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Photogenerated Lectin Sensors Produced by Thiol-Ene/Yne Photo-Click Chemistry in Aqueous Solution

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Abstract

The photoinitiated radical reactions between thiols and alkenes/alkynes (thiol-ene and thiol-yne chemistry) have been applied to a functionalization methodology to produce carbohydrate-presenting surfaces for analyses of biomolecular interactions. Polymer-coated quartz surfaces were functionalized with alkenes or alkynes in a straightforward photochemical procedure utilizing perfluorophenylazide (PFPA) chemistry. The alkene/alkyne surfaces were subsequently allowed to react with carbohydrate thiols in water under UV-irradiation. The reaction can be carried out in a drop of water directly on the surface without photoinitiator and any disulfide side products were easily washed away after the functionalization process. The resulting carbohydrate-presenting surfaces were evaluated in real-time studies of protein-carbohydrate interactions using a quartz crystal microbalance flow-through system with recurring injections of selected lectins with intermediate regeneration steps using low pH buffer. The resulting methodology proved fast, efficient and scalable to high-throughput analysis formats, and the produced surfaces showed significant protein binding with expected selectivities of the lectins used in the study.

Keywords

Photochemistry; Click chemistry; Thiol-ene/-yne; Carbohydrates; Lectins; Quartz Crystal Microbalance

Introduction

More and improved strategies towards the synthesis of oligosaccharides, glycoproteins, and glycoconjugates, as well as an increased number of high-throughput analysis methods, have led to increased knowledge of the biological functions of glycans. (Kiessling and Splain 2010; Lepenies and Seeberger 2010; Li and Richards 2010; Seeberger 2009; Varki et al. 2009; Wu and Wong 2011) For example, different carbohydrate array methodologies have recently been designed and evaluated for high-throughput analysis of protein-carbohydrate

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interactions. (Horlacher and Seeberger 2008; Krishnamoorthy and Mahal 2009; Lee and Shin 2005; Oyelaran and Gildersleeve 2009; Park et al. 2008; Park and Shin 2007; Pei et al. 2007c; Tyagi et al. 2010; Wu et al. 2009) Glycan arrays have thus been used to identify proteins involved in cancer metastasis, (Hatakeyama et al. 2009) enzymes involved in wound healing, (Saravanan et al. 2010) and glycans modulating T cell death; (Earl et al. 2010) to evaluate blood serum glycan binding, (Huflejt et al. 2009) antibodies towards HIV, (Luallen et al. 2010) and antibodies for use in cancer treatment; (Huang et al. 2006; Nagre et al. 2010; Sawada et al. 2011) to evaluate the binding specificity of glycan-binding proteins and receptors; (Feinberg et al. 2010; Gout et al. 2010; Hoorelbeke et al. 2011; Horlacher et al. 2011; Pipirou et al. 2011; Porter et al. 2010; Singh et al. 2009) to investigate the binding specificities of disease causing bacteria, (Hu et al. 2011) viruses, (Krishnamoorthy et al. 2009; Neu et al. 2010; Nilsson et al. 2011) and fungi;(Chachadi et al. 2011) as well as for the in-depth investigation of avian and swine influenza viruses.(de Vries et al. 2011; Lao et al. 2011; Liao et al. 2010; Pappas et al. 2010; Stevens et al. 2010; Xu et al. 2010) Although such glycan array methodologies yield significant knowledge of glycan interactions, there are still obstacles to overcome in the production of universally valid array methodologies. Since the produced glycan surfaces aim to mimic those of cells, proteins or other biomolecules, the glycan presentation is important to achieve correct binding. The ligation techniques therefore need to be selective, adaptable, and compatible with the target glycans, and since most glycans are polar entities, it is highly desirable that the coupling chemistry can be effectuated in water.

These issues have been addressed in the present study, where the thiol-alkene (thiol-ene) and thiol-alkyne (thiol-yne) photoradical reactions have been evaluated for the fabrication of carbohydrate-functionalized surfaces. Although the radical reaction between thiols and alkenes/alkynes has been known since the beginning of the last century, (Posner 1905) it is only recently that it has been applied to functionalization methods with biomolecules.(Bader 1956; Bader et al. 1949) The thiol-ene reaction has since its discovery been thoroughly investigated in the field of polymer chemistry, but the thiol-yne reaction has only recently been explored. (Chan et al. 2009; Fairbanks et al. 2009; Fairbanks et al. 2010; Hensarling et al. 2009; Hoogenboom 2010; Konkolewicz et al. 2009; Yu et al. 2009) In an article from 2010, Fairbanks *et al.* reported a detailed study of reaction rates and mechanisms for various thiol-yne reactions, pinpointing important factors when designing the reaction. (Fairbanks et al. 2010) Since then, the thiol-yne reaction, along with the thiol-ene reaction, has been investigated for glycan coupling, and biomolecular functionalization of diverse materials. (Gupta et al. 2010; Lo Conte et al. 2010; Semsarilar et al. 2010; Wang et al. 2011; Wendeln et al. 2010)

