Rapid Development of Hydrophilicity and Protein Adsorption Resistance by Polymer Surfaces Bearing Phosphorylcholine and Naphthalene Groups

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In order to provide a protein adsorption resistant surface even when the surface was in contact with a protein solution under completely dry conditions, a new phospholipid copolymer, poly (2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-2-vinylnaphthalene (vN)) (PMvN), was synthesized. Poly(ethylene terephthalate) (PET) could be readily coated with PMvN by a solvent evaporation method. Dynamic contact angle measurements with water revealed that the surface was wetted very rapidly and had strong hydrophilic characteristics; moreover, molecular mobility at the surface was extremely low. When the surface came in contact with a plasma protein solution containing bovine serum albumin (BSA), the amounts of the plasma protein adsorbed on the dry surface coated with PMvN and that adsorbed on a dry surface coated with poly(MPC-*co*-*n*-butyl methacrylate) (PMB) were compared. Substantially lower protein adsorption was observed with PMvN coating. This is due to the rapid hydration behavior of PMvN. We concluded that PMvN can be used as a functional coating material for medical devices without any wetting pretreatment.

1. Introduction

Excellent blood compatibility is the most essential property for many medical devices that are in constant contact with blood, such as vascular catheters, disposable blood bags, and so forth. Adsorption of plasma proteins causes not only thrombus formation but also a decrease in the performance of the device. Therefore, to effectively prevent the adsorption of plasma proteins, it is necessary to obtain blood-compatible materials. Many attempts have been made to improve blood compatibility of medical devices by surface modification.[1−4]

Previously, we have prepared several polymers by surface modification using the 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer.[5,6] The chemical structure of MPC is based on that of a cell membrane. Polymers composed of MPC and hydrophobic alkylmethacrylate units have been extensively utilized in many medical devices as coating materials to improve the blood compatibility of these devices.[7−9] In particular, poly(MPC-*co*-*n*-butyl methacrylate (BMA)) (PMB) has been utilized as a surface coating material in an implantable blood pump that is currently undergoing clinical trials.[10] However, this coating requires a long wetting pretreatment time to achieve equilibrium hydration by the reorientation of the phosphorylcholine groups.[11] The mechanism of blood compatibility observed with regard to MPC polymers is essentially a function of the phosphorylcholine groups present at the interface between the polymer and the biological environment.[12−16] Thus, the surface density of the phosphorylcholine groups is an important factor. The phosphorylcholine group is extremely hydrophilic; however, under dry conditions, it is rarely located at the air-contacting surface. Mathieu et al. prepared an acrylate polymer bearing phosphorylcholine groups and investigated the reorganization of the phosphorylcholine groups in polar and nonpolar environments.[17] In the polar environment, the phosphorylcholine group was oriented such that its interfacial energy was minimal, and it exhibited cell adhesion resistant properties. Yang et al. reported the reorientation and deep migration of the phosphorylcholine groups within the amphiphilic polymer.[18] Moreover, we have previously discussed the mobility of the phosphorylcholine group in PMB, which was evaluated by dynamic contact angle (DCA)

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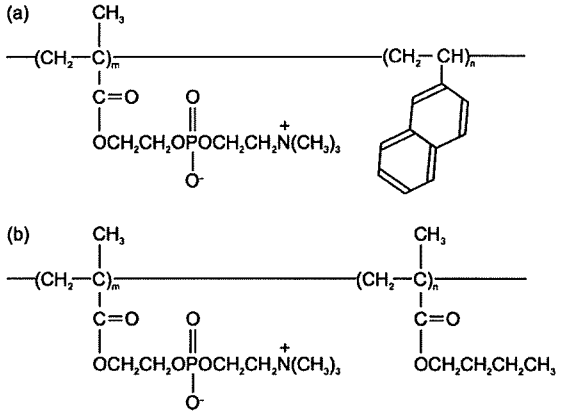

Figure 1. Chemical structure of MPC polymers, (a) PMvN and (b) PMB.

measurements with water.[19] In the case of PMB, there is large hysteresis during immersion and pullup cycles. This is due to the high mobility of the polymer side chains with phosphorylcholine group. Our previous work has shown that under dry conditions, the phosphorylcholine groups of PMB were overlaid by the hydrophobic polymer chains in order to decrease the interfacial free energy, possibly causing a restricted rate of steric rearrangement of this polymer. On the other hand, most single-use medical devices that are in contact with blood are used without the wetting pretreatment. Therefore, the application of PMB to single-use medical devices is disadvantageous. To obtain a hydrophilic surface on the MPC polymer, the chemical structures of the MPC polymer and solvent system for casting the polymer should be considered. In fact, Lewis et al. reported that the coating of the poly(MPC-*co*-lauryl methacrylate) from a water-based solvent system provided a wetting surface.[20,21]

In this study, in order to obtain a high density of phosphorylcholine groups even under dry conditions, we designed the molecular structure of the MPC polymer. First, we considered that the polymer forms an aggregate in a solvent and that the outer layer of the aggregate is covered with phosphorylcholine groups. If this is the case, then in the coating process, the mobility of the phosphorylcholine groups should be fixed during solvent evaporation. The phosphorylcholine group is highly soluble in polar solvents such as water and alcohol. Thus, its amphiphilic nature is one of the important characteristics of this polymer. Moreover, for low mobility of the polymer chains, the glass transition temperature (T_g) of the polymer should be high. On the basis of this molecular design, we synthesized poly(MPC-*co*-2-vinylnaphthalene (vN)) (PMvN). The vN unit possesses high hydrophobicity and low mobility of naphthalene rings. Therefore, this polymer has a high T_g. In this study, we evaluated the molecular mobility and the wetting property of PMvN, which was coated on the substrate; moreover, its properties under wet and dry conditions were compared. Additionally, protein adsorption onto the polymer surface was evaluated.

2. Materials and Methods

2.1. Materials. MPC obtained from NOF Co. (Tokyo, Japan) was synthesized based on a previously reported method,[5] and vN was purchased from Kanto Chemical (Tokyo, Japan) and used without further purification. BMA was purchased from Nakalai Tesque Co., Ltd. (Kyoto, Japan), and purified by distillation under a reduced pressure in an argon atmosphere; fractions collected at a bp of 60 °C/30 mmHg were used. All other solvents used were of extra-pure reagent grade and were used without further purification. Commercially available plates with poly(ethylene terephthalate) (PET) as the substrate were purchased.

2.2. Synthesis and Characterization of Phospholipid Polymers. PMvN and PMB were synthesized by conventional radical polymerization of their corresponding monomers by using α,α′-azobisisobutyronitrile (AIBN) as the initiator.[5] The monomer and AIBN concentrations used for polymerization were 1 mol/L and 5 mmol/L in ethanol, respectively. The formed polymers were purified by a reprecipitation method. We used chloroform as the solvent for reprecipitation in the case of PMvN. The chemical structures of the MPC polymers were confirmed by proton nuclear magnetic resonance (^{1}H NMR; JNM-GX 270, JEOL Co., Ltd., Tokyo, Japan) and Fourier transform infrared spectroscopy (FT-IR 615; Jasco Co., Ltd., Tokyo, Japan) measurements. Figure 1 shows the chemical structures of PMvN and PMB. The composition of the MPC unit in PMvN was determined by ^{1}H NMR measurements. The weight-averaged and number-averaged molecular weights (Mw and Mn, respectively) of these MPC polymers were determined by gel-permeation chromatography (GPC; Jasco Co., Ltd., Tokyo, Japan) with poly(ethylene oxide) standards in a methanol/water mixture (volume fraction, 70/30). The T_g of the polymer was determined using differential scanning calorimetry (DSC; Seiko Instruments, Chiba, Japan). The temperature range from 0 to 200 °C was scanned at a heating rate of 10 °C/min. The average diameter of PMvN was determined by dynamic light scattering (DLS-7000; Otsuka Electronics Co., Ltd., Tokyo, Japan).

2.3. Coating Method. The PET plates (20 × 30 × 0.5 mm^3) were cleaned by sonication in ethanol for 30 min. A mixed solvent of ethanol/water (volume fraction, 20/80) was used as the coating solvent. The PMvN-coated PET plates were treated at 40 °C for 100 min for solvent evaporation and subsequently dried in vacuo overnight. For coating the PMB on the PET plate, the plates were immersed into an ethanol solution containing 0.5 wt % PMB. The solvent was evaporated slowly under ethanol atmosphere over 1 day and subsequently dried in vacuo.[22]

2.4. Surface Characterization. Surface elemental analysis of the PMvN-coated PET plate was carried out by X-ray photoelectron spectroscopy (XPS; AXIS-Hsi; Shimadzu/KRATOS, Kyoto, Japan). The X-ray source used for XPS measurements was a Mg Kα source. The takeoff angles of the photoelectrons were 15° and 90°. Dynamic contact angle (DCA-100; Orientec Co., Ltd., Tokyo, Japan) measurements based on the Wilhelmy plate method were carried out to determine the surface mobility and evaluate the hydrophilicity of the polymer surfaces. The mobility factor (Mf) of the surface was calculated from the advanced and receding contact angles (θ_A and θ_R, respectively) by using the following equation: $Mf = (\theta_A - \theta_R)/\theta_A$.[23]

2.5. Measurement of the Amount of Protein Adsorbed on the Surface under Dry Conditions. Bovine serum albumin (BSA) was used for evaluating protein adsorption. Under dry conditions, the noncoated PET plate and PMvN-coated and PMB-coated PET plates were immersed in phosphate buffered saline (PBS) (pH, 7.4; ion strength, 0.15 M) containing 0.45 g/dL BSA. There was no pretreatment for the hydration and wetting procedures. The plates were maintained at 37 °C for 60 min in the BSA solution. After the plates were removed from the BSA solution and rinsed 5 times with fresh PBS, they were immersed in an aqueous solution containing 1 wt % sodium dodecyl sulfate (SDS) for 20 min and sonicated for desorption of the adsorbed BSA on the plates. A protein analysis kit (Micro bicinchoninic acid (BCA) protein assay reagent kit; Pierce, Rockford, IL) was used to determine the concentration of the BSA in the SDS solution.

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Table 1. Synthetic Results of the MPC Polymers

abbreviation	MPC unit mole fraction: in feed	MPC unit mole fraction: in composition[a]	polymerization time (h)[b]	yield (%)	Mw[c]	M_w/M_n[c]	T_g[d] (°C)
PMvN	0.50[e,f]	0.50	46	88	2.4×10^4	1.7	154.2
PMB	0.30[e,g]	0.28	15	86	1.5×10^5	1.6	56.2

[a] Determined by ^{1}H NMR. [b] Polymerization temperature is 60 °C. [c] Determined by GPC. [d] Determined by DSC. [e] [Monomer] = 1 M, [AIBN] = 5 mM in ethanol. [f] Reprecipitation solvent is chloroform. [g] Reprecipitation solvent is ether/chloroform (volume fraction, 70/30).

Table 2. Solubility Characteristics of PMvN, poly(MPC), and poly(vN)[a]

polymer	solvent SP value ($MPa^{1/2}$)	water 47.9	MeOH 29.7	EtOH 26	acetone 20.3	dichloromethane 19.8	chloroform 19	benzene 18.8	THF 18.6	toluene 18.2	hexane 15.1	ether 15.1
PMvN		−	+	+	−	−	−	−	−	−	−	−
Poly(MPC)		+	+	+	−	−	−	−	−	−	−	−
Poly(vN)		−	−	−	−	+	+	+	+	+	−	−

[a] 1 mg of polymer dissolved (+) and could not be dissolved (−) in the 1 mL solvent at room temperature.

