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Photo-Click Immobilization on Quartz Crystal Microbalance Sensors for Selective Carbohydrate−Protein Interaction Analyses

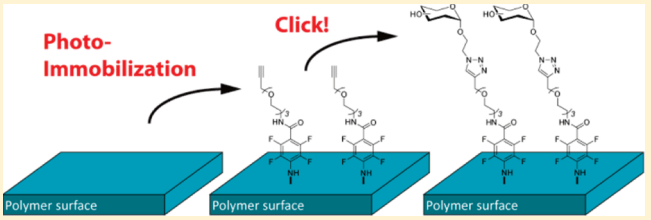
Oscar Norberg,[†,‡] Lingquan Deng,[†] Teodor Aastrup,[‡] Mingdi Yan,[*,§] and Olof Ramström[*,†]

[†]Department of Chemistry, KTH - Royal Institute of Technology, Teknikringen 30, S-10044, Stockholm, Sweden

[‡]Attana AB, Björnnäsvägen 21, S-11347, Stockholm, Sweden

[§]Department of Chemistry, Portland State University, P.O. Box 751, Portland, Oregon 97207-0751, United States

ABSTRACT: A photoclick method based on azide photoligation and Cu-catalyzed azide−alkyne cycloaddition has been evaluated for the immobilization of carbohydrates to polymeric materials. The biomolecular recognition properties of the materials have been investigated with regard to applicable polymeric substrates and selectivity of protein binding. The method was used to functionalize a range of polymeric surfaces (polystyrene, polyacrylamide, poly(ethylene glycol), poly(2-ethyl-2-oxazoline), and polypropene) with various carbohydrate structures (based on α-D-mannose, β-D-galactose, and *N*-acetyl-β-D-glucosamine). The functionalized surfaces were evaluated in real-time studies of protein−carbohydrate interactions using a quartz crystal microbalance flow-through system with a series of different carbohydrate-binding proteins (lectins). The method proved to be robust and versatile, resulting in a range of efficient sensors showing high and predictable protein selectivities.



Glycobiology is a rapidly growing research field encompassing the effects of carbohydrates and glycoconjugates (glycans) in biological systems and their interactions with proteins, cells, and other biomolecules. The complex functions of glycans have so far been difficult to explore due to several challenges, most notably the synthesis and/or purification of glycans and the following analysis of the biomolecular interactions.[1−4] In order to robust and efficient high-throughput analysis methods are required. Several potent glycan array formats have for this reason been developed, where more or less specific chemical ligation techniques are used to selectively immobilize glycans in an array format.[5−11] These techniques have accelerated the development of the field, and glycan arrays have for example been used to discover multivalency effects,[12] evaluate blood serum glycan binding[13] and antibodies toward cancer,[14] determine detailed binding specificity for glycan-binding proteins[15] and macrophage lectins,[16] investigate the receptor binding properties of the influenza virus H2N2,[17] and identify cellular markers,[18] enzymes involved in wound healing,[19] proteins involved in cancer metastasis,[20] glycans modulating galectin-1 T cell death,[21] and the glycome of HIV-1 virions.[22]

Herein we present the exploration of the photoclick immobilization technique previously developed in our laboratory as a general polymeric platform for protein−carbohydrate interactions.[23] Polymeric materials are both cheap and easily moldable, qualities which are highly desirable in life sciences. Methodologies based on polymeric materials are thus potentially useful for large scale production. In addition, the physicochemical properties of polymers can be easily tuned. Copper-catalyzed azide−alkyne cycloaddition (CuAAC) is a highly selective reaction between azides and alkynes, chemical moieties that are both rare and generally inert in biological systems, which forms a strong nonhydrolyzable triazole linkage between the two molecules.[24−26] Additionally, azides are easy to incorporate synthetically in organic compounds and are extensively used as precursors to obtain amine-functionalized molecules, a feature which has resulted in large libraries of available carbohydrate azides.[27] The use of CuAAC to immobilize carbohydrate azides in array formats have also been applied to alkyne-functionalized microtiter plates,[28] alkyne-linkers on glass slides,[29] and alkyne-functionalized gold surfaces.[30]

New methodologies for glycan arrays require rigorous validation procedures to ensure that the measured response indeed correlates to the biological response. Most methodologies are evaluated using glycans and receptors with known and corresponding specificity for the chosen glycans. Lectins (unmodified and FITC-labeled)[10−12,23,28−35] and antibodies (mono- and polyclonal)[14,18,35−37] are the most commonly used validation targets for glycan array methodologies. Lectins are nonenzymatic proteins that bind oligosaccharides with high specificity, often in di- or polyvalent complexes. Many lectins are selective for monosaccharides (e.g., α-D-mannose, β-D-galactose, or *N*-acetyl-β-D-glucosamine) but they show up to a 1000-fold increase in affinity for the corresponding di-, tri-, and tetrasaccharides.[38]

Glycan array methodologies are primarily designed for use in high-throughput microarray printing and analysis with fluorescently tagged targets, or targets with fluorescently tagged antibodies known to bind specifically to the target. To avoid the need for fluorescently tagged targets, which are both expensive and demanding to produce, many glycan array methodologies are evaluated using techniques such as surface plasmon resonance (SPR)[30,39−43] and quartz crystal microbalance (QCM)[23,32,44,45]