Development of new glycan array methodologies is usually accomplished using glycans/proteins with known and complimentary specificities, to ensure accurate binding results and good validation of the systems. Glycan immobilization techniques for glycan array purposes are typically evaluated by biosensors, such as Quartz Crystal Microbalance (QCM),(Lyu et al. 2008; Mahon et al. 2010; Norberg et al. 2009b; Pei et al. 2007a) and Surface Plasmon Resonance (SPR), (de Boer et al. 2008; Dhayal and Ratner 2009; Linman et al. 2008; Muñoz et al. 2009; Uzawa et al. 2008; Zhang et al. 2006) or by high quality imaging using FITC-labeled proteins. (Branderhorst et al. 2008; Michel and Ravoo 2008; Park et al. 2009; Park and Shin 2007; Sun et al. 2006) QCM and SPR, which rely on the physical properties of quartz and gold to detect target binding, have the advantage of enabling the use of unlabeled proteins in the analyses. Conversely, both QCM and SPR are relatively low-throughput analysis tools compared to traditional microarrays, and are primarily used to obtain advanced kinetic data rather than large scale analysis of protein specificity.

Herein, we explore the thiol-ene/-yne reactions as surface functionalization approaches for the fabrication of carbohydrate-presenting surfaces. Carbohydrate-thiols with and without short spacers were coupled to alkene-/alkyne-functionalized surfaces in aqueous solution, and unlabeled lectins of corresponding known specificities were used to evaluate the methodology using a QCM flow-through instrumentation.

Materials and Methods

General

All commercially available starting materials were of reagent grade and used as received. *Ricinus communis* Agglutinin I (RCA-I), Concanavalin A (Con A) and bovine serum albumin (BSA) were purchased from Vector Labs and Sigma-Aldrich. Polystyrene-coated QCM crystals were obtained from Attana AB. ^{1}H and ^{13}C NMR data were recorded on a Bruker Avance 400 instrument at 400 MHz (^{1}H) or a Bruker DMX 500 instrument at 500 MHz (^{1}H) or 125 MHz (^{13}C). Chemical shifts are reported as δ values (ppm) with either $CDCl_3$ (^{1}H: δ = 7.26, ^{13}C = 77.16) or D_2O (^{1}H: δ = 4.79) as internal standard. *J* values are given in Hz. ^{1}H peak assignments were made by first order analysis of the spectra supported by standard ^{1}H-^{1}H correlation spectroscopy (COSY). Thin layer chromatography (TLC) was performed on precoated Cromatofolios AL Silica gel 60 F254 plates (Merck). Flash column chromatography was performed on silica gel 60, 0.040–0.063 mm (SDS). Mass Spectrometry was performed in positive mode on a ThermoElectron LTQ-Orbitrap XL from Thermo Scientific, Bremen, Germany. Spincoating was performed using a Cookson Electronics Specialty Coating Systems Spincoater model P6708D. Photoreactions were performed at either 254, 300 or 350 nm using a photochemical reactor from Rayonet Srinivasan - Griffin. QCM analyses were performed using a flow-through Attana A100 C-Fast QCM system.

Synthesis (cf. Scheme 1)

Compound **2** and **6** were synthesized according to Davis *et al.*(Davis et al. 2000) Compound **4** and **8** were synthesized according to slightly modified procedures by Revell *et al.*(Revell et al. 1998) and Schofield *et al.* (Schofield et al. 2008) respectively. Compounds **14** was synthesized according to a modified procedure of Lim *et al.* (Lim et al. 2007) Compounds **15**–**18** were synthesized as previously reported. (Norberg et al. 2009b; Pei et al. 2007b; Pei et al. 2006) More details about the synthesis along with spectroscopic data can be found in supplementary information.

Surface functionalization (cf. Figure 1)

The polystyrene-coated QCM-crystals were spincoated with a solution of PFPA-NHS (**15**, 10 mM, 10 μL) in ethanol at 1500 rpm for 180 s followed by UV-irradiation at 254 nm for 5 min. The crystals were then immersed in a solution of either alkene-linker **14** or alkyne-linker **16** (200 μL, 53 mM) in acetonitrile for 8 h and subsequently washed with acetonitrile and dried under a gentle stream of nitrogen. The crystal surfaces were then functionalized by applying solutions of thiols **4**, **8**, **17**, **18** or **19** (50 μL, 312 mM) directly on the surface, followed by UV-irradiation at 350 nm for 30 min. The crystals were then thoroughly rinsed with water and methanol, dried under a gentle stream of nitrogen and mounted in the QCM flow-through system.

