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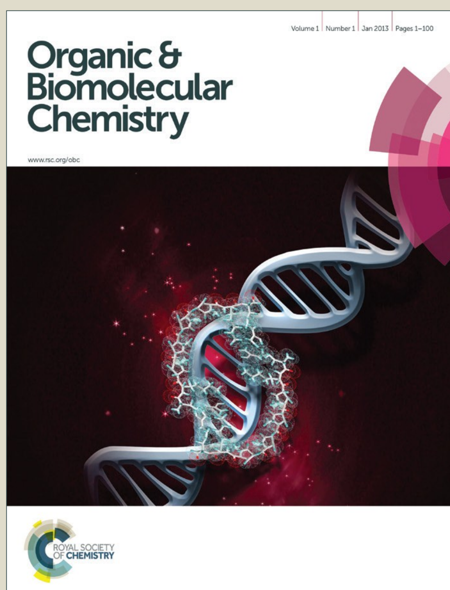

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ARTICLE

Fabrication of Carbohydrate Microarrays on Poly(2-Hydroxy ethyl methacrylate)-Based Photoactive Substrate

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ABSTRACT: We report the fabrication of carbohydrate microarrays on a photoactive polymer, poly(HEMA-*co*-HEMA-PFPA) synthesized by RAFT copolymerization of 2-hydroxyethyl methacrylate (HEMA) and perfluorophenyl azide (PFPA)-derivatized HEMA (HEMA-PFPA). PFPA allows the covalent immobilization of carbohydrates whereas HEMA polymer provides an antifouling surface thus the microarrays can be used directly without pretreating the array with a blocking agent. The microarrays were prepared by spin-coating the polymer followed by printing the carbohydrates. Subsequent irradiation simlutaneously immobilized carbohydrates and crosslinked the polymer matrix. The obtained 3D carbohydrate microarrays showed enhanced fluorescence signals upon treating with a fluorescent lectin in comparison to a 2D microarray. The signals were acquired at lower lectin concentration and shorter incubation time. When treated with *E. coli* bacteria, the carbohydrate microarray showed results that were consistent with their binding patterns.

Introduction

Carbohydrates play important roles in a variety of biological processes, including intracellular signaling, cell-cell interactions, bacterial and viral infections.[1-3] The study of carbohydrate-mediated processes is often hampered by the availability of carbohydrate ligands and the relatively weak carbohydrate-lectin interaction.[1] The carbohydrate microarray in this context provides a high-throughput means to study carbohydrate-mediated interactions. It uses minute amounts of carbohydrate ligands, provides a multivalent scaffold to enhance the carbohydrate-lectin interactions by orders of magnitude, and thus has greatly improved the efficiency of the analysis.[4-7] The microarray fabrication generally involves the covalent immobilization of carbohydrate ligands on a substrate to avoid the removal of the highly water soluble carbohydrates from the substrate.[8] A variety of methods have been developed to fabricate carbohydrate microarrays. An earlier example by Houseman *et al.* used the Diels-Alder reaction to immobilize diene-derivatized monosaccharides on the benzoquinone-functionalized gold surface.[9] The click chemistry was also used in carbohydrate microarray fabrication. For example, azide-derivatized monosaccharides or oligosaccharides can be readily immobilized on alkynylated substrates.[10-14] Methods that can directly immobilize a diverse range of unmodified carbohydrates are highly desirable. For example, Park and colleagues[15] fabricated a monosaccharide microarray by directly attaching unmodified monosaccharides on hydrazide-modified and aminoxy-modified glass slides. We have developed a photocoupling method which allowed the immobilization of unmodified monosaccharides and oligosaccharides on perfluorophenyl azide (PFPA)-modified surface.[16-18]

A critical issue in microarray application is the assay sensitivity, that is, the signal-to-noise ratio. To increase the sensitivity, two strategies are generally applied: to increase the signal by amplifying the specific binding interactions, and to decrease the background noise by reducing the non-specific adsorption of the analyte. The 3D microarrays have shown to be an effective strategy to amplify signals by increasing the ligand density.[19] A high ligand density provides stronger binding than traditional 2D microarray fabricated on a flat surface.[1-3] For example, Branderhorst *et al.*[20] used glycodendrimers to generate a 3D-structured carbohydrate microarray. Dyukova[21] and Godula[22] created 3D microarrays where glycans were immobilized on porous polymer scaffolds. Glycan conjugated on bovine serum albumin (BSA) was also used to fabricate 3D arrays to increase glycan-lectin affinity.[23] To reduce the non-specific adsorption of target biomolecules, especially proteins, protein-based blocking agents,[24-26] antifouling polymers,[27-36] and antifouling SAMs[37, 38] are frequently used.

