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A Versatile Method for Functionalizing Surfaces with Bioactive Glycans

Fang Cheng[†], **Jing Shang**[†], and **Daniel M. Ratner**[*]
Department of Bioengineering, University of Washington, Seattle, Washington 98195

Abstract

Microarrays and biosensors owe their functionality to our ability to display surface-bound biomolecules with retained biological function. Versatile, stable, and facile methods for the immobilization of bioactive compounds on surfaces have expanded the application of high-throughput 'omics'-scale screening of molecular interactions by non-expert laboratories. Herein, we demonstrate the potential of simplified chemistries to fabricate a glycan microarray, utilizing divinyl sulfone (DVS)-modified surfaces for the covalent immobilization of natural and chemically derived carbohydrates, as well as glycoproteins. The bioactivity of the captured glycans was quantitatively examined by surface plasmon resonance imaging (SPRi). Composition and spectroscopic evidence of carbohydrate species on the DVS-modified surface were obtained by X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS), respectively. The site-selective immobilization of glycans based on relative nucleophilicity (reducing sugar vs. amine- and sulfhydryl-derived saccharides) and anomeric configuration was also examined. Our results demonstrate straightforward and reproducible conjugation of a variety of functional biomolecules onto a vinyl sulfone-modified biosensor surface. The simplicity of this method will have a significant impact on glycomics research, as it expands the ability of non-synthetic laboratories to rapidly construct functional glycan microarrays and quantitative biosensors.

Introduction

Versatile methods for surface modification and bioconjugation are essential to the biosensor and microarray communities. A variety of strategies have been used to achieve non-covalent and covalent attachment of biomolecules on a solid support (1-6). Non-covalent adsorption is the most straightforward immobilization method, but it is often most suitable for high molecular weight biomolecules (e.g. proteins, DNA) (7). Water-soluble ligands (e.g., sugars, biotin, small peptides) tend to desorb due to weak interactions with the surface and high solubility (2,8,9). As such, covalent bonds between biomolecules and the surface can yield more reliable attachment. For example, carbodiimide chemistries are frequently involved in surface activation to form stable amide/carbamate bonds with biomolecules bearing primary amine groups (10-14). Isocyanate, epoxy, and acyl chloride groups (15-17), which are highly reactive towards sulfhydryl, amine, and hydroxyl groups present on biomolecues,

[*]To whom correspondence should be addressed. Address: 3720 15th AVE NE, Box 355061, Seattle, WA, 98195, USA, Phone: 206-543-1071, Fax: 206-685-3925, dratner@u.washington.edu.
[†]These authors contributed equally to this work

Supporting Information Available: Trifluoroethyl derivation of DVS activated surfaces, synthesis of ethylvinyl sulfone mannosides, table of theoretical vs. observed XPS comparison of SAMs, XPS spectra for SAMs before and after DVS modification, XPS spectra for DVS-modified SAMs before and after Fluorine derivation, SPR performance of one hundred continuous binding-regeneration cycles, SPR results of Con A non-specific uptake to the inactivated DVS surface, NMR spectra of DVS and mannose surfaces. This material is available free of charge via the Internet at http://pubs.acs.org.

have also been utilized for surface modification. These reactive groups have expanded the pool of biomolecules that can be immobilized for array, sensor and biomaterials applications. However, the intrinsic moisture sensitivity of most immobilization chemistiries requires specialized handling, freshly distilled/dried reagents, anhydrous storage and sophisticated techniques for surface modification—this is neither convenient nor always possible for the end-user or nonchemist (18,19).

In the field of glycomics, alternative methods for array fabrication have been proposed to simplify the process of array/biosensor fabrication. These methods have been developed to address the significant synthetic burden associated with traditional glycan array fabrication. For instance, Cummings and colleagues have developed a novel strategy based on the reductive amination of isolated glycans with an amine-functionalized linker for covalent immobilization to NHS-ester and epoxy activated slides (5,17). This method is being used by the Cummings group to perform ‘shotgun glycomics’ to profile glycan structure and biological function using tissue- and cell-specific glycan libraries. Alternate approaches have also been developed based on photoactivatable functional groups (20,21). Upon UV radiation, biomolecules are conjugated onto the surface through random bond insertions, resulting in unbiased conjugation. The ultimate aim of these alternative methods for array and biosensor functionalization is to facilitate expansion of the diversity of immobilized glycan, and lower the barrier to enter the field of glyomics research.

Along these lines of simplifying the immobilization of carbohydrates for array/biosensor fabrication, we explore the use of divinyl sulfone (DVS), an efficient, robust, and commercially available homobifunctional linker. Since the 1970's, DVS has been utilized to conjugate biomolecules to various materials for applications in biotechnology and biomedicine (10). For instance, chromatographic resins (e.g., Sepharose) have been activated and crosslinked (22) using DVS and conjugated to a variety of ligands (e.g., antigen, enzyme, carbohydrate) for protein affinity purification (23). DVS has been widely used to couple proteins and carbohydrates to polymers (24-27), labels for detection (e.g., fluorophores and biotin) (28,29) and solid supports (28,30,31) for enzyme recovery, drug delivery and immunoassay development. In addition, vinylsulfone-based chemistries have been used in biosensor and microarray fabrication (32,33), including applications of N-hydroxysuccinimide-polyethyleneglycol-vinylsulfone, for the construction of DNA and protein microarrays (34,35). The versatility of vinylsulfone chemistries has also been extended to include glycoconjugate synthesis to study the biology of carbohydrate-mediated interactions (36,37).

