

Patterned Immobilization of Unprotected Carbohydrates on an Aminooxy Polymer-Grafted Solid Surface

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We describe a graft-type modification of a solid surface to be able to immobilize unprotected carbohydrates with a desired pattern. Water soluble carbohydrate-trapping polymers were grown from a solid surface through surface-initiated polymerization. The polymerization of an *N-tert*-butonyloxycarbonyl (*N*-Boc)-protected aminooxy monomer **3** was carried out from the immobilized initiator on silicon or glass surfaces. The *N*-Boc protecting group was then removed in an acidic solution to generate an aminooxy group, which reacts with free carbohydrates through oxime bonds on immersion of the plate in a carbohydrate solution. Sugar attachment on the surface polymer was confirmed by using infrared reflection absorption spectroscopy (IR-RAS) and fluorescent images after sugar-specific binding of fluorescein-labeled lectin proteins. The polymer was also grafted in a desired pattern by partial UV-irradiation over a photomask, giving a new material to create carbohydrates arrays.

Several materials possessing carbohydrate moieties, in particular synthetic carbohydrate-containing polymers (glycopolymers), have been developed to clarify cell–carbohydrate interactions.[1–3] It has been reported that some types of cells bind to glycosylated surfaces in a carbohydrate-specific and concentration-dependent manner.[4–6] Akaike et al. have reported that lactose-grafted polystyrene enables hepatocyte adhesion due to the specific adhesion between the galactose moieties and the asialoglycoprotein receptors on hepatocytes.[7] There are several approaches to the synthesis of artificial glycopolymers.[8] The most widely used technique is the polymerization of carbohydrate-containing monomers (glycomonomers), such as the polymerization of vinyl sugars and the cationic polymerization of anhydro sugars.[9,10] However, one disadvantage is that most glycomonomers must be synthesized via a repeating cycle of protection and deprotection to avoid unwanted side reactions. Moreover, organic synthesis of oligosaccharide-attached monomer is difficult and time consuming. Therefore, a new approach has been needed to make possible the simple utilization of oligosaccharides as widely applicable biomaterials.

Nishimura et al. have reported a technique, referred to as the glycoblotting method, which uses aminooxy-functionalized materials for rapid glycoform analysis of biological samples[11,12] and for cell behavior analysis.[13] This method is based on the chemoselective ligation of hemiacetal groups of carbohydrates with reactive aminooxy groups attached to polymer surfaces. Every carbohydrate has a hemiacetal group at the reducing terminus that provides a single reactive moiety for covalent capture. Hindsgaul and co-workers have reported the application of this sugar-specific reaction for the attachment of tag molecules.[14] Shin and Lee have shown that oligosaccharides can be captured on glass slides coated with a hydrazide- or aminooxy-functionalized monolayer for the rapid analysis of protein–carbohydrate interactions.[15] In past studies using glycoblotting polymers, the aminooxy residues are on the surface of the nanoparticles. However, for further application to biomaterials, an aminooxy polymer-immobilized flat surface is also required.

Here, we report a solid surface modification method with carbohydrate-trapping aminooxy-polymers to create the carbohydrate pattern. The carbohydrate-trapping polymers were covalently attached to a solid surface via surface-initiated atom-transfer radical polymerization (ATRP). ATRP is a well-developed controlled living polymerization technique suitable for a variety of monomers and solvents.[16–19] Surface-initiated ATRP has the advantage of producing well-defined dense polymers from the solid/liquid interface. The merit of the graft polymerization on the solid surface is also that the pattering is relatively easy combined with conventional photo-patterning. The surface-initiated polymerization was carried out using a Si(100) or glass surface (Scheme 1). The aminooxy polymers grafted on the solid surface could readily trap unprotected carbohydrates. Our method of simple immobilization of carbohydrates on a solid surface enabled us to prepare various carbohydrate-related materials without any complicated synthesis of carbohydrate derivatives.

