Image Printing on the Surface of Anti-Biofouling Zwitterionic Polymer Brushes by Ion Beam Irradiation[a]

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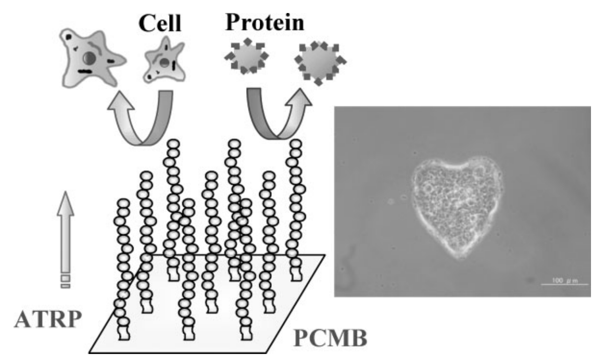
A CMB monomer was polymerized on a glass plate with a surface-confined ATRP initiator containing a 2-bromoisobutyryl group. The glass plate modified with a PCMB brush was highly hydrophilic and showed a strong resistance against non-specific adsorption of proteins and cell adhesion. Upon ion beam irradiation, furthermore, the PCMB brush was ablated and a hollow space with a designed shape could be made to which HEK293 cells (from human embryonic kidney) and Hep G2 (from human hepatoma) cells non-specifically adhered, while no adhesion of these cells to the non-treated area on the brush was observed. The present results clearly indicate the usefulness of ion beam-printed patterns of anti-biofouling zwitterionic polymer brushes in the biomedical field.



Introduction

The modification of solid surfaces with a functional moiety drastically enlarges the usefulness of solid materials. In recent years, various kinds of "polymer brushes", those are polymer chains accumulated on solid surfaces, have been extensively investigated. It has been clarified that by the introduction of polymer brushes, changes in surface properties such as wettability, adhesiveness, etc., can easily be made.[1] The reason for these properties has been attributed to the condensed structure of the well-defined brush. Furthermore, polymer brushes which resist against non-specific adsorption of proteins and cells are expected to be biocompatible materials.[2–6]

There are mainly two strategies for constructing polymer brushes on the surface of solid materials. One is surface-initiated polymerization, the so-called "grafting-from" method.[7–15] The other is the grafting of preformed polymers on the surface of solid materials via covalent bonds, the so-called "grafting-to" method.[15–23]

These grafting procedures can easily be carried out by the application of the preparation procedure of self-assembled monolayers (SAMs) which conjugate organic and inorganic components. Organosilane compounds such as alkyl silane, for example, form a SAM on inorganic material surfaces (silicon and glass) via covalent Si–O bonds,[24–26] while organosulfur compounds such as alkyl or aromatic thiols and disulfides form a SAM on noble metal surfaces via chemisorptive Au–S and Ag–S bonds.[27–30]

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Atom-transfer radical polymerization (ATRP) belongs to the living radical polymerizations and can be applied to a wide variety of monomers, varying the topology of polymer (linear, branched, etc.) and the composition of polymeric chains (block or graft copolymers, etc.).[31–37] The ATRP method has also been applied to the polymerization of various zwitterionic monomers.[2–6,38–48]

Zwitterionic polymers have been designed to mimic phosphatidylcholine (lecithin) that is abundant in cell membranes, and their applicability to the biomedical field has extensively been investigated. For example, polymer films composed of butyl methacrylate (BMA) and zwitterionic monomers such as 2-methacryloyloxyethylphosphorylcholine (MPC, phosphobetaine), 3-sulfo-*N,N*-dimethyl-*N*-(3'-methacrylamidopropyl)propanaminium inner salt (SPB, sulfopropylbetaine with an amide form) and 1-carboxy-*N,N*-dimethyl-*N*-(2'-methacryloyloxyethyl)methanaminium inner salt [CMB, caboxymethylbetaine, Scheme1 (a)] were found to be highly biocompatible.[49–53] The CMB monomer can very easily be prepared by the simple coupling of 2-(dimethylamino)ethyl methacrylate with potassium chloroacetate in water and subsequent electric dialysis.[54] Not using harmful chemicals in the preparation of CMB monomer is quite advantageous in comparison with other zwitterionic vinyl monomers: β-propiolactone used for the preparation of carboxyethylbetaine (CEB)[2] is anticipated to be carcinogenic, and 1,3-propanesultone used for the preparation of sulfopropylbetaine is a cancer suspect agent. Phosphorus trichloride used for the preparation of 2-chloro-1,3,2-dioxaphospholane as an intermediate in the synthesis of MPC causes [55] irritation of eyes, nose and throat, and pulmonary edema.

