratio of 635 nm/532 nm were quantified with use of the GenePix Pro 3.0 image analysis software package.

SPR spectroscopy: SPR measurements were performed with a Biacore X™ instrument (Biacore AB, Uppsala, Sweden). The Sensor Chip Au (bare gold surface) was cleaned with H_2O_2 (30%), NH_3 (30%), and MilliQ water in a 1:1:5 ratio for 10 min and was then thoroughly washed with water, before being covered with a solution of hydrazide-modified C17 thiol linker (0.1 mg mL^{-1}, 100 μL in 50% methanol) and incubated at room temperature for 24 h. The chip was then sonicated in ethanol and washed with MilliQ water and treated with heparin solution (dp-10, 33 μM, 0.1 mg mL^{-1}, 100 μL in 1 M betaine) at room temperature for 16 h in a Parafilm-sealed chamber. After washing with water, the chip was docked into the instrument. FGF2 protein (100 nM in PBS, pH 7.4) was injected onto monolayers presenting immobilised heparin dp-10 or hydrazide-only control surfaces (nonspecific binding), and measurements were carried out with PBS-T as running buffer. The sensor surface was regenerated by two consecutive treatments (30 s) with HCl (100 mM) or NaOH (100 mM).

Acknowledgements

This work was supported by an RCUK Basic Technology Programme grant to the UK Glycoarray Consortium (GR/S79268). We acknowledge assistance from Prof. A. Cossins (Liverpool Microarray Facility), Y. Ahmed (for heparin saccharides), Dr. T. Rudd (for help with QCM), Dr. B. Tissot (ESI-MS analysis), and Dr. J. Satherley (advice on contact angle measurements), and helpful suggestions from Profs. Milan Mrksich and Injae Shin.

Keywords: arrays · carbohydrates · fluorescence · MALDI-MS · surface plasmon resonance

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Received: December 31, 2007
Published online on June 18, 2008