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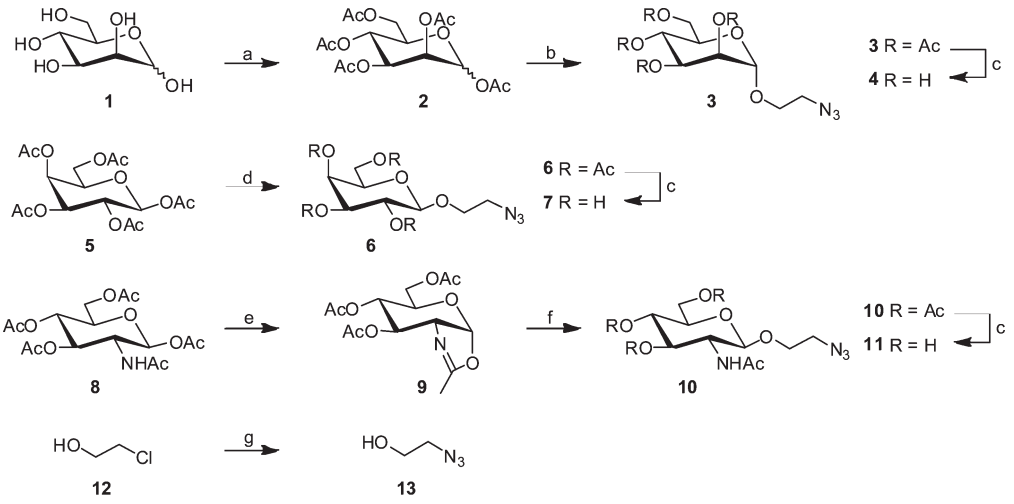
Scheme 1. Synthesis of Azide-Functionalized Ligands for Photoclick Immobilization[a]



[a] (a) Ac_2O, pyridine, r.t., 46 h (quant.); (b) 2-azidoethanol, $BF_3 \cdot Et_2O$, DCM, 0 °C, 18 h (83%); (c) NaOMe, MeOH, rt, 2.5 h (95%); (d) 2-azidoethanol, $BF_3 \cdot Et_2O$, DCM, −40 °C, 24 h (69%); (e) (i) TMSOTf, 50 °C, 30 min, (ii) TEA, r.t., 10 min (98%); (f) 2-azidoethanol, H_2SO_4, r.t., 19 h (23%); (g) NaN_3, TBABr, 110 °C, 18 h (99%).

which instead rely on the physical properties of surface plasmon absorption and mass change to detect target binding.

In this publication we explore the versatility of the platform and protein selectivity of the photoclick immobilization technique. The methodology was evaluated by protein binding analyses on a range of polymeric materials functionalized with a series of monosaccharides, using a QCM flow-though instrumentation.

MATERIALS AND METHODS

General. All commercially available starting materials were of reagent grade and used as received. Lectins were purchased from Sigma-Aldrich and Vector Laboratories. QCM crystals were obtained from Attana AB. ^1H and ^{13}C NMR data were recorded on a Bruker Avance 400 instrument at 400 MHz (^1H) or a Bruker DMX 500 instrument at 500 MHz (^1H) or 125 MHz (^{13}C). Chemical shifts are reported as δ values (ppm) with either $CDCl_3$ (^1H: δ = 7.26, ^{13}C = 77.16) or D_2O (^1H: δ = 4.79) as internal standard. J values are given in Hertz. ^1H peak assignments were made by first-order analysis of the spectra supported by standard $^1H-^1H$ 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).

Synthesis. All compounds were synthesized in a few steps with high yields according to previously published procedures (Scheme 1). Compounds **4** and **7** were synthesized from the corresponding pentaacetates (**2** and **5**) using a procedure by Wong et al.[28] Compound **11** was prepared from the peracetylated *N*-acetyl-β-D-glucosamine (**8**) through the oxazoline intermediate according to Nakabayashi et al.[46] and Åberg et al.[47] 2-Azidoethanol (**13**) was synthesized from 2-chloroethanol according to Pfaendler and Weimar.[48] Compounds **14**, **15**, and **16** were synthesized as previously reported by our group.[23,32]

1-(2-Azidoethyl)-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (3). D-Mannose (**1**, 4.64 g, 25.7 mmol) and pyridine (1.0 mL, 12.9 mmol) were mixed in acetic anhydride (Ac_2O, 150 mL) and stirred at r.t. for 46 h, after which the solution turned clear. The mixture was then poured on ice, and the water phase was extracted with dichloromethane (DCM). The organic phase was washed with saturated $NaHCO_3$ (aq) and brine and dried over $MgSO_4$. The solvent was evaporated under reduced pressure yielding D-mannose pentaacetate (**2**) as a pale yellow oil (10.3 g, quant.), with an α/β ratio of 1.37:1. A solution of 2-azidoethanol (**13**, 424 mg, 4.88 mmol) in DCM (20 mL) was subsequently added to compound **2** (1.59 g, 4.06 mmol). The mixture was stirred at 0 °C, and boron trifluoride diethyl etherate ($BF_3 \cdot Et_2O$, 2.04 mL, 16.2 mmol) was added slowly. After 1 h, the mixture was allowed to reach r.t., and after 18 h added to ice−water (20 mL). The resulting mixture was extracted with DCM (2 × 20 mL), and the combined organic phase was washed with ice−water, saturated $NaHCO_3$ (aq), and ice−water. The organic phase was dried over Na_2SO_4 and the solvent evaporated under reduced pressure yielding product **3** (1.41 g, 83%). ^1H NMR (500 MHz, $CDCl_3$): δ 5.34 (dd, 1 H, J = 3.5 and 9.8 Hz, H-3), 5.28 (d, 1 H, J = 10.1 Hz, H-4), 5.25 (dd, 1 H, J = 1.9 and 3.5 Hz, H-2), 4.85 (d, 1 H, J = 1.9 Hz, H-1), 4.26 (dd, 1 H, J = 5.4 and 12.3 Hz, H-6), 4.10 (dd, 1 H, J = 2.2 and 12.3 Hz, H-6′), 4.02 (ddd, 1 H, J = 2.5, 5.4, and 9.8 Hz, H-5), 3.85 (ddd, 1 H, J = 3.8, 6.9, and 10.7 Hz, $CH_2CH_2N_3$), 3.65 (ddd, 1 H, J = 3.9, 6.2, and 10.3 Hz, $CH_2CH_2N_3$), 3.47 (ddd, 1 H, J = 3.5, 6.9, and 13.2 Hz, CH_2N_3), 3.42 (ddd, 1 H, J = 3.8, 6.0, and 13.2 Hz, CH_2N_3), 2.14 (s, 3 H, Ac), 2.08 (s, 3 H, Ac), 2.03 (s, 3 H, Ac), 1.97 (s, 3 H, Ac). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.74, 170.13, 169.93, 169.88, 97.88, 69.53, 69.00, 68.98, 67.18, 66.14, 62.59, 50.49, 21.00, 20.87, 20.83, 20.78.

