Nanometer-Scale Immobilization of Polysaccharides on Hydrophobic Polymer Plates in Supercritical Fluoroform/Water Emulsions

Toshiaki Mori, Yoshimi Sekine, Mirei Hasegawa, and Yoshio Okahata*

Department of Biomolecular Engineering, Frontier Collaborative Research Center, Tokyo Institute of Technology, 4259 Nagatsuda, Midori-ku, Yokohama 226-8501, Japan

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Hydrophilic polysaccharides such as dextran and hyaluronan were immobilized on a hydrophobic polystyrene (PSt) plate by a nanometer-scale surface penetration method in the emulsion of aqueous solutions in supercritical fluoroform ($scCHF_3$). Since a supercritical fluid has high diffusiveness, water emulsions of polysaccharides can penetrate into the polymer surface. Dextran was surface-penetrated by two different methods: (1) the penetration of sucrose as a glucose donor and then the enzymatic polymerization to dextran near the surface catalyzed by dextransucrase, and (2) the direct penetration of dextran polymer into the PSt plate. The contact angle for water of the dextran-penetrated PSt plate was decreased to 78° from 95° of the untreated plate. The surface coverage and the penetration depth of polysaccharides could be obtained to be 10−30% and 10−20 nm, respectively, by X-ray photoelectron spectroscopy. These values could be controlled by the pressure of $scCHF_3$. The transparency of the PSt dish did not change after the dextran penetration. Dextrans on the PSt plate could be elongated enzymatically by dextransucrase in the presence of sucrose as a glucose donor, and be detected by the enzyme-linked biotin−avidin assay. When anionic hyaluronan was surface-penetrated on the PSt plate instead of the neutral dextran, the plate showed the specific adhesion for human T-cells having hyaluronan receptors.

Introduction

It is very important to create and control a junction of interfaces between biomolecules and artificial materials. In fact, preparations of biocomposite compounds are significantly focused on biomedical materials.[1] For example, the hydrophobic surface of polyurethane was coated with heparin to obtain antithrombogenicity in biomaterials.[2] Many biopolymer-coated polymer substrates are used as biomedical materials.[3−8] It is, however, difficult to cover hydrophobic polymer substrates with hydrophilic biopolymers such as polysaccharides. Simple coating from aqueous solution causes the exfoliation of biopolymers. Surface modifications such as graft-polymerizations and polymer blends are not suitable for polysaccharides because of the difficulty of chemical modification of or compatibility with hydrophobic materials.[9]

Recently, supercritical carbon dioxide ($scCO_2$) has been used as a clean and moderate reaction media, because CO_2 is inexpensive and safe and has the distinctive properties of supercritical fluids. The physical properties (e.g., density, diffusiveness, and viscosity) of supercritical fluids are intermediate between those of gases and liquids. The large diffusiveness of supercritical fluids compared to liquids can be expected to increase the reaction rate[10] and the penetration depth of the substrate. DeSimone and co-workers applied $scCO_2$ as polymerization media for various monomers.[11] We developed the polymerization of tetrafluoroethylene (TFE) in supercritical fluoroform ($scCHF_3$), whose reactivity could be controlled by pressure changes.[12] We accomplished enzyme reactions in supercritical fluids[13] and molecular recognitions to study solvation effects in supercritical fluids.[14] Shim and co-workers reported that polymer plates swell in supercritical fluids because of their high diffusiveness.[15] McCarthy and co-workers applied $scCO_2$ for the modification of a surface by polymer blends.[16] We previously prepared poly(tetrafluoroethylene) (PTFE)-penetrated polymer substrates using a penetrated photopolymerization of TFE monomers in $scCHF_3$.[17] We have also reported that the CHF_3 containing water can form microemulsions without detergent, and it was effected in the reaction media as the emulsion polymerization of TFE.[12]

In this study, we prepared polysaccharide-penetrated polystyrene (PSt) plates utilizing the high diffusiveness of supercritical fluids in emulsions of the aqueous solution of polysaccharides in $scCHF_3$. Dextran was surface-penetrated by two methods: the penetration of sucrose monomers and then the enzymatic polymerization in the substrate, and the direct penetration of dextran polymer into the substrate (see Figure 1). Anionic hyaluronan could also be surface-penetrated into the PSt plate. The reason we chose $scCHF_3$ as the reaction media is the high polarity of $scCHF_3$ in comparison with that of $scCO_2$; hence, the dielectric constant of $scCHF_3$ can be changed from 1 to 7 (corresponding to the value of hexane to tetrahydrofuran as the organic media) by manipulating the pressure of $scCHF_3$.[18] In addition, $scCHF_3$ can form stable microemulsions of water in $scCHF_3$ without detergents.[19] Wettability was analyzed by contact angles for water. The surface coverage and the penetration depth of penetrated dextran on the PSt surface were analyzed by X-ray photoelectron spectroscopy (XPS). These values could be controlled by changing the pressure of $scCHF_3$. Biochemical assays for the surface polysaccharides were achieved via enzyme reactions and cell adhesions on the surface.