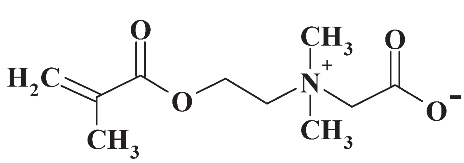
We have reported that the amount of proteins adsorbed and the number of platelets adhered onto a film of random copolymer of CMB and BMA were much less than that to PBMA.[56,57] The usability of CMB/BMA copolymer as dressings for wound healing was also reported.[58]

In addition, the solution behavior of zwitterionic polymers has received much attention due to the unique properties of typical polyelectrolytes.[59–61] Raman and IR spectroscopy indicated that the hydrogen-bonded network structure of water in the vicinity of zwitterionic polymers is not largely disturbed.[56,57,62–64] Based on these findings, we were convinced that the small perturbation effect of zwitterionic polymers on the structure of water at polymer/water interfaces is one of the important factors for their excellent biocompatibility.

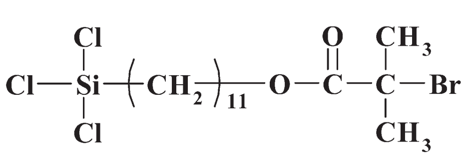
Besides it has been found that zwitterionic telomer brushes (PMPC, PSPB and PCMB) constructed on a gold surface via Au–S bonds resist non-specific adsorption of proteins by using an electrochemical method (cyclic voltammetry) and localized surface plasmon resonance spectroscopy.[38,39,65] In a recent study, it was reported that a CEB polymer grafted on a glass substrate highly resists against protein adsorption and cell adhesion.[2]

In this report, a zwitterionic polymer brush-protected glass substrate was prepared by the ATRP of CMB from surface-confined initiating sites, and a resistance against non-specific adsorption of proteins to the surfaces of PCMB brush was examined. Furthermore, the brush was irradiated by an ion beam for a short time, and the adhesion of cells to the hollow space has appeared on the glass substrate due to the ablation of the brush. Recently an image imprinting of polymer surfaces has extensively been examined by many researchers.[5,66–69] Among various techniques for fabricating micro-patterns, the ablation of polymer materials using ion beam irradiation is quite advantageous due to its ease of use. The usefulness of the ion beam to modulate the surface of polymer materials has clearly been shown in this work.

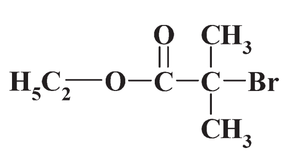
(a)



(b)



(c)



Scheme 1. Chemical Structure of (a) CMB, (b) Br-PUCS and (c) Et-Br.

Experimental Part

Materials

CMB [Scheme 1 (a)] was prepared by the coupling of 2-(dimethylamino)ethyl methacrylate and potassium chloroacetate in water at room temperature (r.t.) for 24 h, and subsequent electric dialysis.[54] Ethyl 2-bromo-2-methylpropionate [Et-Br, 98%, Scheme 1 (c)] and 2,2'-bipyridine (Bpy, 99.5%) were purchased from Merck. 2-Bromoisobutyryl bromide (98%), copper (I) bromide (99.999%), tetrahydrofuran (THF, 99.5%) and *N*-methyl-2-pyrrolidinone (NMP, 99.0%) were purchased from Wako Pure Chemicals, Osaka, Japan. Bovine serum albumin (BSA) and lysozyme from egg white were obtained from Sigma-Aldrich. Slide glass and micro cover glass from Matsunami Glass (Kishiwada, Osaka, Japan) were cut into the most suitable size (38×26 mm^2). Silicon wafers [N(100), having 0.001–0.005 $\Omega \cdot cm^{-1}$ resistivity, and 0.525 ± 0.025 mm thickness] from Furuya Metal Co., Ltd. (Tokyo, Japan) were cut into the most suitable size (20×10 mm^2). All aqueous solutions were prepared with Ultrapure water (18 $M\Omega \cdot cm^{-1}$, Millipore System). Other reagents used were commercially available.