Herein we describe a simple two-step method to functionalize hydroxyl-terminated surfaces with unmodified and modified saccharides (thiolated and aminated) as well as proteins and glycoproteins via a DVS conjugation strategy. The process involves two sequential nucelophilic 1,4-additions (i.e., Michael reactions) in basic solution at ambient temperature, wherein DVS serves as a linchpin between the surface and captured biomolecules. The first step modifies hydroxyl groups present on the surface to generate an activated vinyl sulfone-modified substrate. In the second step, the DVS is reacted with biomolecules bearing sulfhydryl, amino, or hydroxyl groups. The functional biomolecules are thus covalently bound to the surface in a site selective manner based on the nucleophilicities of the reactive groups. This strategy enables immobilization of a variety of biomolecules on a single array/chip and simplifies the process for arraying synthetically challenging biomolecules (e.g. carbohydrates) by covalent capture via endogenous nucleophiles.

A variety of analytical and biophysical methods were employed to validate this conjugation strategy. X-ray Photoelectron Spectroscopy (XPS) was used to characterize each surface modification step. Spectroscopic evidence of saccharide-related species on the surface was

also obtained by Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS). Surface Plasmon Resonance imaging (SPRi) was used to screen carbohydrate-specific protein binding on the saccharide-functionalized array; lectin binding affinities on unmodified and modified mannose were determined. In addition, Nuclear Magnetic Resonance (NMR) spectroscopy was used to establish the site selectivity of a model coupling reaction between a reducing sugar and ethyl vinyl sulfone, simulating the reaction of free sugars to DVS-modified surfaces.

Experimental Procedures

Reagents and Materials

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Acros Organics (West Chester, PA) and used as received without further purification. Aminopropyl mannoside (38) was provided by Prof. Rodrigo B. Andrade at Temple University. Mannose thiol and OEG thiol were synthesized as previously described (39). Bovine serum albumin (BSA) and wheat germ agglutinin (WGA) were purchased from Sigma-Aldrich (St. Louis, MO). Concanavalin A (Con A) was purchased from MP Biomedicals (Solon, OH). RNase A and RNase B were purchased from New England Biolabs (Ipswich, MA). Ricin (RCA120) was purchased from EY Laboratories (San Mateo, CA) and dialyzed overnight against PBS prior to use. Ethanol (200 proof, USP) was purchased from Decon Labs (King of Prussia, PA). Culture-Well Silicone sheets were purchased from Grace Bio-Labs (Bend, OR). SF-10 glass substrates were purchased from SCHOTT Glass Technology (Duryea, PA). Silicon wafers were purchased from Silicon Valley Microelectronics (San Jose, CA). Millipore-filtered water was used for all aqueous solutions and rinsing. Unfunctionalized bare gold SIA Biacore kits were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

Preparation of Au substrates

Titanium (2 nm) and gold (45 nm) films were deposited onto cleaned SF-10 glass (18 mm × 18 mm) and silicon wafers (10 mm × 10 mm) for SPRi and surface analysis, respectively. Metal films were prepared via electron beam evaporation at the Washington Technology Center (Seattle, WA).

DVS-modified surfaces

Fresh Au substrates were immersed in 11-mercaptoundecanol (0.1 mM in EtOH) for 2 h at room temperature to form a hydroxyl-terminated self-assembled monolayer surface. The surfaces were dipped in a stirring ethanol bath for 1 min. The cleaned surfaces were dried under a stream of argon for 1 min and then immersed in 10% DVS (v/v, 0.5 M carbonate buffer, pH 11) solution for 1 h at ambient temperature. The DVS-modified surfaces were thoroughly rinsed with ~10 mL water, dried by a stream of argon and stored in the dark at 4 °C under an inert atmosphere.

Glycan-functionalized surfaces

The DVS-modified surfaces on silicon wafer substrates were immersed in mannose (20% w/v, pH 10 carbonate buffer) solution at ambient temperature for 16 h unless specified. The surfaces were then thoroughly rinsed with ~10 mL water and ~5 mL ethanol, dried under a stream of argon for 1 min and stored in dark at 4°C.

Glycan-functionalized arrays

Nine-element silicone masks were placed onto the DVS-modified surfaces. 2 μL of dissolved glycan (pH 10 carbonate buffer for natural glycans and aminated glycans, and pH

7.4 PBS for thiolated glycans, RNase A, and RNase B) was spotted onto each spot. To prevent oxidation of thiolated glycan, 0.5 stoichiometric equivalents of tris(2-carboxyethyl)phosphine (TCEP) were added to the aqueous thiol solutions prior to spotting. The arrays were incubated in a 75% relative humidity chamber. After 16 h incubation, surfaces were immersed in oligo(ethylene glycol)$_3$ thiol (OEG-thiol)(40) (1mM in carbonate buffer, pH 8.5) for 2 h to deactivate remaining vinyl sulfone groups, then rinsed with water, dried under a stream of argon and stored at 4 °C.

XPS analysis

XPS composition data was acquired on a Kratos AXIS Ultra DLD instrument equipped with a monochromatic Al-Kα X-ray source (hν = 1486.6 eV). XPS data were collected at 0° take-off angle in the hybrid mode with approximately 10 nm sampling depth. Compositional survey and detailed scans (N 1s, O 1s, and S 2p) were acquired using a pass energy of 80 eV. Three spots on two or more duplicates of samples were analyzed. Reported compositional data were averaged over multiple spots. Data analysis was performed on the CasaXPS software (Casa Software Ltd.).