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†Electronic Supplementary Information (ESI) available: Fabrication schemes of polymer films and 2D array, ^{1}H NMR spectra of polymers and calculation of molecular weight, AFM images of the polymer film, fluorescence imaging results, and IR spectra of the polymer. See DOI: 10.1039/x0xx00000x

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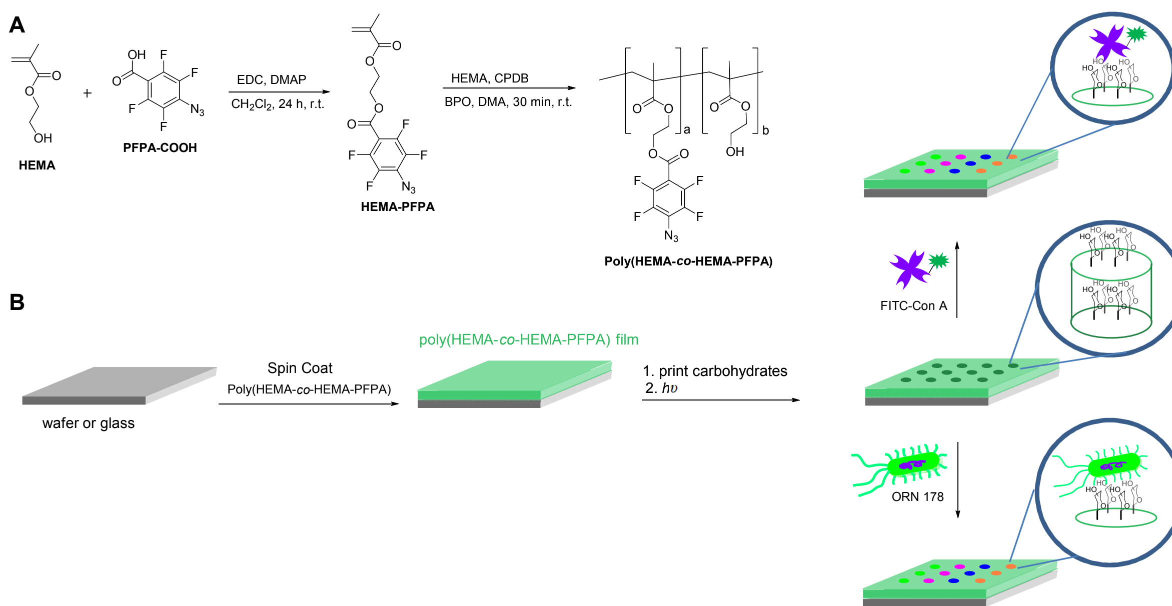

Scheme 1. (**A**) Synthesis of poly(HEMA-co-HEMA-PFPA). (**B**) Farbication of carbohydrate microarray followed by treatment with FITC-Con A or *E. coli* ORN 178.

In this study, we report the fabrication of 3D carbohydrate microarray using a photoactive polymer as the substrate matrix. The polymer was prepared by copolymerizing 2-hydroxyethyl methacryalte (HEMA) and 2-(methacryloyloxy)ethyl 4-azido-2,3,5,6-tetrafluorobenzoate (HEMA-PFPA) to yield poly(HEMA-*co*-HEMA-PFPA). The photoactive group, PFPA, allows the covalent attachment of carbohydrates upon photoactivation.[39, 40] PHEMA is known to resist protein adsorption, and is a widely used biomaterial such as in soft contact lenses.[41-44] Therefore, HEMA provides the anti-fouling surface to reduce non-specific adsorption, whereas PFPA in the copolymer matrix allows the immobilization of carbohydrate ligands. The combination of increased ligand density and reduced non-specific adsorption is expected to result in higher signals and lower background noise, and thus higher array sensitivity. The obtained 3D carbohydrate microarray was further used to probe lectin and bacteria interactions.

