Orientated glyco-macroligand formation based on site-specific immobilization of *O*-cyanate chain-end functionalized glycopolymer†

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We report an oriented glyco-macroligand formation and its glyco-affinity capturing and glycoarray application by combining an *O*-cyanate chain-end functionalized glycopolymer with commercially available amine-functionalized silica gel and glass slides *via* isourea bond formation. The *O*-cyanate chain-end functionalized glycopolymer was synthesized *via* cyanoxyl-mediated free-radical polymerization (CMFRP) in a one-pot fashion. The oriented glyco-macroligand formation was conducted under mild aqueous conditions at room temperature and was confirmed with fluorescence imaging after specific lectin binding, and will find important biomedical applications such as in biosensors and glycoarrays. In addition, the *O*-cyanate chain-end functionalized glycopolymer can also be used for site-specific polymer-protein conjugation.

Introduction

Cell surface carbohydrates act as receptors for a variety of protein ligands and thereby play a significant role in a wide range of biological processes, including immune recognition events[1] and the interaction of viruses and bacteria with host cells,[2] as well as tissue growth and repair.[3] Therefore, carbohydrate–protein binding interactions have provided a starting point for the development of new therapies,[4] protein and cell isolation,[5] and targeted drug and gene delivery concepts[6] in biomedical research and applications. It is notable that the low affinity and specificity that are typical of monomeric carbohydrate–protein interactions are dramatically enhanced when the carbohydrate component is presented as a multivalent ligand: a phenomenon referred to as the "cluster glycoside effect".[7] Glycopolymers, as a multivalent clustering of carbohydrate derivatives, have proven to be an effective tool in the study of carbohydrate-based cellular processes and show great potential in biomedical applications such as glycomics, biotechnology, biosensors, and medicine. For example, glycopolymers have been explored for the development of therapeutic agents,[8] in bio- and immunochemical assays,[9] and as biocapture reagents.[10] It has been found that the shape and size of glycopolymers,[11] as well as the density and relative positioning of their glycan appendages,[12] are of great importance with regard to their effectiveness in bio-interactions. Therefore, a large variety of various glyco-homo- and (block) copolymers, linear or branched, have been reported realizing high control over the molar mass and the structure of the products.[13] Furthermore, surface-bound multivalent glycan ligands were shown to exhibit higher avidity to protein receptors compared to immobilized monomeric glycans.[14] We first reported a biotin chain-end functionalized glycopolymer for oriented glyco-surface engineering based on specific non-covalent interactions.[15–18] Since then, several groups have demonstrated the potential of biotin chain-end functionalized glycopolymers for bioconjugation applications.[19–23] Most recently, reactive groups such as aldehyde,[24] azide,[25] *N*-hydroxysuccinimide (NHS) active ester,[26] maleimide,[27] and pyridyl disulfide[28] chain-end functionalized glycopolymers have been explored for site-specific covalent glycopolymer conjugations. Most of these chain-end functionalized glycopolymers were synthesized through atom transfer radical polymerization (ATRP) of the glycomonomer by using an aldehyde, azide, NHS active ester, maleimide or pyridyl disulfide-modified initiator. On the other hand, a trithiocarbonate[23,29] chain-end functionalized glycopolymer was synthesized by reversible addition fragmentation chain transfer polymerization (RAFT). Most recently, Bertozzi and co-workers reported a trithiocarbonate/alkyne dual chain-end functionalized mucin-like glycopolymer by RAFT polymerization for microarray applications.[30] As they pointed out, a key element of their approach was that the densities and orientations of the glycan ligands were determined by the polymer structure and were more controllable than in the case of conventional glycan arrays. Therefore, the utility of glycopolymer microarrays as a platform for profiling glycan-binding proteins is a matter of considerable future interest. In addition, we reported a facile synthesis of amine and carboxylic acid chain-end functionalized glycopolymers *via* cyanoxyl mediated free radical polymerization (CMFRP) by using amine and carboxylic acid functionalized aryl amines as initiators, respectively.[18] Interestingly, all these glycopolymers have an *O*-cyanate group at the other chain-end, which might serve as a reactive chain-end group, but this has not yet been investigated. During the past decades, *O*-cyanate-based isourea bond formation has been proven to be a very useful tool in bioconjugate chemistry, such as in protein and antibody immobilization.[31–33] Therefore, we envisioned that the chain-end *O*-cyanate group of the glycopolymer provides an anchor for site-specific and covalent immobilization of the glycopolymer onto an amine surface *via* isourea bond formation, and thereby facilitates an oriented glyco-macroligand formation (Fig. 1). Herein, we report the oriented glyco-macroligand formation and its glyco-affinity capturing and glycoarray applications by combining an *O*-cyanate chain-end functionalized glycopolymer with commercially available