1-(2-Azidoethyl)-α-D-mannopyranoside (4). 1-(2-Azidoethyl)-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**3**, 66 mg, 0.16 mmol) was dissolved in MeOH (30 mL). Then sodium methoxide (NaOMe, 25 mg, 0.47 mmol) was added, and the reaction mixture was stirred at r.t. for 2.5 h until TLC indicated full conversion. Then Amberlyst 15 was added until pH reached ∼7. The Amberlyst was then filtered off, and the solvent was evaporated under reduced pressure giving the pure product **4** (37 mg, 95%). ^1H NMR (500 MHz, D_2O): δ 4.93 (d, 1 H J = 1.6 Hz, H-1), 3.99 (dd, 1 H, J = 1.6 and 3.5 Hz, H-2), 3.93 (ddd, 1 H, J = 3.4, 7.0, and 10.8 Hz, $CH_2CH_2N_3$), 3.91 (dd, 1 H, J = 1.3 and 12.3 Hz, H-6), 3.85 (dd, 1 H, J = 3.5 and 9.1 Hz, H-3), 3.77 (dd, 1 H, J = 5.7 and 12.0 Hz, H-6′), 3.73 (ddd, 1 H, J = 3.2, 6.3, and 11.0 Hz, $CH_2CH_2N_3$), 3.70−3.65 (m, 2 H, H-4 and H-5), 3.56 (ddd, 1 H,

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J = 3.1, 6.9 and 13.6, CH_2N_3), 3.50 (ddd, 1 H, J = 3.2, 6.4 and 13.6, CH_2N_3). ^{13}C NMR (125 MHz, D_2O): δ 99.80, 72.88, 70.37, 69.92, 66.67, 66.29, 60.90, 50.19.

1-(2-Azidoethyl)-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (6). β-D-Galactose pentaacetate (**5**, 652 mg, 1.67 mmol) was dissolved in DCM (10 mL), after which 2-azidoethanol (**13**, 250 μL, 3.34 mmol) was added. The mixture was then stirred at −40 °C and $BF_3 \cdot Et_2O$ (1.07 mL, 8.52 mmol) was added slowly. After 24 h, the temperature had reached 10 °C. The mixture was then added to ice−water (20 mL) and extracted with DCM (2 × 20 mL). The combined organic phases were washed with ice−water (20 mL), saturated $NaHCO_3$ (aq), and ice−water. The organic phase was dried ($MgSO_4$) and the solvent evaporated under reduced pressure. The crude product was separated by flash column chromatography with solvent system hexanes/ethyl acetate (Hex/EtOAc) 1:1 giving pure **6** (482 mg, 69%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.40 (d, J = 3.4 Hz, 1H, H-4), 5.24 (dd, J = 8.0 and 10.4 Hz, 1H, H-2), 5.03 (dd, J = 3.4 and 10.5 Hz, 1H, H-3), 4.56 (d, J = 8.0 Hz, 1H, H-1), 4.19 (dd, J = 6.6 and 11.3 Hz, 1H, H-6), 4.13 (dd, J = 6.8 and 11.2 Hz, 1H, H-6′), 4.04 (dt, J = 4.2 and 10.6 Hz, 1H, CH_2), 3.93 (t, J = 6.7 Hz, 1H, H-5), 3.72−3.67 (m, 1H, CH_2), 3.53−3.48 (m, 1H, CH_2), 3.31 (dt, J = 4.0 and 13.4 Hz, 1H, CH_2), 2.16 (s, 1H, Ac), 2.07 (s, 1H, Ac), 2.05 (s, 1H, Ac), 1.99 (s, 1H, Ac). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.50, 170.35, 170.27, 169.60, 101.26, 71.02, 70.95, 68.66, 68.50, 67.14, 61.38, 50.69, 20.88, 20.78, 20.77, 20.68.

1-(2-Azidoethyl)-β-D-galactopyranoside (7). 1-(2-Azidoethyl)-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**6**, 480 mg, 1.15 mmol) was dissolved in MeOH (10 mL) under nitrogen atmosphere. Then sodium methoxide (74 mg, 1.4 mmol) in methanol was added with syringe, and the reaction mixture was stirred at r.t. for 4 h after which TLC indicated full conversion. Then Amberlyst 15 was added under gentle stirring until the pH reached ∼7. The Amberlyst was then filtered off, and the solvent was evaporated under reduced pressure to give pure **7** (286 mg, quant.). ^{1}H NMR (500 MHz, D_2O): δ 4.43 (d, J = 7.9 Hz, ^{1}H, H-1), 4.06 (dt, J = 4.9 and 11.3, 1H, CH_2), 3.92 (d, J = 3.4 Hz, 1H, H-4), 3.86−3−73 (m, 3H, CH_2, H-6 and H-6′), 3.69 (dd, J = 4.4 and 7.8 Hz, 1H, H-5), 3.65 (dd, J = 3.5 and 10.0 Hz, 1H, H-3), 3.56−3.51 (m, 3H, 2 × CH_2 and H-2). ^{13}C NMR (125 MHz, D_2O): δ 152.92, 102.88, 99.77, 75.17, 72.69, 70.67, 68.61, 68.37, 60.93, 56.11, 50.54, 20.27.