* To whom correspondence should be addressed. Fax: +81-45-924-5836. E-mail: yokahata@bio.titech.ac.jp.

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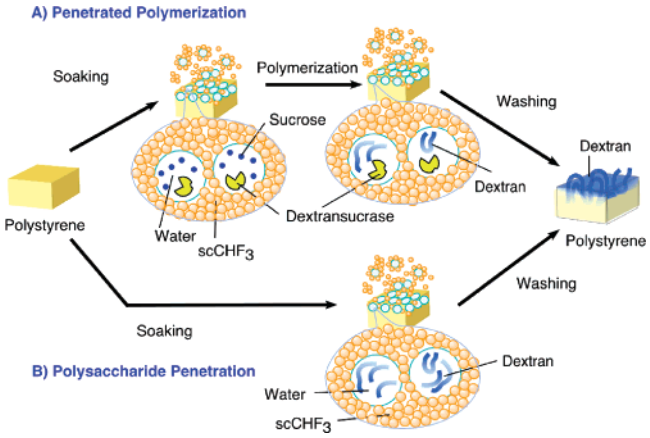


Figure 1. Schematic illustrations of (A) the penetrated polymerization of sucrose by dextransucrase, and (B) the penetration of dextran on a surface of a PSt plate in the aqueous solution/$scCHF_3$ emulsions.

Experimental Section

Materials. Glucose, dextran, and hyaluronan were purchased from Seikagaku Kogyo Co. (Tokyo). Dextransucrase from *Leuconostoc mesenteroides* was purchased from Wako Chemicals Co. (Tokyo). Fluoroform (purity: 99.999%) was kindly provided by Asahi Glass Company (Tokyo). PSt laboratory dishes were used as the polymer substrate and were obtained from Taiatsu Glass Co. (Tokyo). Horseradish peroxidase-fused avidin (HRP-avidin) and 6-[6-(biotinylamino)-hexanoylamino]hexanoylhydrazide were purchased from Dojindo Co. (Kumamoto, Japan). Other reagents for modifications and the dyeing reagent containing nitrotetrazolium blue (NTB), NADH, and hydrogen peroxide were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI) or Tokyo Kasei Co. (Tokyo), and all reagents were used without further purification.

Surface Penetration of Polysaccharides. Reaction vessels made of stainless steel were purchased from Taiatsu Glass Co. (Tokyo), and other pressure-resistant lines (tube, bulb, union, and adaptor) were purchased from Swagelok Co. (Solon, OH). Penetrations of polysaccharides into polymer substrates were performed in a 10 mL pressure-resistant stainless vessel equipped with sapphire glass windows for visual observation during the reactions. The vessel was charged with 0.5 mL of the buffer solution (20 mM acetate, pH 5.2, and 1 mM $CaCl_2$) containing dextransucrase (30 mU mL^{-1}) as an enzyme and sucrose (1 mg mL^{-1}) as a glucose donor or Milli-Q water containing dextran polymer (D_p = 1−5000; 1 mg mL^{-1}) and a piece of PSt plate (5 × 5 × 1 mm) in advance. Then liquid CHF_3 at −5 °C was injected at 5−25 MPa from an LC pump (JASCO PU-980 HPLC pump) connected to a CHF_3 gas cylinder. The vessel was warmed with magnetic stirring to 30 °C to keep a supercritical state, and then the pressure was kept constant (±0.1 MPa) by a back-pressure regulator (SCE-Bpg/M, JASCO, Co., Tokyo). After 24 h, the solvent was evacuated from the vessel carefully under cooling at 0 °C. The PSt plate was take up from the vessel and washed thoroughly with buffer solution and then Milli-Q water, in order to remove the physically adsorbed polysaccharides and enzymes from the substrate.

Stability of Enzymes in Buffer/$scCHF_3$ Emulsion. Dextransucrase was incubated in the emulsion of the buffer (20 mM acetate, pH 5.2, 1 mM $CaCl_2$) in $scCHF_3$, in the emulsion of the buffer in $scCO_2$, or in the simple aqueous buffer solution for 1 h at 30 °C. Then, the residual enzyme activities (productions of dextran from sucrose) were measured using the conventional Somogyi−Nelson method[20] in the aqueous buffer solution (20 mM acetate, pH 5.2, 1 mM $CaCl_2$) at 30 °C. One unit (U) was defined as the amount of enzyme that released reducing sugars equivalent to 1.0 μmol of D-glucose from the substrate per minute under the assay conditions.