General procedure for QCM analysis

A continuous flow of running buffer (TRIS 10 mM, pH 7.4, 25 μL/min) was used throughout the experiments, and samples of RCA-I and Con A were prepared in the same buffer. The protein solutions were desalted on PD-10 columns and the final protein

concentrations were determined by UV-Vis. The crystals were washed/equilibrated with buffer solution prior to manipulations/measurements. After equilibration of the crystals in the flow-through system, they were subjected to ten injections of BSA (20 μM), three injections of low pH buffer (pH 1.5) and finally two additional injections of BSA (20 μM) to block non-functionalized surfaces. Solutions of lectins were then injected on the system. Binding to the surfaces was monitored by frequency logging with Attester 3.3.4 (Attana), and adsorption/desorption to the surface recorded as the resulting frequency shifts. Bound lectins were released from the surfaces between measurements by two successive injections of low pH buffer (TRIS 10 mM, pH 1.5). The procedure was then repeated three times for each lectin concentration (32 nM - 1 μM) to give an average value and determine the surface stability over time.

Results and Discussion

The reported reaction conditions for both the thiol-ene and thiol-yne reactions, *i.e.* nonpolar solvents or solvent-free systems, conventional nonpolar radical initiators and commonly high temperatures, were not applicable in the present study due to the instability of carbohydrates at elevated temperatures and their poor solubility in organic solvents. Therefore, model starting materials, allyl/propargyl alcohol and 2-mercaptoethanol (Figure 2) were initially evaluated to test the reactions in aqueous solutions and at room temperature. The reactions were carried out under UV-irradiation at 254, 300 or 350 nm, of which 350 nm proved optimal and was deemed the most suitable for the forthcoming applications. The results showed that the reactions proceeded smoothly in water (from pH 3 to pH 10) and, as expected, that the thiol-ene reaction proceeds at a higher rate than the thiol-yne reaction. NMR-analyses for the model systems are displayed in Figure 2, showing the expected mono- and difunctionalized alkene/alkynes. Reactions carried out in the presence or absence of a water-soluble photoinitiator (4,4'-azobis(4- cyanovaleric acid), ACVA) showed similar reaction times, in principle allowing for initiator-free reaction conditions. In some cases, disulfide side products were obtained in varying concentrations, a side effect that is insignificant in the surface reactions where the disulfide can be easily washed away.

The photo-click immobilization method presented in Figure 1 is based on initial photochemical insertion of perfluorophenyl azides (PFPAs) into the polymeric material, as previously reported, (Norberg et al. 2011; Norberg et al. 2009a) and subsequent amide coupling to produce the corresponding active materials. In the present case, the polystyrene surfaces were functionalized with triethyleneglycol-linked alkynes or alkenes in a two-step procedure, starting with spincoating of PFPA-NHS structure **15** from an ethanol solution, and subsequent nitrene-mediated photoligation under UV-irradiation at 254 nm. The resulting activated ester-functionalized surfaces were then submerged in solutions of alkyne- (**16**) or alkene-linkers (**14**) in acetonitrile, where stable amide bonds were formed. Carbohydrate-thiols were then coupled to the surfaces by photoinitiated radical addition in aqueous solution, yielding the corresponding carbohydrate-presenting surfaces. The crystals were thus placed on a horizontal surface and a drop of water containing the respective carbohydrate-thiol was placed directly on the surface, followed by UV-irradiation at 350 nm (Figure 3).

The overall methodology makes use of relatively simple organic molecules which can be synthesized in few steps in good yields. The compounds used to produce the alkene-/alkyne-surfaces can also be stored in prepared solutions at low temperature for repeated use over long time. The procedure is in addition fast and straightforward and can easily be expanded to high-throughput analysis methods using fluid robotics. The photochemical steps furthermore enable spatial and temporal control of the ligation process, in principle allowing for various patterning applications.

Initial surface experiments were carried out in the presence and absence of the water-soluble ACVA photoinitiator, with very similar product outcome in accordance with the solution-phase results. Thus, the surfaces could be efficiently prepared also in the absence of any photoinitiator, albeit requiring somewhat longer reaction times. It was also found that carbohydrate-thiols **4** and **8** were prone to oxidation to disulfides during storage in water-solution, and for this reason fresh solutions of all thiol-ligands were prepared prior to functionalization. In addition to carbohydrate-thiols **4**, **8**, **17** and **18**, 2-mercaptoethanol **19** was used to produce control-surfaces for both alkyne- and alkene-surfaces. The resulting control-surfaces were used to evaluate and correct for non-specific binding of each lectin in the binding analysis.