2,3-Dihydrooxazole-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside (9). 2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**8**, 210 mg, 0.539 mmol) was dissolved in dichloroethane (15 mL). Then trimethylsilyl trifluoromethanesulfonate (TMSOTf, 97 μL, 0.59 mmol) was added, and the mixture was stirred at 50 °C for 30 min after which TLC indicated full conversion. The mixture was then removed from the heat, and triethylamine (TEA, 225 μL, 1.62 mmol) was added. The mixture was then stirred at r.t. for 10 min after which it was passed through a short plug of silica and washed carefully with DCM and EtOAc. The solvent was then evaporated under reduced pressure, and the crude product was separated by flash column chromatography with EtOAc as solvent giving **9** as a colorless oil (174 mg, 98%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.86 (d, J = 7.4 Hz, 1H, H-1), 5.14 (app. t, J = 2.1 Hz, 1H, H-5), 4.80 (d, J = 9.3 Hz, 1H, H-4), 4.07−3.99 (m, 3H, H-2, H-6 and H-6′), 3.51−3.57 (m, 1H, H-3), 2.00 (s, 3H, Ac), 1.98 (s, 6H, 2 × Ac), 1.97 (s, 3H, Ac). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.41, 169.39, 169.08, 166.51, 99.27, 70.24, 68.27, 67.38, 64.81, 63.22, 20.76, 20.69, 20.59, 13.80.

1-(2-Azidoethyl)-2-acetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside (10). 2,3-Dihydrooxazole-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside (**9**, 90 mg, 0.27 mmol) was dissolved in dichloromethane (5 mL) containing molecular sieves (4 Å). The mixture was stirred for 30 min at r.t. after which concentrated sulfuric acid (three drops) was added. The mixture was then stirred at r.t. for 19 h after which it was diluted with dichloromethane and filtered through Celite. The organic phase was washed with saturated $NaHCO_3$ (aq) and ice−water. The organic phase was dried ($MgSO_4$) and the solvent evaporated under reduced pressure. The crude product was separated by flash column chromatography with solvent system Hex/EtOAc 1:8 giving pure **10** (26 mg, 23%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.63 (d, J = 8.6 Hz, 1H, NH), 5.36 (t, J = 9.9 Hz, 1H, H-3), 5.07 (t, J = 9.6 Hz, 1H, H-4), 4.83 (d, J = 8.3 Hz, 1H, H-1), 4.25 (dd, J = 4.8 and 12.3 Hz, 1H, H-6), 4.15 (dd, J = 2.4 and 12.3 Hz, 1H, H-6′), 4.04 (app. dt, J = 3.9 and 10.8 Hz, 1H, CH_2), 3.81 (app. q, J = 9.3 Hz, 1H, H-2), 3.74−3.68 (m, 2H, H-5 + CH_2), 3.53−3.48 (m, 1H, CH_2), 3.26 (app. dt, J = 3.9 and 13.2 Hz, 1H, CH_2), 2.08 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.95 (s, 3H, Ac). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.93, 170.84, 170.61, 169.57, 100.64, 72.22, 72.14, 68.72, 68.57, 62.15, 55.09, 50.77, 23.53, 20.90, 20.83, 20.79.

1-(2-Azidoethyl)-2-acetamido-β-D-glucopyranoside (11). 1-(2-Azidoethyl)-2-acetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside (**10**, 101 mg, 0.241 mmol) was dissolved in MeOH (15 mL). Then sodium methoxide (14 mg, 0.27 mmol) was added, and the reaction mixture was stirred at r.t. for 4 h until TLC indicated full conversion. Then Amberlyst 15 was added until the pH reached ∼7. The Amberlyst was then filtered off, and the solvent was evaporated under reduced pressure giving the pure product **11** (67 mg, 95%). ^{1}H NMR (500 MHz, D_2O): δ 4.58 (d, 1H, J = 8.6 Hz, H-1), 4.05 (ddd, 1H, J = 2.8, 5.3, and 11.2 Hz, $CH_2CH_2N_3$), 3.92 (app. dd, 1H, J = 1.6 and 12.5 Hz, H-6), 3.87−3.70 (m, 3H, H-2, H-5 and $CH_2CH_2N_3$), 3.54 (app. t, 1H, J = 9.2 Hz, H-3), 3.50−3.39 (m, 4H, H-4, H-6′ and 2 × CH_2N_3), 2.04 (s, 3H, NAc). ^{13}C NMR (125 MHz, D_2O): δ 169.63, 95.96, 70.83, 68.79, 64.81, 63.65, 55.62, 45.28, 25.14, 17.15.

2-Azidoethanol (12). 2-Chloroethanol (2.09 g, 26.0 mmol), sodium azide (5.1 g, 78 mmol), and tetrabutylammonium bromide (TBABr, 836 mg, 2.60 mmol) were mixed in a round-bottom flask equipped with a reflux condenser and stirred at 110 °C for 18 h (using a safety shield). The mixture was then diluted with diethyl ether, and the solid byproducts were filtered off. The solvent was evaporated under reduced pressure (no heating), giving a yellow oil. The crude product was purified by distillation at 12 mbar, yielding **13** as a colorless oil (2.23 g, 99%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.78 (app. q, 2H, J = 5.2 Hz, $HOCH_2$-CH_2N_3), 3.45 (t, 2H, J = 5.2 Hz, $HOCH_2CH_2N_3$), 1.77 (t, 1H, J = 5.7 Hz, $HOCH_2CH_2N_3$). ^{13}C NMR (125 MHz, $CDCl_3$): δ 61.71, 53.71.