ToF-SIMS analysis

ToF-SIMS data was acquired using an ION-TOF TOF.SIMS 5-100 system (ION-TOF GmbH, Münster, Germany). Positive spectra were recorded by rastering a pulsed 25-keV Bi_3^+ primary ion source over a (100 × 100) μm^2 area, and keeping the total ion dose below the static limit (i.e., 10^{12} ions/cm^2). The mass resolution ($m/\Delta m$) was typically 6000 at m/z = 27 for all spectra, which were mass calibrated using the CH_3^+, $C_2H_3^+$, $C_3H_7^+$, and $AuC_2H_4^+$ peaks. Maximum calibration errors were kept below 15 ppm.

SPRi

Glycan-specific protein binding was performed on a SPRimagerII (GWC Technologies). The SPRimagerII was operated at room temperature using a standard flow cell and a peristaltic pump (BioRad-EconoPump) at 100 μL/min. All surfaces were passivated with 0.1% BSA in PBS buffer for 30 min and equilibrated in protein buffers prior to protein binding. HEPES buffer (10 mM HEPES, 150 mM NaCl, 1 mM Ca^{2+} and Mn^{2+}, pH 7.4) was used for Con A binding, PBS (2.7 mM KCl, 137 mM NaCl, 10 mM phosphate, pH 7.4) was used for RCA and WGA binding. Unless specified, lectins were used at 500 nM. Data acquisition consisted of the averaging of 30 images over a short duration to create an average image. The SPR signal given by the average image (in pixel intensity) was subsequently converted to normalized percentages change in reflectivity according to GWC protocol. Urea (8 M) was used to strip bound protein and regenerate the array surfaces. For sensorgram acquisition, a 500 μm × 500 μm of the image was selected as the region-of-interest (ROI). For visual clarity, the contrast and brightness of SPR difference image was adjusted by ImageJ software (U. S. National Institutes of Health, Bethesda, MD). Two spots on two or more duplicates of samples were analyzed. Reported changes in SPR reflectivity (Δ %R) were averaged over multiple spots and background subtracted.

SPR

Surface performance of the glycan-functionalized chip was carried on a Biacore T100 (LSDF Center for Intracellular Delivery of Biologics). The SPR was operated at room temperature using a syringe pump at 30 μL/min. All saccharide-functionalized surfaces were passivated with 0.1% BSA in PBS buffer for 30 min and equilibrated in protein buffers prior to protein binding. HEPES buffer was used for Con A binding. Glycine (10 mM, pH 2) was used to remove captured lectin and regenerate the array surfaces; to avoid damaging the

microfluidic channels in the Biacore T100, 8.0 M urea was not used for this regeneration step. Data analysis was performed using Biacore evaluation software.

Data analysis

Three spots on two or more duplicate samples for surface analysis and two spots on two or more duplicates of samples for SPRi were obtained and analyzed. The values reported are averaged over the multiple spots and presented with standard deviations. Statistical analysis was performed using the Student's t-test to calculate significance.

Results and Discussion

Surface modification and characterization

The simple stepwise process for DVS-based immobilization of modified and unmodified glycans is illustrated in Scheme 1. A hydroxyl-terminated self-assembled monolayer (SAM) on Au was used for DVS modification under alkaline conditions followed by incubation with the sugar. Each surface modification step and subsequent incubation was characterized using XPS to obtain the organic composition (Figure 1, Figure S1, and Table S1). Due to XPS sulfur signal attenuation by the outermost organic monolayer, the O/S ratio (2.4 ± 0.2) in the starting hydroxyl-terminated surface is higher than the value predicted by stoichiometry (1.0), this is expected (41,42). For sequential DVS- and glycan-treated surfaces, a slight excess of oxygen was observed and attributed to DVS, glycan, in addition to surface-bound water (40,42). Although oxygen content from DVS surfaces incubated with 10% mannose and 5% mannose appears to change abruptly, their C/O ratios do not differ significantly ($p > 0.05$). XPS sulfur 2p peaks were assigned to the Au-bound thiol (160 eV) and oxidized sulfur species (168 eV), respectively (Figure S1b) (18,43). To establish that the oxidized sulfur species observed on the DVS-modified SAMs originates from the vinyl sulfone and not from oxidation of the Au-thiol during handling, the SAM was incubated in buffer with and without divinyl sulfone. The absence of an oxidized sulfur signal on the buffer treated control SAM established that oxidation of thiols in the SAM is not observed by XPS. These results suggest that the observed oxidized sulfur species at 168 eV can be attributed to the vinyl sulfone modification of the SAM. The XPS ratio of sulfone to Au-thiol is approximately 1:8. Taking into account signal attenuation due to the monolayer thickness, the DVS coupling efficiency is estimated to be 10%. The XPS results indicated that all surfaces are of high quality and contain the expected elemental composition with minimal amounts of contamination. Elemental information obtained by XPS, however, is not sufficient to determine whether carbohydrate is in fact present on the surface (see comparison of theoretical vs. observed XPS composition measurements in Table S1). Therefore, to verify the existence of surface-bound glycan, ToF-SIMS was employed to identify molecular fragments unique to immobilized carbohydrate species. Consistent with the reported positive ion fragments, a series of surface species related to saccharides were identified by ToF-SIMS (44,45). As illustrated in Figure 2, peak 73 ($C_3H_5O_2^+$) is a positive ion fragment characteristic of a pyranose moiety (44,45), therefore confirming the presence of mannose on the surface.