Synthesis

Synthesis of [11-(2-Bromo-2-methyl)propionyloxy]-undecyltrichlorosilane [Br-PUCS, Silane ATRP Initiator, Scheme 1(b)]

Br-PUCS was prepared from 10-undecen-1-ol by the two-step reaction as described in the Supporting Information (Scheme S1 and S2).[9]

Preparation of Initiator-Coated Glass via Silane Coupling (Scheme 2)

To toluene (40.0 mL) in a sample vial was added Br-PUCS at 4×10^{-3} M. A glass plate ($38 \times 26\ mm^2$), which had been washed with water, methanol and acetone and subsequently cleaned by UV/ozone method (UV/ozone cleaner UV253E, Filgen, Nagoya, Japan), was immersed into the solution, and after replacing the atmosphere with Ar gas, the vial was tightly sealed. After 18 h at r.t., the glass plate was washed twice with pure toluene. Furthermore, the plate was immersed in toluene and washed by ultrasonication for 1 min, and after further rinsing with toluene, the plate was dried in N_2, and stored in a sample vial filled with Ar.

Preparation of PCMB Brushes on a Glass Plate via ATRP (Scheme 2)

A magnetic stirrer chip and a home-made Teflon rack for glass plates were placed into a sample vial (50 mL). A solution of CMB (4.67 g, 20.0 mmol) in methanol (40.0 mL) was degassed with Ar and added into the vial. While Ar was continuously purged, the glass plate was put into the vial. While stirring, CuBr (57.4 mg, 0.40 mmol), 2,2'-bipyridine (125 mg, 0.80 mmol), and Et-Br (78.0 mg, 0.40 mmol) were added, and the vial was tightly sealed. After the reaction at 30 °C for 48 h, the glass plate was removed and washed with ethanol, NMP, water, methanol and chloroform. The reaction conversion was determined by the analysis of small aliquots using ^1H NMR. The Cu(I) in the solution mixture was converted to Cu(II) by bubbling air. After drying in vacuo, the product in the liquid phase was dissolved in water, and passed through a chelate resin column (IRC748 Amberlite, Organo Ltd., Tokyo, Japan). The solution obtained was purified by ultrafiltration (Amicon; membrane, YM-1, MWCO 1 000) and lyophilized to give a white powder (E-PCMB). The molecular weight and its dispersity were determined by gel-permeation chromatography (GPC) using pullulan standards [column, Shodex OHpak SB-803HQ (Showa Denko, Tokyo, Japan); mobile phase, 0.1 M aqueous NaBr solution].