Experimental

Materials. Silicon wafers (100, N-type) were purchased from Nilaco Co. (Tokyo, Japan). Acrylamide was obtained from

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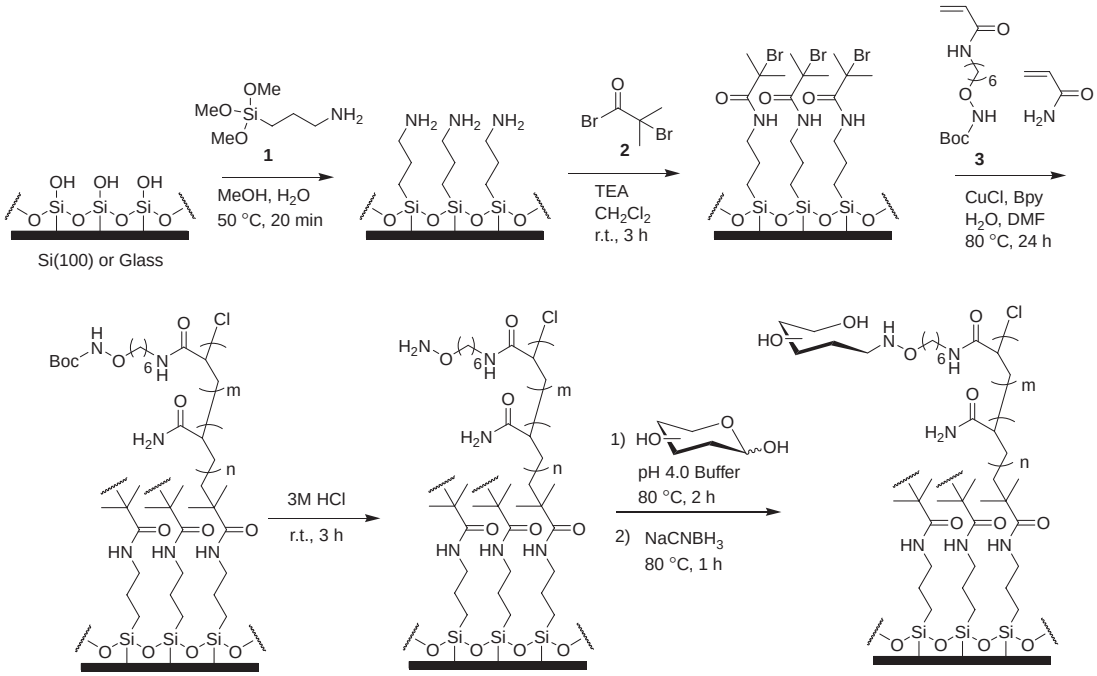

Scheme 1. Synthetic scheme of carbohydrate-grafted polymers on silicon or glass surfaces using ATRP and the sugar-trapping method.

Bio-Rad (Japan) and used without further purification. All commercial reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used as received. *N*-[6-(*N*-*tert*-Butoxycarbonylaminooxy)hexyl]acrylamide (**3**) was synthesized according to a previous report.[20] Reactions were monitored by TLC on 250 μm silica gel plates (E. Merck, 60F254) using UV light and a cerium molydate solution (10% Cerium(IV) sulphate, 15% H_2SO_4 aqueous solution). NMR spectra were recorded on a JEOL 400 spectrometer. MALDI-TOF-MS spectra were measured with a Voyager-DE STR-H spectrometer (Applied Bio Systems) using 2,5-dihydroxybenzoic acid (Bluker, Germany) as the matrix.

Pretreatment and Silanization with APTS of Silicon or Glass Substrates. Silicon wafers or glass substrates were cut into 1.5 cm × 1.5 cm pieces. The substrates were sonicated for 10 min in acetone, ethanol and deionized water, and dried in a stream of nitrogen. The substrates were cleaned with UV light (185 and 254 nm) and ozone gas by using a UV/ozone cleaner (NL-UV253, Nippon Laser & Electronics Lab.). Silanization was carried out in a 1 vol % solution of (3-aminopropyl)trimethoxysilane (APTS) (**1**) in methanol/H_2O (95:5) containing 1 mM acetic acid for 20 min at 50 °C. The substrates were then rinsed with methanol and baked at 120 °C for 5 min.