3. Results and Discussion

3.1. Molecular Design of the Phospholipid Polymer by Surface Modification. In biomaterials research, protein-adsorption resistance is the most important issue that remains to be resolved. In general, it has been believed that surface wettability by water is very closely related to protein-adsorption resistance. There are many hydrophilic polymers that prevent protein adsorption. However, although wettability by water is a very important factor, it is not a sufficient factor because some hydrophilic polymers were not effective in reducing protein adsorption. Our research demonstrates that in such instances, the phosphorylcholine group is a promising polar group that could be used. We have been systematically synthesizing MPC polymers and investigating their protein-adsorption resistance.[7–9] Other research groups have also synthesized derivatives of MPC polymers and demonstrated the important role of phosphorylcholine groups.[12–16] The density of the phosphorylcholine group affects the protein-adsorption resistance of the polymer.[11] Thus, it is necessary to increase the density of these groups at the surface prior to protein adsorption. Therefore, we designed the MPC polymer for coating from its solution; that is, the phosphorylcholine group becomes concentrated at the surface by evaporation of the solvent. The functional groups at the surface are mobile and reorient easily in response to changes in the surrounding environment. In fact, in the dry state, the hydrophilic phosphorylcholine groups are embedded under the hydrophobic polymer chains; however, once the surface comes in contact with an aqueous medium, the phosphorylcholine groups migrate to the surface. This implies that the wetting pretreatment of the MPC polymer surface is necessary before it comes in contact with blood or plasma. Thus, we considered modifying the molecular structure of the MPC polymer to eliminate the need for pretreatment.

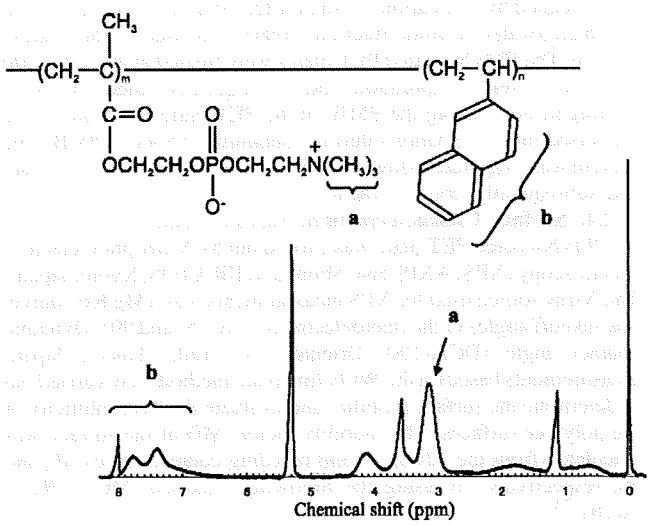

Figure 2. ^{1}H NMR spectrum of PMvN showing the signals attributed to the protons of the phosphorylcholine group (a) and naphthalene ring (b).

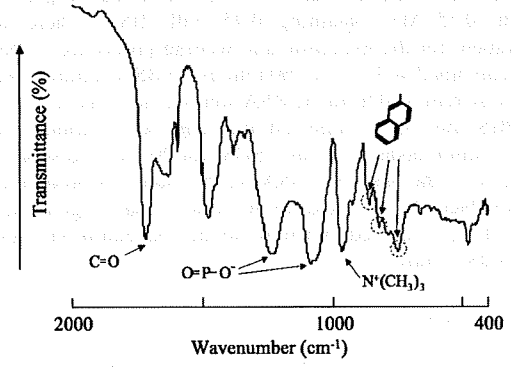

Figure 3. The FT-IR spectrum of PMvN.

PMvN is an amphiphilic polymer with bulky naphthalene groups as hydrophobic moieties. PMvN synthesis was carried out by conventional radical polymerization. The synthetic results of the MPC polymers are summarized in Table 1. The polymerization time for PMvN was much longer than that for PMB. This is due to the effect of steric hindrance of the PMvN polymer. The MPC unit mole fraction in PMvN was identical to that in the feed. The solubility characteristics of PMvN, poly(MPC), and poly(vN) were shown in Table 2. PMvN could be dissolved in ethanol but not in water. The averaged diameter

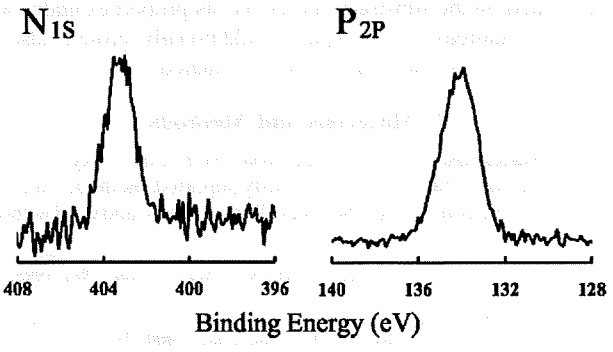

Figure 4. XPS Spectra of the PMvN-Coated PET Plate.

Table 3. Atomic Ratios of Phosphorus (P) vs Carbon (C) (P/C) of PMvN Measured by XPS at 15° and 90° of the Takeoff Angles of the Photoelectrons

	P/C	
sample	15°	90°
PMvN	0.034	0.040
PMB	0.024	0.030

of PMvN in the mixture of ethanol/water (volume fraction, 20/80) was 10.5 nm. These results suggest that PMvN formed an aggregate in the coating solvent, a mixture of ethanol/water (volume fraction, 20/80), and that the outer layer of the aggregate was covered with phosphorylcholine groups. The T_g of PMvN was 154 °C, which was moderately higher than that of PMB.[5] This indicates that at room temperature, the mobility of the polymer chains of PMvN is restricted. Figures 2 and 3 show the ^{1}H NMR and the FT-IR spectra of PMvN, respectively. In the NMR spectrum, there was a distinctive peak at 3.2 ppm of PMvN due to the $-N^+(CH_3)_3$ in the MPC and another characteristic broad peak at 6.7−8.1 ppm due to the $-C_{10}H_7$ of the vN moiety. In the FT-IR spectrum, prominent peaks were observed at 966, 1078, 1242, and 1718 cm^{-1} that indicate the presence of $-N^+(CH_3)_3$, P−O−C, O=P−O$^-$, and C=O, respectively. In addition, there were 3 peaks at 752, 821, and 858 cm^{-1} due to the adsorptions of β-displacement naphthalene.

3.2. Surface Characterization of PMvN on PET Plate. Surface elemental analysis of PMvN-coated PET plate was performed using XPS. As shown in Figure 4, the signals observed at 133 and 403 eV were attributed to phosphorus and nitrogen atoms in the MPC unit, respectively. The atomic ratios of phosphorus (P) vs carbon (C) (P/C) of PMvN and PMB measured by XPS at 15° and 90° of the takeoff angles of the photoelectrons were summarized in Table 3. The escape depths at 15° and 90° of the takeoff angles of the photoelectrons are approximately <2 nm and 5−10 nm, respectively. The P/C ratios of PMvN analyzed at 15° and 90° of the takeoff angles of the photoelectrons were much higher than those of PMB. Moreover, the P/C ratio of PMvN analyzed at 15° was greater than that of PMB analyzed at 90°. It has been reported that the P/C ratio of PMB analyzed for the photoelectrons takeoff angle of 15° becomes identical to that for 90° when PMB had been hydrated and freeze-dried prior to analysis.[11] These results suggest that the phosphorylcholine groups of PMvN are enriched on the extreme surface of the sample even under dry conditions. Figure 5 shows images of water droplets on the PET surface and the PMvN-coated and PMB-coated PET surfaces after 2 s of contact. On the noncoated PET plate and PMB-coated PET plate, the droplets almost formed a semicircle shape, i.e., the water contact angle was very large. On the other hand, the droplet on the PMvN-coated PET plate spread rapidly, indicating that the contact angle was extremely small. Coating with PMvN provided superior hydrophilicity without the wetting pretreatment. Figure 6 shows the DCA curves

Table 4. Advancing and Receding Contact Angles of Water Applied under the Dry Condition (PET plate)

	contact angle (deg)		
sample	advancing	receding	MF[a]
PMvN	24	22	0.083
PMB	101	16	0.84
noncoating	90	60	0.33

[a] Mobility factor.

of the PMvN-coated and PMB-coated PET plates. Table 4 summarizes the values of θ_A and θ_R obtained from Figure 6. As shown in Table 4, the PMB-coated PET plate had large hysteresis (Figure 6) corresponding to a large θ_A and a small θ_R. Under dry conditions, the phosphorylcholine groups of PMB were covered with the hydrophobic polymer chains; this induced a decrease in the surface free energy.[11] On the other hand, the phosphorylcholine groups should be exposed to the aqueous environment in order to reduce the interfacial free energy. Thus, the observed large hysteresis for PMB may be related to the reorientation of the phosphorylcholine groups. In this method, a long pretreatment time is required for the phosphorylcholine groups to achieve equilibrium. Therefore, if the PET plate was coated with PMB, the surface of the plate did not show good blood compatibility without the wetting pretreatment prior to contact with blood.[11] On the other hand, no hysteresis was observed in the DCA curve of the PMvN-coated PET plate. Moreover, its values of both θ_A and θ_R were small. This may be attributed to the ability of the PMvN network to enrich the phosphorylcholine groups at the surface and immobilize them even under dry conditions. On the basis of the Mf value, the mobility of the polymer chains in PMvN was expected to be quite low due to the high T_g of PMvN. Figure 7 shows the expected surface structure of PMvN under dry conditions. We suggest that the process of development of this surface structure is as follows. PMvN in the mixed coating solvent of ethanol/water (volume fraction, 20/80) results in the formation of aggregates, and the outer layers of the aggregates are covered with phosphorylcholine groups. Subsequently, during solvent evaporation, the surface orientation of the phosphorylcholine groups is strictly immobilized due to the rather low mobility of PMvN. Therefore, as shown in Figure 7, even under the dry conditions, the PMvN coating provides a surface enriched with phosphorylcholine groups.

3.3. Protein Adsorption under Dry Conditions. Figure 8 shows the amount of BSA adsorbed on the PET plates coated with the MPC polymers. The PMB-coated PET surface was not in equilibrium with phosphorylcholine groups because the incubation time was merely 60 min. It is reported that the PMB coating requires 300 min of wetting to reach equilibrium hydration by reorientation of the phosphorylcholine groups.[11] On the PET surface, BSA adsorption was significant. When BSA solution was in contact with the PET plate coated with MPC polymers under dry conditions without the wetting pretreatment, the amount

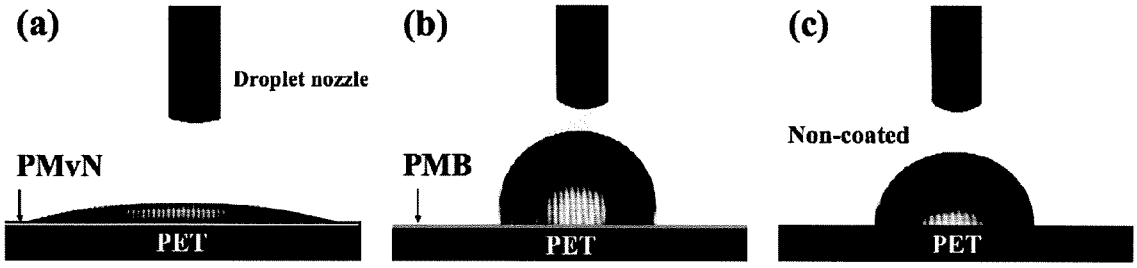

Figure 5. Water droplets on the surfaces under dry condition after contact for 2 s: (a) PMvN-coated PET, (b) PMB-coated PET, (c) noncoated PET.