The carbohydrates D-mannose and D-galactose, along with the lectins Con A, specific for α-D-mannosides, and RCA-I, specific for β-D-galactosides, were used as model pairs for evaluation of the method. These lectins were chosen based on their binding specificities towards the chosen carbohydrates, their sizes and physical properties. RCA-I and Con A thus display similar molecular weights and pI values, (Bhattacharyya and Brewer 1990; Norberg et al. 2011; Ready et al. 1984; Sweeney et al. 1997) leading to similar behavior towards the surfaces in the QCM setup. Furthermore, two different types of carbohydrate thiols were utilized to investigate the versatility of the method as well as the potential implications of different divalent ligands on protein binding: 1-thio-carbohydrates **17**–**18** and thioethyl-linked carbohydrates **4** and **8**. The methodology was evaluated using a QCM flow-through instrumentation which allowed for repetitive injections of different proteins/concentrations of proteins and subsequent binding analysis to the same surface. BSA was used to block any potential unreacted/blank surface and consequently minimize the non-specific binding of the lectins in the study. BSA was injected only in the initial phase of the flow-through experiment, which was found to be sufficient to obtain stable blocking throughout the whole experiment. (Norberg et al. 2009b; Pei et al. 2005; Pei et al. 2006) Regeneration of the carbohydrate surfaces was easily accomplished by injections of low-pH buffer (pH 1.5), and the resulting binding was found to be very stable and reproducible over time (Figures 4 and 5).

The analysis resulted in binding well in accordance with the known specificities for both lectins. The quantitative binding was found to be similar for both alkyne- and alkene-originated surfaces, although varying slightly within series of the two types of carbohydrate thiols. In general, higher binding signals were found for carbohydrate surfaces produced from the thioethyl-linked carbohydrates (**4** and **8**) compared to surfaces based on the corresponding 1-thio-carbohydrates **17**–**18** (Figure 4), an effect which may be due to differences in lectin affinity towards 1-*O*- or 1-*S*-carbohydrates or differences in reactivities between the carbohydrate-thiols in the study. Interestingly, analysis of the binding curves furthermore suggested similar residual binding to both the alkyne- and the alkene-based surfaces (Figure 5), indicating comparable presentation patterns of the surfaces and potential monofunctionalization of the alkyne-based surfaces. This effect, together with the fact that the thiol-ene/-yne reactions proceed at higher rates, render the alkene-based surfaces more appropriate under the present conditions. More detailed studies are however of interest in order to elucidate the substrate dependency in the alkyne-thiol photoradical reaction when utilizing carbohydrate thiols for future applications.

Conclusions

A methodology to functionalize polymeric materials with carbohydrates using thiol-ene and thiol-yne photoinitiated radical chemistry has been demonstrated. The functionalization of intermediate alkene/alkyne surfaces was successfully performed with both 1-thio-carbohydrates and thioethyl-linked carbohydrate structures in pure water, and at room

temperature using long-range UV-irradiation. The results showed that no addition of radical initiator was necessary, and that the reactions proceeded well in aqueous solutions. The method is fast and straightforward, and can easily be employed in other biosensing/glycan array formats. As expected, the produced carbohydrate-presenting surfaces showed good selectivity towards the model lectins used in the study. Generally, the surfaces produced from 1-thio-carbohydrates showed slightly lower responses compared to the corresponding thioethyl-linked structures in the interaction studies. Inexpensive materials and relatively simple organic compounds were used throughout the study and the photochemical steps of the method enable the use of photolithography to produce patterned functionalized surfaces, important factors which further prompt the potential of the method in high-throughput analysis formats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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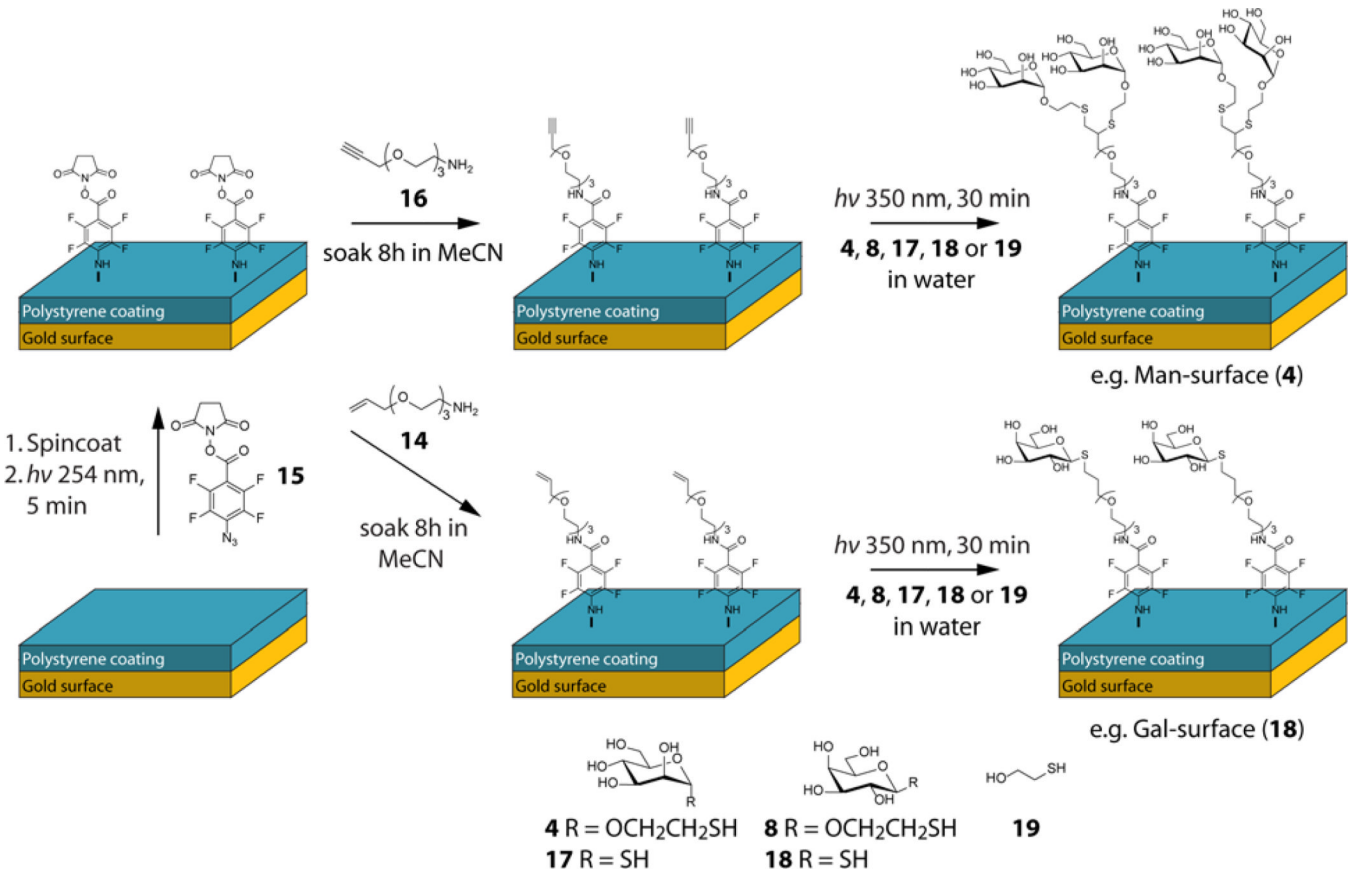