X-ray photoelectron Spectroscopy. Surface analyses of the substrate as elements were performed by XPS. XPS spectra were obtained with a Shimadzu XPS spectrometer ESCA 3400 using Mg Kα excitation (8.0 kV, 10 mA), equipped with an Ar etching setup (1.0 kV; pre-etching: 1 min, post-etching: 2 min, and start-etching: 1 min), and were recorded at a takeoff angle of 90°. The charging shift was corrected with the C_{1S} line emitted from neutral hydrocarbons. The analyzed area was 500 × 500 μm. At least five different spots were analyzed on each sample to check for homogeneity. A Gaussian curve-fitting program was used to treat the C_{1S} signals. The surface coverage by polysaccharides was estimated from the relative area of the $C_{1S(C-O)}$-showing polysaccharides against the C_{1S}-showing PSt plate surface. The etching depth by the argon beam (1.0 kV, pre-etching: 1 min, post-etching: 2 min, and start-etching: 1 min) was calibrated to be 7 ± 2 nm on average using an untreated PSt plate and a dextran-cast film. The polymer substrate was hardly etched (less than 1 nm) during XPS measurements.

Contact Angle Measurements. The wettabilities of the PSt plate were estimated from contact angles for water using a FACE auto contact angle meter (CA-V, Kyowa Interface Science, Co., Saitama). Each Θ value used for calculations was the average of six determinations. All quoted angles were subject to an error of ±2°.

Biochemical Evaluation of Surface-Penetrated Dextran on a PSt Plate. The surface-penetrated dextran on the PSt plate was estimated by two different methods: enzymatic elongation and the enzyme-linked biotin−avidin assay of polysaccharides.

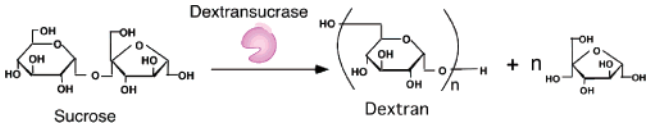
The dextran-penetrated PSt plate was soaked in an aqueous buffer solution (20 mM acetate, pH 5.2, 1 mM $CaCl_2$) with 30 mU/mL of dextransucrase and 1 mg/mL of sucrose for 24 h (see Figure 6A). After washing the surface, the elongated dextran on the plate was analyzed by XPS.

Dextran on the plate was oxidized by 0.1 M $NaIO_4$ in 1 mL of the buffer solution (10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.05% Tween20) for 1 h (see Figure 6B). After washing the plate, aldehyde side chains were reacted with 6-[6-(biotinylamino)hexanoylamino]hexanoylhydrazide (10 mM) in the buffer solution for 30 min. Biotins on the dextran side chain were marked with HRP-avidin in the buffer solution for 1 h, and the dyeing solution containing NTB, NADH, and hydrogen peroxide was added to the solution and incubated for 10 min. Finally, the substrate surface was thoroughly washed with Milli-Q water, and the blue enzymatic product of diformazan was analyzed by the NIH-Image assay.

Cell Adhesion Experiments on Hyaluronan Immobilized on a PSt Plate. The hyaluronan-immobilized PSt plate was incubated in human T-cells (ca. 10^5) with IRPM1940 medium containing 10% fetal calf serum (FCS) at 37 °C for 3 days. After washing the substrate, cells adhered on the surface (where hyaluronan receptors on cells recognized hyaluronan on the plate) were observed by an optical microscope, and the number of the cells adhered on the plate was counted.

Results and Discussion

Penetrated Polymerization of Sucrose Monomers to Dextran in a Buffer/$scCHF_3$ Emulsion. Dextransucrase is known to catalyze elongations of dextran from sucrose, releasing fructose.[21] Therefore, when dextransucrase and sucrose are solubilized in the aqueous buffer phase of an $scCHF_3$ emulsion and penetrate into the surface of polymer substrates because of the high diffusiveness of supercritical fluids, the enzymatically polymerized dextran is expected to be immobilized near the surface of the substrate.