Results and discussion

The photoactive polymer, poly(HEMA-*co*-HEMA-PFPA), was synthesized as shown in Scheme 1A. HEMA-PFPA, prepared by coupling HEMA and PFPA-COOH, was copolymerized with HEMA at 1:10 mole ratio by RAFT polymerization at room temperature to give poly(HEMA-*co*-HEMA-PFPA) (Figure S-1).[45] The polymer films were prepared on silicon wafers by spin-coating the polymer solution in acetone followed by irradiation with a medium-pressure Hg lamp in the presence of 280 nm long path optical filter to minimize the potential photodegradation of the polymer.[46] The polymer films were fairly uniform, as indicated by the film thickness 100 ± 2.5 nm by ellipsometry and the root-mean-square (RMS) roughness value of 1.4 nm measured by AFM (Figure S-2). The static water contact angle of the polymer film was 47.5 ± 1.8^{o}, which was comparable to the reported value for PHEMA (~49°).[47] In addition, the resulting polymer films were stable and robust. The films remained on wafers and glass slides, and did not peel off from the surface after soaking in pH 7.5 HEPES buffer for one week.

PHEMA is known to resist protein adsorption.[48-50] To investigate the fouling property of our synthesized polymer, we synthesized PHEMA by RAFT polymerization (Figure S-3), and tested the non-specific adsorption of the polymer towards Concanavalin A (Con A), a lectin which was used in the subsequent studies. Two well-known non-fouling polymers, poly(ethylene oxide) (PEO) and poly(2-ethyl-2-oxazoline) (PEOX), and a fouling polymer, polystyrene (PS), were used as controls. The polymers were fabricated into an array following the procedure developed in our laboratory.[51-53] PFPA was introduced on wafers or glass slides by treating with a silane-functionalized PFPA (Scheme S-1).[54, 55] Polymer solutions were then manually printed on the PFPA-functionalized surfaces followed by irradiation to covalently attach the polymer with the aid of PFPA. The polymer arrays were then treated with FITC labeled Con A (FITC-Con A), and the fluorescence intensities were measured using a microarray scanner. PHEMA showed low fluorescence signals, which were comparable to PEO and lower than PEOX (Figure S-4).

Carbohydrate microarrays were fabricated as shown in Scheme 1B. Poly(HEMA-*co*-HEMA-PFPA) thin films were prepared on a silicon wafer or glass slide by spin-coating. Aqueous solutions of carbohydrate were printed on the films

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using a robotic printer, and the samples were then irradiated. The characteristic azide peak at 2134 cm^{-1} disappeared after irradiation (Figure S-5).[55, 56] PFPA in the polymer also crosslinked the film to form a 3D polymer scaffold. Crosslinking additionally enhanced the adhesion of the polymer film to the substrate. The films could withstand solvent washing without falling off the substrate. Carbohydrate microarrays were obtained after excess carbohydrates were removed by washing the sample in water.

To determine the optimal array fabrication conditions, varying concentrations of D-mannose (Man) (0.09 – 90 mg/mL) was printed, and the obtained array was then treated with 0.5 µg/mL of FITC-Con A. The fluorescence intensity increased with the concentration of Man (Figure 1), consistent with the 3D nature of the substrate. Only a low concentration of FITC-Con A (0.5 µg/mL) and a short incubation time (30 min) were needed. This is a drastic improvement over the 2D carbohydrate microarray fabricated on PFPA surface, which needed 500 µg/mL of FITC-Con A and overnight incubation time.[56, 57] At low concentrations (0.09 – 3 mg/mL), the signals were low and there were large variations in the fluorescence intensities. To minimize the signal variations and carbohydrate consumption, the concentration of 9 mg/mL was used for the subsequent studies.

To confirm that the carbohydrates were indeed covalently attached to the substrate, a control experiment was carried out. PHEMA was used instead of poly(HEMA-*co*-HEMA-PFPA), and the carbohydrate array was prepared following the same protocol. The fluorescence image of the array showed almost no signals from the Man spots (Figure S-6). Therefore the signal obtained in Figure 1 can only be from covalently immobilized carbohydrates on the photoactive polymer.