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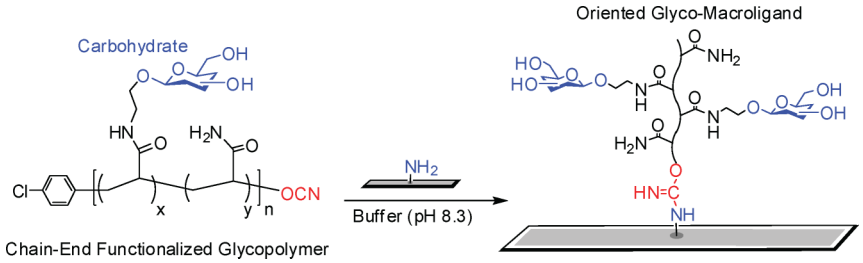


Fig. 1 Chemical structure of the *O*-cyanate chain-end functionalized glycopolymer and its oriented glyco-macroligand formation *via* isourea bond formation.

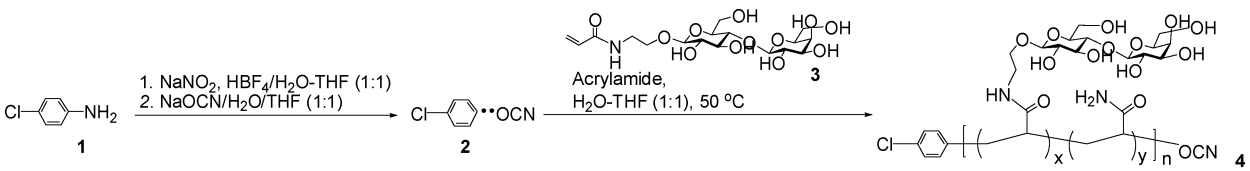
amine-modified silica gel and glass slides *via* isourea bond formation. In this report, the multivalent lactose units serve as model affinity carbohydrate ligands for lectins[34] and cell surface carbohydrates such as GM3 for carbohydrate–carbohydrate interaction-based cell–cell interactions.[35]

Results and discussion

1. One-pot synthesis and characterization of the *O*-cyanate chain-end functionalized glycopolymer

The synthesis of chain-end functionalized glycopolymers still poses significant challenges, including a requirement for serial protection/deprotection steps or further polymer derivatization after the initial synthesis. In the present study, the *O*-cyanate chain-end functionalized glycopolymers were synthesized *via* our previously developed cyanoxyl free radical mediated copolymerization scheme in a one-pot fashion (Scheme 1).[18] Briefly, 4-chloroaniline (**1**) was used as an initiator for the copolymerization of acrylaminoethyl lactoside **3** and acrylamide. Specifically, cyanoxyl radicals were generated by an electron-transfer reaction between cyanate anions from a sodium cyanate aqueous solution and aryldiazonium salts prepared *in situ* through a diazotization reaction of arylamine in water. In addition to cyanoxyl persistent radicals, aryl-type active radicals were simultaneously produced, and only the latter species was capable of initiating chain growth. By altering glycomonomer (GM) and acrylamide (AA) concentrations, a series of *O*-cyanate chain-end functionalized glycopolymers of varying sugar density were prepared in good conversion yield (60–70%) and low polydispersity ($1.1 < M_w/M_n < 1.6$) as described previously.[18]

The chain-end *O*-cyanate functional group of glycopolymer **4** was confirmed by its IR spectrum, in which *O*-cyanate absorption was observed at 2157 cm^{-1} (Fig. 2A). It is also noteworthy that the *O*-cyanate group could be converted to a hydroxyl group by treatment with pyridine in water.[36] Therefore, the disappearance of the *O*-cyanate absorption band at 2157 cm^{-1} after pyridine treatment of glycopolymer **4** (to form **5**) confirmed the existence of the *O*-cyanate chain-end group and its complete hydrolysis (Fig. 2B). Comparing the ^{1}H NMR spectra of **4** and **5** (Fig. 3), there was no apparent polymer structural change observed during the hydrolysis reaction.