Grafting of the Aminooxy Polymer from the Glass or Si(100) Surface by ATRP. The aminopropylsilane (APS)-coated glass and Si(100) plates were immersed in CH_2Cl_2 (20 mL), and then triethylamine (1.2 mL, 8.54 mmol) and 2-bromo-2-methylpropanoyl bromide (**2**) (1.05 mL, 8.54 mmol) were added. The reaction was carried out for 3 h at room temperature. The substrates were washed extensively with CH_2Cl_2 and dried under a stream of nitrogen. *N*-[6-(*N*-*tert*-butoxycarbonylaminooxy)hexyl]-acrylamide (**3**) (114 mg, 400 μmol) and acrylamide (256 mg, 3.60 mmol) were dissolved in 4 mL of a mixture solvent of H_2O and *N*,*N*-dimethylformamide (DMF) (1:1 v/v). After bubbling with nitrogen for 30 min, CuCl (1.38 mg, 14.0 μmol) and bipyridine (4.36 mg, 28.0 μmol) were added to the mixture. The initiator-functionalized substrate was then soaked in the reaction mixture. Polymerization was carried out for 24 h at 80 °C under a nitrogen gas atmosphere. The polymer-grafted substrate was rinsed with DMF and water.

Characterization of the Polymer-Grafted Surface. X-ray photoelectron spectroscopy (XPS) measurements were made on a JPS-9200 spectrometer (JEOL, Japan) with monochromatic Al Kα radiation ($E = 1486.6$ eV). The X-ray source was run at a reduced power of 150 W, and the take-off angle was 90°. Wide-scan spectra were recorded at a constant dwell time of 200 ms and a pass energy of 50 eV, and narrow scans were recorded at 300 ms and 10 eV. The thickness of the APS layer and grafted-polymer layer were measured using an ellipsometer equipped with a He-Ne laser (632.8 nm) (MARY-102, FiveLab Co., Ltd., Kanagawa, Japan) and X-ray diffraction system (SmartLab., Rigaku Corporation, Tokyo, Japan). The incident angle of the ellipsometer was fixed at 70°, and thickness measurements were performed at least four spots on each samples. The X-ray source (Cu Kα) of the X-ray diffraction system was run at 45 kV–200 mA. The scan angle was from 0 to 10° (sampling distance was 0.01°), and the scan speed was 0.67° min^{-1}.

Lactose-Trapping on the Aminooxy Polymer-Grafted Glass Plate. The *N*-Boc-protected aminooxy polymer-grafted substrate was treated with 3 M (1 M = 1 mol dm^{-3}) for 3 h at room temperature and then rinsed with deionized water. The substrate was immersed into a 1 mM lactose solution (10 mM acetate buffer, pH 4.0) for 2 h at 90 °C, then soaked with 1 mM $NaBH_3CN$ for 1 h, and subsequently rinsed with deionized water.

IR-RAS Measurement of the Lactose-Displaying Surface. Infrared reflection absorption spectra were measured using an FT/IR-660 Fourier transform infrared (FT-IR) spectrometer (JASCO, Japan) equipped with a PR-510 reflection absorption spectroscopy (RAS) unit and an MCT detector. Single-channel transmittance spectra (1000 scans) were collected at a spectral resolution of

4 cm^{-1}. All spectra shown in this paper are the result of spectra subtraction of modified samples with a cleaned silicon sample.

Hybridization with Fluorescence-Labeled Lectin. A fluorescein isothiocyanate (FITC)-labeled RCA120 (SEIKAGAKU Co., Tokyo, Japan) solution was prepared by diluting a stock solution to a concentration of 0.2 μg mL^{-1} with PBS buffer (pH 7.4, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.1% Tween 20). Lactose-displaying substrates were immersed in the lectin solution and gently shaken for 1 h. The substrate was then soaked in a PBS buffer solution with gentle shaking for 30 min. The substrate was rinsed with deionized water and dried in a stream of nitrogen. Fluorescent images were recorded on a microscope (FV300, OLYMPUS, Japan).