We first studied the stability of dextransucrase in a buffer/$scCHF_3$ emulsion. Figure 2 shows time courses of enzymatic

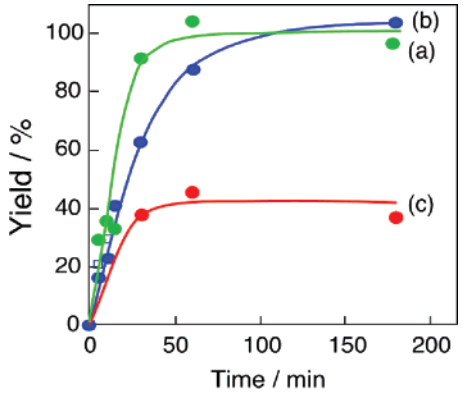


Figure 2. Reaction time courses of the enzymatic polymerization of sucrose catalyzed by dextransucrase in the aqueous buffer solution after the incubation of enzymes (a) in the buffer/scCHF$_3$ emulsion, (b) in the aqueous buffer solution, and (c) in the buffer/scCO$_2$ emulsion. (Buffer solution: 0.5 mL of scCHF$_3$ or scCO$_2$, 10 mL of [Sucrose] = 100 mg μL^{-1}, [dextransucrase] = 0.03 unit mL^{-1}, at 6 MPa, at 30 °C; buffer solution: 20 mM acetate, pH 5.2, 1 mM CaCl$_2$).

sucrose polymerizations in an aqueous buffer solution (20 mM acetate, pH 5.2, 1 mM CaCl$_2$) after the incubation of dextransucrase in a buffer/scCHF$_3$ emulsion, in a buffer/scCO$_2$ emulsion, and in an aqueous buffer solution. The enzyme activity, after incubation in the scCHF$_3$/buffer emulsion, was still similar to that observed after incubation in the aqueous buffer solution alone. The increase in activity observed after ~30−50 min for the treatment in the buffer/CHF$_3$ emulsion (curve a) compared to that for the treatment in the aqueous solution (curve b) in Figure 2 may be due to experimental error, because the activities indicated by curves a and b became similar after ~200 min. The average degree of polymerization of dextran sucrose was determined to be 150 ± 50 from the ratio of the reducing end glucoses (obtained from the Somogyi−Nelson method)[20] to other glucose units (the phenol-sulfuric acid method). On the other hand, the enzyme activity was largely reduced after incubation in the buffer/scCO$_2$ emulsion. This is explained by the reduction in buffered pH from 5.2 to 4.0 due to the solvation of CO$_2$ in the aqueous phase of the scCO$_2$ emulsion.[22] Thus, dextransucrase is not inactivated in the buffer/scCHF$_3$ emulsion for at least 24 h.

Figure 3A shows typical XPS charts of the carbon 1S region of the surface-penetrated polymerization of sucrose on a PSt plate in the buffer/scCHF$_3$ emulsion. The untreated PSt plate showed a simple peak of the neutral C−C and C−H bonds (C_{1S}) at 285 eV, and the PSt on which sucrose was penetrated and polymerized to dextran showed the new peak of the C−O bond ($C_{1S(C-O)}$) at 287 eV in addition to the peak at 285 eV. Peak area gives the atomic composition for each component. The peak area ratio of $C_{1S(C-O)}/C_{1S}$ was 0.15, and the surface coverage of dextran was calculated to be 18% from the $C_{1S(C-O)}/C_{1S}$ = 0.83 calculation of the surface of a dextran-cast film as a reference.

In order to get the information for the depth of the penetrated dextran, etching treatments to the surface by an argon beam were performed. Figure 3B shows the effect of etching cycle numbers for the dextran-penetrated PSt plate (curve a). The etching depth was calibrated in advance, by using an untreated PSt plate and a dextran-cast film, to be 7 ± 2 nm on average. The polymer substrate was hardly etched (less than 1 nm) during XPS measurements. The ratio of the $C_{1S(C-O)}/C_{1S}$ peak areas gradually decreased with increasing etching cycles (the depth from the surface), and almost diminished at a depth of 20 nm from the surface. Thus, sucrose can penetrate and is polymerized