Figure 1.

General surface functionalization procedure. The polymeric surfaces were spincoated with a solution of compound **15** and irradiated with UV-light (254 nm) for 5 min. The NHS-activated ester surfaces were linked through an amidation process with amine **14** or **16** to produce the alkene/alkyne functionalized surfaces. The alkene/alkyne surfaces were then differentiated with thiol-functionalized molecules (**4**, **8**, **17**, **18** or **19**) through radical photo-click chemistry.

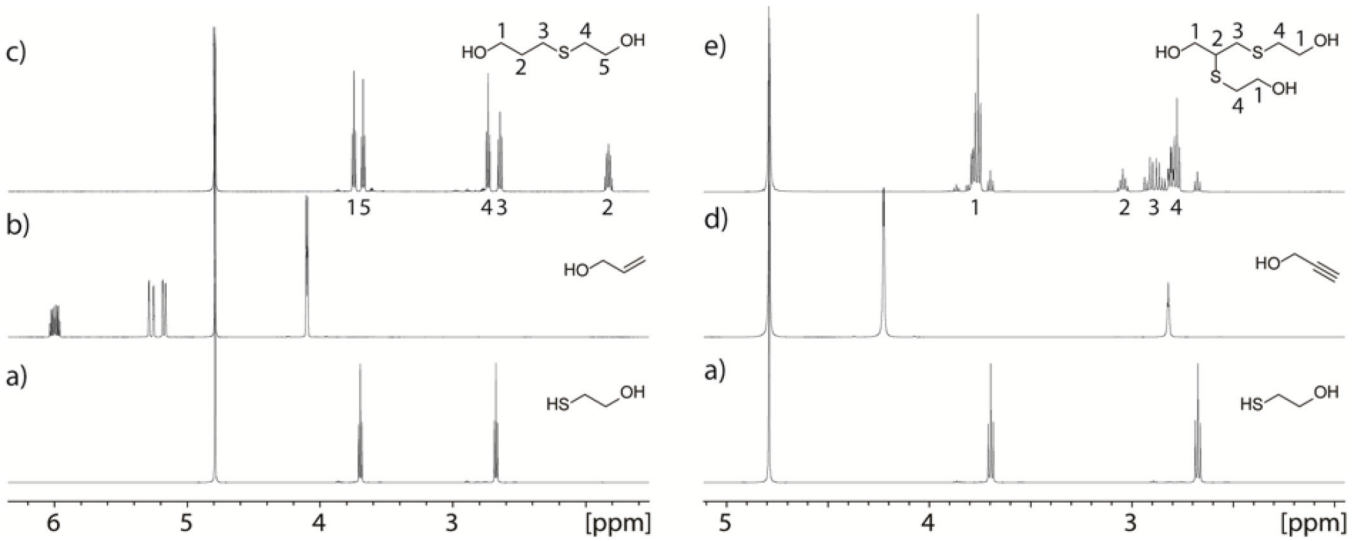


Figure 2.