Surface Patterning with UV Irradiation of APS-Coated Substrates. Surface-patterned amine degradation by UV light was carried out according to a previously reported procedure.[21] APS-coated substrates were exposed to UV light (184.9 nm) for 2 h through a mesh photomask for transmission electron microscopy (Cu mesh, hole 85 mm, bar 40 mm; Okenshoji, Tokyo, Japan) under a nitrogen atmosphere. The substrates were rinsed with methanol. Initiator functionalization, ATRP, and sugar-trapping were then performed under the same conditions as those mentioned above.

Results and Discussion

ATRP Synthesis of Sugar-Trapping Polymer in a Solution. A mixture of monomer **3** and acrylamide (1:9 mol/mol) was polymerized using ethyl 2-bromoisobutyrate as a free initiator in the presence of a CuCl/bpy catalyst in a DMF/H_2O solution at 80 °C (Supporting Fig. 1). After 24 h at 80 °C, the polymerization was terminated by exposure to air. Precipitation of the obtained polymer was performed with acetonitrile.

The molecular weight distribution of the polymer was analyzed by gel-permeation chromatography (GPC). The number-average molecular weight (M_n) and the weight-average molecular weight (M_w) were 2.6 and 4.7 kDa, respectively, and the dispersity ($=M_w/M_n$) was 1.8. Monomer conversion was about 15%, based on polymer weight. Brittain and co-workers have reported that the polymerization process during the ATRP of acrylamide-type monomers is difficult to control.[22] This is due to the Cu salt complex-mediated trapping of reactive radicals by the amide group, resulting in the deactivation of the ATRP reaction. Therefore, the molecular weight distributions of the acrylamide-type polymers synthesized by ATRP have been reported to be large, and their molecular weights are relatively small.

From NMR spectroscopy of the copolymer, the ratio of methylene protons in the polymer backbone and the protons in the *tert*-butyl group in monomer **3** was 34:9. This result suggests that the ratio of monomer **3** and acylamide in the copolymer is 1:10 mol/mol. This ratio in the copolymer corresponds closely to the starting monomer ratio (1:9 mol/mol). Considering the average molecular weight, the single chain is thought to contain about 2.5 aminooxy residues.

The Boc group protecting the aminooxy group of the polymer side chain was removed in aqueous HCl (3 M) for 3 h at room temperature. The potential for chemoselective ligation between the aminooxy groups of the polymer and glucose (model sugar) was tested under the appropriate conditions for oxime formation determined in the previous report (pH 4.0 at 90 °C for 2 h).[12] Both the deprotection and sugar-immobilization processes were observed by NMR analysis (Supporting Fig. 2). Deprotection of the Boc groups was confirmed by the disappearance of a singlet corresponding to *tert*-butyl at 1.40 ppm. After the chemoselective ligation, new signals appeared at 7.52 and 6.84 ppm. The peaks corresponded to the *E*- and *Z*-oxime isomers,[23] respectively, indicating successful sugar-immobilization via oxime bonds.

Grafting of Aminooxy Polymers from Si(100) or Glass Surfaces via Surface-Initiated Polymerization. Synthesis of the aminooxy polymer from the substrate surface and the immobilization of carbohydrates are outlined in Scheme 1. The silicon or glass surface was covered with the amino group through silane-coupling reaction with APTS **1**. Some synthetic approaches for introducing the ATRP initiator have been reported.[24–26] We chose the reaction of an acidic halide with the displayed amino groups on the substrate. This method is simple and does not require the purification of unstable and active initiators, such as trimethoxysilane derivatives. The amine on the surface was connected with the polymer initiator, 2-bromo-2-methylpropanoyl bromide (**2**), which is widely employed as an ATRP initiator, under basic conditions. Initiator immobilization and polymer grafting were first ascertained by XPS measurement. Figure 1A shows the XPS spectrum of the initiator-treated silicon substrate. Successful display of the initiator was indicated by the appearance of a bromide 3d signal, not detected for the bare substrate (Fig. 1C).