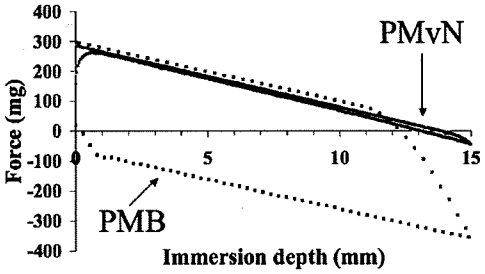

Figure 6. DCA curves of the PMvN-coated and PMB-coated PET plates dipped in water at a speed of 80 μm/s. The curve of the PMvN-coated PET plate shows an extremely hydrophilic and motionless surface.

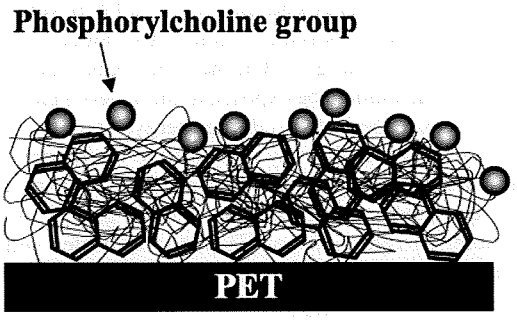

Figure 7. Expected surface structure of PMvN on the PET surfaces under dry conditions. The phosphorylcholine groups are enriched on the surface even in the dry condition.

of adsorbed BSA reduced significantly compared with the amount that was adsorbed on the PET plates. However, more effective protein adsorption resistance was observed with the PMvN coating. This difference between the two MPC polymers may be their state of hydration due to the density and orientation of the phosphorylcholine groups in the polymer. The amount of BSA adsorption to achieve equilibrium on the PMB-coated PET

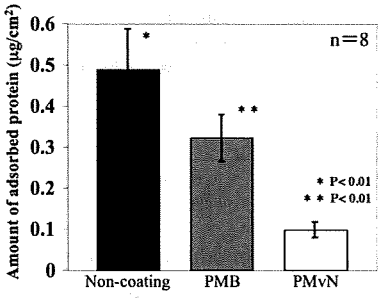

Figure 8. The amount of adsorbed BSA on the polymer surfaces under dry conditions. The sample plates were in contact with 0.45 g/dL BSA solution for 60 min at 37 °C.

plate after pretreatment was 0.12−0.30 μg/cm^2.[24] This implies that the PMvN-coated surface was the same as this equilibrated surface. Thus, we could develop an MPC polymer surface that provided rapid hydrophilicity and protein adsorption resistance when in contact with protein solutions even under dry conditions.

4. Conclusion

PMvN is capable of forming a phosphorylcholine group-enriched surface from its solution during the polymer-coating process. Under dry conditions, these surfaces immediately showed hydrophilicity and protein-adsorption resistance on contact with protein solutions. Therefore, PMvN may have a potential application as a coating polymer in blood-contacting medical devices, which require excellent blood compatibility.

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Protein adsorption resistant surface on polymer composite based on 2D- and 3D-controlled grafting of phospholipid moieties

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ABSTRACT

To prepare the biocompatible surface, a phosphorylcholine (PC) group was introduced on this hydroxyl group generated by surface hydrolysis on the polymer composite composed of polyethylene (PE) and poly (vinyl acetate) (PVAc) prepared by supercritical carbon dioxide. Two different procedures such as two-dimensional (2D) modification and three-dimensional (3D) modification were applied to obtain the steady biocompatible surface. 2D modification was that PC groups were directly anchored on the surface of the polymer composite. 3D modification was that phospholipid polymer was grafted from the surface of the polymer composite by surface-initiated atom transfer radical polymerization (SI-ATRP) of 2-methacryloyloxyethyl phosphorylcholine (MPC). The surfaces were characterized by X-ray photoelectron spectroscopy, dynamic water contact angle measurements, and atomic force microscope. The effects of the poly(MPC) chain length on the protein adsorption resistivity were investigated. The protein adsorption on the polymer composite surface with PC groups modified by 2D or 3D modification was significantly reduced as compared with that on the unmodified PE. Further, the amount of protein adsorbed on the 3D modified surface that is poly(MPC)-grafted surface decreased with an increase in the chain length of the poly(MPC). The surface with an arbitrary structure and the characteristic can be constructed by using 2D and 3D modification. We conclude that the polymer composites of PE/PVAc with PC groups on the surface are useful for fabricating biomedical devices due to their good mechanical and surface properties.

1. Introduction

The adsorption of proteins on the surface is recognized as the first event in determining subsequent biological events, including thrombus formation, foreign body reaction, bacterial infection, and other undesirable bioresponses [1]. Thus, there is considerable interest in surfaces that might inhibit or reduce protein adsorption [2]. One of the approaches to prepare a "protein resistant" surface is the incorporation of the phosphorylcholine (PC) group that is a phospholipid polar group of a major component of the outer membrane of cells. Ishihara et al. synthesized one of the PC group-bearing monomers, 2-methacryloyloxyethyl phosphorylcholine (MPC) [3]. Since MPC-based polymers provide resistance to protein adsorption and cell adhesion, they use the surface of blood-contacting and implantable medical devices [4–15].

For preparing well-defined surface, surface-initiated atom transfer radical polymerization (SI-ATRP) is particularly useful because of its versatility with respect to the monomer type, its tolerance of impurities, and the typically mild reaction conditions under which it is conducted [16]. Feng et al. and Iwata et al. reported the graft polymerization of MPC by SI-ATRP from silicon surfaces that were functionalized with 2-bromoisobutyl derivatives [17–19]. The surface showed excellent protein adsorption resistance when the polymer chain length and density were optimized. The grafting of poly(MPC) instead of silicone on the surface of a soft polymer material is considerably useful for developing new biomaterials and biomedical devices.

Recently, we succeeded in the preparation of a polymer composite composed of polyethylene (PE) and poly (vinyl

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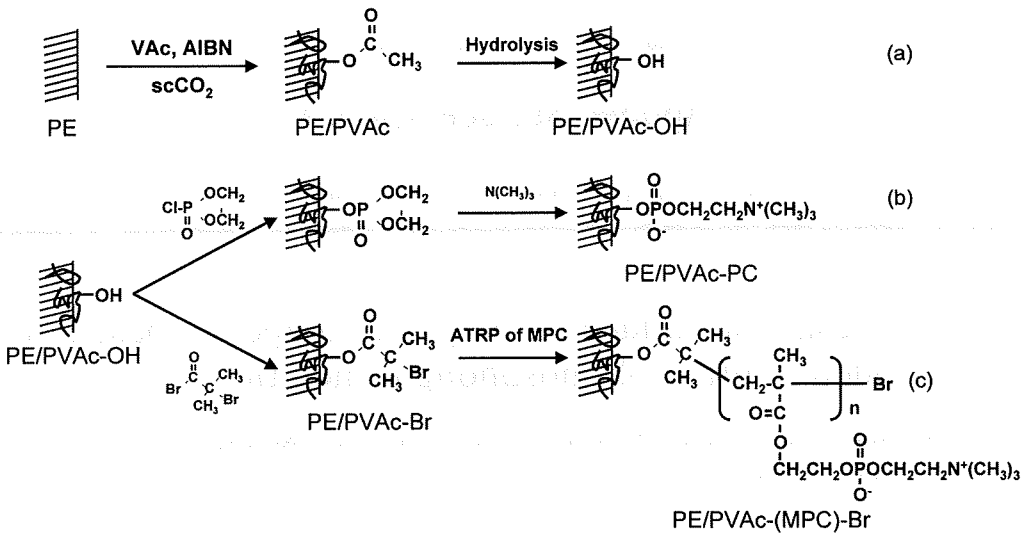

Fig. 1. Synthetic route of poly(MPC)-grafted polymer composite through SI-ATRP.

acetate) (PVAc) by *in situ* polymerization of vinyl acetate in the PE substrate by using supercritical carbon dioxide ($scCO_2$) fluid [20,21]. Further, we converted the acetyl groups in PVAc on the surface of the polymer composite to hydroxyl groups following the PC groups (Fig. 1(a) and (b)). In this way, the surface prepared by two-dimensional (2D) correction in which the PC group was anchored directly on the surface of the polymer composite suppressed effectively the protein adsorption. It is expected that new polymer biomaterials could be created by the new method that is the surface modification of polymer composite prepared by $scCO_2$.

In this study, the biocompatible surface was constructed by three-dimensional (3D) modification, that is, the phospholipid polymer was grafted from the surface of the polymer composite by SI-ATRP of MPC. The surface initiator was immobilized by the reaction between 2-bromoisobutyryl bromide and the hydroxyl group on the polymer composite surface. Subsequently, graft polymerization of MPC was carried out by the SI-ATRP method (Fig. 1(c)). We reported the 3D-controlled poly(MPC)-grafted surface on the PE/PVAc polymer composite. Further, we discussed that the effect of the poly(MPC) chain length on the surface properties and protein adsorption behavior compared with those of PC group directly immobilized surface.

2. Experiment

2.1. Materials

PE/PVAc, PE/PVAc-OH, and PE/PVAc-PC were synthesized and purified by a method reported previously [20,21], as shown in Fig. 1(a) and (b). 2-Bromoisobutyryl bromide, 2,2′-bipyridyl (Bpy), ethyl-2-bromoisobutyrate, copper(I) bromide (CuBr), and fluorescein isothiocyanate-labeled bovine serum albumin (FITC–BSA) were purchased from Sigma–Aldrich Co., Saint Louis, USA, and used as-received. The other reagents were purified by conventional distillation.

2.2. Preparation of poly(MPC)-grafted polymer composite: PE/PVAc-(MPC)-Br

On the surface of PE/PVAc-OH, 2-bromoisobutyryl bromide was reacted to introduce the initiator of SI-ATRP (PE/PVAc-Br). Argon gas was purged in methanol to eliminate oxygen before the polymerization. MPC (0.01 mol) was added to the Schlenk flask containing a magnetic stir bar and was subsequently dissolved in 10 mL of methanol bubbled with argon for 15 min to eliminate oxygen. CuBr and Bpy were added to the MPC solution with stirring under argon. After being stirred for 30 min under an argon gas atmosphere, the PE/PVAc-Br plate was submerged to the flask. Ethyl-2-bromoisobutyrate was then added as a sacrificial initiator ([I]). The graft polymerization was performed at 25 °C with stirring under an argon gas atmosphere. After 24 h, the poly(MPC)-grafted polymer composite (PE/PVAc-(MPC)-Br) plate was removed from the polymerization mixture and rinsed with methanol and water. Subsequently, the PE/PVAc-(MPC)-Br plate was dried *in vacuo* at room temperature after being extracted with methanol for 24 h to remove the unreacted reagents and homopolymer by using a Soxhlet-extractor. Four different [MPC]/[I] ratios, 50, 100, 150, and 200, were applied to prepare poly(MPC)-grafted polymer composites with different poly(MPC) chain lengths (MPC monomer units). The polymerization condition is as follows: [MPC] = 1.0 M, [CuBr]:[Bpy]:[I] = 1:2:1.

The molecular weight of free poly(MPC) in solution was measured by gel permeation chromatography (GPC) using a Shodex SB-804HQ column (upper limit of the molecular weight was $\sim 1.0 \times 10^7$ g/mol, the flow rate was 0.40 mL/min).

Surface characterizations of the sample were carried out by X-ray photoelectron spectroscopy (XPS) (AXIS-HSi, Shimadzu/KRATOS, Kyoto, Japan), dynamic water contact angle (DCA) measurement (CA-W, Kyowa Interface Science Co., Tokyo, Japan), and atomic force microscopy (AFM) (NanoScope IIIa Multimode SPM, Veeco Instruments, Tokyo, Japan).