Scheme 1 Synthesis of *O*-cyanate chain-end functionalized glycopolymers.

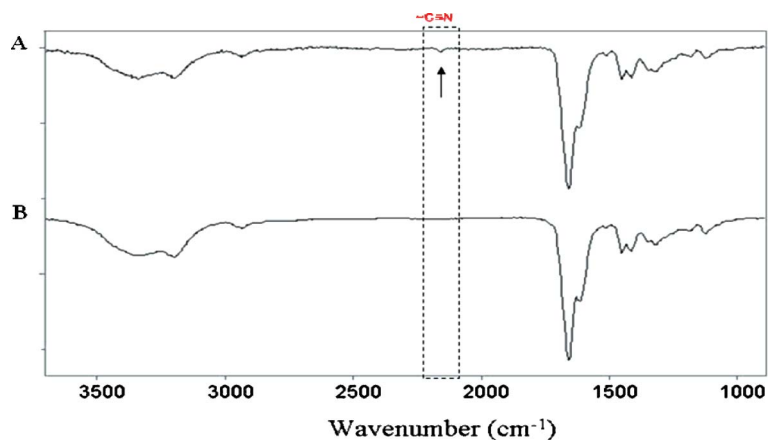


Fig. 2 FT-IR spectra of *O*-cyanate chain terminated glycopolymer **4** (**A**) and hydroxy chain terminated glycopolymer **5** (**B**).

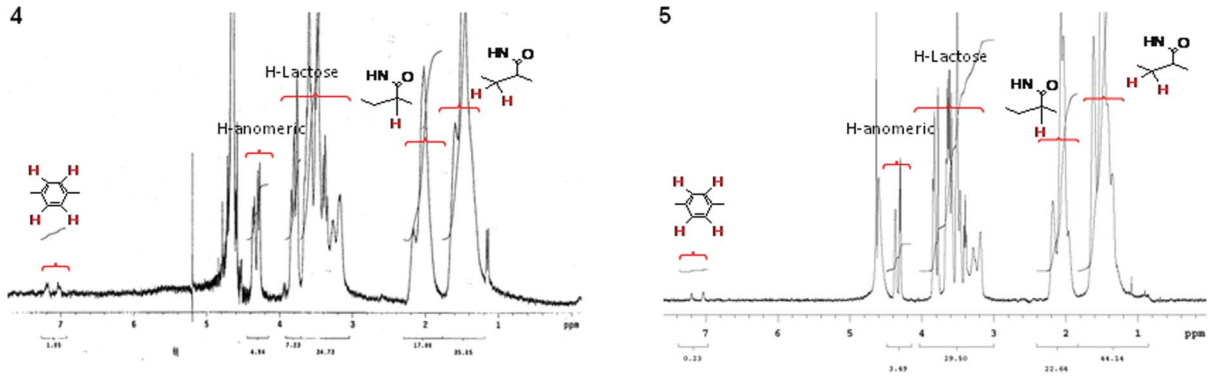


Fig. 3 ^{1}H NMR spectra of *O*-cyanate chain terminated glycopolymer **4** (**A**) and hydroxyl chain terminated glycopolymer **5** (**B**) in D_2O.

The ^{1}H NMR spectrum was used to calculate polymer component content and length by using the signals of the phenyl group at the other polymer chain terminal. As shown in the ^{1}H NMR spectrum of glycopolymer **4** (Fig. 3A), comparison of the integrated signal from the phenyl protons (7.04 ppm, $H_{2',6'}$ and 7.22 ppm, $H_{3',5'}$) with that of the anomeric protons of lactose (4.36 ppm, $H_{1'\text{-Lact}}$ and 4.43 ppm, $H_{1\text{-Lact}}$), as well as that of the polymer backbone methine (2.10 ppm, CH) and methylene (1.55 ppm, CH_2), indicated an average glycopolymer composition of 10 lactose and 70 acrylamide units. The molecular weight (M_n) was about 7,000 as determined by ^{1}H NMR. Furthermore, the grafted carbohydrate on glycopolymer **4** was quantified *via* the phenol–sulfuric acid method.[37] Briefly, concentrated H_2SO_4 was added directly into a solution of phenol and glycopolymer **4**. The mixture was then vortexed, and allowed to stand for 30 min at room temperature. The optical density was then recorded at 490 nm. The results indicated there was an average of 8 lactose units per polymer, which is close to the value determined by ^{1}H NMR above.