The mixture of aminooxy derivative monomer **3** and acrylamide was polymerized from the initiator in the presence of a CuCl/bpy catalyst in a DMF/H_2O solution at 80 °C. After approximately 24 h at 80 °C, the polymerization was terminated by exposure to air. XPS analysis showed that the polymer was grafted onto the substrate. The XPS spectrum of the polymer-grafted surface, which is shown in Fig. 1B, showed a significant increase in the carbon and nitrogen signals and a large decrease in the Si signals. The narrow spectra of nitrogen 1s indicated the presence of three different types of nitrogen atoms that were assigned as the two types of amide groups and the aminooxy group of the polymer layer (Fig. 1D). The spectrum of carbon 1s indicated the presence of three different types of carbon: the amide group, ether group, and alkane backbone of the polymer (Fig. 1E). These signals support the conclusion that the polymer was grafted from the surface as shown in Scheme 1. The thickness of the polymer layer was measured by using a ellipsometer. Figure 2 shows the thickness of the polymer layer synthesized under various conditions. The thicknesses of the grafted polymers made from monomer **3**/acrylamide at ratios of 10/90 and 5/95 were 6.69 and 8.20 nm, respectively. This indicates that polymerization proceeds more smoothly with the addition of the acrylamide monomer. The average thickness of the APS layer was 1.5 nm; therefore, ca. 5 nm of polymer layer was grown from the APS surface. We also measured the thickness of the grafted polymers made from monomer **3**/acrylamide in a ratio of 10/90 using X-ray diffraction analysis. This also had a thickness of 4.75 nm, which is similar to the value obtained from the ellipsometric analysis. These values are reasonable considering the molecular weight ($M_n = 2.6$ kDa) of the corresponding polymer grown in the solution. Increasing the monomer concentration from 1.0 to 2.0 M caused only a 0.71 nm increase in polymer