2.3. Protein adsorption test

Polymer composites with poly(MPC)-grafted surface were exposed to 4.5 mg/mL FTIC-BSA in Dulbecco's phosphate-buffered saline (PBS) at 37 °C for 60 min and then rinsed five times with fresh PBS. The FITC–BSA concentration was 10% of the plasma concentration. The sample was dried in an argon stream and observed with a fluorescence microscope. The relative fluorescent intensity based on the adsorbed protein on the surface was calculated by comparison with the adsorbed protein on the unmodified PE surface.

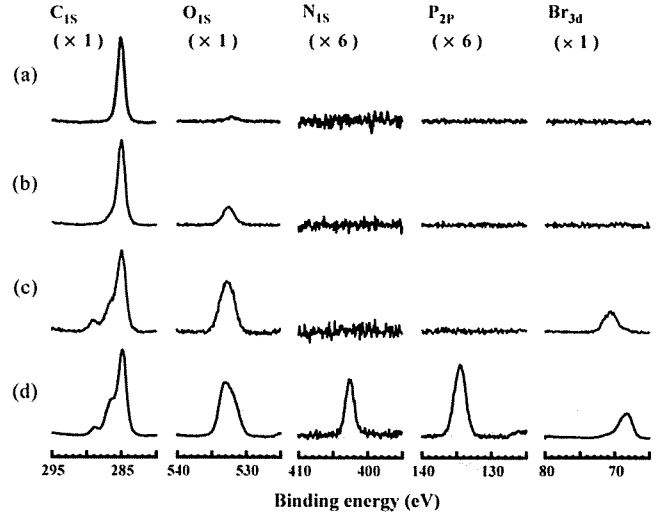

Fig. 2. XPS charts of (a) unmodified PE, (b) PE/PVAc-OH, (c) PE/PVAc-Br, and (d) PE/PVAc-(MPC)-Br ([MPC]/[I] = 100). The intensities of each atom were normalized by the intensity of C 1s at 285 eV.

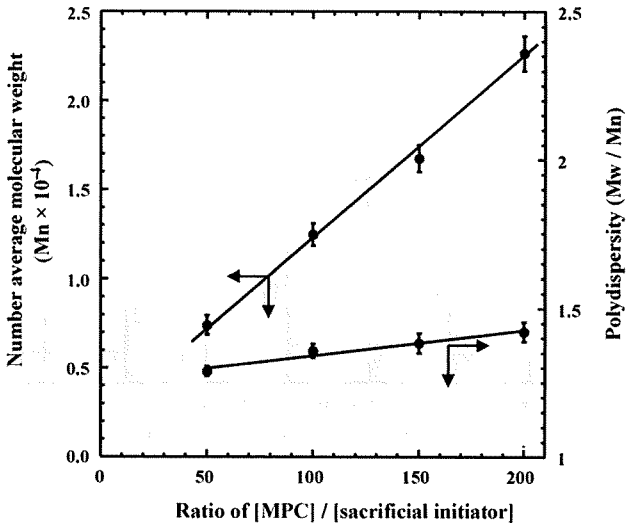

Fig. 3. Relationship between [MPC]/[sacrificial initiator] values and molecular weight of the poly(MPC) formed.

3. Results and discussion

3.1. Preparation of poly(MPC)-grafted polymer composite

Fig. 2 shows the XPS charts of unmodified PE, PE/PVAc-OH, PE/PVAc-Br and PE/PVAc-(MPC)-Br. In the unmodified PE, a strong intensity was observed at 285 eV (Fig. 2(a)). This is attributed to the carbon atoms in the methylene chain. The XPS peaks of PE/PVAc-OH broadened in the C 1s region and new peaks corresponding to the hydroxyl group were observed in the O 1s region (Fig. 2(b)). After the immobilization of the ATRP initiator on the polymer composite (Fig. 2(c)), the XPS peaks became broad in the C 1s region, the peak intensity of the O 1s region increased and the new peak was observed in the Br 3d region. This broad peak was attributed to the 2-bromoisobutyl groups in the C 1s region. An increase in the intensity of the O 1s region and the new peak in the Br 3d region were attributed to the carbonyl group (C=O) and bromine on the 2-bromoisobutyl groups, respectively. After the grafting of poly(MPC) (Fig. 2(d)), the increase in the peak intensity is caused by the increase in the amount of oxygen in the O 1s region, moreover, the nitrogen and phosphorus peaks were observed at 403 eV and 134 eV, respectively, and these peaks were attributed to the PC groups. In addition, the position of the bromine peak was shifted from a binding energy of 71–69 eV by the SI-ATRP of MPC monomer. These results confirmed that the surface initiator for the SI-ATRP was immobilized on the PE/PVAc polymer composite and the SI-ATRP of MPC succeeded had been successfully performed.

In this study, we added ethyl-2-bromoisobutyrate ([I]) as the sacrificial initiator to the polymerization solution to provide a deactivator that was concentration sufficiently high for the control of ATRP grafting from the surface of PE/PVAc-Br. The added sacrificial initiator also facilitated the control of the polymer chain length through the variation of the [MPC]/[I] ratio, assuming that the chains grown from the surface and in solution have similar molecular weights. This assumption was proved to be valid for the PMMA-grafted PE films [22]. Figs. 3 and 4 show the relationship between the molecular weight of poly(MPC) and the atomic surface composition with the [MPC]/[I] ratio, respectively. The number average of the molecular weight (Mn) of poly(MPC) and the phosphorus composition (P 2p/C 1s ratio determined by XPS) increased linearly with the [MPC]/[I] ratio, suggesting that the

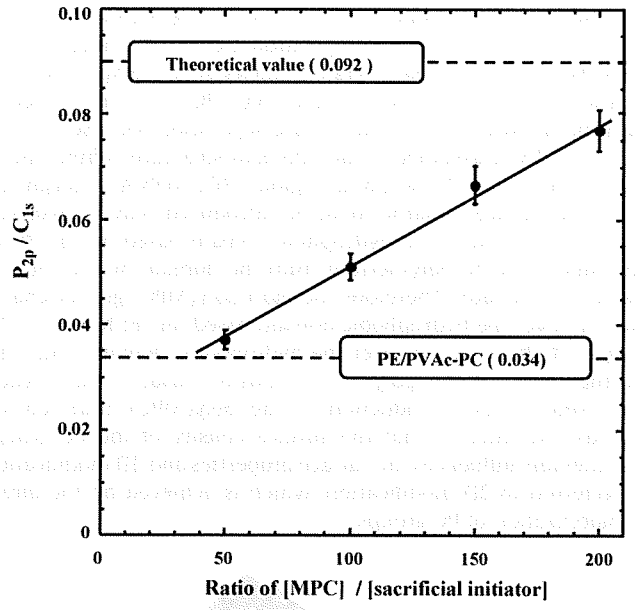

Fig. 4. Relationship between [MPC]/[sacrificial initiator] values and P 2p/C 1s values determined by XPS analysis.

ATRP grafting of MPC from the polymer composite surface was well-controlled process. In addition, the value of the P 2p/C 1s ratio approaches the theoretical value (0.092) as the chain length of poly(MPC) increases. This fact indicates that the density of the PC group on the surface increases with the chain length of the grafted poly(MPC). Further, the value of the P 2p/C 1s ratio of PE/PVAc-PC (0.034) was almost the same in the case of small graft chain lengths ([MPC]/[I] = 50). In this case, it is thought that the poly(MPC)-grafted surface ([MPC]/[I] = 50) and PE/PVAc-PC have similar surface properties.

3.2. Contact angle measurements

Dynamic water contact angle measurement has commonly been used to characterize the relative hydrophilicity or hydrophobicity of surfaces [23]. For surfaces with comparable structures, a relatively low contact angle value generally implies high

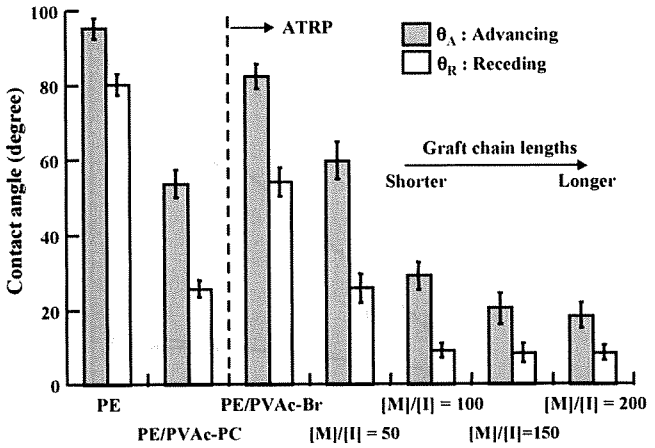

Fig. 5. Advancing and receding water contact angles of poly(MPC)-grafted surfaces of the polymer composite.

hydrophilicity. Fig. 5 shows the results of the DCA measurements. The advancing (θ_A) and receding (θ_R) contact angles decreased with the introduction of PC groups on the surface. Moreover, the poly(MPC)-grafted surface ([MPC]/[I] = 50) and PE/PVAc-PC with almost the same surface composition exhibited similar hydrophilicity. In the case of large graft-chain lengths, the hydrophilicity of the surface increased considerably. PE/PVAc-OH caused a hydrophilic/hydrophobic microphase separation because the surface of PE/PVAc assumed a microdomain structure comprising of crystalline of PE and amorphous regions (PE and PVAc amorphous) [21]. The surface initiator could be introduced only in hydroxyl groups generated in the hydrophilic domain based on the PVAc, and MPC could be polymerized from the domain by introducing the surface initiator. Therefore, the short poly(MPC)-grafted chains did not cover the hydrophobic domain based on PE; however, the long graft chains could cover the hydrophobic domain. Thus, the hydrophilicity of the polymer composite surfaces was greatly improved by the introduction of the poly(MPC) graft. Consequently, we inferred that the surface density of the PC groups considerably influences the surface properties and 3D modification is preferred to 2D modification, which is achieved by the direct immobilization of PC groups.

3.3. Surface morphology

The wet condition topology of the surfaces was examined by employing fluid tapping mode AFM in pure water. Fig. 6 shows height images of the unmodified PE and grafted poly(MPC) surfaces. The unmodified PE surface was smooth with a root mean square (RMS) roughness of 1.60 nm. The surface of the introduced surface initiator had an RMS roughness of 4.78 nm (Fig. 6(d)–(f)) indicate that the morphology of poly(MPC)-grafted surface was dependent on the poly(MPC) chain length. The surface containing chains with small lengths showed a greater roughness (Fig. 6(e)) as compared to the surfaces comprising chains with large lengths (Fig. 6(f)), as is evident from the RMS data. Further, on the surface of longer chain length ([MPC]/[I] = 100), regular peaks and valleys were observed within the measurement areas. These results indicated that the surface was covered with long poly(MPC)-graft chains.