General Surface Modification. All QCM experiments were performed on gold-plated 10 MHz quartz crystals (Attana) coated with polymeric layers. Polystyrene-coated crystals were purchased from Attana. All other surfaces were spin-coated using a Cookson Electronics Specialty Coating Systems Spincoater model P6708D and solutions of the selected polymers. The photoreaction step was performed at 240−400 nm at a measured intensity of 13.3−13.5 mW/cm^2 with a LC8 equipped Hg−Xe UV-lamp from Hamamatsu Photonics. The fabricated crystals were mounted in a flow-through QCM system (Attana A100 C-Fast).

Surface Functionalization (cf. Figure 1). All polymeric surfaces except for commercial polystyrene-coated surfaces were produced according to Figure 1 by a modified procedure previously developed in our laboratory.[32] The gold-plated quartz crystals were soaked in a solution of PFPA-disulfide (**14**, 14 mM)

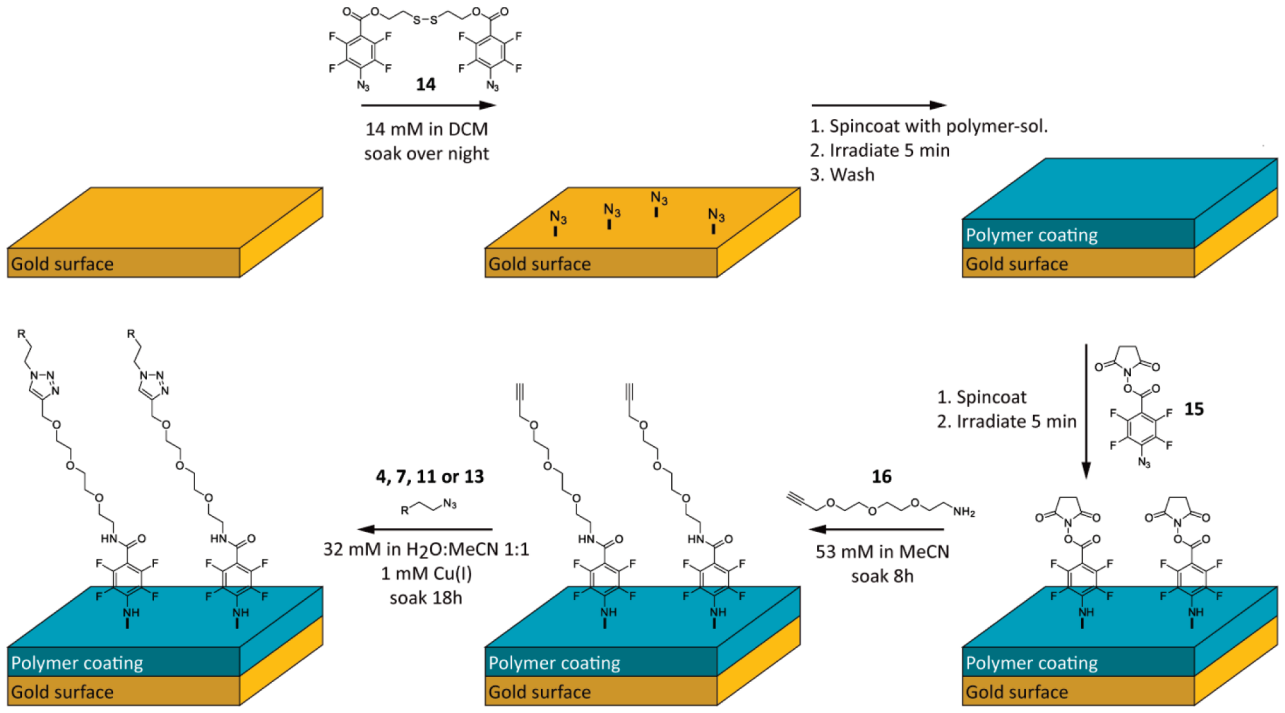

Figure 1. General surface functionalization procedure. Gold surfaces were soaked in a solution of compound **14** overnight, after which they were spin-coated with polymer solutions, irradiated with UV light for 5 min and subsequently washed. The polymeric surfaces were then spin-coated with a solution of compound **15** and irradiated with UV light for 5 min. The NHS-activated ester surfaces were linked through an amidation process with amine **16** to produce the alkyne functionalized surfaces. The alkyne surfaces were then differentiated with several azide functionalized molecules through CuAAC chemistry.

Table 1. Polymer Spin-Coating Parameters

polymer	M_w	solvent	concn, mg/mL	drop size, μL	wash solvent
polyacrylamide (PAAm)	$5-6 \times 10^6$	ethylene glycol	10	10	EtOH
poly(ethylene glycol) (PEG)	20×10^3	dimethoxyethane	10	15	EtOH
poly(2-ethyl-2-oxazoline) (PEOX)	200×10^3	chloroform	10	15	$CHCl_3$
polypropylene (PP)	250×10^3	1,1,2,2-tetrachloroethane (R-130)	10	10	R-130

in dichloromethane overnight (protected from light). The next day, they were removed from the solution and dried under a gentle stream of nitrogen, spin-coated with a solution of the selected polymer (see Table 1) at 2000 rpm for 120 s, and irradiated for 5 min with UV light. The crystals were then washed (see Table 1) and dried under a gentle stream of nitrogen. Subsequently, the crystals (including polystyrene) were spin-coated with a solution of PFPA-NHS (**15**, 10 mM, 10 μL) in ethanol at 1500 rpm for 180 s followed by immersion in a solution of linker **16** (53 mM) in acetonitrile for 8 h. The crystals were then washed with acetonitrile before the CuAAC-step. The crystals were soaked in a solution of azide **4**, **7**, **11**, or **13** (32 mM), CuI (1 mM), and diisopropylethylamine (DIPEA, 13 mM) in water:acetonitrile (1:1) for 18 h after which they were thoroughly rinsed with water:acetonitrile (1:1) and methanol, dried under a gentle stream of nitrogen, and placed in the QCM flow-through system.