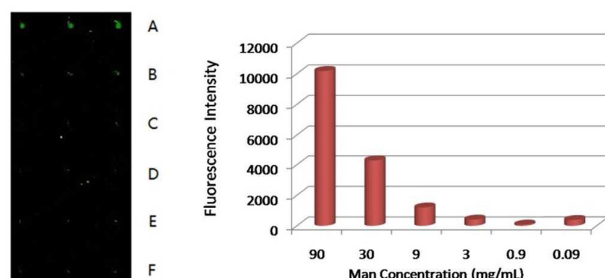

Figure 1. Fluorescence image (left) and intensities (right) of Man array after treating with FITC-Con A. The concentrations of Man used in array fabrication were (A) 90, (B) 30, (C) 9, (D) 3, (E) 0.9 and (F) 0.09 mg/mL, respectively. On the fluorescence intensity plot (right), each data was the average of the 3 spots on the array, and the error bars were omitted for clarity.

Following the procedures developed above, a microarray consisting of 3,6-di-O-(α-D-mannopyranosyl)-D-mannopyranose (Man3), 2-O-α-D-mannopyranosyl-D-mannopyranose (Man2), Man, and D-galactose (Gal) was fabricated, and was then treated with FITC-Con A. Results show that the signal was the highest from Man3 followed by Man2 and Man (Figure 2). This affinity rank order is consistent with that reported in the literature.[58, 59] The non-binding ligand Gal showed low intensities as expected. The signal-to-noise ratio for Man3, Man2, Man and Gal was 6.4, 5.1, 6.7 and 1.8 respectively (Figure S-8). As a comparison, a 2D microarray was prepared by printing Man3, Man2, Man, and Gal on a PFPA-functionalized wafer, and the array was then treated following the same protocol (Scheme S-2). The fluorescence signals from this sample were much lower (Figure S-9) in comparison with those fabricated on poly(HEMA-*co*-HEMA-PFPA) films which gave clear and high signals (Figure 2).

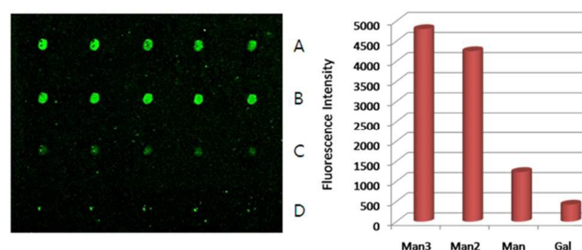

Figure 2. Fluorescence image (left) and intensities (right) of carbohydrate array after treating with FITC-Con A. The array contained immobilized Man3 (A), Man2 (B), Man (C), and Gal (D). On the fluorescence intensity plot (right), each data was the average of the 5 spots on the array, and the error bars were omitted for clarity.

The 3D carbohydrate microarray was tested for its ability to screen for carbohydrate-binding bacteria. First, PHEMA film was tested for its non-specific interactions with the bacteria used in our studies, *E. coli* ORN 178 and ORN 208. PS, PEO, and PEOX were used as the controls. The polymers was spin-coated and subsequently immobilized on PFPA-functionalized glass slides following the same procedure described previously. The polymer films were then incubated with *E. coli* ORN178 that has been stained with SYTO 61, a red fluorescent nucleic acid staining dye, to give the bacterial cells red fluorescence. Almost no bacteria cells were seen on PHEMA (Fig. 3A). On the contrary, PS, a fouling polymer, had many bacteria adhered to the surface (Figure 3B). PEO gave a similar result as PHEMA (Figure 3C), and slightly more bacteria were seen on the PEOX films (Figure 3D). Similar results were obtained from *E. coli* ORN 208 (Figure S-10). These results are consistent with the literature report on the antifouling property of PHEMA against bacteria.[48, 60-62] The polymer film can therefore be used as the substrate to fabricate arrays for bacteria studies without pre-treating the arrays with a blocking agent.

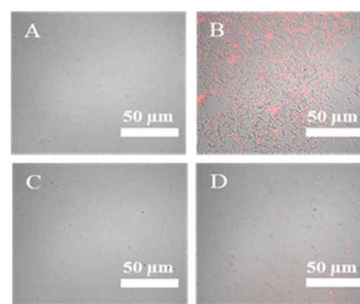

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Figure 3. Optical images of PHEMA (A), PS (B), PEO (C), and PEOX (D) after incubating with *E. coli* ORN 178. The bacteria were stained with Syto61.