3.4. Protein adsorption test

Fig. 7 shows the fluorescent intensity of the FITC–BSA adsorbed on the surfaces. The fluorescent intensity is proportional to the amount of adsorbed protein on the surface. The amount of the FITC–BSA adsorbed on the surface was in good agreement with the contact angle with water depended on the density of the PC groups, as shown in Fig. 5. The PC group immobilized surface effectively reduced protein adsorption as compared with unmodified PE; further, the amount of protein adsorbed on the poly(MPC)-grafted surface decreased with an increase in the chain length of poly(MPC). Both the hydrophilicity and surface morphology are significant factors for the protein adsorption resistance properties. The suppression of protein adsorption by the poly(MPC)-grafted surface was considerably greater than that on PE/PVAc-PC because of the higher density of PC groups. The amount of protein adsorbed on the poly(MPC)-grafted surface decreased with an increase in the chain length of grafted poly(MPC). We determined the amount of adsorbed BSA on the unmodified PE and PE/PVAc-PC surface quantitatively and it was 1.12 μg/cm^2 and 0.22 μg/cm^2, respectively [20]. The value obtained on the PC group immobilized surface was sufficient to suppress thrombus formation even when the surface was in contact with blood [24]. Thus, such significant reduction in protein adsorption that was observed in the case of

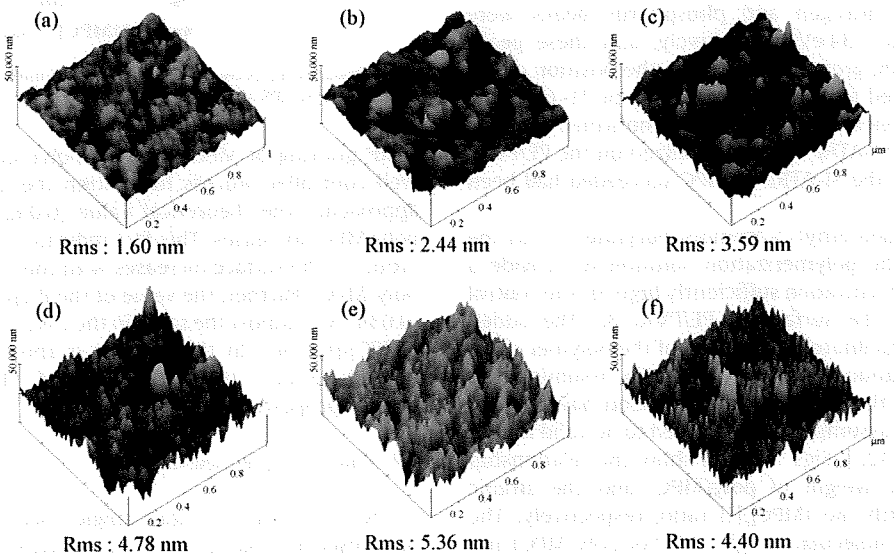

Fig. 6. AFM 3D height images of (a) unmodified PE, (b) PE/PVAc-OH, (c) PE/PVAc-PC, (d) PE/PVAc-Br, (e) PE/PVAc-(MPC)-Br ([MPC]/[I] = 50), and (f) PE/PVAc-(MPC)-Br ([MPC]/[I] = 100) (image size: 1 μm × 1 μm × 50 μm).

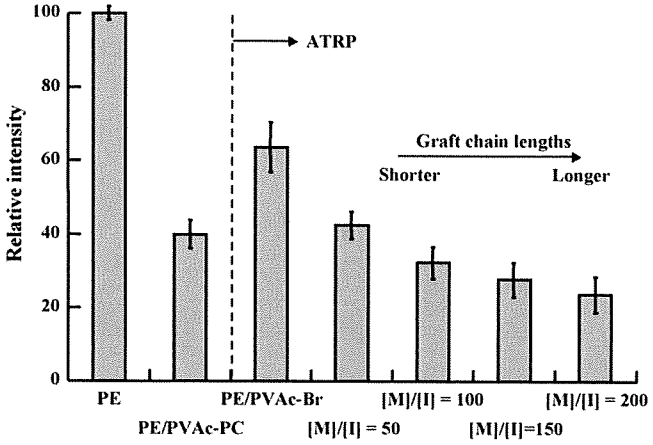

Fig. 7. Relative fluorescence intensity based on the FITC–BSA adsorbed on the various surfaces.

the poly(MPC)-grafted surface may be good for the inhibition of thrombus formation. Therefore, the 3D regulated aliment of PC groups on the surface of the polymer composite has excellent potential in the fabrication of biomedical devices.

4. Conclusion

The surface modification of polyolefins such as PE is very difficult because of these low surface tension and chemical stability. If the grafts are covalently attached to the substrate, the modified layer is stable and does not delaminate. To prepare for the biocompatible surface, phospholipid moieties were directly anchored on the new polymer composite with hydroxyl groups on the surface through the surface reaction of hydroxyl groups. Two different procedures such as 2D modification and 3D modification were applied to obtain the steady biocompatible surface. Our previous article demonstrated the 2D modification that PC groups were directly anchored on the surface of the polymer composite. In this study, we have reported a versatile approach that is 3D modification for preparing poly(MPC)-grafted substrates based on a novel synthetic method of polymer composites and SI-ATRP. 3D modification was that phospholipid polymer were grafted from the surface of the polymer composite by SI-ATRP of MPC. By varying the [MPC]/[sacrificial initiator] ratio, a set of surfaces corresponding to various graft chain lengths was prepared. The density of the PC groups on the surface, which are closely related to the hydrophilicity, increased with the length of the grafted poly(MPC) chains. The surfaces with high poly(MPC) chain lengths showed a drastic reduction in protein adsorption. The surface with an arbitrary structure and the characteristic can be constructed by using 2D and 3D modification. In addition, another monomer enables to polymerize from the terminal halogen group of graft MPC polymer chain using SI-ATRP. The various functional bio-interface is constructed by the choice of next monomer with the functional groups such as carboxylic acid, amine, active ester, epoxy, and cell adhesion molecule. We conclude that the present process for preparing protein adsorption resistant surfaces on polymer composites by the SI-ATRP of MPC will provide an excellent method for developing new biomedical devices.

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Surface immobilization of biocompatible phospholipid polymer multilayered hydrogel on titanium alloy

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ABSTRACT

The aim of this study is to improve the biocompatibility of titanium alloy (Ti) implants by immobilization of multilayered phospholipid polymer hydrogel able to reduce protein adsorption and cell adhesion. We fabricated and characterized a multilayered hydrogel on Ti substrate via a layer-by-layer self-assembly deposition method using a phospholipid polymer bearing a phenylboronic acid moiety and poly(vinyl alcohol) (PVA). The water-soluble phospholipid polymer (PMBV) was synthesized from 2-methacrylocyloxyethyl phosphorylcholine, *n*-butyl methacrylate, and 4-vinylphenylboronic acid (VPBA). The PMBV reacted with PVA and formed a hydrogel due to covalent linkage between the VPBA units and hydroxyl groups of PVA. The hydrogel layer growth on the Ti surface was initialized by the deposition of one layer of photoreactive PVA bonded by UV irradiation to the Ti surface, which was modified with an alkylsilane compound. The multilayered hydrogel was built up by alternating the deposition of the PMBV and PVA; this was monitored by several methods: static contact angle measurement, X-ray photoelectron spectroscopy, and attenuated Fourier-transform infrared spectroscopy. The results revealed clearly the progressive construction of the multilayered hydrogel on the Ti substrate. The PMBV/PVA multilayer prepared on the Ti substrate reduced the adhesion of L929 cells compared with that on an untreated Ti substrate. Thus, we concluded that the formation of the multilayered hydrogel is effective to improve the biocompatibility on Ti-based medical devices.

1. Introduction

When medical implants come into contact with the biological environment, various biological responses ranging from the adsorption of biomolecules to cell attachment and tissue response should occur [1]. The design of biomedical devices for controlling the physical, chemical and biochemical properties of an implant surface is important since the unfavorable interaction at the interface between an implant and biological environment might lead to implant failure, complications with high morbidity and treatment costs [2,3]. Among the various biomaterials used for load-bearing applications, such as orthopedic and cardiovascular implants, titanium and its alloys (Ti) is the key material owing to its excellent tissue compatibility and corrosion resistance that stems from the native oxide layer. This oxide layer sometimes is beneficial to the adsorption of many proteins for healing process. However, in blood-contacting devices, the bare Ti surface activates the intrinsic pathway of coagulation and promotes unnecessary protein adsorption due to the negative surface charge [4]. Therefore, the achievement of an implant surface which enhances the biocompatibility while inhibiting protein adsorption and cell adhesion for preventing undesirable responses towards implants in living systems can potentially have biomedical application.

Modifications of biomaterial surfaces have a long history in implantology and form a major area of research. Among the considerable number of biomaterials that have been developed thus far, the phospholipid polymers prepared from 2-methacryloyloxyethyl phosphorylcholine (MPC) and other vinyl compounds have shown the greatest promise; they mimic the natural cell membrane surface and are, therefore, inherently resistant to biofouling by proteins. Moreover, it is known as they perform better than most other types of polymeric materials with respect to their resistance to protein adsorption, cell adhesion, and whole blood coagulation. For these reasons, MPC polymers are currently being widely used in the biomedical field for surface modification [5–16].

Since its introduction by Decher in 1992 [17,18], the process of building up organic multilayer films through layer-by-layer self-assembly (LbL) has been attracted a great deal of attention and allows the formation of interpolymer complexes by the deposi-

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tion of oppositely charged polyelectrolytes. The LbL method is renowned for being a convenient, versatile, and efficient technique to generate biologically active surfaces. In addition, compared with conventional polymer coating, it affords more stable coating and various applications because of chemical bonding between layer to layer, such as controlled drug release [19], cell–surface interaction [20] and surface modification [21]. Furthermore, various driving forces have been introduced for forming multilayers by the LbL method, such as electrostatic interaction [22–24], hydrogen bonding [25–28], and covalent bonding [29].

This study employed covalent bonding-driven self-assembly to produce polymer hydrogel multilayers on Ti surfaces. We adopted a phospholipid polymer (PMBV) containing 2-methacryloyloxyethyl phosphorylcholine, *n*-butyl methacrylate (BMA), and 4-vinylphenylboronic acid unit (VPBA). The hydrophobic BMA unit can regulate the solubility of MPC polymer and also form the hydrophobic domain in aqueous condition, which dissolve hydrophobic bioactive agents, such as paclitaxel [11]. Phenylboronic acid in a tetrahedral anionic structure is known to rapidly form a cyclic boronic complex with cis-diols [30], for example, carbohydrates such as glucose, catechol derivatives such as dopamine, and some polymers such as poly(vinyl alcohol) (PVA) [31,32]. The interpolymer complexation of a polymer comprising boronic acid with PVA has been reported to form a hydrogel due to the covalent linkage in both constituent polymers and this hydrogel is reversibly dissociated by the addition of glucose [33–35].

We expected that the LbL deposition method would enable the combination of PMBV and PVA to produce a polymer hydrogel multilayer bonded to Ti. Therefore, the objective of this study was to fabricate and characterize Ti surfaces modified with PMBV and PVA via the LbL method for improving biocompatibility of Ti implant. By constructing a multilayered hydrogel on the Ti surface, the surface may become much more biocompatible, and moreover, the multilayered hydrogel layer can have a functional stage for the sustained release of biologically active molecules.

2. Experimental

2.1. Materials

MPC was obtained from NOF (Tokyo, Japan); it was synthesized using a method proposed by Ishihara et al. [5]. *n*-Butyl methacrylate was purchased from Nacalai Tesque Co. Ltd. (Tokyo, Japan). VPBA and PVA (degree of polymerization: 1500) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Octadecyltriethoxysilane (ODS) were purchased from ShinEtsu Chemical Co. Ltd. (Tokyo, Japan). Photoreactive PVA (AWP, azide-unit pendented water-soluble PVA) was purchased from Toyo Gosei Co. Ltd., Japan. Titanium alloy (Ti) substrates were obtained from DENISPY-Sankin K.K. (Tokyo, Japan). The other reagents and solvents were of the extra-pure grade and were used without further purification.

2.2. Synthesis of PMBV

PMBV was synthesized by the conventional radical polymerization of the corresponding monomers: the desired amounts of MPC, BMA, and VPBA were dissolved in ethanol taken in an ampoule. The total concentration of monomer was adjusted to 1.0 mol/L. An initiator, α,α′-azobisbutyronitrile (AIBN), was added to the ampoule at a concentration of 1.0 mmol/L. Next, argon gas was bubbled into the solution for 10 min to eliminate oxygen and the ampoule was then sealed. Polymerization was carried out at 60 °C for 2.5 h. After cooling, the contents were poured into a large amount of diethylether and chloroform (8:2 by volume) to remove any unreacted monomers and to yield PMBV. The precipitant was collected and dried *in vacuo*. The structure of the copolymer was confirmed with ^{1}H-NMR (α-300, JEOL, Tokyo, Japan) and a Fourier-transform infrared spectrometer (FT-IR; FT/IR-615, JASCO, Tokyo, Japan). The molecular weight was determined by gel permeation chromatography (GPC, JASCO, Tokyo, Japan). The chemical structure of PMBV is shown in Fig. 2.