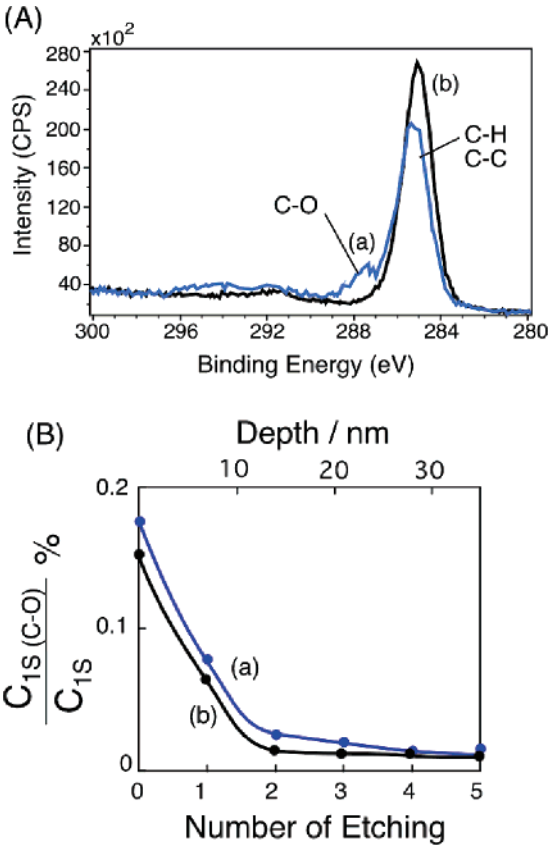


Figure 3. (A) Typical XPS charts of the carbon 1S region of (a) the PSt plate, in which sucrose was penetrated and enzymatically polymerized to dextran, and (b) the untreated PSt plate (scCHF$_3$: 10 mL; buffer solution: 0.5 mL, [Sucrose] = 100 mg μL^{-1}, [dextransucrase] = 0.03 unit mL^{-1}, at 6 MPa, at 30 °C; buffer solution: 20 mM acetate, pH 5.2, 1 mM CaCl$_2$). (B) Etching effects on PSt plates, in which (a) sucrose was penetrated and then polymerized to dextran (average D_p = 150 ± 50), and (b) dextran polymer (average D_p = 220) was penetrated. Etching depth was calibrated to be 7 ± 2 nm per etching cycle.

to dextran at a depth near 20 nm from the surface (curve a). We tried to extract the penetrated dextran from the PSt substrate; however, it was difficult to extract it because of the small amount of the penetrated dextran and the strong interaction of dextran and the PSt substrate. Therefore, we assumed that the molecular weight of the dextran polymerized in the substrate may be similar to that of the dextran polymerized in the bulk buffer/scCHF$_3$ emulsion (D_p = 150 ± 50).

Penetration of Dextran Polymer in a Water/CHF$_3$ Emulsion. As it was found that the sucrose monomer penetrated and polymerized enzymatically to dextran near the PSt surface, we also applied the direct penetration of dextran polymer (average D_p = 220) into the PSt surface in a water/scCHF$_3$ emulsion in the similar manner. After washing the physically adsorbed dextran, the dextran-penetrated PSt surface was analyzed by XPS. We observed XPS charts very similar to those generated by the penetrated polymerization method shown in Figure 3A. The effect of etching on the dextran-penetrated PSt is shown in curve b of Figure 3B. These results indicate that dextran polymer (average D_p = 220) can penetrate to a ca. 20 nm depth from the surface, and the surface coverage was calculated to be 15%, similar to the 18% calculated for the sucrose-penetrated and polymerized to dextran (average D_p = 150 ± 50) plate. Thus, we could prepare the dextran-penetrated PSt plate by both methods; that is, the monomer penetration and then polymerization, and the polymer penetration. We chose the simple

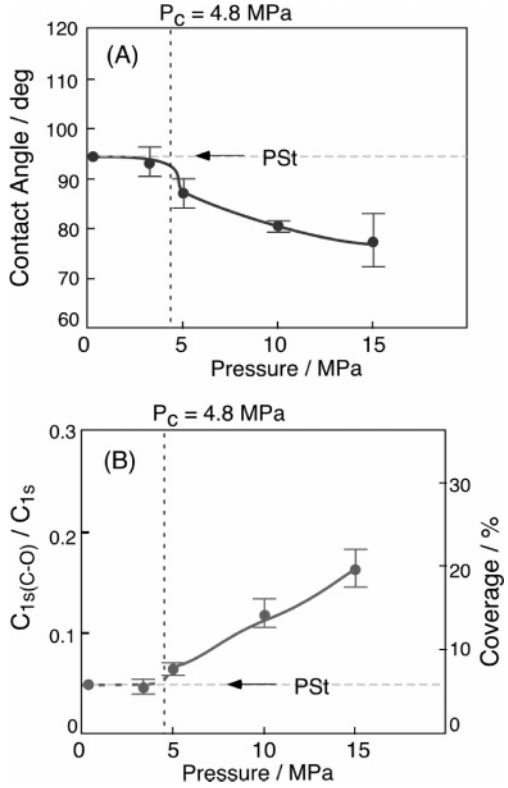


Figure 4. Effect of pressure in the dextran penetration to the PSt plate in the water/scCHF$_3$ emulsion on (A) contact angels for water (the contact angle for water of the untreated PS plate was 95°), and (B) the $C_{1S(C-O)}/C_{1S}$ ratio obtained from XPS (the $C_{1S(C-O)}/C_{1S}$ ratio of 0.15 corresponded to a ca. 20% surface coverage of dextran on the surface). Dotted lines show the contact angle and the $C_{1S(C-O)}/C_{1S}$ ratio of the untreated PSt plate, respectively.