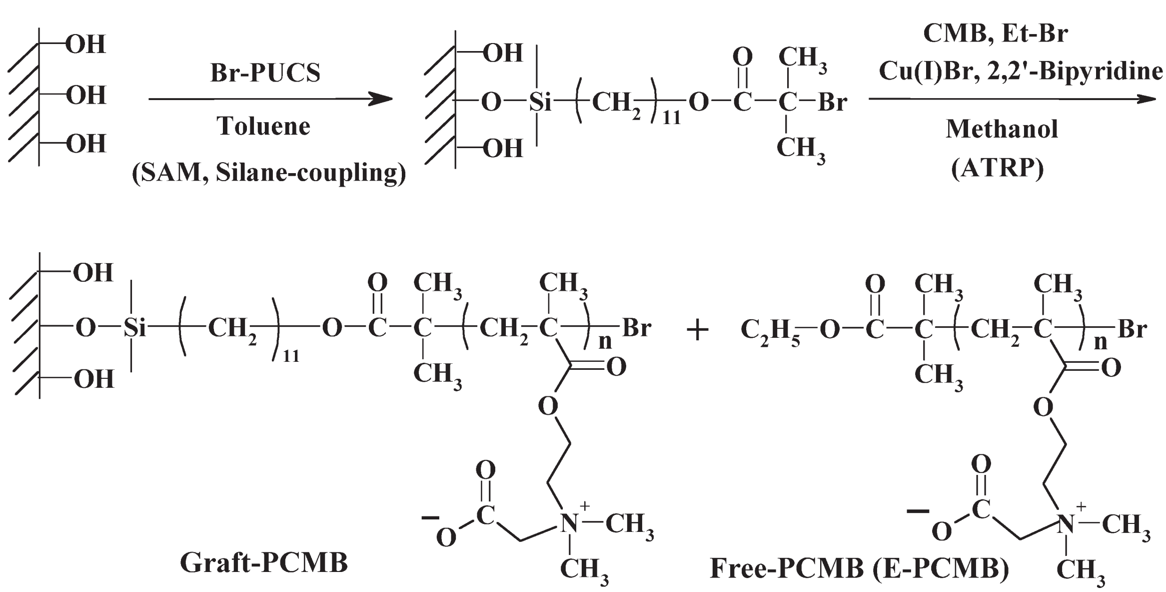
Characterization

Measurement of Contact Angles

Static contact angles, θ, of a droplet of water (3–4 μL) on the surface of various polymer brushes constructed on the glass substrates were determined fifteen times to obtain a reliable average value (sessile drop method). Similarly, the θ values of air bubble (10 μL) attached to the surface of the polymer brush immersed in water were also determined (air-in-water method).

Measurement of Brush Thickness

The thickness of initiator SAM and polymer brushes on a silicon wafer was determined by a spectroscopic ellipsometer (M-2000U, J. A. Woollam Co., Inc., USA). Measurements were taken at an incident angle of 70°. The thickness was calculated from the ellipsometric angles recorded in a wavelength range from 242 to 999 nm assuming that the refractive index of the graft layer is 1.49, which is the experimental value for a poly(methyl methacrylate) film.[70,71] All measurements were conducted in air at r.t.



Scheme 2. Preparation of surface-confined PCMB brush and free PCMB (E-PCMB) using ATRP.

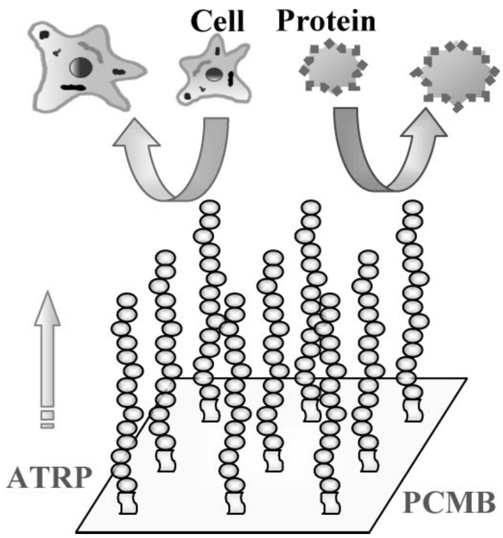


Figure 1. Schematic illustration of the anti-biofouling properties of the PCMB brushes.

Adsorption of Proteins to the Surfaces of Polymer Brushes

Two pieces of the PCMB-modified glass plates were attached to a U-shaped silicon spacer to give a glass cell. The polymer-modified surface of each glass chip was facing inside. Various kinds of protein solutions [BSA and lysozyme; 4.5 mg · mL^{-1} in PBS (pH = 7.4)] were filled into the cell at 37 °C, and it was incubated for 90 min. The protein solution was discarded, and the cell was rinsed with PBS. For desorption, a 5 wt.-% SDS solution was filled into the cell, and after ultra-sonication of the cell for 60 min (at 28 and 40 kHz alternately every 1 min), the solution mixture was recovered, and mixed with a solution of bicinchoninic acid (BCA). The absorbance at 560 nm was measured using a microplate reader (see Figure 1 and S1, Supporting Information).[57,58]