General Surface Analysis. A continuous flow of running buffer (PBS 10 mM, pH 7.4, 25 μL/min) was used throughout the experiments, and samples of concanavalin A (Con A), wheat germ agglutinin (WGA), *Pisum sativum* agglutinin (PSA), *Ricinus communis* agglutinin I (RCA-I), and *Bandeiraea simplicifolia* lectin II (BS-II) were prepared in the same buffer. The crystals were washed/equilibrated with buffer solution prior to manipulations/measurements. After the crystal was equilibrated in the flow-through system, it was subjected to five injections of Bovine Serum Albumin (BSA, 0.5 mg/mL), three injections of low pH buffer (pH 1.5), and finally two additional injections of Bovine Serum Albumin (BSA, 0.5 mg/mL) to fully block any nonfunctionalized surface. Binding to the surface was monitored by frequency logging with Attester 1.1 (Attana), and adsorption/desorption to the surface recorded as the resulting frequency shifts. Solutions (0.2 mg/mL) of each lectin were then injected into the system, where the resulting shift in frequency corresponds to the binding to the surface. Bound lectin was released from the surface between measurements by eight successive injections of low pH buffer (PBS 10 mM, pH 1.5). The procedure was then repeated two times for each lectin to give an average value and determine the surface stability over time.

RESULTS AND DISCUSSION

The photoclick immobilization procedure presented in Figure 1 is a convergent approach that consists of three steps;

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Table 2. Properties of Lectins Used in the Binding Study

name	M_w, kDa	binding valency	carbohydrate specificity	family	reported pI	measured pI	refs
Con A	104	homotetramer	Man, Glc	legume	8.35[52]	6.5−8	52−55
WGA	36	homodimer	GlcNAc	cereal	8.7[56]	n.d.[a]	56
PSA (seeds)	48	heterotetramer	Man, Glc	legume	5.9 and 7.0[57]	4.5−7.3	57,58
RCA-I (seeds)	120	heterotetramer	Gal	*Euphorbiaceae*	−	6.6−9.3	59,60
BS-II (seeds)	120	homotetramer	GlcNAc	legume	−	5−7.3	61

[a] n.d. = not detectable.

photochemical insertion of perfluorophenyl azides into the polymeric material, amide coupling to produce a triethylene glycol-linked alkyne surface, and, the last step, copper-catalyzed azide−alkyne cycloaddition, which is the diversifying step of the procedure. Since only the last step is dissimilar for production of different carbohydrate-functionalized surfaces, the methodology allows for large scale production of the intermediate alkyne surfaces, which, after production, can be stored for a long time before use (no detectable reduction in activity after weeks of storage at +4 °C in water). The photochemical step being very fast, the overall surface preparation time is governed primarily by the amidation and the CuAAC reaction steps. In the present setup, the crystals were submerged during these steps in a solution of the reactants (≥300 μL) to ensure full coverage of the crystal surface, and the reaction times can likely be further reduced with smaller crystal formats and higher concentrations.

One of the advantages of the functionalization method is that relatively simple organic molecules, which require short and facile synthesis, can be employed. The NHS-activated ester (**15**), the alkyne linker (**16**), and the azide derivatives (**4**, **7**, **11** and **13**) were all synthesized in few steps with good overall yields. Both the NHS-activated ester (**15**) and the alkyne linker (**16**) can be stored in solution at low temperature for extended times, enabling repeated use of one batch of compounds. Since the functionalization is only diversified in the final step, expansion of the format can be efficiently achieved using arrays of carbohydrate azides.

For instrumentation reasons, gold-plated quartz-crystals were used as platforms for the functionalization methodology, thus requiring the production of polymeric layers on the gold surfaces. The polymeric surfaces used in the study were therefore produced by the spin-coating of dissolved polymeric materials on PFPA-derivatized polished gold surfaces and subsequent photoirradiation to covalently attach the polymers to the gold surface (Figure 1).[32] The polymers were chosen to cover a range of polymeric materials to illustrate the versatility of the method. Both hydrophobic (PS and PP) and hydrophilic (PAAm, PEG, and PEOX) polymers with a wide range of molecular weights were used (Table 1). The solvents used to dissolve the polymers for spin-coating were chosen based on previous experience in spin-coating (PEG and PEOX[49]) and solubility properties of the polymers. The solvation of the hydrophobic polymer polypropene proved in this context especially difficult. Only with a combination of 1,1,2,2-tetrachloroethane and heating could the polymer be dissolved. After the spin-coating, the crystals were irradiated with UV light for 5 min before washing with a suitable solvent. This time, the solvent was chosen so that it would be possible to wash off any unbound polymer as well as solvent residues from the spin-coating (ethylene glycol and dimethoxyethane) and at the same time dry the surface on a reasonable time scale.