The carbohydrate arrays were subsequently used to study carbohydrate-bacteria interactions. An array consisting of three different carbohydrates, Man3, Man and Gal, was prepared and incubated with bacteria overnight (Scheme 1B). Two *E. coli* strains were used, ORN178, which has FimH, a mannose-binding lectin, on its pili and a mutant strain, ORN208, which lacks FimH.[63-65] Microscopic images showed densely clustered ORN178 on Man3 (Figure 4A) and Man surface (Figure 4B). On the other hand, very little bacteria were observed on Man3 and Man when the array was incubated in ORN208 (Figure 4D and 4E). For Gal, the printed spots were free of *E. coli* ORN 208 (Figure 4F), however, some *E. coli* 178 were seen on Gal surface (Figure 4C). This also explains the higher background seen on Man3 (Figure 4A) and Man (Figure 4B) spots.

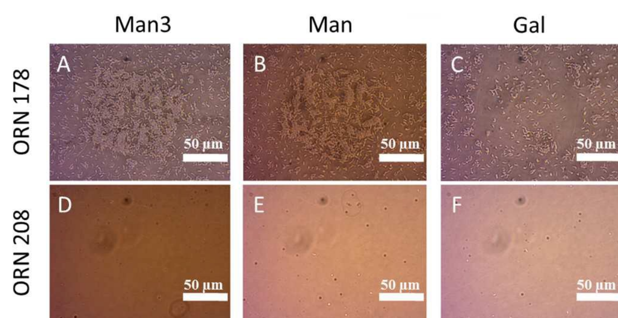

Figure 4. Optical images of the carbohydrate microarray after treating with *E. coli*. A, B, C were treated with *E. coli* ORN 178 and D, E, F were treated with *E. coli* ORN 208. Only one spot of Man 3 (A, D), Man (B, E) and Gal (C, F) were shown for clarity.

Conclusions

In summary, a photoactive polymer, poly(HEMA-*co*-HEMA-PFPA), has been successfully developed for the fabrication of 3D carbohydrate microarrays. We demonstrated that a single step of light activation covalently immobilized carbohydrate ligands, and at the same time, crosslinked the polymer substrate to form the 3D matrix. The 3D microarray gave higher signals which were acquired at lower lectin concetration and shorter incubation time than its 2D counterpart. The fabrication was carried out by a fast and simple procedure, and no substrate functionalization or chemical modification of the carbohydrates was required. Furthermore, the excellent antifouing property of PHEMA allowed the microarrays to be used with lectins or bacteria directly without the need for the pretreatment with a blocking agent. The fabricated carbohydrates retained their binding selectivity towards lectins and bacteria.

Experimental

Materials and Instrumentation

3-Aminopropyltrimethoxysilane, benzoyl peroxide (BPO, 98%), Concanavalin A from *Canavalia ensiformis* (Jack bean) FITC conjugate type IV, 2-cyano-2-propyl benzodithioate (CPBD, >97%), *N*,*N*-dimethylaniline (DMA), 4-dimethylaminopyridine (DMAP), dichloromethane, dimethylsulfoxide (DMSO, HPLC grade), diethyl ether, ethyl acetate, fructose (Fru), D-galactose (Gal), D-glucose (Glc), *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride, hexanes, 2-hydroxyethyl methacrylate (HEMA, 97%), hydrochloric acid, D-mannose (Man), 2-O-α-D-mannopyranosyl-D-mannopyranose (Man2), 3,6-di-O-(α-D-mannopyranosyl)-D-mannopyranose (Man3), methanol, polyethyleneoxide (PEO) (M_v= 1,000,000), poly(2-ethyl-2-oxazoline) (PEOX) (M_w= 200,000) and polystyrene (PS) (M_w=280,000) were obtained from Sigma-Aldrich. Luria-Bertani (LB) medium was prepared from LB (2 g, Sigma-Aldrich) in Milli-Q water (100 mL), and was sterilized before use. The phosphate buffer saline (PBS) (pH 7.4) was prepared by dissolving a PBS tablet (Sigma-Aldrich) in 200 mL of Milli-Q water. Syto 61 was obtained from Invitrogen. 4-Azido-2,3,5,6-tetrafluoro-*N*-(2-(triethoxysilyl)ethyl)benzamide (PFPA-silane) was synthesized following to a previously published procedure.[66, 67] Silicon wafer was cleaned by soaking in the piranha solution (H_2SO_4/30% H_2O_2) at 100 °C with stirring for 1 hour followed by D.I. water 100 °C for 30 minutes (C*aution*: the piranha solution react violently with most organic materials and must be handled with extreme care.). It was kept inside D.I. water until use. ^{1}H (500 MHz) and ^{13}C NMR (126 MHz) spectra were recorded on a Bruker Avance Spectrospin–500 spectrometer. ^{1}H (200 MHz) spectra were recorded on a Bruker Avance Spectrospin–200 spectrometer.