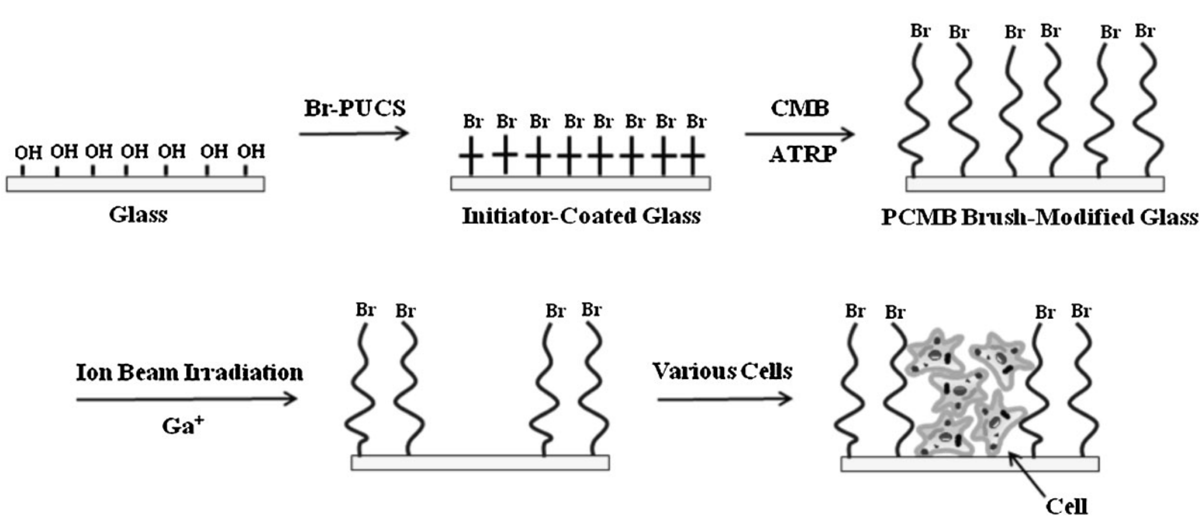
Irradiation of the Polymer Brushes with an Ion Beam (Scheme 3)

The polymer-brush-modified cover glass was soaked in a 70 vol.-% ethanol, and dried in a desiccator. The PCMB polymer brush on the cover glass was irradiated by an ion beam using a focused ion beam system (Hitachi FB-2100; ion beam, Ga^+). The acceleration voltage, the diameter of aperture, and the standard beam current were 40 kV, 30 μm and 0.01–0.03 nA, respectively. The dwelling time and the processing time were 10 μs and 30 s, respectively, in the experiments with HEK293 cells, and 10 μs and 60 s, respectively, with Hep G2 cells. After the ion beam irradiation, the cover glass was washed with PBS (-) and put in a Petri dish (plasma-treated polystyrene; diameter: 60 mm). HEK293 cells and Hep G2 cells in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics were seeded in the dish (5×10^5 cells per dish), and incubated at 37 °C and 5% CO_2 for 12 h. After rinsing with PBS (-), the medium was changed with the new one, and the glass substrate was further incubated for 24 h. After rinsing with PBS (-), the cover glass was observed with a microscope (DP-71, Olympus, Tokyo, Japan).

Results and Discussion

Preparation of Initiator-Coated Glass via Silane Coupling

The construction of a SAM of ATRP initiator conjugated with a silane coupling reagent onto a glass and a silicon substrate was previously reported using the same or similar compounds.[9] The silane coupling reaction was pursued with 4×10^{-3} M of Br-PUCS in toluene at r.t. for 18 h. The progress of the modification reaction was confirmed by the increase in contact angle of the glass plate (sessile drop



Scheme 3. Schematic of adhesion of cells to a hollow space in the surface-confined PCMB brush. The length of the PCMB brush was largely magnified.

Table 1. Contact angle of various glass substrates determined by the sessile drop and air-in-water methods.

Sample	Contact Angle		
	°		
	Sessile Drop[a)]	Air-in-water[a)]	Total
glass	8.2 ± 0.8	155.3 ± 2.8	163.5
Br-PUCS	86.6 ± 2.0	97.3 ± 2.2	183.9
PCMB50[c)]	10.5 ± 1.2	159.8 ± 2.8	170.3

[a)]Measurement conditions: temperature of air 25.0 °C, relative humidity 45.5%, temperature of water 25.0 °C, indicated error is one standard deviation; [b)] Preparation conditions: [CMB]:[Et-Br]:[CuBr]:[Bpy] = 50:1:1:2 in methanol at 30 °C for 48 h.

method) from 8.2 to 86.6° (Table 1) due to the exchange from OH groups to 2-bromoisobutyroylundecyl groups on the glass plate.

Preparation of PCMB Brush via ATRP

Previously, it was reported that the use of oligomeric methoxypoly(ethylene glycol) 2-bromoisobutyrate (OEG-Br) initiator for polymerization of MPC by ATRP was successful.[4–6] It was also reported that the polymerization of SPB using the 2-bromoisobutyryl-group-carrying initiator, CuBr and Bpy catalyst system at r.t. in methanol or a methanol-water mixture had a good correlation between the theoretical and experimental molecular weights.[38–48] Based on these previous results, CMB was polymerized in methanol (40.0 mL) at 30 °C for 48 h using the surface-confined ATRP initiator with the initial molar ratio of [CMB]:[Et-Br]:[Cu(I)Br]:[Bpy] = 50:1:1:2 in this report.