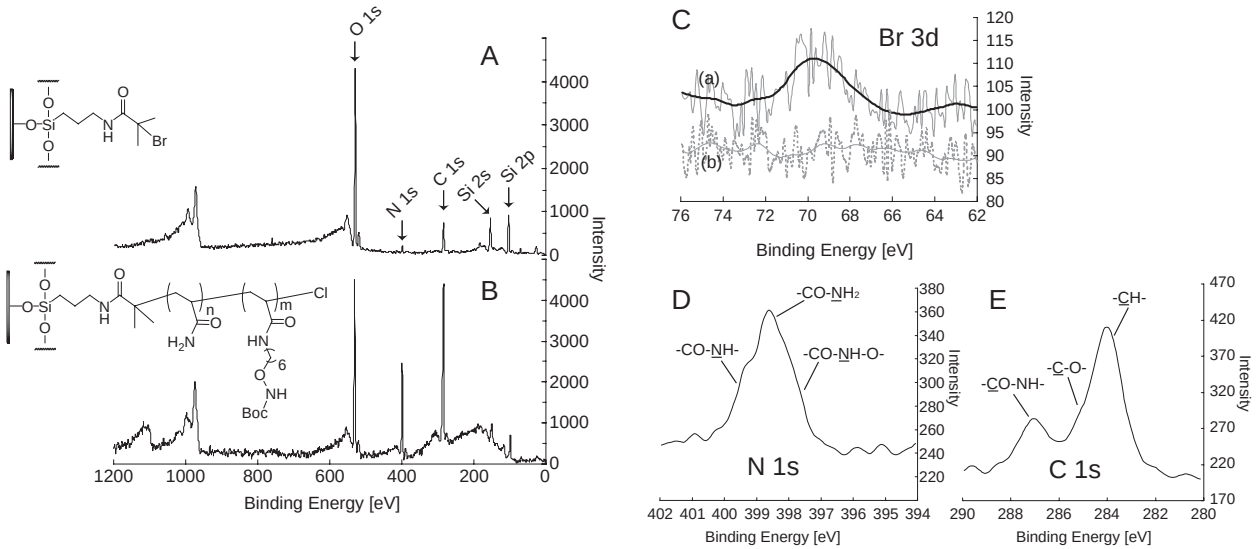

Fig. 1. XPS survey spectra and selected elemental scans before and after ATRP. (A) Initiator-immobilized surface. (B) After ATRP. (C) Bromo 3d signal (a) before initiator functionalization and (b) after ATRP. (D) and (E) Nitrogen 1s and carbon 1s signals of the grafted polymer.

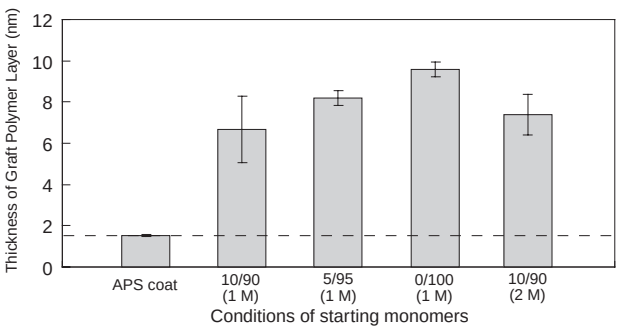

Fig. 2. Ellipsometric analysis of the thickness of the grafted polymer (APS coat, **3**/acrylamide: 10/90, 5/95, 0/100, and 10/90). The monomer concentration is shown in parentheses.

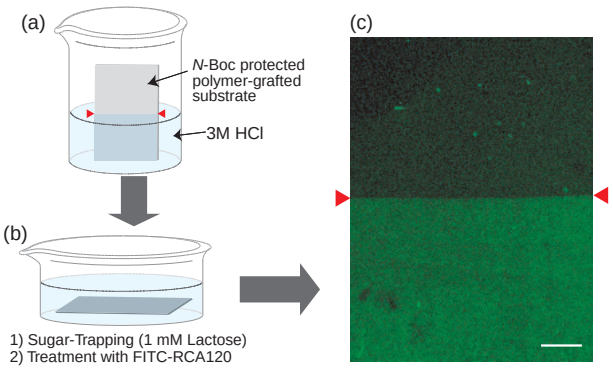

Fig. 3. Sugar-immobilization on the grafted sugar-trapping-polymer and hybridization with fluorescence-labeled lectin. (a) Half of the glass plate (shown by the red arrows) was suspended in aqueous HCl for the deprotection of *N*-Boc-protected aminooxy groups. (b) The entire glass plate was immersed in a lactose-containing buffered solution, and then treated with FITC-labeled RCA120. (c) Fluorescent image of lactose-displaying grafted polymer and *N*-Boc-protected aminooxy polymer after treatment with FITC-labeled RCA120. Bar is 250 µm.

thickness, indicating that a monomer concentration of 1.0 M is sufficient for effective polymer grafting. Assuming the M_w of the graft chain to be the same as that of the free chain from the thickness of the graft-polymer layer (5.0 nm), the molecular weight (2.6 kDa), and the bulk poly(acrylamide) density (1.3 g cm^{-3}), the graft density was estimated to be ca. 1.5 chains nm^{-2}. The density of the aminooxy group was calculated to be 0.27 nm^2 residue^{-1}. PMMA polymers grafted by ATRP on the silicon surface have been reported to have a density of 0.4–1.0 chains nm^{-2}.[27] Our results agreed with these reported values, implying that the aminooxy polymers are well-packed on the substrate.