Bioactivity of saccharide-functionalized array surface by unmodified saccharides

To assess the bioactivity of DVS immobilized glycoprotein, unmodified mono- and oligosaccharides, a spotted glycan array was fabricated for SPRi binding studies. Solutions of monosaccharides (glucose, mannose, and galactose), oligosaccharides (maltose, lactose, chitobiose, and maltotriose), RNase B, and RNase A were included in the spotting array. Carbohydrate-binding proteins concanavalin A (Con A), ricin (RCA120), and wheat germ agglutinin (WGA) were used to screen carbohydrate bioavailability. As shown in Figure 3a and 3b, those spots treated with mannose, maltotriose, maltose, glucose, and RNase B

showed expected SPR reflectivity increases due to Con A binding, whereas the rest of the spots which were galactose, lactose, chitobiose, and RNase A spots did not show appreciable change in reflectivity. RNase B is a glycoprotein modified with highly-branched mannose, while RNase A has the identical peptide sequence but lacks the glycan. These binding results are consistent with the fact that Con A has specific affinity towards α-mannose, α-glucose, maltose (which displays a terminal α-glucose residue) and other mannose-containing glycoproteins, but not towards galactose, lactose, or RNase A. RCA120, a protein toxin, selectively binds to β-galactose and lactose. When a solution of RCA120 is passed over the surface, only the spots with galactose and lactose showed changes in SPR reflectivity (Figure 3c and 3d). Lastly, WGA, which has affinity to chitobiose, was screened on the array. The SPRi results showed expected chitobiose-WGA binding specificity (Figure 3e and 3f). In addition to these simple mono-, disaccharide and glycoprotein arrays, SPRi protein binding assays also showed that the array surface functionalized by polysaccharides (amylose and amylopectin) exhibited their predicted specificity to Con A (data not shown).

By varying glycan immobilization conditions (solution concentration, pH, and incubation time), high protein bioactivity can be achieved; using mannose as an example, Figure 4 illustrates the effects of these variables on Con A binding (as measured by SPR). Compared to the XPS composition study, SPRi binding (Figure 4a) showed a dose dependent change as the function of mannose solution concentrations. This can be explained by the fact that SPR is more sensitive to changes of very low concentrations of surface-bound ligand, which cannot be observed accurately by XPS. SPR measures the response of protein (analyte) binding to immobilized ligand, which can be easily detected at low ligand density (sub-monolayer). As expected, high concentration of mannose (20 - 30% w/v), alkaline immobilization buffer (pH 10 - 11), and longer incubations (20 h or more) increase subsequent protein binding. These results can be used to determine optimal fabrication conditions for printed glycan arrays prepared via DVS capture. Based on these results, we determined that microarrays printed using this method require only 150 - 300 ng of isolated glycan for each array element (0.5 - 1 nanoliter of sugar solution per spot). Many isolated glycan purification strategies yield microgram to milligram quantities of material, which would be sufficient to print hundreds to thousands of microarray spots without the need for further purification (5,17,46,47).

During surface activation and glycan functionalization steps by using DVS, it is possible that crosslinking of terminal hydroxyl groups on the alkanol SAM and hydrolysis of vinylsulfone could reduce the coupling efficiency, but it doesn't significantly affect our ability to use the surfaces for glycan conjugation and bioactivity detection. Our results showed that glycan-functionalized surfaces are highly stable and reusable for biosensing experiments. Printed arrays retained 90% of their maximum protein-binding capacity after one hundred continuous binding-regeneration cycles on SPR (Figure S3) or after three months of storage (4 °C, dark, desiccated). Under the same storage conditions, the unreacted DVS-modified surfaces maintained 90% maximal activity to glycan immobilization after 1 week and 80% after 1 month of storage. These results demonstrate the potential for long-term stability of activated surfaces (48) greatly simplifying array fabrication by making it possible to create and store activated surfaces.

DVS is an especially versatile method for surface functionalization, and can easily be adapted to a variety of hydroxyl-bearing substrates (25,26). To demonstrate the flexibility of this approach, we have begun to explore a variety of surfaces for DVS modification with great success. For instance, in addition to utilizing hydroxyl-terminated alkane-thiols (11-mercaptoundecanol), DVS immobilization of carbohydrates was also achieved on surfaces bearing hydroxyl-terminated oligo(ethylene glycol) (OEG). Compared to the alkanethiol

SAM, we observed lower nonspecific adsorption (Figure 5a) and a similar Con A binding profile (Figure 5b) on the DVS modified OEG substrate.

To investigate Con A non-specific uptake onto inactivated DVS surfaces, we examined Con A binding to a mannose-functionalized chip without prior blocking with BSA (Figure S4). The results showed a small refractive index change (~0.5 Δ%R) due to Con A non-specific adsorption during the first binding cycle. However, nonspecific uptake was not observed in following cycles.

Bioactivity of saccharide-functionalized array surfaces

The site selectivity of the 1,4-conjugate addition, or Michael reaction, between biomolecules and the vinyl sulfone moiety of DVS is determined by the nucleophilicity of the pendant functional groups on the biomolecule (i.e., hydroxyl, amino or sulfhydryl) (49-51). In weakly alkaline solutions, the sulfhydryl and amino groups are more nucleophilic than the hydroxyl (51). Hence, thiolated and aminated mannoses can be conjugated onto the DVS-modified array surface via their sulfhydryl and amino groups, orienting the bioactive glycan moiety at the array surface. SPRi Con A binding (Figure 6) shows that the amine- and thiol-modified mannose exhibited high Con A binding capacity, as they needed lower solution concentration and shorter immobilization time than unmodified mannose. To evaluate the possibility of physical adsorption of mannose species (especially thiolated mannose) on SAM surfaces, unmodified, aminated and thiolated mannoses were spotted on the hydroxyl-terminated SAM without DVS activation. Con A specific binding was not detected on these surfaces (data not shown), further demonstrating the importance of DVS for glycan immobilization.