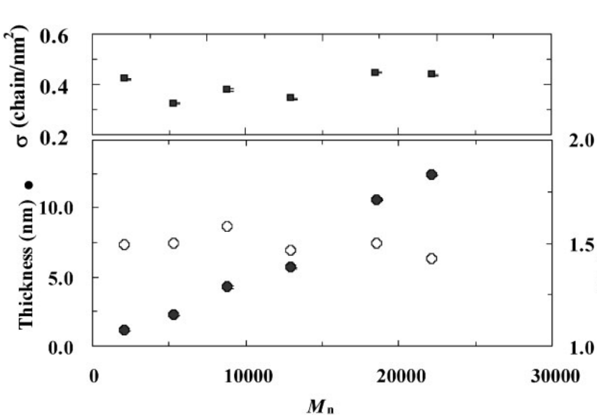


Figure 2. Plot of $\overline{M}_w/\overline{M}_n$ (○), brush thickness (determined by ellipsometry, ●) and graft density (■) vs. $\overline{M}_n$ of free PCMB. [CMB]:[Et-Br]:[CuBr]:[Bpy] = x:1:1:2 (x = 10, 30, 50, 100, 150, 200) in methanol at 30 °C for 48 h. The graft density was obtained by using Equation (1).

The evaluation of both the living behavior and the correlation of thickness and molecular weight of the brush was carried out under the conditions of [CMB]:[Et-Br]:[Cu(I)Br]:[Bpy] = x:1:1:2 (x = 10, 30, 50, 100, 150 and 200) in methanol (40.0 mL) at 30 °C for 48 h.

Previously, it was observed that when the polymerization was pursued both in solution and at the solid surface simultaneously, $\overline{M}_w$ and $\overline{M}_n$ at the solid surface were similar to that in the solution phase.[71] Figure 2 shows the plots of $\overline{M}_w/\overline{M}_n$ and brush thickness (determined by ellipsometry) vs. evolution of $\overline{M}_n$ for the polymerization of CMB. The $\overline{M}_n$ and $\overline{M}_w$ values of the polymer brush were assumed to be equal to that for the PCMB produced in liquid phase (E-PCMB). The $\overline{M}_w/\overline{M}_n$ ratios of E-PCMB were around 1.50, and seemed to decrease with the increase in molecular weight, suggesting the progress of living polymerization.

The graft density of the brushes, σ, in the figure was determined according to[5]

$$\sigma = \frac{d\rho N_A}{\overline{M}_n} \tag{1}$$

where d is the layer thickness determined by ellipsometry, ρ is the density of dry polymer layer (1.30 g · cm^{-3} for PMPC was adopted),[5] N_A is the Avogadro number, and $\overline{M}_n$ is the number-average molecular weight of polymer chains on the surface.

The ellipsometric measurements were carried out to confirm the formation of various kinds of polymer-brushes on solid substrates. Figure 2 indicated that the thickness of the PCMB brushes on the glass substrate increased linearly with the evolution of $\overline{M}_n$, which also supports the living behavior of the polymerization reaction.

Since the graft density was almost constant irrespective of $\overline{M}_n$ and the thickness of the polymer brushes (Figure 2), the graft density was affected only by the surface density of starting points, and the brushes grew equally above the glass plate. Taking into account that the graft density of PCMB was 0.38 chains · nm^{-2}, and that of ethynyldimethylchlorosilane SAM, which is analogous to the Br-PUCS SAM, was about 1.8 residues · nm^{-2} on the average,[24] the effectiveness of the initiation was estimated to be 21%. The graft density of the PCMB brush was larger than 0.1 chains · nm^{-2}. This value reaches the region where the introduction rate of the polymer chain to the introduction rate of the initiator residue is balanced.[1] Taking account of the bulkiness of CMB monomer, it is thought that a polymer brush of satisfactorily high density could be constructed.

Characterization of the Brush Surface

The contact-angle measurement was carried out to confirm the formation of polymer brushes on glass substrates (Table 1). Both the sessile drop method and the air-in-water method indicated that the modification with Br-PUCS (initiator) largely decreased the hydrophilicity of the glass substrate in comparison with the bare glass, whereas that with the PCMB brush largely increased, indicating that the zwitterionic polymer brush was very hydrophilic.