2. Immobilization of the glycopolymer onto silica gel *via* *O*-cyanate-based isourea bond formation and its application to affinity chromatography

Silica gel has been widely used as a small, rigid particle for high performance affinity chromatography since it is capable of withstanding high flow rates and/or pressures. The introduction of functional groups on a silica surface to yield modified silica gel has received great attention for the development of functionalized silica gel for chromatography applications.[38] In particular; methods such as covalent grafting of polymers or coating with hydrophilic polymers have succeeded in passivating the silica surface and conferring specificity. Recently, silica materials containing covalently bound sugars have been explored for boron removal[39] and isolation and purification of lectins.[40,41] In the present study, oriented immobilization of multivalent carbohydrates onto a silica gel surface was investigated for efficient and inexpensive isolation and purification of carbohydrate-binding proteins (Fig. 4). Briefly, *O*-cyanate end-terminated glycopolymer **4** was immobilized onto the surface of silica gel by incubation of **4** with 3-aminopropyl-functionalized silica gel (Sigma) in sodium bicarbonate buffer solution (pH 8.3) at room temperature for 2 h, followed by thorough washing with the same buffer three times. A control experiment with the hydroxyl chain-end functionalized glycopolymer **5** was conducted on the same scale. The amount of glycopolymer immobilized on the silica gel was determined by the phenol–sulfuric acid method as described above.[37] Briefly, phenol solution was added to a solution of glycopolymer–silica gel, and mixed. Then concentrated H_2SO_4 was added directly into the solution. The mixture was then vortexed, and allowed to stand for 30 min at room temperature and finally centrifuged. The optical density of the supernatant was then recorded at 490 nm. The results suggested there was an average of 276 nmol of *O*-cyanate chain terminated glycopolymer **4** per gram of silica gel, while there was no detectable glycopolymer determined with hydroxyl chain terminated glycopolymer **5** treated silica gel. This result indicated specific immobilization of glycopolymer through isourea bond formation.

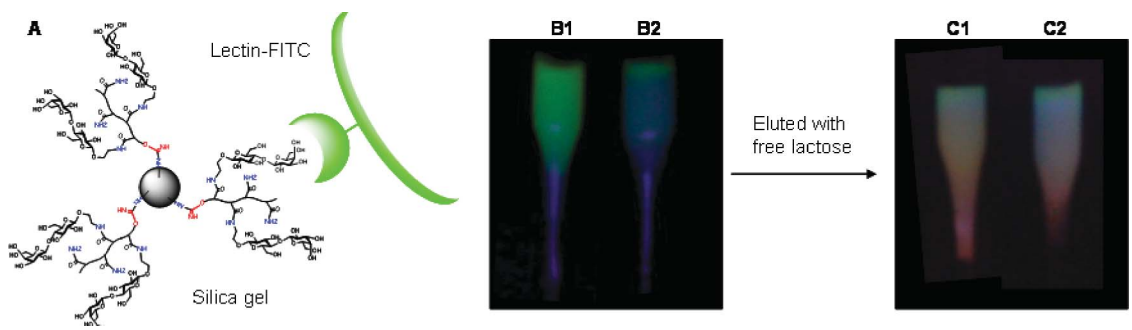


Fig. 4 Illustration of glycopolymer immobilization onto silica gel and its lectin capture (**A**), Lectin capture onto the glycopolymer–silica gel column (**B1**) and raw silica gel (**B2**), after elution with free lactose (**C1** and **C2**).