Unmodified versus modified glycan: A comparison of adsorption isotherm and surface density

To establish the role of thiolated and aminated linker on the bioactivity of DVS-immobilized glycans the adsorption isotherms of Con A were constructed for surfaces composed of unmodified-, thiolated-, and aminated mannose, and their interactions were analyzed with the Langmuir adsorption model(52). SPR response at equilibrium was related to Con A concentration according to the following equation, where R_{eq} is the SPR response to Con A with concentration of C at equilibrium and $R_{\max}$ is the equilibrium response when C is infinity (3,52,53)

$$\frac{1}{R_{eq}} = \frac{1}{R_{\max} K_A} \times \frac{1}{C} + \frac{1}{R_{\max}} \quad (1)$$

The association constant (K_A) and the maximum response ($R_{\max}$) of Con A binding with unmodified and modified mannose are listed in Table 1. These binding parameters indicate that the interaction is greater for the thiolated and aminated mannose, suggesting higher bioactivity – and possibly higher densities – of glycan species on the array surface (3,54). We are currently exploring the role of surface glycan density on these results. Our measured association constants agree with the reported values, $10^5 \cdot M^{-1}$ to $10^6 \cdot M^{-1}$ (53-55).

To obtain a more accurate sense of the immobilized surface density of thiol-, amine- and hydroxyl-bearing molecules, DVS-modified surfaces were reacted with trifluoroethanol, trifluoroethanethiol and trifluoroethylamine, and analyzed by XPS (See supporting information). The XPS fluorine to oxygen (F/O) ratio obtained from these modified surfaces can be used to establish the density of the trifluoroethane-nucleophile, since the presence of surface-bound fluorine is unique to the trifluoro-species, while oxygen is mainly attributed

to hydroxyl-terminated surface. The F/O ratio was determined to be 0.08 ± 0.01 for trifluoroethanol, 0.20 ± 0.06 for trifluoroethanethiol, and 0.19 ± 0.03 for trifluoroethylamine (See Figure S2). Based upon a starting density of 4.67 hydroxyls (or less) per nm^2 (56), the estimated density for trifluoroethanol on DVS-modified surface is roughly 0.1 molecule/nm^2, while the stronger amino- and thiol- nucleophiles yielded nearly 0.3 molecules/nm^2. These results, combined with the Con A binding to different functional mannosides (i.e. free sugar, aminated and thiolated), suggest that thiolated and aminated biomolecules conjugate more efficiently to DVS and result in a higher surface density, in agreement with the well-established reactivity of these nucleophilic groups (10, 57).

Vinyl sulfone reaction preference for hydroxyl groups in saccharides

By definition, carbohydrates display multiple hydroxyl groups and reducing sugars present a ratio of the α and β anomeric configuration at equilibrium in solution. Therefore, free sugars may conjugate to the DVS-modified surface via any one of its multiple hydroxyl sites and can exist as both the α and β-diastereomers, making it necessary to carefully characterize the mode of unmodified carbohydrate-binding to DVS surfaces. To establish reaction sites and the ratio of α and β- isomers conjugated with vinyl sulfone, proton and carbon NMR spectroscopy were used to examine a model reaction based on the reaction of ethyl vinyl sulfone (EVS) with free mannose (Scheme S1). As predicted by the increased nucleophilicity of the anomeric hydroxyl, NMR characterization of the mannose-EVS products suggests that the glycan reacts with EVS predominantly via the anomeric hydroxyl group, resulting in a mixture of α and β products (Figure S5). From the ^1H NMR spectrum of mannose/EVS reaction products, the peaks at 4.85ppm and 4.62ppm are assigned to α- and β- isomers respectively, which is consistent with the reported chemical shift difference (~0.2) between the two anomeric protons (58). By integration of the area under these two peaks, the ratio of α- and β- isomers is estimated around 3:1, as predicted by the anomeric effect in aqueous solution. This model reaction suggests that reducing sugars, like mannose, react with vinyl group on a surface via the more nucleophilic hydroxyl group at its anomeric position (the hemiacetal). In the case of mannose, this reaction showed alpha selectivity, and the resulting immobilized glycan was specifically recognized by Con A.