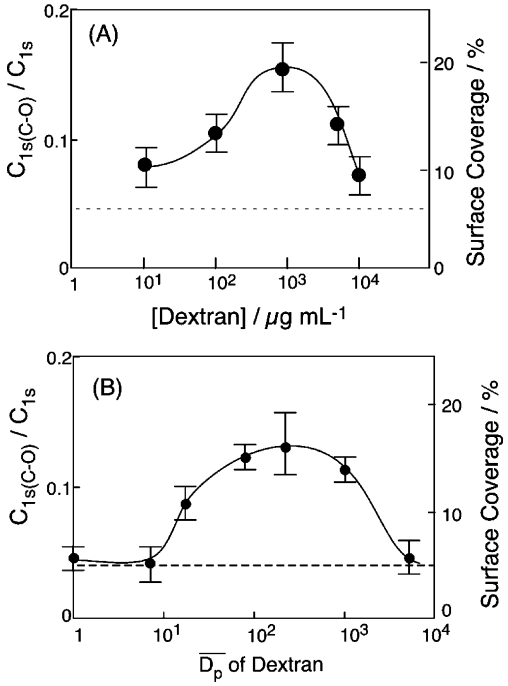


Figure 5. Effect of (A) concentration (average D_p of dextran: 220) and (B) degree of polymerization of dextran (concentration: 1 mg mL^{-1}) on $C_{1S(C-O)}/C_{1S}$ ratios and surface coverages of the dextran-penetrated PSt plate in the water/scCHF3 emulsion (0.5 mL of aqueous dextran solution in 10 mL of scCHF$_3$ at 30 °C, 6 MPa).

polymer penetration method in the following section. We have confirmed that polysaccharides were stable and not hydrolyzed in the buffer/scCHF$_3$ emulsion for at least several days under these experimental conditions.

Effect of Pressure on Dextran Penetration in a Water/scCHF$_3$ Emulsion. One of the features of using supercritical fluids as a reaction medium is that their physicochemical properties such as the density and the dielectric constant (ϵ) can be manipulated by continuous changes in temperature or pressure.[18,22] Figure 4A shows the effect of pressure in CHF$_3$ on the contact angles for water of the dextran-penetrated PSt plate. Below the critical pressure (P_c = 4.8 MPa), where the media exists as gaseous CHF$_3$, the contact angle was very close to that of the untreated PSt. Thus, surface penetration hardly occurred in gaseous CHF$_3$. On the contrary, above P_c = 4.8 MPa, the contact angle for water decreased with increasing pressure. Thus, the macroscopic wettability of the dextran-penetrated PSt plate became hydrophilic. However, the minimum value of 77° was much higher than that of the dextran-cast films (ca. 10°).

Figure 4B shows the effect of pressure in CHF$_3$ on the $C_{1S(C-O)}/C_{1S}$ ratio of the dextran-penetrated PSt plate. Below P_c = 4.8 MPa in the gaseous CHF$_3$, the penetration of dextran hardly occurred. This is due to the fact that, below the critical pressure, the aqueous solution and gaseous CHF$_3$ do not form an emulsion. Above the critical pressure of the supercritical fluid, the dextran peak ratio gradually increased upon increasing the pressure to 15 MPa. As the density of fluoroform increased with increasing pressure, the improvement in the efficiency of the formation of the emulsion and the degree of swelling would cause the amount of the immobilization to increase. In summary, a long dextran (average D_p = 220) can penetrate into the PSt plate to a depth of ~20 nm and cover ca. 18% of the surface of the plate at a pressure of 15 MPa in the water/scCHF$_3$ emulsion.

Effect of Concentration and Length of Dextran on Penetration into the PSt Plate. Figure 5A shows the effect of the concentration (10 μg mL^{-1} to 10 mg mL^{-1}) of dextran in the water/scCHF$_3$ emulsion on surface coverages calculated from the $C_{1S(C-O)}/C_{1S}$ peak ratios of XPS. The surface coverage showed a maximum of 18% at 1 mg mL^{-1} of the dextran aqueous solution. At a low concentration, it is reasonable to show a low penetration amount. On the contrary, at a high concentration of dextran, the surface coverage decreased because of the low stability of the emulsion due to the high viscosity of dextran. Thus, the proper concentration of dextran was determined to be 1 mg mL^{-1} in the emulsion of 0.5 mL of water in 10 mL of scCHF$_3$ at 6 MPa.