2.3. Fabrication of multiplayer

2.3.1. Preparation and cleaning of Ti

Two types of Ti substrates were used. The Ti substrates were prepared from a Ti plate (thickness: 0.5 mm) by cutting it into 10 mm × 10 mm square pieces.

These were rinsed in acetone and ethanol using sonification for 15 min each. After drying in air, the samples were oxidized as follows: They were immersed in a 3:1 (v/v) mixture of concentrated H_2SO_4 and 30% H_2O_2 for 1 h at 25 °C. The samples were then rinsed three times with distilled water and dried in an oven at 60 °C. To evaluate the thickness of the multilayered hydrogel, quartz substrates (10 mm × 15 mm × 0.8 mm; Matsunami, Tokyo, Japan) were coated with a 100-nm-thick Ti layer by RF magnetron sputtering (Ulvac Kiko, Inc., Tokyo, Japan, duration: 4 min, pressure: ~2×10^{-3} Pa). The quartz substrates were cleaned using an oxygen plasma apparatus (PR500 plasma reactor, Yamato Science, Tokyo, Japan) for 20 min. Finally the Ti-sputtered substrates were oxidized using the oxygen plasma apparatus for 10 s, and silanization was carried out immediately afterward.

2.3.2. Silanization on Ti substrate

A monolayer of ODS was prepared by the following procedure: First, 10 mM ODS was dissolved in anhydrous toluene. The Ti substrates were then immersed in the ODS solution and reacted for 24 h at 80 °C. And finally, the Ti substrates were rinsed in toluene three times and dried *in vacuo* at room temperature.

2.3.3. Fabrication of multilayer on Ti substrate

The ODS-treated Ti substrates were coated with an aqueous solution of AWP (1 wt%) by dip coating. The AWP-coated Ti substrates were air-dried under a fume hood at room temperature. After applying the AWP coating, the Ti substrates were irradiated with UV light (135 mW/cm^2) using a UV Spot Cure (SP-7, Ushio Inc., Yokohama, Japan) for 40 s.

PMBV solutions with concentrations of 50 and 25 mg/mL and PVA solutions with concentrations of 15 and 30 mg/mL were prepared with distilled water. The combinations of PMBV and PVA solutions examined are as follows: PMBV 50 mg/mL and PVA 15 mg/mL (PMBV50/PVA15), PMBV 25 mg/mL and PVA 30 mg/mL (PMBV25/PVA30), and PMBV 25 mg/mL and PVA 15 mg/mL (PMBV25/PVA15). The multilayer construction was accomplished by alternately dipping the Ti substrates with bonded AWP in the PMBV and PVA solutions for 10 min each and subsequently rinsing them with distilled water for 1 min. Six layers (3-bilayers) terminated with a layer of PMBV were obtained by the LbL method.

2.4. Characterization of multilayers

The thickness of the polymer multilayer was characterized by field emission scanning electron microscopy (FE-SEM, S-4200, Hitachi, Japan). Multilayers were built up as described above on the surface of the Ti-sputtered quartz substrates. And the cross-section of the quartz substrate was analyzed after the formation of each layer. Gravimetric measurements were performed after each successive layer was constructed. The swelling ratios of the multilayered hydrogel on the Ti surface were obtained at room

temperature. Multilayered hydrogels in triplicate were incubated in phosphate-buffered saline (pH 7.4) and their dry weights were measured beforehand. Then, the swelling ratios were calculated by the following equation:

$$\text{Swelling ratio}\,(\%) = \left[\frac{(W_s - W_d)}{W_d}\right] \times 100$$

where W_s and W_d are the weights of the swollen and dry hydrogel, respectively.

The static water contact angle on the prepared surfaces was measured using the sessile drop method at the ambient temperature using a contact angle goniometer (G-1, Erma, Tokyo, Japan). Images of water spreading on the sample surfaces were recorded by a camera and then analyzed using the software supplied by the manufacturer. Five measurements were made for each sample. For captive bubble method, the glass cell was filled with ultrapure water and 1 cm^2 samples of test solids were placed in it. A special L shaped syringe needle containing air releases bubbles beneath the sample. A computer screen provided an image of the captive bubble and then analyzed using the software supplied by the manufacturer. In addition, infrared spectral analysis was performed by attenuated Fourier-transform infrared spectroscopy (ATR-FTIR, IMV-4000, FT/IR-6300, JASCO, Tokyo, Japan). The spectra were examined visually, with special interest given to the spectral range of 4000–850 cm^{-1}.

For chemical composition analysis, specimens were characterized using X-ray photoelectron spectroscopy (XPS; AXIS-His165 Kratos/Shimadzu, Kyoto, Japan) with a focused monochromatic Mg Kα X-ray source (1253.6 eV) for excitation. The electron take-off angle was 60° in the dry state and the analyzer was operated in the constant energy mode for all measurements.

2.5. Cell culture and cell morphology

For cell adhesion, L929 cells were cultured in a culture medium (D-MEM (Gibco), supplemented with 10% (v/v) fetal calf serum (FBS)) at 37 °C in an atmosphere of 5% CO_2 at 95% humidity. The cells were seeded at a density of 4×10^4 cells/mL on the experimental substrates and were cultured for 1 day. Subsequently, cell fixation was carried out for 90 min in 2.5% glutaraldehyde solution at 4 °C. Then, the samples were washed twice with PBS and dehydrated using a graded series of ethanol solutions (70%, 90%, and 100%) for 15 min each and were subsequently dried with 1,1,1,3,3,3-hexamethyldisilane. The morphology of the adherent cells was observed using a scanning electron microscope (SEM, SM-200, Topcon, Tokyo, Japan) after depositing gold.

3. Results

3.1. Characterization of PMBV

The copolymerization of MPC, BMA, and VPBA proceeded well and a polymer containing these monomer units was obtained; the obtained PMBV was water-soluble. The spectral data from ^{1}H-NMR and FT-IR revealed the chemical structure of the obtained PMBV.

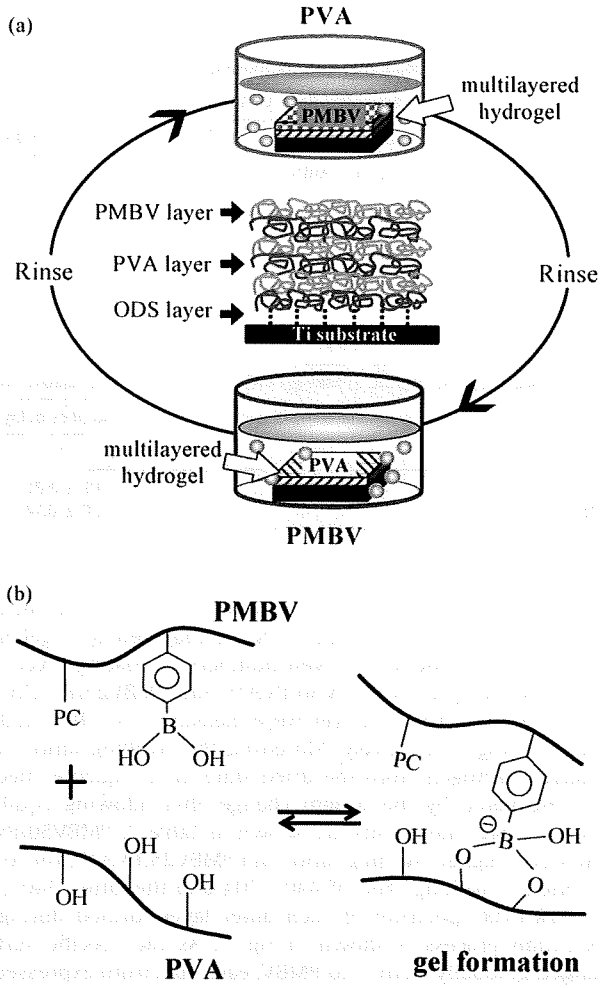

Fig. 1. (a) Schematic representation of construction procedure for the multilayered hydrogels on the Ti substrates. (b) Reaction between the phenylboronic acid moiety in PMBV in an aqueous solution and a polyol moiety in the presence of PVA.

Fig. 1 shows a schematic illustration of the process for fabricating the multilayered hydrogels on the Ti substrates via the LbL method (a) and the mechanism of PMBV/PVA hydrogel formation (b). The chemical structure and synthetic results of the obtained PMBV are summarized in Fig. 2 and Table 1.

3.2. Multilayered hydrogel formation from PMBV/PVA system

The preparation of the PMBV/PVA hydrogel layers on the Ti substrates via the LbL method was followed by a change in the weight

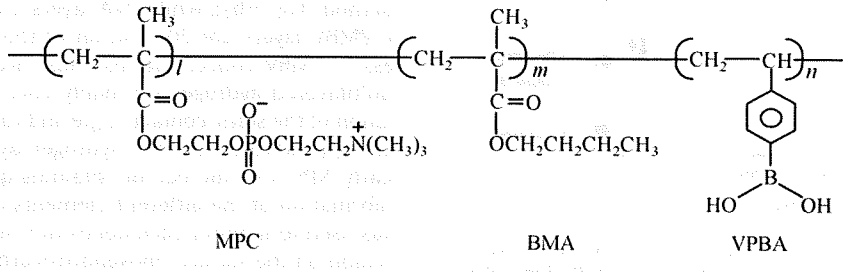

Fig. 2. Chemical structure of PMBV.

Table 1
Synthetic result of PMBV.

Abb	Monomer unit composition (mol%)[a]		Yield(%)	Molecular weight Mw($\times 10^4$)[b]
	In feed MPC/BMA/VPBA	In polymer MPC/BMA/VPBA		
PMBV	60/30/10	57/25/18	70	6.5

$[Monomer]_{total}$ = 1.0 mol/L in EtOH; [AIBN]=1 mmol/L; copolymerization time: 2.5 h, polymerization temperature 60 °C.
[a] Determined by ^{1}H-NMR.
[b] Determined by GPC.

Table 2
Characterization of polymer multilayer.

Polymer concentration (mg/mL) [PMBV]/[PVA]	Cumulative weight increase (mg) ± SD			Swelling ratio ± SD (%)
	Number of layer			
	2	4	6	
50/15	0.19 ± 0.01	0.96 ± 0.10	1.98 ± 0.23	440 ± 70
25/15	0.20 ± 0.14	0.31 ± 0.23	0.74 ± 0.42	340 ± 50

of the substrates; the results are shown in Table 2. Weight measurements showed an increase in the multilayered hydrogel on the Ti surface as a function of even numbers of coating layers. The cumulative weights of PMBV50/PVA15 and PMBV25/PVA15 after the formation of the 3-bilayer were measured as 1.98 ± 0.23 and 0.74 ± 0.42 mg, respectively. Moreover, the swelling ratios of the 3-bilayer hydrogels from the dried state in an aqueous medium were estimated by the weight change after allowing equilibration for 1 day; the results are shown in Table 2. PMBV50/PVA15 exhibited a higher swelling ratio than PMBV25/PVA15; the former exhibited a swelling ratio of 440 ± 70% and the latter, 340 ± 50%. The ATR-FTIR spectrum of each outer layer formed during the fabrication process is shown in Fig. 3. As the specific surfaces changed gradually from Ti to PMBV, each spectrum expressed the particular peaks associated with the functional groups of that surface. After the bare Ti surface (Fig. 3(a)) treated the silanization, the ODS-treated Ti surface (Fig. 3(b)) exhibited the band corresponding to ≡Ti—O—Si≡ in the region of 970–1000 cm^{-1}. And after bonding the AWP layer (Fig. 3(c)) to the ODS-treated Ti surface, the spectrum exhibited characteristic vibration at 1720 cm^{-1} for the carbonyl group, and at 1650 and 1463 cm^{-1} for the C=C of aromatic ring. However, no azide peak was observed around 2200 cm^{-1}. After complexation with PMBV (Fig. 3(d)), the phosphate group in the MPC unit could be seen at 1080 and 970 cm^{-1}, and peaks at 1720 and 1460 cm^{-1} corresponding to C=O carbonyl stretching and $-CH_2-$ bending were also observed, respectively. The appearance of peaks at 1330 and 1350 cm^{-1} is attributed to the B–O stretching modes in phenylboronic acid. In Fig. 3(e), the peak around 3417 cm^{-1} is associated with the –OH bands in PVA. The thickness of the PMBV/PVA hydrogel increased as a function of the number n of deposited layers (Fig. 4). The final thicknesses of PMBV50/PVA15, PMBV25/PVA30, and PMBV25/PVA15 were determined as 8, 7, and 6 μm by SEM measurements of the cross-sections of the hydrogel layers on the Ti-sputtered quartz, respectively.