Synthesis of HEMA-PFPA

HEMA-PFPA was synthesized according to a modified method by Ferrar, et. al.[68] Briefly, 4-azido-2,3,5,6-tetrafluorobenzoic acid (PFPA-COOH) (300 mg, 1.28 mmol) was added to a solution of HEMA (491.5 mg, 3.84 mmol) and DMAP (62.6 mg, 0.512 mmol) in dichloromethane. After stirring for 30 minutes, EDC (318.7 mg, 1.66 mmol) was added to the reaction mixture which was then stirred further for 12 hours at room temperature. The reaction mixture was washed for 3 times with 10% HCl, 3 times with 10% $NaHCO_3$ and brine, respectively. The organic layer was dried over anhydrous Na_2SO_4, filtered, and was concentrated under reduced pressure. The product was purified by flash chromatography using 20% ethyl acetate in hexanes to give HEMA-PFPA as a pale yellow liquid (236.8 mg, 53%). ^{1}H NMR (200 MHz, DMSO) δ 6.04 (s, 1H), 5.77 – 5.64 (m, 1H), 4.61 (dd, *J* = 5.6, 3.1 Hz, 2H), 4.41 (dd, *J* = 5.7, 3.1 Hz, 2H), 1.87 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) = 166.1, 158.5, 145.5, 143.4, 141.1, 139.2, 135.4, 125.8, 123.7, 106.1, 63.9, 61.8, 17.6.

Synthesis of PHEMA

The polymer were synthesized using the RAFT polymerization method reported by Li *et al.*[69] CPBD (8.0 mg, 0.036 mmol), HEMA (1.0 g, 7.8 mmol), and BPO (63.0 mg, 0.26 mmol) were stirred in a 10-mL three-neck round bottom flask for 30 minutes under argon gas bubbling. DMA (34.2 mg, 0.28 mmol)

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was then added and was stirred at room temperature for additional 1 hour. The viscous solution mixture was dissolved in DMSO, and diethyl ether was added to precipitate the product. This process was repeated 3 times, and the precipitate was collected and dried under vacuum to give PHEMA as a yellow gel (0.65 g, 65%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.14 (s), 6.59 (s), 4.79 (s), 3.89 (s), 3.59 (s), 1.86 (s), 0.87 (d, J = 34.0 Hz). IR (neat)/cm^{-1}: 3404.20, 2949.15, 1705.89, 1437.36, 1361.43, 1273.06, 1222.10, 1153.77, 1079.05, 1021.99, 951.55, 900.99, 851.94, 747.76.

Synthesis of poly(HEMA-*co*-HEMA-PFPA)

The copolymer was synthesized using the RAFT polymerization method reported.[69] CPBD (8.0 mg, 0.036 mmol), HEMA-PFPA (267.7 mg, 0.78 mmol), HEMA (1.0 g, 7.8 mmol), and BPO (63.0 mg, 0.26 mmol) were stirred for 30 minutes under argon bubbling. DMA (34.2 mg, 0.28 mmol) was then added and was stirred at room temperature for additional 1 hour. The viscous solution mixture was dissolved in DMSO, and diethyl ether was added to precipitate the product. This process was repeated 3 times, and the precipitate was collected and dried under vacuum to give poly(HEMA-*co*-HEMA-PFPA) as a yellow gel (1.05 g, 82%). ^{1}H NMR (200 MHz, DMSO-d_6) δ 7.15 (s), 6.60 (s), 4.83 (s), 4.57 (s), 4.21 (s), 3.91 (s), 3.59 (s), 1.85 (s), 0.87 (d, J = 34.0 Hz). IR (neat)/cm^{-1}: 3342.31, 2946.04, 2359.60, 2133.55, 1720.52, 1650.39, 1601.18, 1487.80, 1437.03, 1404.59, 1312.89, 1259.68, 1151.38, 1017.81, 950.56, 898.56, 850.88, 747.54.