The ζ-potential of the glass surface indicated that the PCMB brushes were slightly negative, which is in contrast to the largely negative values for the bare glass and the Br-PUCS-SAM-modified glass (Figure 3). From the changes in contact angle and ζ-potential, the progress of the modification was definitely confirmed.

Meanwhile, the state of electric charges of zwitterionic polymers is influenced by the pH values, and it is necessary to consider the effect of pH on the properties of PCMB. The pK_a value of the carboxyl groups in the PCMB molecules was reported to be 2.9.[56] Therefore, PCMB seemed to be almost zwitterionic under the conditions adopted for the experiments of protein adsorption and adhesion of cells discussed below (pH = 7.4). In other words, the effect of pH is not worthy to be considered for PCMB in the experiments under the physiological conditions. The slightly negative ζ-potential of the PolyCMB brushes might be due to the effect of glass surface beneath the brush.

Adsorption of Proteins to the Polymer Brushes on the Glass Surface

Using the BCA method, the non-specific adsorption of proteins (BSA and lysozyme) to the surfaces of the PCMB brushes was investigated. The PCMB brushes for the measurement of protein adsorption and cell adhesion were

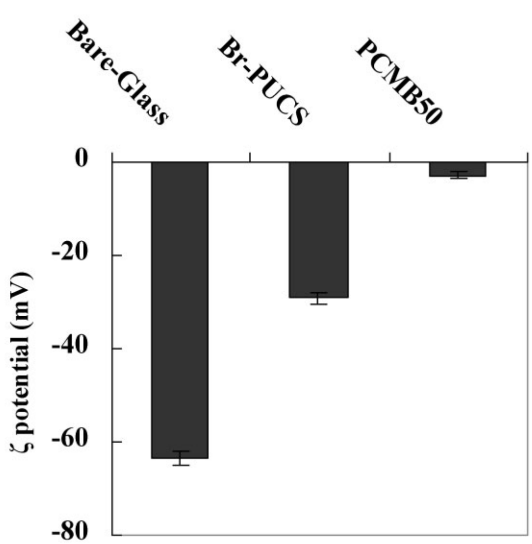


Figure 3. Surface ζ-potentials of various substrates in a 0.010 M NaCl solution. Br-PUCS: Glass substrate modified with SAM of Br-PUCS. PCMB50: Glass substrate modified with PCMB brushes. Preparation conditions: [CMB]:[Et-Br]:[CuBr]:[Bpy] = 50:1:1:2; in methanol at 30 °C for 48 h.

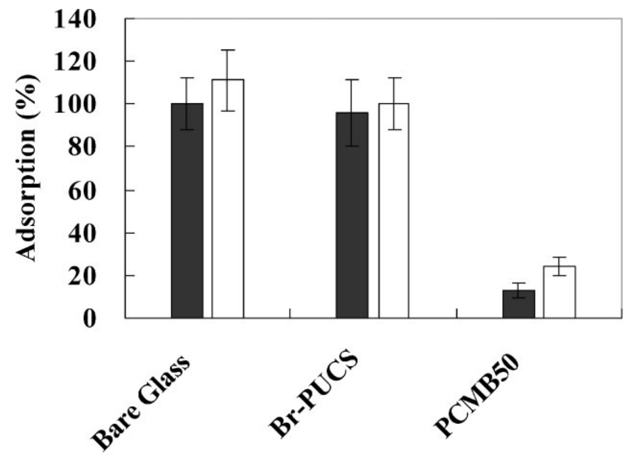


Figure 4. Protein adsorption to various polymer brushes at 37 °C. The *y*-axis expresses the relative amount of adsorbed BSA or lysozyme when the relative quantity of BSA adsorption to bare glass was 100%. BSA (■) and lysozyme (□); 4.5 mg · mL^{-1} in PBS.

prepared under the same conditions as that for the brushes indicated in Table 1. Figure 4 indicates that the PCMB brushes showed only a very slight non-specific adsorption of proteins, which is in good contrast to the significant adsorption to bare glass and the initiator-modified glass.