Once the glycopolymer–silica gel (GP–SG) was synthesized, its ability to capture lectin was evaluated as an adequate methodology to characterize and determine potential applicability as a chromatographic support. The characterization was carried out by packing the glycopolymer–silica gel in a column followed by saturating the column with a β-galactose-specific lectin (*Arachis hypogae*, FITC-Labeled, 0.5 mg, 4.16×10^{-9} mol, Sigma) in PBS buffer solution (pH 7.4), and washing with PBS (pH 7.4) buffer to remove the unbounded lectin (Fig. 4A). Lectin capturing was confirmed by visualization of FITC-labeled lectin on the glycopolymer–silica gel column under a UV lamp (Fig. 4B1), while raw silica gel showed little non-specific capturing of the lectin (Fig. 4B2). Furthermore, no capture was observed for the non-specific lectin (glucose/mannose-specific Concavalin A) on the glycopolymer–silica gel (data not shown). Next, eluting the column with 1 M free lactose in PBS buffer (pH 7.4) released the specifically captured lectin on the glycopolymer–silica gel (Fig. 4C1). A similar protocol was followed with raw silica as a control assay (Fig. 4C2). The lectin-capturing capacity was 13.9 nmol per mg of silica gel modified with the glycopolymer, and 2.9 nmol per mg of raw silica gel due to non-specific binding, as determined by fluorescence intensity analysis. Finally, the specifically captured lectin was confirmed with SDS-PAGE (Fig. 5). The captured lectin on glycopolymer-modified silica gel was confirmed on protein staining gel (Gel A, lane 4) and silver staining gel (Gel B, lane 4), while no non-specific lectin (glucose/mannose-specific Concavalin A) on the glycopolymer–silica gel was observed on protein staining gel (Gel A, lane 5) and silver staining gel (Gel B, lane 5). These results demonstrated the successful oriented glyco-macroligand formation and its carbohydrate-binding protein purification and identification capacity.

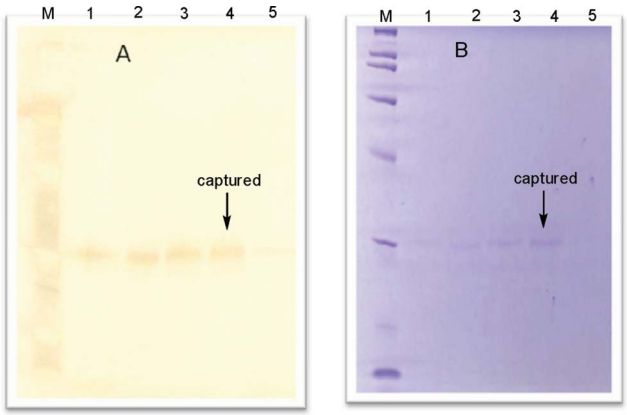


Fig. 5 SDS-PAGE identification of unbound and released lectins from silica gel beads (**A**: silver staining; **B**: Protein staining): M: MW marker, 1: lectin alone, 2: unbound lectin from glycopolymer–silica gel, 3: unbound lectin from control raw silica gel, 4: captured lectin eluted from glycopolymer–silica gel, 5: non-specific lectin eluted from glycopolymer–silica gel.

3. Oriented glycopolymer glycoarray *via O*-cyanate-based isourea bond formation

Surface-bound multivalent glycan ligands showed higher avidity to protein receptors compared to immobilized monomeric glycans.[14] Inspired by this example, we sought to develop an oriented glycopolymer array that spatially positions the pendant glycans similarly to natural glycoproteins. Specifically, integration of such constructs into arrays may create a more physiologically authentic platform for probing glycan binding proteins. In the present study, a glycopolymer microarray (spot size 500 μm diameter) was fabricated by microcontact stamping glycopolymer **4** onto amine functionalized glass slides (Xenopore Co.) in $NaHCO_3$ buffer (pH 8.3) (Fig. 6). The glass slide was incubated in a humidifier chamber for 4 h and then washed for 30 min (3 times) with respective buffers followed by washing with PBS containing 0.2% Tween 20 (PBST) to minimize nonspecific binding of proteins onto the surface. Then the glass slides were incubated with lectin-FITC (*Arachis hypogae*, FITC-Labeled, Sigma) solution in PBST buffer for 3 h, followed by extensive washing with PBST buffer for 30 min, and finally subject to fluorescent imaging. A control was prepared by incubating the glycopolymer arrayed glass slide with lactose (200 mM) pre-incubated lectin-FITC instead of free lectin. Fluorescence microscopy analysis of the surfaces revealed specific binding of lectin only to the immobilized glycopolymer (Fig. 6B). The lectin binding to the arrayed glycopolymer was inhibited in the presence of free lactose (Fig. 6C), further confirming the specific lectin binding to the glycopolymer. Moreover, arrays with different concentrations of glycopolymer showed concentration-dependent lectin binding to the glycopolymer (Fig. 6D). These