^{1}H-NMR-spectra of model thiol-ene and thiol-yne reactions in water; a) 2-mercaptoethanol; b) allyl alcohol; c) thiol-ene reaction mixture (1:1 ratio) after 30 min UV-irradiation at 350 nm; d) propargyl alcohol; e) thiol-yne reaction mixture (1:2.1 alkyne:thiol) after 2 h UV-irradiation at 350 nm.

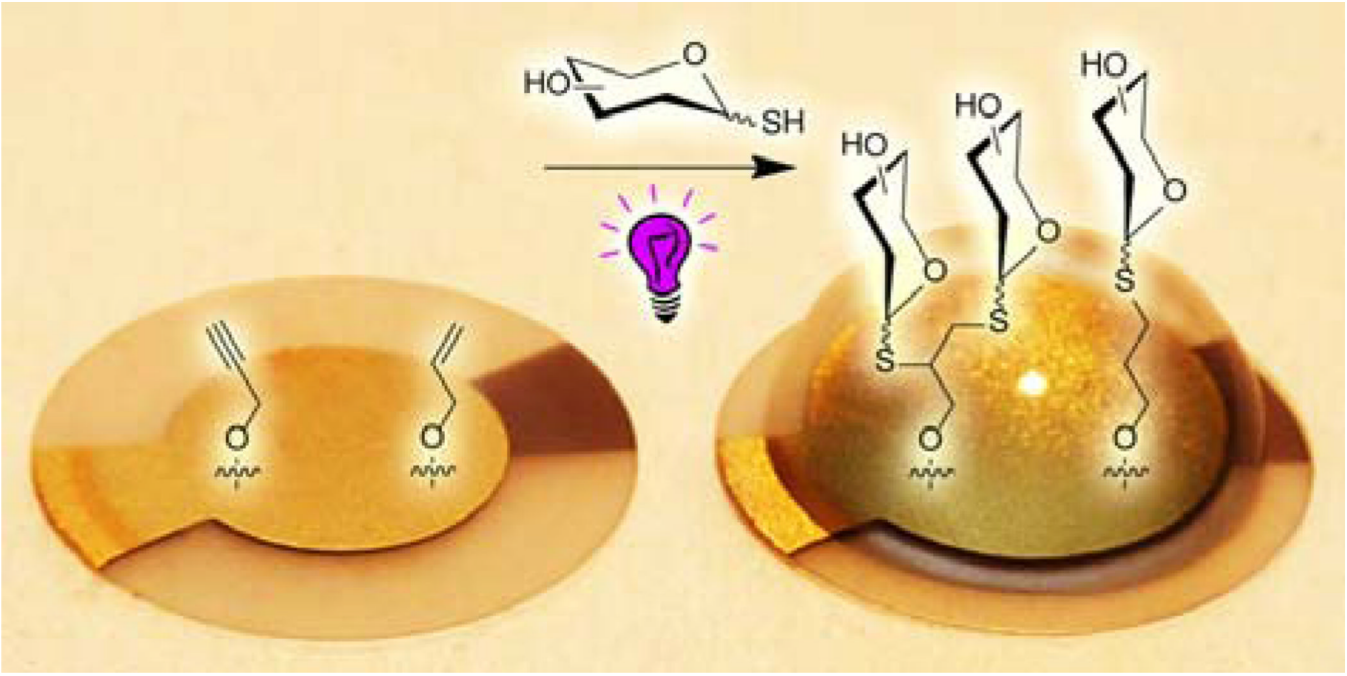


Figure 3.
Illustration of thiol-ene/-yne reactions performed directly on alkene/alkyne functionalized quartz crystals in a drop of pure water.