The tendencies in Figure 4 could be explained by the hydrophilicity of the substrate, electric charges and the freedom of the brushes on the surfaces. The tendency that the proteins were extremely adsorptive to the bare glass and not to the PCMB brushes is consistent with the hydrophobicity of the substrates. BSA and lysozyme have different pI values (BSA: 4.7–4.9;[72] lysozyme: 11.0),[73] and are negatively and positively charged at pH = 7.4, respectively. The polymer brushes reduced the adsorption of these proteins, indicating the decisive role of zwitterionic brushes irrespective of the charge of proteins. The proteins attached to the PCMB brushes might be smoothly detached, keeping their native structures.[49,50]

Previously, it was revealed that PMPC brushes of high graft density showed a dramatic reduction of the protein adsorption as compared to that of lower density.[4] Therefore, it is understandable that the PCMB chains with a high graft density are also resistant against non-specific adsorption of proteins.

Ion Beam Irradiation of the Polymer Brush Surface

Next, we examined ion beam irradiation of the PCMB surface. The irradiated area was designed to be heart-shaped. The micrograph of the PCMB brushes indicated that the irradiated area was covered with HEK293 cells while no cells attached to other areas, definitely indicating anti-biofouling properties of the PCMB brushes [Figure 5 (A)]. The same tendency was observed for Hep G2 cells, too [Figure 5

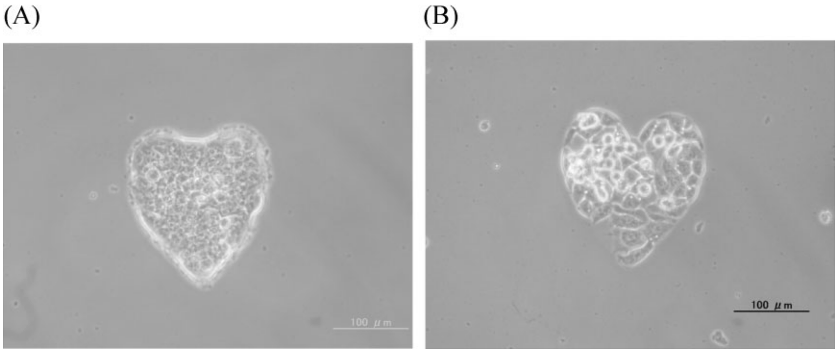


Figure 5. Micrographs of (A) HEK293 cells and (B) Hep G2 cells adhered to the heart-shaped area to which ion beam had been irradiated beforehand. Substrate: PCMB brushes (PCMB50) on a glass plate. The length of bar is 100 μm.

(B)]. The diamond-shaped hollow area with a similar size to that in the figure could also be fully covered with both HEK 293 and Hep G2 cells (data not shown), indicating that image printing with ion beam irradiation can easily be pursued on the surface of the PCMB brushes.

When the other living radical polymerization method, reversible addition-fragmentation chain transfer (RAFT) using butylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid as a chain transfer reagent,[74] was adopted, the PCMB brushes constructed on the glass plate (thickness 5.3 nm, graft density 0.12 chains · nm^{-2}, ζ-potential −4.9 mV) showed strong resistance against the adhesion of HepG2 cells in a similar manner to the brushes prepared by the ATRP method.[75] Although the thickness and density of the PCMB brushes from the ATRP and RAFT methods are not exactly the same, both brushes are largely hydrophilic and charge-balanced. Therefore, these factors (large hydrophilicity and electrostatic neutralization) are considered to be strongly related to the anti-biofouling property of polymer materials.

Based on the experimental results, it can be said that the proteins did not adsorb to the surface of the PCMB brushes significantly. The subsequent introduction of hollow area by the ablation of the brush using ion beam irradiation confirmed the role of zwitterionic brushes in the suppression of cell adhesion. The image printing of anti-biofouling PCMB brushes on the solid substrate using ion beam irradiation may be highly useful for biomedical applications.

Conclusion

PCMB brushes prepared with the surface-confined initiator for ATRP were resistant against the non-specific adsorption of proteins (BSA and lysozyme) on the surface, which is consistent with the previously reported blood- and biocompatibilities of the PCMB polymers. The irradiation of the PCMB brushes with ion beam provided a hollow space to which HEK293 and Hep G2 cells significantly adhered, while these cells did not adhere to the non-treated area on the brushes. Therefore, the ion beam-printed patterning of anti-biofouling zwitterionic polymer brushes may be appropriate for diverse biomedical applications.

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