Sugar-Immobilization on the Grafted Aminooxy Polymer. ATRP graft polymerization of monomer **3**/acrylamide (10/90 mol/mol) was carried out on a glass plate. After polymerization, half of the plate was suspended in an aqueous 3 M HCl solution for deprotection of the aminooxy group (Fig. 3a). The entire glass plate was then soaked in a 1 mM lactose-containing buffered solution (pH 4.0) for 2 h for trapping, and the plate was immersed in a solution of sodium cyanotrihydridoborate Na[B(CN)H$_3$], which is a soft reducing agent, to form the non-reversible covalent bonds. In our previous studies using aminooxy-displaying nanoparticles, we have found that 80% of aminooxy groups formed oxime bonds with 1 mM carbohydrates at pH 4.0.[12] The oxime bond formation between the sugar and side chain aminooxy groups of the polymer was identified by NMR analysis of the free copolymer (see Supporting Information). Sugar immobilization on the surface was confirmed by the specific binding of lectin, which is a sugar-recognizing protein (Fig. 3b). We confirmed the specific binding of FITC-labeled RCA120 lectin to lactose using fluorescence microscopy (Fig. 3c). Fluorescent images showed clear differentiation between the deprotected and protected areas, supporting the conclusion that lactose is specifically anchored to the aminooxy group via oxime bonds. As a control

experiment, when FITC–ConA, which is a mannose-specific lectin and has weak affinity for lactose, was added to the lactose-immobilized surface, no binding was observed. Normally, proteins tend to bind to the glass plate nonspecifically. However, non-specific binding of the lectin was suppressed by the grafted polymer (see the dark area in Fig. 3c). These results show that polymerization from a solid surface produced a layer sufficiently hydrophilic to block nonspecific protein binding to the bare glass plate. Further confirmation of sugar-immobilization on the grafted polymer was obtained from IR-RAS spectra (Fig. 4). After treatment with lactose, a broad band at 3400 cm^{-1}, which is due to a stretching vibration mode of the hydroxy group, was observed, indicating sugar-anchoring to the polymer. Our aminooxy-based sugar-trapping polymer greatly simplifies the immobilization process of carbohydrates onto the solid surface through mounting or soaking in a sugar-containing solution alone. The carbohydrates trapped on the polymer layer were recognized by specific lectins, and non-specific binding of proteins was inhibited. These facts suggest the polymer on the surface is sufficiently dense to avoid non-specific adsorption of proteins. This advantage is important and should allow for the use of these sugar-trapping plates to create a sugar-array for the detection of specific sugar-binding proteins. Finally, as a demonstration of the application of this sugar-displaying substrate as a biomaterial, we observed the binding behavior of human fibroblast cells. The fibroblast cells on the lactose-displaying surface were well-stretched compared to the cells on the APS surface (Supporting Fig. 3), indicating that the sugar-immobilized substrate will be useful as a scaffold in the study of sugar-dependent cellular adhesion.

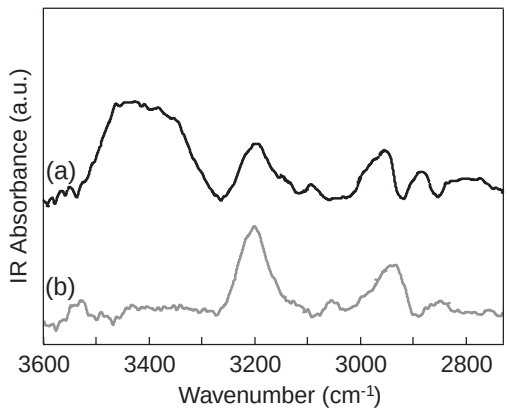

Fig. 4. IR-RAS spectra of the grafted polymer (b) before and (a) after sugar-trapping of the lactose.

Patterning of Sugar-Grafted Polymers on Si(100). The technique for patterned immobilization of desired carbohydrates can be widely applied to create various biomaterials, such as cell-patterned films and sugar arrays. Using our sugar-trapping approach, we aimed to produce the patterned immobilization of carbohydrates. Figure 5a shows the process for grafted polymer micropatterning. UV light was irradiated onto an APS layer over a photomask to cause partial degradation of amines on the substrate. The subsequent grafted polymerization and lactose-immobilization were as described above. Using this method, the grafted polymer was only grown over the photomask-protected areas. Treatment with FITC-labeled RCA120 lectin showed that successful patterned immobilization of sugars on the substrate was achieved (Fig. 5b).