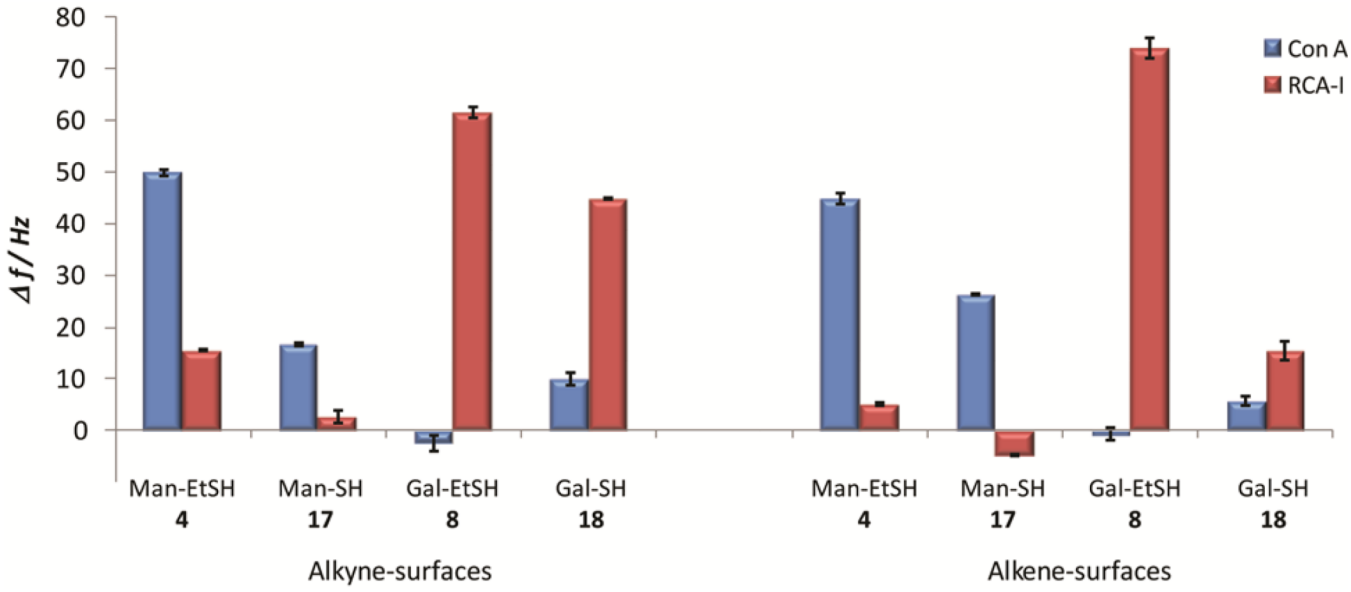


Figure 4.

Corrected lectin binding to all functionalized surfaces. Negative values are due to slightly higher binding to control surfaces. Error bars represent the standard errors of the mean (SEM) of triplicate injections of lectin at the same concentration (1 μM).