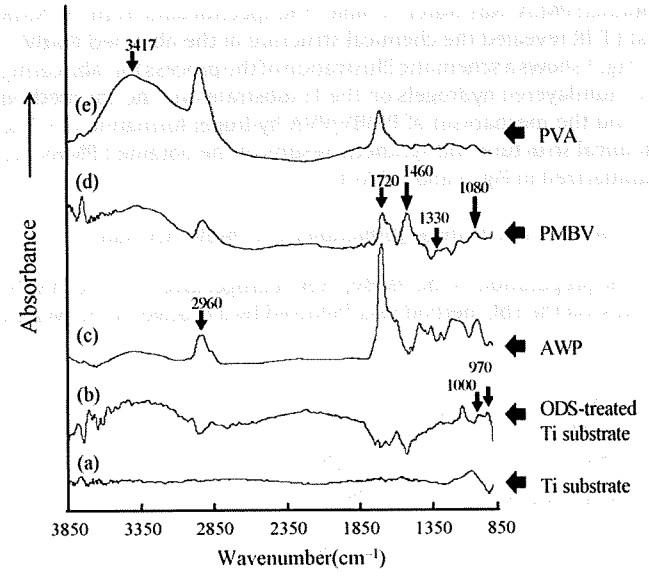

Fig. 3. ATR-FTIR spectra of (a) Ti substrate, (b) ODS-treated Ti substrate, (c) AWP, (d) PMBV, and (e) PVA. In this case, the multilayered hydrogel of PMBV25/PVA30 was used.

3.3. Surface property of the multilayered hydrogel

The contact angle depends on the character of surface wettability, and measurements were performed to confirm the alternate deposition of PMBV and PVA. Polymer hydrogel layers made by layer-by-layer methods on Ti surfaces are expected to alternately change surface wettability. Fig. 5 shows the results of the static water contact angle by sessile drop method and by the captive bubble method for three polymer combinations. In case of the sessile drop method (Fig. 5(a)), the static water contact angle for PVA 15 and 30 mg/mL is approximately 60°, and that for PMBV 25 and 50 mg/mL is 80°. The subsequent adsorption of PMBV made the surface slightly hydrophobic compared to that with the PVA surface in dry condition. However, in the case of captive bubble method (Fig. 5(b)), while PVA layers are approximately 40°, most of PMBV layers are 20° and all of the 6th layers are 0° regardless of PMBV concentrations. This means the outmost layers of multilayered hydrogel are totally covered with PMBV. The alternation of the static contact angle and captive bubble contact angle strongly indicates that the hydrogel layers were constructed regularly. XPS is useful tool for obtaining qualitative and quantitative information of the different elements at a substrate surface. XPS was used to monitor each deposition step as it can provide information on the surface phosphorus/carbon ratio (P/C ratio) on the Ti samples coated with different deposition layers; the results are

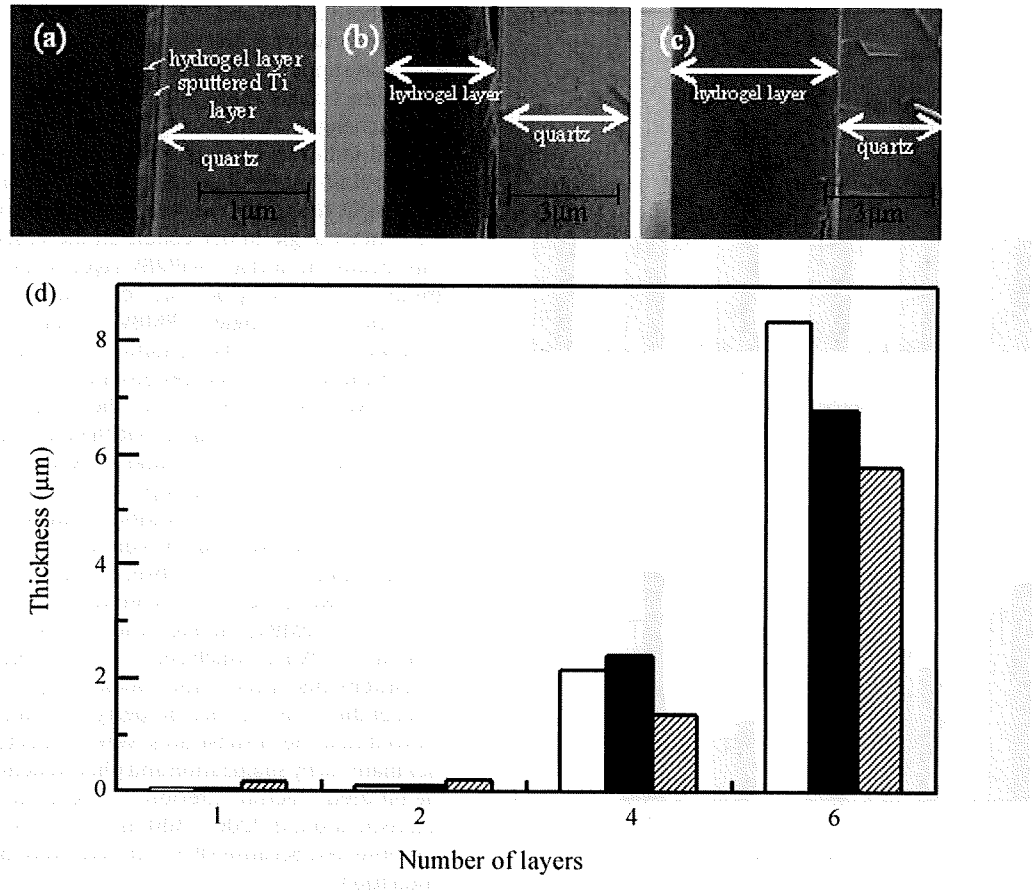

Fig. 4. Cross-sectional SEM images of multilayered hydrogel with (a) second layer (b) fourth layer, and (c) sixth layer of PMBV25/PVA30. (d) Hydrogel layer thickness of PMBV/PVA measured by SEM as a function of layer number. In this case, samples with an even number of layers have PMBV, whereas samples with an odd number of layers have PVA: (□) PMBV50/PVA15, (■) PMBV25/PVA30, and (▨) PMBV25/PVA15. Multilayered hydrogels were constructed on Ti-sputtered quartz substrates. The results are the means of the results of three independent experiments.

presented in Fig. 6. As the deposition cycle was repeated, P/C ratio could discriminate the presence or absence of a PMBV layer; that is, PMBV-terminated layers (the even number) exhibited 4 times larger P/C values.

3.4. Cell adhesion on the multilayered hydrogel

The morphological aspects of L929 cells grown on the differently loaded multilayered hydrogel coatings were evaluated using an SEM (Fig. 7). On a plain Ti substrate (Fig. 7(a,b)) and on one modified with AWP (Fig. 7(c,d)), the adhesion and proliferation of L929 cells was observed during a 1-day culture, as usual. However, apparent differences in cell morphology were found on PMBV (Fig. 7(e,f)) and PVA (Fig. 7(g,h)) as compared to that on the Ti substrate and AWP-modified substrate. That is, the number of L929 cells that adhered to the PMBV and PVA surfaces was significantly lesser than those on the Ti substrate and photoreactive PVA. In addition, the L929 cells did not preserve their normal spindlelike shape, but exhibited circular, round shapes.

4. Discussion

This study focused on the fabrication and characterization of multilayered hydrogels using water-soluble MPC polymers bearing a phenylboronic acid moiety and PVA via the LbL deposition method. Phenylboronic acid is one of the most familiar reactive groups used to synthesize stable covalent complexes with polyol compounds, including PVA. Because of the strong binding, it can be used as a recognition moiety in sugar fractionation. However, besides sugar recognition, the usage of polyols such as PVA has permitted the present system to form hydrogels without any chemical crosslinking treatment. Moreover, the use of PVA in hydrogels has attracted considerable attention because of its inherent low toxicity and high degree of swelling in water [33]. The LbL method is a desirable and versatile tool for assembling PMBV and PVA into a multilayered hydrogel. In this study, we have chosen the concentration of polymer capable of forming hydrogel upon mixing aqueous solutions in physiological condition, whose conditions depend on the concentrations of PMBV and PVA. The gelation did not affect the ionic strength [34]. After constructing multilayered hydrogel, we observed the chemical bonding between layer to layer through unique peaks of FT-IR. However, it was not clear to confirm the bonding between phenylboronic acid and polyol because the change of peak corresponding to –B–O stretching appeared from 1330 to 1350 cm^{-1}.

Based on the increases in both the weight and thickness of the substrates, we confirmed that the LbL method was feasible with the PMBV/PVA system. The build-up of multilayered films arises from covalent bonding between alternately deposited PMBV and PVA. In case of thickness, the concentration of AWP coated on Ti substrate was lower than that of PMBV and PVA constructing multilayer by LbL method, that is, the number of binding sites are different. This is why the difference of thickness between 1st layer and 2nd layer

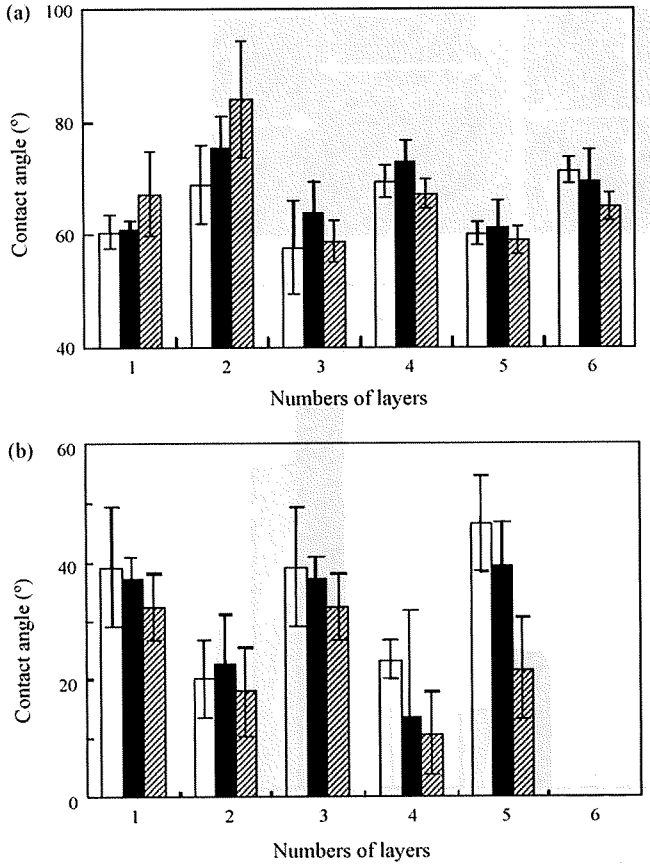

Fig. 5. The static water contact angles (a) by sessile drop method and (b) by captive bubble method on the PMBV/PVA multilayered hydrogel surface as a function of the layer number. In these cases, samples with an even number of layers have PMBV, whereas samples with an odd number of layers have PVA: (□) PMBV50/PVA15, (■) PMBV25/PVA30, and (▨) PMBV25/PVA15. The results are expressed as the mean ± SD for three independent experiments.