Fabrication of Polymer Films

A solution of poly(HEMA-*co*-HEMA-PFPA) in acetone (10 mg/mL) was dropped on a cleaned silicon wafer and it was spinned at 2000 rpm for 20 seconds. The substrate was then irradiated with a 450-W medium-pressure (Hg lamp Ace Glass Inc., Vineland, NJ) for 30 min. A 280-nm long-path optical filter was placed on the film surface during irradiation. The thicknesses of the obtained polymer films were measured by a Wyko NT2000 Optical 3D Profiling System (Veeco Metrology Group, Plainview, NY). Surface roughness of the polymer films was determined using AFM (Park Xe-150 System, Park Systems Co., Santa Clara, CA).

Evaluation of Antifouling Property of PHEMA

A previously developed procedure was used to evaluate the interaction of proteins with polymers.[51-53] The piranha-cleaned wafers were soaked in a solution of PFPA-silane in toluene (12.6 mM) under ambient conditions for 4 hours. Polymer arrays were generated by manually spotting solutions of polymers (PS, PHEMA, PEO, PEOX) (10 mg/mL) onto the PFPA-functionalized silicon wafers using a pipette tip followed by irradiating with a medium-pressure Hg lamp (Ace Glass Inc., Vineland, NJ) for 10 min. A 280-nm long-path optical filter was placed on the film surface during irradiation to avoid crosslinking of the polymer films. The wafers were then sonicated in chloroform, acetone and Milli-Q water for 5 min each, and finally dried under nitrogen.

The wafers containing the polymer arrays were incubated in a solution of FITC-labeled Concanavalin A (FITC-Con A, 0.5 µg/mL) in HEPES buffer (pH 7.5, 10 mM) containing NaCl (90 mM), $MnCl_2$ (1 mM) and $CaCl_2$ (1 mM) for 1 hour, and were washed with HEPES buffer followed by Milli-Q water. The wafers were then imaged with a fluorescence array scanner (GenePix 4100A, Axon Instruments Inc., Foster City, CA) at 488 nm excitation and 532 nm emission.

Fabrication of Carbohydrate Microarrays

Piranha-cleaned silicon wafers or glass slides were spin-coated (2,000 rpm, 1 min) with a solution of poly(HEMA-*co*-HEMA-PFPA) in acetone (20 mg/mL). Carbohydrate microarray was fabricated by printing aqueous solutions of carbohydrates on the polymer coated wafers or glass slides using a robotic printer (Arrayit Spot 2, Arrayit, Inc.) or a micropipettor (Thermofisher Finnpipette F2 single channel, Fisher Scientific, Inc.). The array was irradiated with the 450 W medium pressure Hg lamp for 30 min in the presence of a 280-nm long path optical filter. The excess carbohydrates were removed by soaking the samples in HEPES buffer (pH 7.5) for 1 hour.

Interaction of Carbohydrate Microarrays with Lectin

The carbohydrate microarray was incubated in a solution of FITC-labeled Con A (0.5 µg/mL) in HEPES buffer (pH 7.5, 10 mM) containing NaCl (90 mM), $MnCl_2$ (1 mM) and $CaCl_2$ (1 mM) for 30 min, and were then washed 3 times with HEPES buffer. The wafers were then imaged using a fluorescence array scanner (GenePix 4100A, Axon Instruments Inc., Foster City, CA) at 488 nm excitation and 532 nm emission.

Interaction of Carbohydrate Microarrays with Bacteria

The bacteria, *E. coli* ORN 178 or ORN 208, were inoculated in Mueller Hinton Broth and incubated at 37 °C until OD_{625} of 0.3 was reached, which corresponded to ~10^8 CFU/mL. The carbohydrate microarray printed on a glass slides were incubated with the bacteria (4 mL) at 37°C for 18 h in a Stuart S1505 microtitre plate shaker incubator while shaking at 250 rpm, and was then rinsed with HEPES buffer for 3 times. The glass slides was then imaged using a Zeiss Primo Vert microscope (Carl Zeiss Microscopy, LLC., Thornwood, NY) and an Olympus FluoView laser scanning confocal microscope (Olympus America Co., Center Valley, PA) in the bright field optical mode.

Acknowledgements

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