This study demonstrates a simple method to functionalize hydroxyl-terminated surfaces with bioactive glycans and glycoproteins via DVS chemistry. Surface analysis using SPRi, XPS and ToF-SIMS show that DVS results in covalent immobilization of biomolecules on the activated surface and natural and modified carbohydrates retain their protein-binding specificity. DVS provides a facile method to capture biomolecules with nucleophilic hydroxyl, sulfhydryl or amino groups onto a solid surface, such as an array or biosensor. In the case of the carbohydrate microarray, the direct immobilization of natural sugars dramatically reduces the challenge of glycan modification and will have significant impact on research in the burgeoning field of glycomics by making the array paradigm more amenable to non-synthetic laboratories. The DVS immobilization method can be applied to biosensor development, microarray fabrication, and biomaterial functionalization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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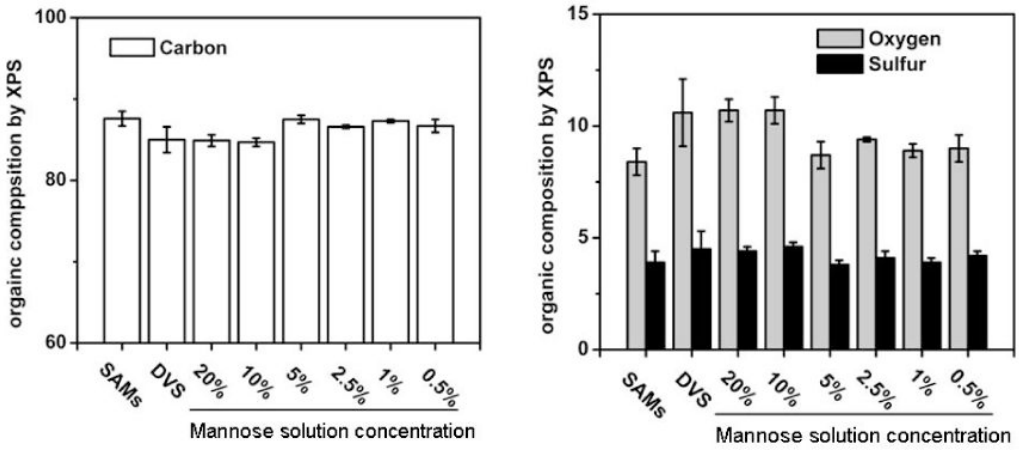


Figure 1.

Average XPS composition (with standard deviations) of hydroxyl-terminated SAM, DVS-modified surface, and mannose-functionalized surfaces under various immobilization conditions.

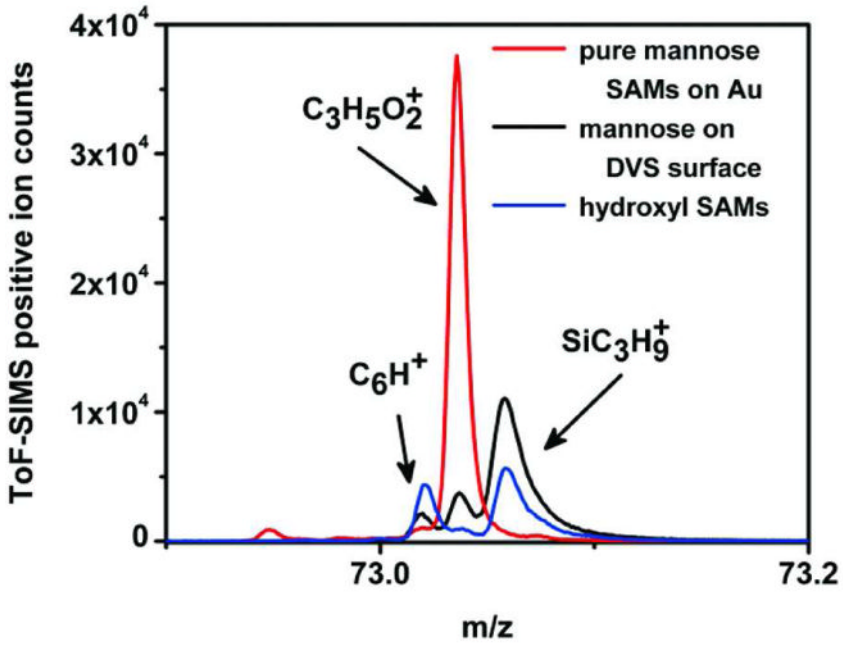


Figure 2.

ToF-SIMS positive ion spectra of a mannose-functionalized DVS surface, a thiolated-mannose SAM, and a hydroxyl-terminated alkanethiol SAM, indicating the presence of immobilized mannose on the surface (as evidenced by the carbohydrate-associated fragment $C_3H_5O_2^+$ (44,45)). Two positive ion fragments, C_6H^+ and $SiC_3H_9^+$, are believed to originate from adventitious hydrocarbon and silicone contamination respectively.