Figure 5B shows the effect of the length of dextran (average $D_p = 1-10^4$) on the surface coverage of the plate. The surface coverage showed a maximum near D_p = 200, showing that the proper length of dextran exists. The short dextran ($D_p < 10$) can easily penetrate into the substrate, but is easily removed from the substrate during the evacuation of the media. The long dextran ($D_p > 10^3$) is difficult to penetrate into the substrate, even into the swollen PSt surface. Thus, the proper size of dextran (average D_p = 200, 70 ± 20 nm length) exists for penetration into the substrate. Although the D_p of the enzymatic polymerized dextran in the penetrated plate was 150 ± 50 in our conditions, we can expect to penetrate a longer dextran by using the penetration−polymerization method.

It is still unclear whether sucrose penetrates to the PSt substrate first and then polymerizes in the substrate or polymerizes to dextran in the bulk emulsion and then penetrates into the substrate for the penetrated polymerization method. Although it is difficult to determine which process occurs in this experiment, we assume that the penetrated polymerization is

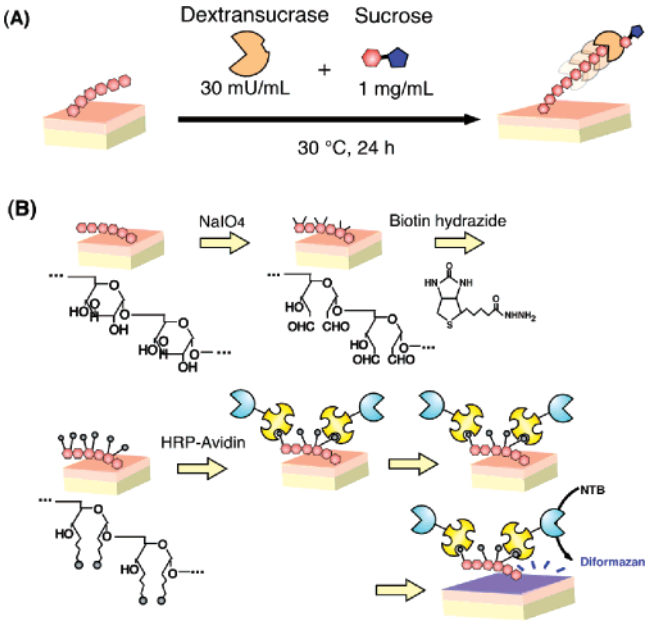


Figure 6. Schematic illustrations of (A) enzymatic elongation of dextran catalyzed by dextransucrase with sucrose on the dextran-penetrated PSt plate, and (B) enzyme-linked biotin−avidin assay on the dextran-penetrated PSt plate.

the main pathway, because the small sucrose monomer can easily penetrate compared to the large dextran polymer.

Enzymatic Elongation and Biochemical Assay of Surface-Penetrated Dextran on a PSt Plate. Since the surface coverage of the penetrated dextran was not enough (10−18% on the surface), we examined biochemical evaluations of the enzymatic elongation of the surface dextran and the enzyme-linked assay of the surface dextran shown in Figure 6.

The dextran-penetrated PSt plate was soaked in an aqueous solution of dextransucrase and sucrose to elongate dextran by enzymatic reactions (Figure 6A). After the elongation, the surface coverage was analyzed by XPS. The surface-penetrated dextran was also analyzed by a biochemical assay (Figure 6B). The dextran on the surface was oxidized by $NaIO_4$ to form reactive bis-aldehyde groups from vicinal alcohols on α-glucan. The resulting aldehyde groups were allowed to react with biotinylated hydrazine to introduce biotin groups in the side chains of dextran. Finally biotinylated dextran was reacted with HRP-avidin, and the appearance of blue diformazan formed by an enzymatic reduction of NTB was analyzed by the NIH-Image technique. The results of the dextran elongation and biochemical assays are summarized in Table 1, together with XPS analyses.

As a negative control, the untreated PSt plate did not show the existence of dextran by the NIH-Image assay (0.1) or XPS analysis (0%). When the surface-penetrated dextran was elongated by dextransucrase, the surface coverage of dextran increased from 14 to 28%, and the density counts of the NIH-Image also increased from 17 to 32. This clearly indicates that enzymes can recognize the surface dextran and elongate the length of dextran on the surface. As a positive control, when dextran was cast on the PSt plate, it was confirmed by both XPS and NIH-Image analyses that the surface was completely covered by dextran. After washing the surface, however, the covered dextran was completely removed from the PSt surface. On the contrary, the surface-penetrated dextran was hardly removed from the surface by washing with water several times. Thus, the surface-penetrated dextran exhibits non-reducing ends of glucose units that are active for enzyme reactions. Dextran also exhibits its side chains on the surface, since oxidized aldehydes on the side chains were recognized by the enzyme-linked assay.