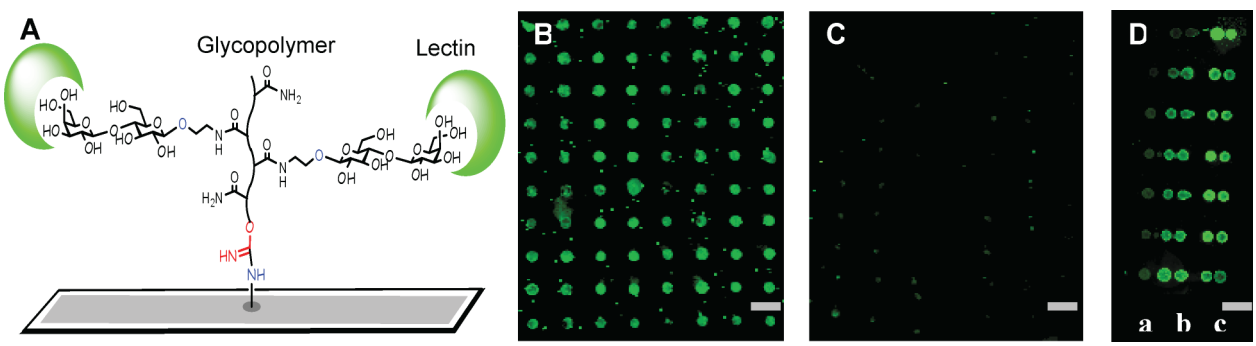


Fig. 6 Glycopolymer microarray for probing specific glycan–protein interactions (**A**), fluorescent imaging of FITC-labeled lectin binding to microarrayed patterns of glycopolymers (**B**), control with lactose pre-incubated lectin-FITC (**C**) and with different concentrations of glycopolymer in the array (a: 0.001 mg mL^{-1}, b: 0.1 mg mL^{-1} and c: 0.5 mg mL^{-1}) (**D**). Bar size: 500 μm.

observations indicated that arrayed glycopolymers can distinguish glycan-binding proteins based on their ligand specificity.

Conclusions

An oriented glyco-macroligand formation based on immobilisation of an *O*-cyanate chain-end functionalized glycopolymer with commercially available amine-modified silica gel and glass slides *via* isourea bond formation was demonstrated. The *O*-cyanate chain-end functionalized glycopolymer was synthesized *via* a one-pot cyanoxyl mediated free radical polymerization. It is notable that the polymerization can be performed under aqueous conditions and is tolerant of a wide range of monomer functionalities, including –OH, –COOH, $–NH_2$ and $–OSO_3^-$ groups.[18] Therefore, there is no protection and deprotection needed and the approach is thus straightforward.

Notably, multivalent lactose units serve as model ligands but can be changed to any specific carbohydrates for specific lectins or cells. The chain-end *O*-cyanate group of the polymer provides an anchor for site-specific and covalent immobilization of the glycopolymer onto any amine surface *via* isourea bond formation under mild conditions, which can be used for biosensor and glycoarray applications. In addition, the chain-end functionalizable glycopolymer can be used for polymer–protein conjugation as well.

Experimental

Materials and methods

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water with a resistivity of 18 MΩ cm^{-1} was used as a solvent in all polymerization reactions. Glycomonomer, 2-*N*-acryoyl-aminoethoxyl 4-*O*-(*β*-D-galatopyranosyl)-*β*-D-glucopyranoside (**3**) was synthesized as described in a reported method.[18] Thin-layer chromatography (TLC) was performed on Whatman silica gel aluminium backed plates of 250 μm thickness, on which spots were visualized with UV light or by charring the plate after dipping in 15% H_2SO_4 in methanol. Dialysis was performed using cellulose membranes with a molecular weight cutoff of 3 kDa with water as solvent. ^{1}H NMR spectra were recorded at room temperature with a Varian INOVA 300 MHz spectrometer. In all cases, the sample concentration was 10 mg mL^{-1}, and the appropriate deuterated solvent was used as an internal standard. UV-vis absorbance spectra were recorded on a Varian Bio50 UV-vis spectrometer. IR spectra were measured on Bruker FT-IR spectrometer. Fluorescence spectra were measured on a Hitachi F-7000 Fluorescence Spectrophotometer.