The surfaces produced were then functionalized with alkyne moieties in two steps. First, the polymeric surfaces were spin-coated with an ethanol solution of PFPA-NHS **15** followed by irradiation with UV light. Second, the QCM-crystals were immersed in an acetonitrile solution of alkyne linker **16**, where the amine was coupled to the activated ester, forming a stable amide bond. The two-step method was chosen over the faster direct alkyne functionalization method (previously reported by our group[23]) since the two-step methodology produced more consistent surfaces with shorter response times in the QCM instrumentation. The alkyne surfaces were then immersed in a water/acetonitrile (1:1) solution of the carbohydrate azides in the presence of a catalytic amount of CuI and diisopropylethylamine. CuI and diisopropylethylamine were chosen over the more traditional catalyst, in situ reduction of $CuSO_4$ by sodium ascorbate, in order to avoid the use of ascorbate in the methodology. Since the reduction of Cu^{II} by ascorbate is a radical two-step oxidation process of ascorbate, possible side reactions of the radical intermediate to the surface are highly undesirable. The functionalized surfaces were subsequently washed with water/acetonitrile and methanol to remove unreacted reagents and dry the surface before analysis. The surfaces were thus functionalized with three different monosaccharide moieties (α-D-mannose, β-D-galactose, or *N*-acetyl-β-D-glucosamine) which encompassed a short spacer between the carbohydrate and the azide in order to reduce potential interference in the subsequent protein binding event, due to a too closely attached triazole moiety. The polymeric surfaces were also functionalized with 2-azidoethanol to produce control surfaces.

The method was evaluated using a QCM flow-through instrumentation with repeated injections of lectins specific for the monosaccharides used in the study. The lectins and carbohydrates were chosen based on binding specificity, molecular weight, and previous use of lectins in surface analysis methodology (Table 2). Con A and PSA were chosen because of their specificity toward α-D-mannose-residues, whereas WGA and BS-II bind selectively to *N*-acetyl-β-D-glucosamine and RCA-I to β-D-galactose. To minimize nonspecific binding, BSA was used to block all nonderivatized surfaces by several injections in the beginning of the experiment. Previous experience with the instrumentation shows that blocking with BSA in the initial phase of the experiment lasts throughout the whole experiment, thus obviating the need for BSA in the running buffer.[23,50,51]

The protein−carbohydrate interactions were monitored in real time, and both association and dissociation of the lectins to the surfaces could be observed (Figure 2). Small levels of potential nonspecific binding of several of the lectins were noticed, hypothetically owing to, for example, surface structure and composition, blocking coverage by BSA, pI values of the lectins, etc. A strategy to correct for this nonspecific binding was therefore

adopted, based on the binding to surfaces produced using 2-azidoethanol, resulting in good specific affinities for all carbohydrate surfaces tested. An average binding value was thus obtained for each lectin on each surface with good reproducibility over time (Figure 3). High binding selectivities were found for all lectins evaluated and followed the predicted trends indicated in Table 2. Thus, Con A and PSA displayed binding to the mannose surfaces, WGA and BS-II to the *N*-acetylglucosamine surfaces, and RCA-I to the galactose surfaces (Figure 3). The difference in quantitative binding between the different lectins is primarily due to parameters such as the lectin molecular weight and the affinity for the monosaccharides used in the study.

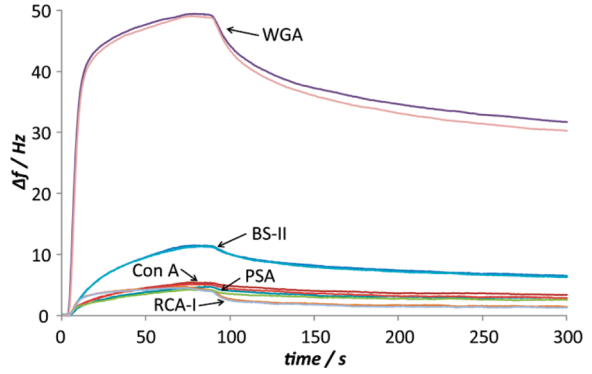

Figure 2. General lectin binding behavior to functionalized surfaces, in this case to GlcNAc-functionalized polystyrene.

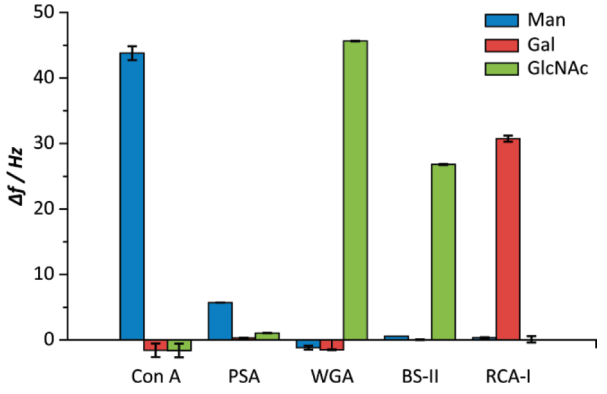

Figure 3. Corrected lectin binding to PEG-functionalized surfaces. Each value represents an averaged binding of two separate injections of protein, subtracted with the averaged binding to the 2-azidoethanol surface.

The lectin binding profiles to all carbohydrate-functionalized sensor surfaces are represented in Figure 4. From the binding analysis it can be noted that Con A proved to be the most consistent lectin in the study with more than 40 Hz corrected binding to all mannose-functionalized surfaces and with insignificant nonspecific binding. PSA followed the pattern of Con A although the selective binding was lower than for Con A. The other lectins showed a variance in binding between the polymeric materials; e.g., low binding of WGA and BS-II to the GlcNAc/PAAm surface and high binding to GlcNAc/PEG. RCA-I also showed some variance in binding to the galactose-functionalized surfaces although less than for WGA/BS-II to the GlcNAc-surfaces. The binding analysis of RCA-I resulted in a low residual negative binding to some of the mismatching carbohydrate surfaces (Man/PS, GlcNAc/PS, and GlcNAc/PAAm), within the limits of sensitivity.