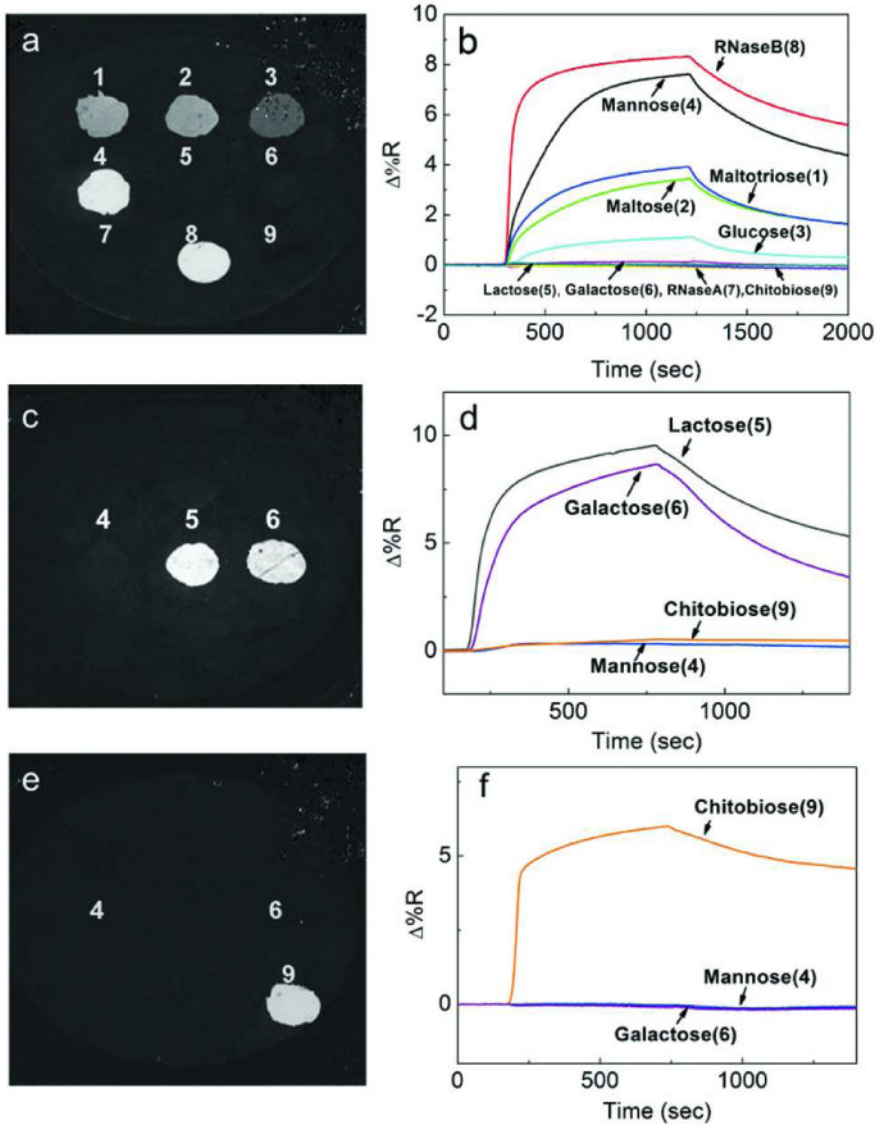


Figure 3.

SPRi reflectivity images and the corresponding sensorgrams on a spotted glycan array. Glycan bioactivity was established via lectin binding with ConA (a and b), RCA120 (c and d), and WGA (e and f). The 9-element array includes (1) maltotriose, (2) maltose, (3) glucose, (4) mannose, (5) lactose, (6) galactose, (7) RNase A, (8) RNase B, and (9) chitobiose.

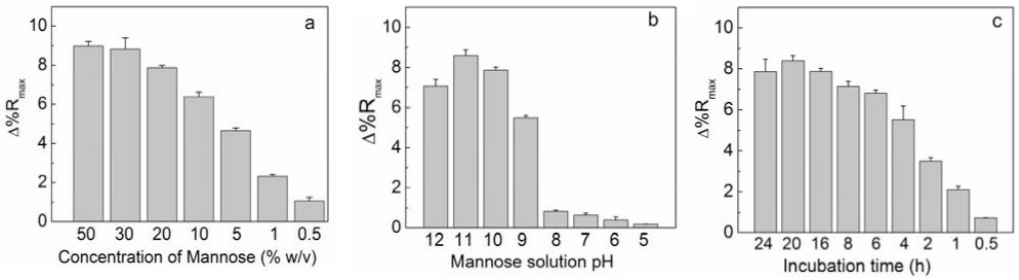


Figure 4.

Glycan immobilization conditions (free sugar concentration in solution, pH and incubation time) play a significant role in determining the bioactivity of the modified surfaces. Average SPRi ConA response (with standard deviation) to mannose-functionalized array surfaces. (a) pH 10 carbonate buffer, 16 h incubation; (b) 20% w/v mannose, 16 h incubation; (c) 20% mannose, pH 10 carbonate buffer.

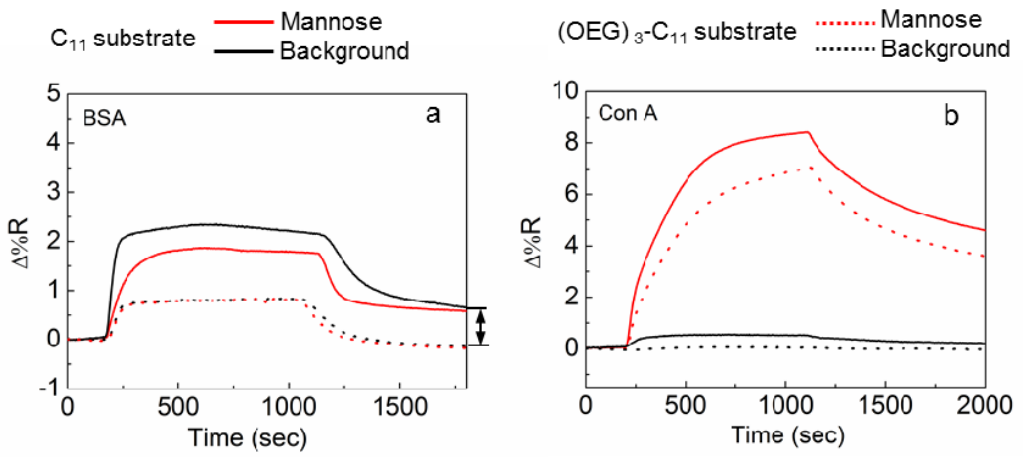


Figure 5.