Synthesis of *O*-cyanate chain-end functionalized glycopolymer 4

In a three-necked flask, 4-chloroaniline (21.6 mg, 1.69×10^{-4} mol) and sodium nitrite (14.1 mg, 2.04×10^{-6} mol) were dissolved in a mixture of 2 mL water and THF (1 : 1, v/v). To the above mixture, HBF_4 (66 mg, 7.51×10^{-4} mol) was added and allowed to react for 30 min at 0 °C under an Ar atmosphere. Following this, a degassed mixture of 2-*N*-acryoyl-aminoethoxyl 4-*O*-(*β*-D-galatopyranosyl)-*β*-D-glucopyranoside **3** (186 mg, 2.65×10^{-5} mol), acryl amide (210 mg, 2.65×10^{-4} mol) and NaOCN (55.2 mg, 8.49×10^{-4} mol) dissolved in 1 mL of water was added into the flask containing the diazonium salt. The reaction solution was thus heated at 65 °C for 16 h, and then filtered to remove any precipitates. The resultant mixture was separated of any inorganic salts and impurities by dialysis against deionized water for 2 days at room temperature to afford glycopolymer **4** (248 mg). The conversion yield was about 60%, which was determined by weight for the resultant glycopolymer.

Quantification of carbohydrate in glycopolymer (Phenol–Sulfuric Acid Assay)

The phenol–sulfuric acid assay was used to quantify the lactose molecules attached to the glycopolymer **4**. To 0.5 mL of sugar solution, 0.5 mL of 5% aq. phenol solution and 2.5 mL of H_2SO_4 (96%) was added and the mixture vortexed. The blank was prepared with distilled water replacing the sugar solution. The above solutions were allowed to stand for 30 min at room temperature and the absorbance measured at 490 nm. A calibration curve of sugar (β-lactose) concentration *versus* absorbance (A) was plotted and the presence of sugar on the polymer was detected by measuring the absorbance of glycopolymer samples (0.1 moles, 0.5 moles and 1 mole).

Immobilization of glycopolymer 4 onto amine-modified silica gel through isourea bond formation and its affinity lectin capture

Glycopolymer **4** (8.5 mg, 1.21×10^{-3} mol) was added to 200 mg of amine functionalized silica gel beads (Sigma) in 2 mL of $NaHCO_3$ buffer (pH 8.3). The mixture was shaken for 4 h at room temperature and was then packed into a column. The column was washed with $NaHCO_3$ buffer (pH 8.3) followed by PBS buffer (pH 7.4). Lectin-FITC solution (*Arachis hypogae*, FITC-Labeled, 0.5 mg, 4.16×10^{-9} mol) in PBS buffer (pH 7.4) (1 mL) was loaded onto the column. The drained solutions were collected and preserved for further analysis. The column was then washed with PBS buffer (pH 7.4) to remove the unbound lectin. Finally, the captured lectin was released by eluting with free lactose (1 M), then analyzed by SDS-PAGE and quantified by fluorescence spectroscopy.

Oriented glycopolymer glycoarray *via* *O*-cyanate-based isourea bond formation

Glycopolymer microarrays were fabricated by microcontact stamping with MicroCaster Arrayer (Whatman). The MicroCaster array tool (spot size 500 μm diameter) was inked with a solution of glycopolymer (**4**, 1 mg mL^{-1}, 1.4×10^{-7} moles) in $NaHCO_3$ buffer (pH 8.3). The MicroCaster tool was pressed onto amine functionalized glass slides (Xenopore Co.) for 10 min. The glass slide was incubated in a humidifier chamber for 4 h and then washed for 1 h (3 times) with respective buffers followed by washing with PBS containing 0.2% Tween 20 (PBST) to minimize nonspecific binding of proteins to the surface. Then the glass slides were incubated with lectin-FITC (*Arachis hypogae*, FITC-Labeled, Sigma) solution (0.2 mg mL^{-1}) in PBST (pH 7.4) buffer for 3 h followed by extensive washing with PBST buffer for 1 h, and subject to fluorescent imaging by using Typhoon 9410 Variable Model Imager (Amersham Biosciences, USA). A control was prepared by incubating the glycopolymer printed glass slide with lactose (200 mM, PBS (pH 7.4)) pre-incubated lectin-FITC.

Acknowledgements

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