Table 1. Physical and Biochemical Evaluations and Enzymatic Elongations of the Surface-Penetrated Dextran on the PSt Plate

substrate plate	surface coverage (%) obtained from XPS[a]	NIH-Image (mean density)[b]
untreated PSt	0 (0.040)	0.1
dextran-penetrated PSt		
before elongation	14 (0.12)	17
after elongation[c]	28 (0.18)	32
after washing[d]	28 (0.18)	32
dextran-cast PSt		
before washing	100 (0.83)	100
after washing[d]	0 (0.040)	1.6

[a] XPS peak ratios of $C_{1S(C-O)}/C_{1S}$ are shown in parentheses. [b] The blue color obtained by enzyme-linked biotin−avidin assay was analyzed by NIH-Image, and mean densities are obtained. [c] The surface dextran was elongated by dextransucrase with an excess of sucrose on the PSt plate. [d] The PSt surface was washed by water several times.

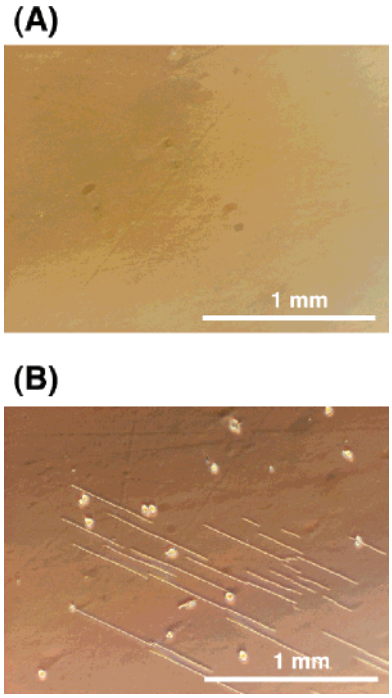


Figure 7. Photographs of (A) the untreated PSt surface, and (B) the hyaluronan-penetrated PSt surface ($C_{1S(C-O)}/C_{1S} = 0.06$, the surface coverage of hyaluronan: 7%), in which the average number of adsorbed cells was determined to be 4.5 mm^{-2} cells/mm^2.

Surface-Penetration of Hyaluronan and Cell Adhesion. We applied the surface-penetration technique to other polysaccharides such as hyaluronan, a negatively charged polysaccharide known as an extracellular matrix.[23] The surface-penetration of hyaluronan was carried out in the water/$scCHF_3$ emulsion (0.5 mL of distilled water in 10 mL of $scCHF_3$, hyaluronan (average $D_p = 600$) 1 mg mL^{-1}, 15 MPa, at 30 °C), similar to the dextran penetration. The surface coverage was obtained by XPS analyses to be 7.2%.

Since hyaluronan is used as an extracellular matrix, we tried to adsorb human T-cells on the hyaluronan-penetrated PSt dish. The plate was soaked in IRPM 1940 medium containing 10% FCS and 1×10^5 human T-cells at 37 °C for 3 days. After washing the surface, the adsorbed cells were observed by an optical microscope. The results are shown in Figure 7. Although cells were hardly adsorbed on the untreated PSt surface, 4.5

pieces/mm^2 of cells adsorbed on the hyaluronan-penetrated surface on average. As the positive control, we confirmed that similar cell adsorption was observed for the substrate on which hyaluronan was simply cast. We should mention that the line-shaped objects in Figure 7B were artifacts due to cracks on the polymer surface.

We emphasize that the transparency of the hyaluronan-penetrated PSt dish hardly changed compared with that of the untreated dish, which shows that the $scCHF_3$ treatment and hyaluronan-penetration hardly change the surface roughness. Since the penetrated hyaluronan was hardly removed from the dish surface, the supercritical method of introducing biopolymers to hydrophobic substrates will become a useful tool to prepare a transparent artificial extracellular matrix.

Conclusion

Hydrophilic carbohydrates such as neutral dextran and acidic hyaluronan could be penetrated into the surface of hydrophobic polymer substrates in the emulsion of an aqueous solution of polysaccharides in $scCHF_3$. Dextran can be penetrated by two methods: the penetration of monomer and then the enzymatic polymerization to dextran, or the penetration of polymeric dextran. The surface coverage and the penetration depth of polysaccharides could be controlled within 0−20% and 0−20 nm, respectively. The surface-penetrated dextran exhibits non-reducing ends and side chains, which was confirmed by the enzymatic elongation experiments and the enzyme-linked assay, respectively. The hyaluronan-penetrated PSt dish is expected to be used as a transparent cell-adhesion matrix.

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