Conclusion

In this paper, we reported a synthetic method of grafting sugar-trapping polymers from solid substrates. Carbohydrates were then trapped on this substrate by simple immersion of the plate in a carbohydrate solution. Our method eliminated the need for any time-consuming and difficult processes related

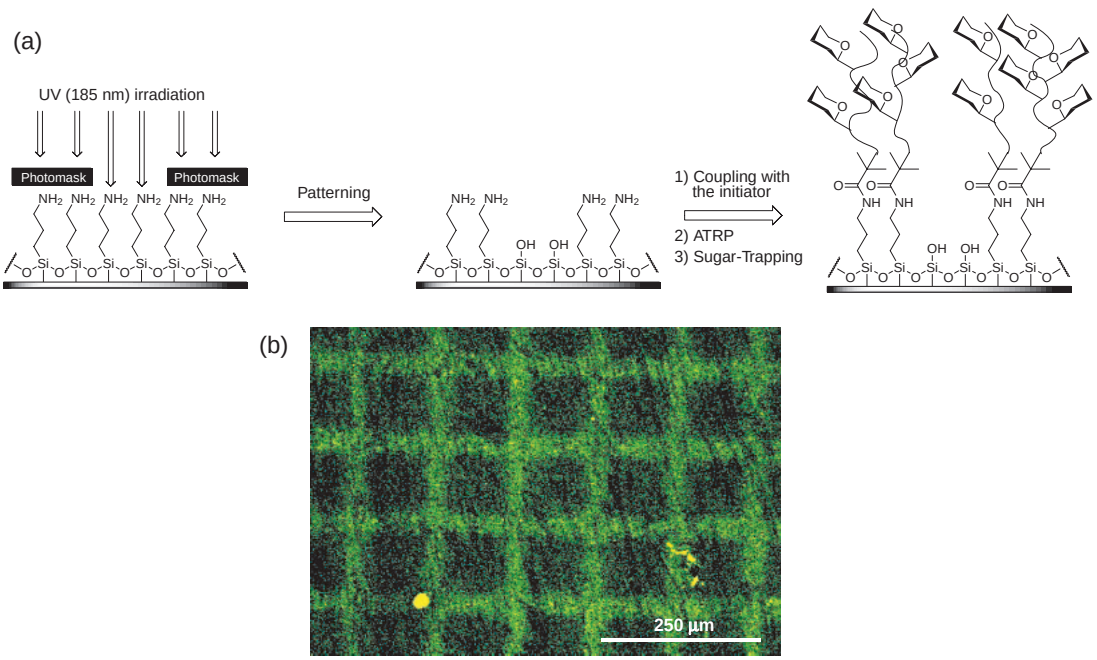

Fig. 5. Patterning of sugar grafted-polymers on Si(100). (a) Fabrication scheme of the patterned carbohydrate-grafted polymer by UV irradiation. (b) Fluorescent image of the patterned lactose-displaying polymer after treatment with FITC-labeled RCA120.

to carbohydrate chemistry, such as protection and glycosylation. Therefore, valuable carbohydrates extracted from cells can be attached to a solid surface, producing a new tool for the determination of the functions of novel carbohydrates. This method could also become the basic technique for the construction of carbohydrate materials to be used for carbohydrate-arrays or as scaffolds for tissue engineering.

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Supporting Information

Experimental section of ATRP synthesis of sugar-trapping polymer in a solution, synthetic scheme of the free copolymer (Supporting Fig. 1), NMR spectra of free polymers (Supporting Fig. 2), Experimental section of cell culture and cell adhesion assay, DIC images of NHDF cell adhesion on APS coat glass and lactose-grafted polymer substrate (Supporting Fig. 3). This material is available free of charge on the Web at: http://www.csj.jp/journals/bcsj/.

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