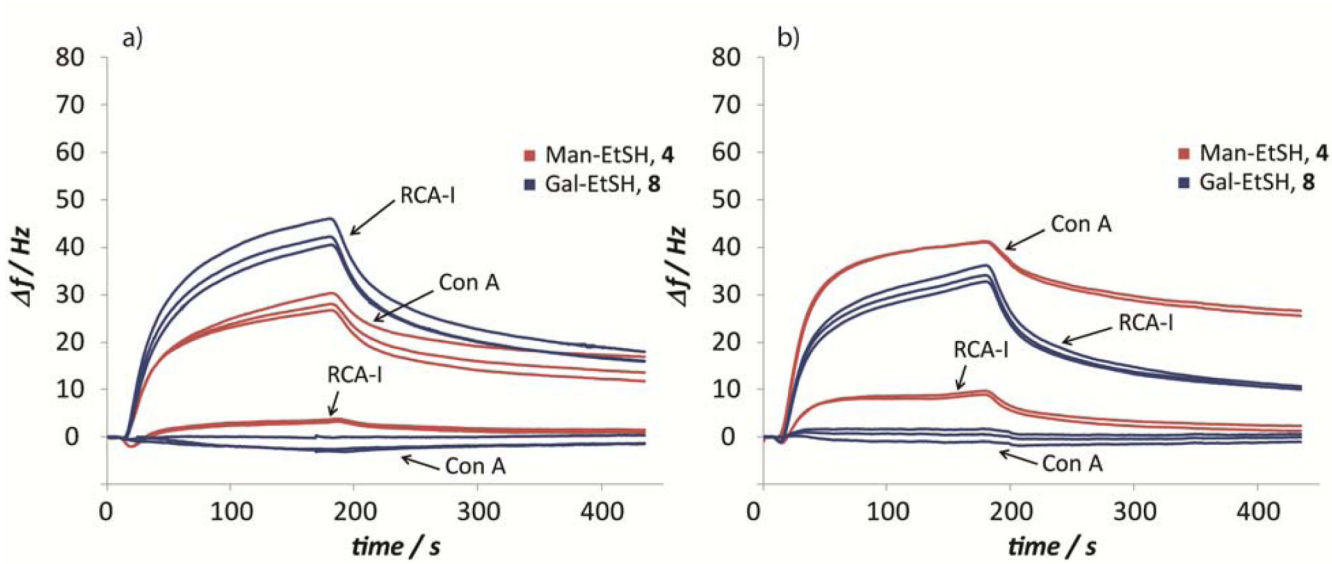
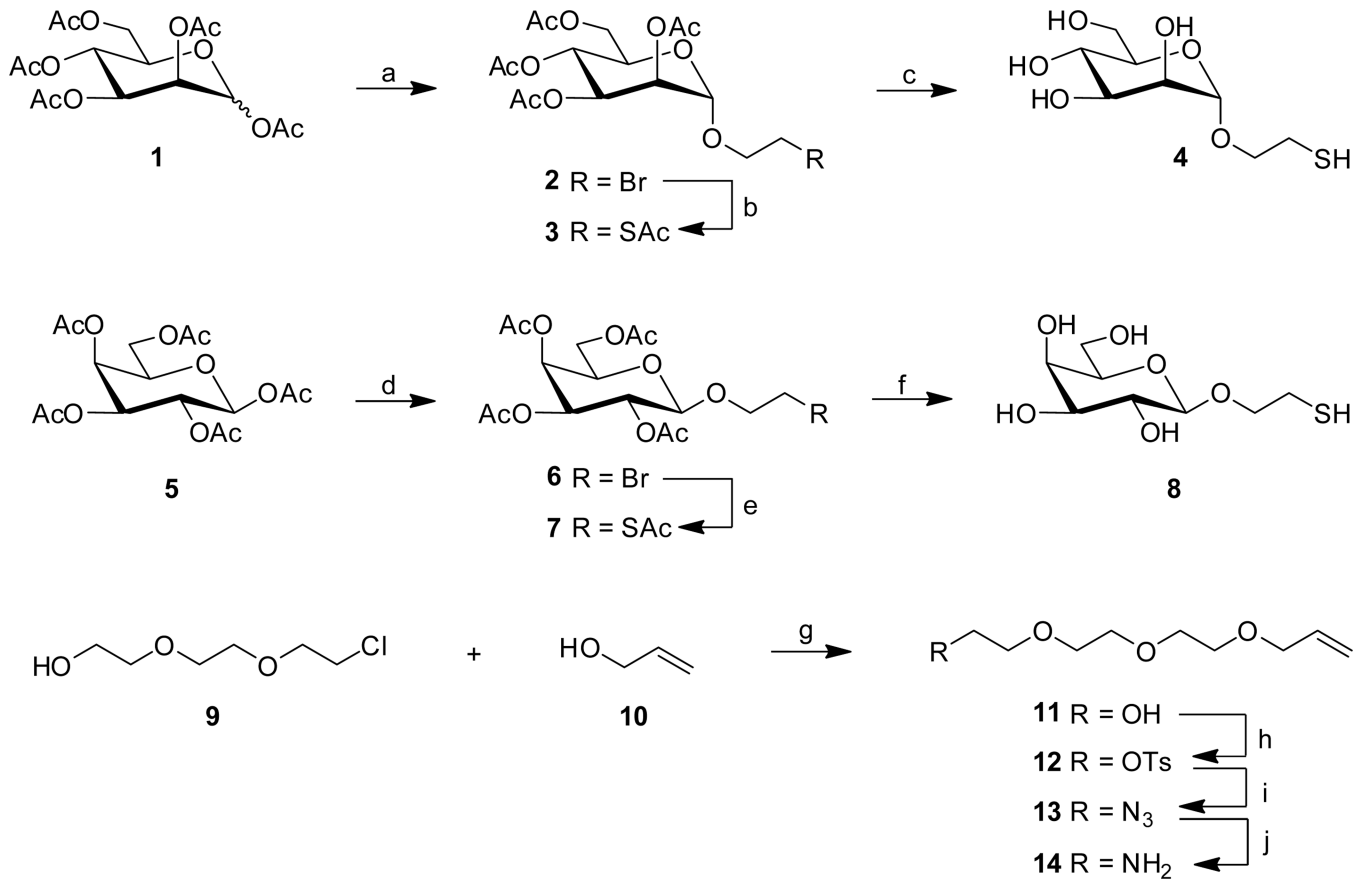


Figure 5.
Illustration of referenced binding curves of triplicate injections of Con A and RCA-I to mannose-/galactose-surfaces on a) alkyne-based surfaces and b) alkene-based surfaces.



Scheme 1.

Synthesis of thio-functionalized carbohydrates and alkene-linker for photo-click immobilization; a) 2- bromoethanol, $BF_3{\cdot}Et_2O$, DCM, 0 °C, 23 h; b) KSAc, DMF, 45 °C, 17 h (72 %); c) NaOMe, MeOH, rt, 3 h (quant.); d) 2-bromoethanol, $BF_3{\cdot}Et_2O$, DCM, −40 °C, 22 h (67 %); e) KSAc, DMF, 45 °C, 7 h (56 %); f) NaOMe, MeOH, rt, 4 h (70 %); g) NaOH, DMF, 45 °C, 2 h (30 %); h) TsCl + KOH, DCM, 0 °C, 5 h (quant.); i) NaN_3 + TBAI, DMF, 45 °C, 22 h (77 %); j) PPh_3, THF + H_2O, 0 °C, 24 h (32 %).