Comparison of the differences in sensitivity between the different polymeric surfaces shows that the polystyrene surfaces in general had a resulting negative binding to the mismatched carbohydrate-functionalized surfaces (e.g., RCA-I to Man and PSA to GlcNAc) whereas the polypropylene surfaces had a resulting positive binding (e.g., PSA to GlcNAc and Con A to Gal). The water-soluble polymeric surfaces showed a more coherent binding than the hydrophobic polymeric surfaces, shown clearly by the more coherent corrected lower binding values to the corrected mismatched surfaces (except for WGA to Man/PEOX and RCA-I to GlcNAc/PAAm).

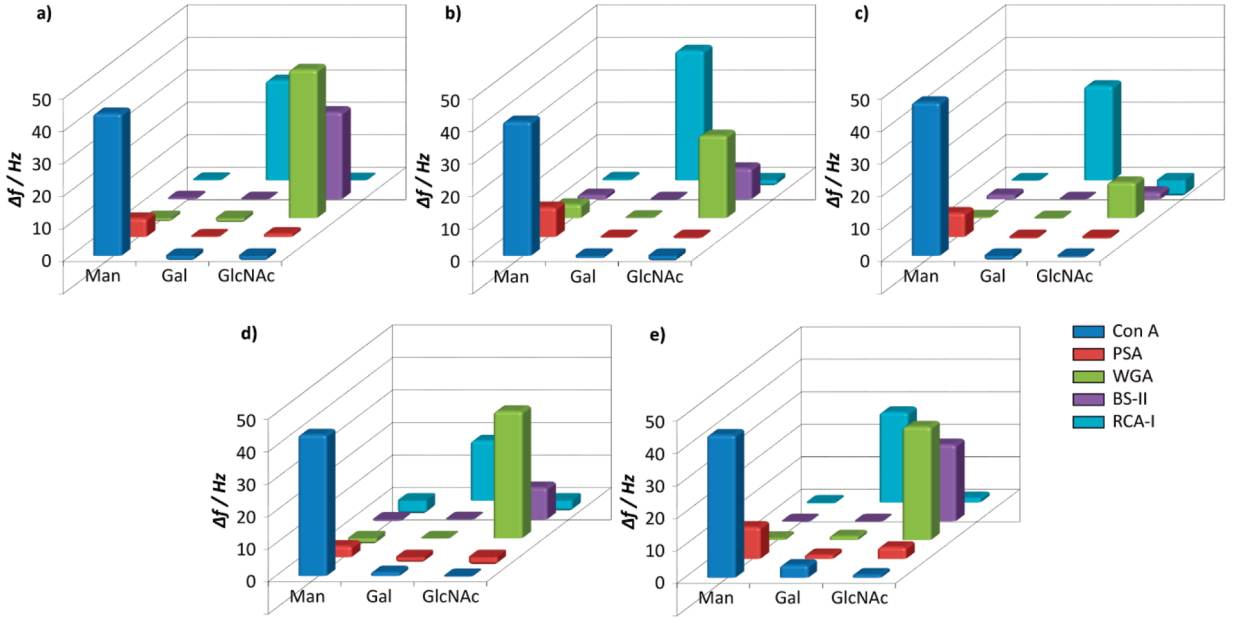

Figure 4. Corrected binding values of lectins to the functionalized surfaces: (a) poly(ethylene glycol) (PEG), (b) poly(2-ethyl-2-oxazoline) (PEOX), (c) polyacrylamide (PAAm), (d) polystyrene (PS), and (e) polypropylene (PP).

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A hypothetical reason for nonspecific binding is ionic binding of the lectins to the blocking agent on the surface (immobilized BSA in the primary blocking step). Since BSA has a relatively low pI value (4.7) it would essentially be positively charged at the buffer pH (7.4), thereby creating a partial positively charged surface. The lectins which showed the most nonspecific binding (WGA, Con A, and RCA-I) all have pI values above 7.4, resulting in a net negative charge at the buffer pH. The lectins with a pI value below the buffer pH (PSA and BS-II) both show a lower nonspecific binding which is expected since there would be no ionic attraction between the lectin and the surfaces. Further, both PSA and BS-II showed a general smaller binding even to the matched carbohydrate surfaces, which fits well with the expected behavior if a repulsive ionic interaction exists, in coherence with recently published material regarding antibody immobilization to functionalized surfaces.[62] Any potential ionic attraction by lectins with a pI value above the buffer pH is however not a problem per se, since it can be easily corrected for. Ionic repulsion, on the other hand, may present a problem if it results in reduced binding below the detection limit. To circumvent this possible problem, the use of other blocking agents such as Tween-20 is currently being investigated.

CONCLUSIONS

It has been shown that the photoclick surface functionalization method is highly versatile, resulting in robust carbohydrate sensor surfaces with high protein selectivities. Sensors were fabricated using a panel of carbohydrate structures and evaluated using a QCM setup for lectin binding. The predicted protein binding could thus be efficiently demonstrated, validating the technique with other formats. A variety of polymeric substrate materials were further evaluated, generally showing good compatibility with the method. As expected, poly(ethylene glycol)-based sensors proved very good, but other formats also showed high potential. The polymeric substrates enable control of the non-specific binding, and only low levels were found in the present study. Further optimization with respect to polymer structure, composition, and size is ongoing. The method is inherently robust, requiring generally low-cost starting materials, and can be performed under ambient conditions. The photochemical step also allows for the use of photolithography to easily produce alkyne-functionalized arrays. In addition, the convergent fabrication approach constitutes a versatile platform for sensing and glycoarray development.

AUTHOR INFORMATION

Corresponding Author

*O.R.: E-mail: ramstrom@kth.se. Fax: +46 8 7912333. M.Y.: E-mail: yanm@pdx.edu. Fax: +1 503 725-9525.

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