SPR sensorgrams (without background subtraction) of non-specific BSA (a) and specific Con A (b) binding to mannose functionalized chips with different underlying chemistries. C_{11} substrate: 11-mercaptoundecanol, $(OEG)_3$-C_{11} substrate: (11-mercaptoundecyl) tri(ethylene glycol). — Mannose, — Background (non-glycan functionalized region) on C_{11} substrate, ····· Mannose, ····· Background on $(OEG)_3$-C_{11} substrate. These results indicate that the $(OEG)_3$-C_{11} SAMs yield surfaces that are more resistant to non-specific protein adsorption, as shown by the difference in non-specific BSA binding in (a).

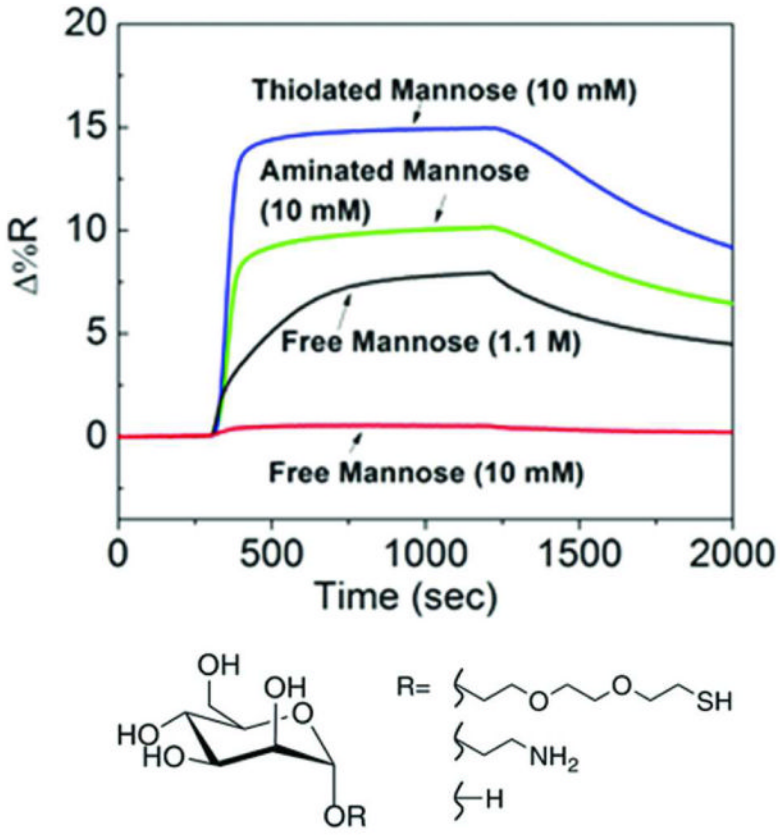
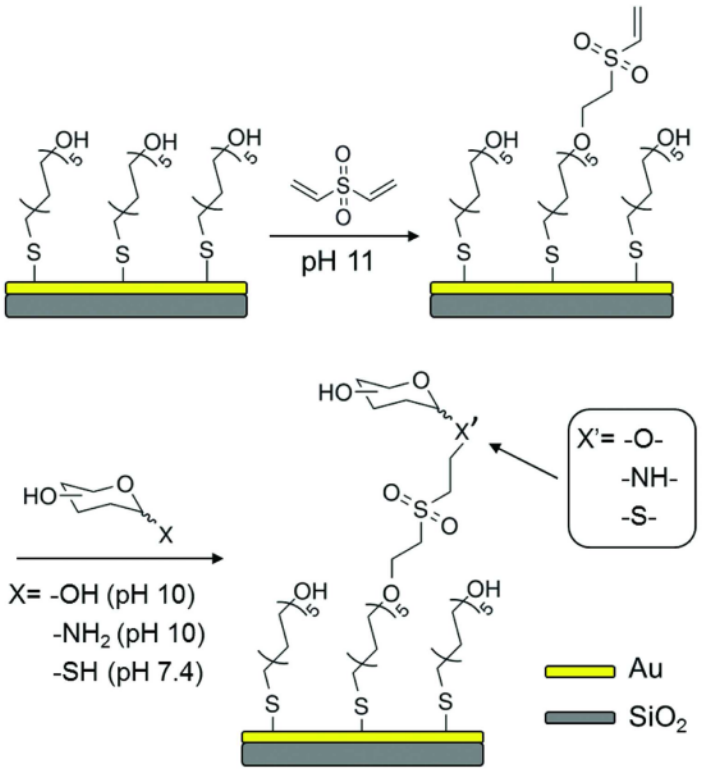


Figure 6.
SPRi shows that the response of Con A varies as a function of the nucleophile used for conjugation of mannose to the surface.



Scheme 1.
Immobilization of natural sugars as well as amine- and sulfhydryl-derived glycans on the DVS-modified surface.

Table 1

Association constant (K_A) and the maximum binding (R_{max}) of Con A to array surfaces composed of unmodified, thiolated, and animated mannose.

Immobilized mannose	K_A ($10^6 \cdot M^{-1}$)	R_{max}	Δ %R [a]
Unmodified [b]	3.1 ± 0.2	11.0	7.9 ± 0.5
Aminated [c]	7.3 ± 0.5	11.9	9.8 ± 0.3
Thiolated [d]	8.6 ± 0.7	17.7	14.6 ± 0.4

[a] Con A 500 nM in HEPES buffer for 50 min flow;

[b] 20% (1.1 M), 16 h, pH 10 carbonate buffer;

[c] 10 mM, 1h, pH 10 carbonate buffer;

[d] 10 mM, 1h, pH 7.4 PBS;

[e] SPRi values represent the averages and standard deviations from at least two spots on two replicates or more.