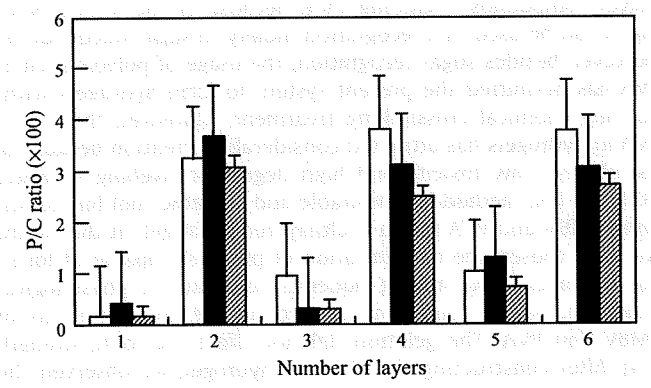

Fig. 6. P/C ratio of multilayered PMBV/PVA hydrogel layer. The even numbers correspond to PMBV as the outmost layer and the odd numbers correspond to PVA as the outmost layer: (□) PMBV50/PVA15, (■) PMBV25/PVA30, and (▨) PMBV25/PVA15. The results are expressed as the mean ± SD for three independent experiments.

cannot observe. From 2nd layer to 6th layer, the thicknesses have increased linearly and have been influenced in the concentration of polymers. The number of cycles and the concentrations of polymer solution modulate the thickness [24,35]. The change in both weight and thickness of the hydrogel layer depend on the polymer concentrations. PMBV/PVA multilayered hydrogel appeared high swelling ratio without regard for polymer concentration. It means both of PVA and PMBV containing hydrophilic PC-group are familiar to water molecules. It is thought that the contact angle data also support hydrophilic property of these polymers. Both of the contact angle measurement revealed that the outmost layer was exchanged alternatively. In particular, as the captive bubble method indicates the wettability of surface in wet condition, we could obtain interesting information by comparing the results of the sessile drop method. When the outermost layer was PMBV, the contact angle in dry condition increased over that with PVA. This means the surface of PMBV layer is covered with hydrophobic group, that is, phenylboronic acid and butyl group of BMA. However, the contact angle of PMBV in wet condition is dramatically opposed to that in dry condition. It is due to the rapid adsorption of the water molecules around the hydrophilic PC-groups in the PMBV [36]. In all of case, the contact angle of 6th layer is zero (Fig. 5(b)). It is thought that the outmost surfaces are covered with PMBV. On the other hand, PVA layers keep similar surfaces covered with hydroxyl groups regardless of whether it is in dry condition or it is in wet condition. From both of contact angle data, although it is known that PC-containing polymer, PMBV, and PVA are hydrophilic, we could observe the change of surface property in dry and wet condition. According to the change of layer surface from PVA to PMBV, the P/C ratio in XPS increased because of the influence of PMBV, which contains a phosphorylcholine group. The measurements of the static contact angle and the XPS results are susceptible to the surface property. In addition, the ATR-FTIR data showed that the Ti substrates were connected to the organic polymer material by silanization and photoreaction with AWP. Although the infrared spectral criterions of the azide structure have a characteristic around 2200–2300 cm^{-1}, it was thought that no trace was observed because ODS and AWP were bonded together by UV irradiation.

The cellular behavior is an important factor for interpreting the biocompatibility of biomaterial. The cell morphology images revealed that Ti and AWP surfaces permitted the adhesion, spreading, and migration of L929 cells to degrees that PVA and PMBV surfaces did not. The adhesion of cells to surfaces is dependant on the adsorption of highly adhesive proteins such as fibronetin and vitronectin, which link cells to the biomaterial surface. Cell–substrate interaction is mainly based on the recognition of Arg-Gly-Asp (RGD) sequences by receptors located on the cell surface. As AWP allows easy immobilization of specific proteins, it has been studied for application to microarray chips and cell adhesion assays [37]. It is generally accepted that hydrophilic polymers such as poly(ethylene oxide)-based polymers and phosphorylcholine-functioned polymers do not allow protein adsorption at their surface. Thus, they can reduce the adhesion of cells, including fibroblasts, platelets, and macrophages [9,10,38]. A wide variety of polymers containing charges, including anionic DNA and cationic chitosan, have been used for the construction of multilayerd coatings with the aim to modulate cell behavior. In particular, various studies with cells, including fibroblast and osteoblast, have shown that polyelectrolyte multilayered coatings are useful tool to regulate biomaterial surface through in vitro experiment of cell proliferation and viability [21,39]. In this study, the MPC polymer-hybridized Ti substrates and multilayered hydrogels offer the potential for preparing blood-contacting materials via the incorporation of the outmost layer, PMBV, which inhibits the adsorption of proteins and the adhesion of cells. Moreover, we note that bioactive reagents may be incorporated in the multilayered hydrogel to allow for sustained release using hydrophobic domain of BMA unit. Hence, the release behavior of a bioactive reagent from a Ti substrate with an immobilized hydrogel is currently under investigation in the laboratory and the results will be reported in the near future.

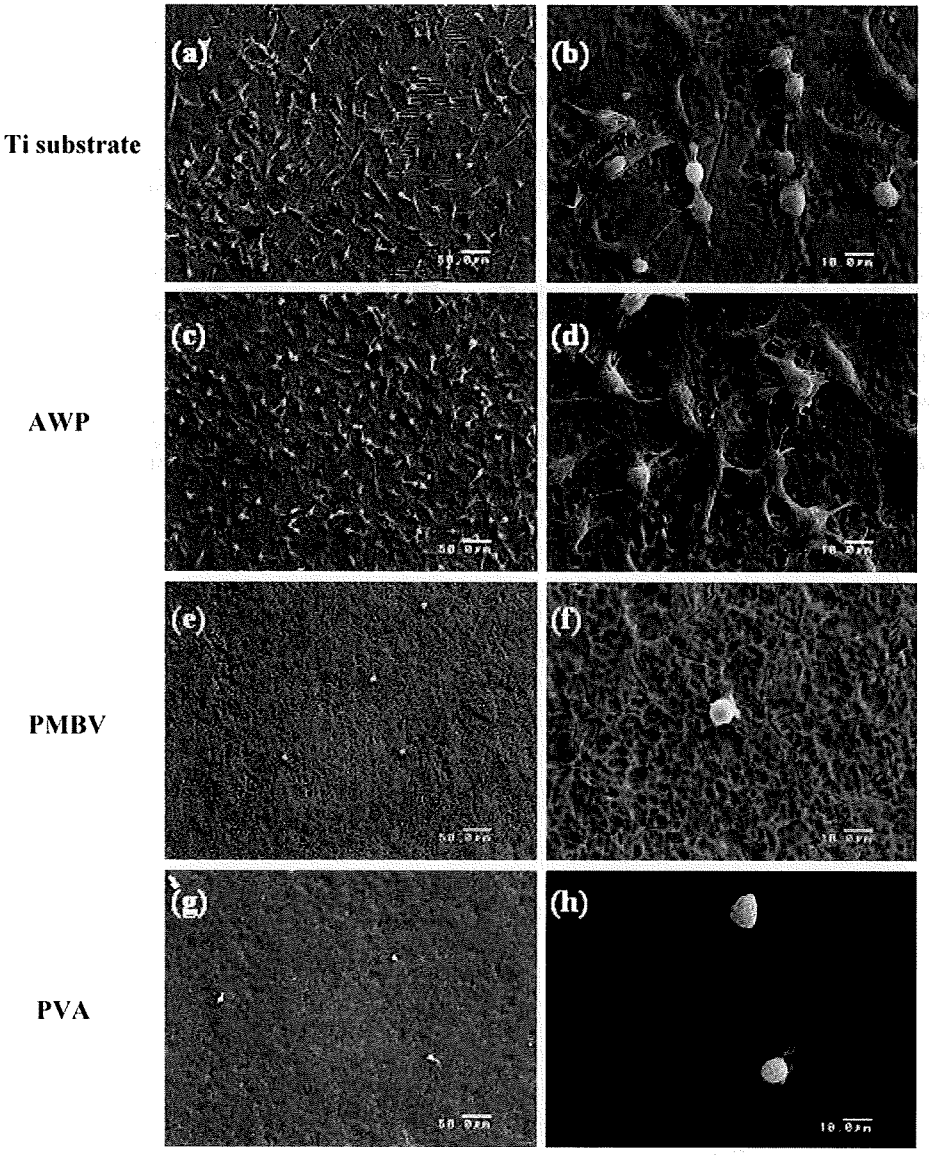

Fig. 7. SEM images of L929 cell morphology adhered on a Ti substrate (a, b) and on Ti substrates modified with AWP (c, d), PMBV (e, f), and PVA (g, h) after a 1-day culture. The PMBV and PVA concentration were 25 and 30 mg/mL, respectively.

5. Conclusions

This study demonstrates the feasibility of the LbL method in constructing multilayered hydrogels composed of the PMBV/PVA system on Ti substrates for improving biocompatibility of implant surface. The basic mechanism for the construction of the multilayered hydrogels was the selective reactions between the boronic acid moiety in PMBV and the hydroxyl groups in PVA. To initiate the LbL process, a silane coupling reaction was used to introduce an alkyl group on the Ti substrates, and AWP could bind covalently to the substrate surface by photoirradiation. Subsequently, the PMBV/PVA hydrogel system was fabricated. The process was followed by contact angle measurements and XPS analyses; these results indicated that the surface properties changed alternatively, reflecting the nature of the polymer on the outer surface. The thickness of the hydrogel increased with the number of layers. These data suggested that PMBV/PVA successfully covered the Ti substrate surface. Furthermore, PMBV, the outmost layer, was found to inhibit cell attachment. Moreover, the hydrogel layer can effectively entrap bioactive reagents and control their diffusivity. Research on this is currently underway in our laboratory and the results will be reported in the near future.

We concluded that the Ti substrates with multilayered polymer hydrogels will be useful in applications such as implantable devices and local drug delivery systems.

Acknowledgment

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Hydration of phosphorylcholine groups attached to highly swollen polymer hydrogels studied by thermal analysis

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ABSTRACT

Hydration of polymer chains plays a key role for determining the extent of protein adsorption on polymeric materials. Here we investigated the hydration of poly(2-methacryloyloxyethyl phosphorylcholine (MPC)) chains, which resist protein adsorption and following cell adhesion effectively. The hydration was compared with that of poly(methoxy oligo(ethylene glycol)-monomethacrylate ($Me(EG)_nMA$)) chains, which also have hydrophilic units. The poly(MPC) and poly($Me(EG)_nMA$) hydrogels with equilibrium water contents (EWCs) in the range from 86 to 97 wt% were prepared. By differential scanning calorimetric measurements, water in both the hydrogels was classified into two states: freezable and nonfreezable water. The poly(MPC) hydrogels had larger nonfreezable water than the poly($Me(EG)_nMA$) hydrogels even when their EWCs were similar, which indicated the higher hydrating ability of poly(MPC) chains. We suggested that the difference in the amount of nonfreezable water around polymer chains may influence the degree of protein adsorption resistance after contact with body fluid for a long period.

1. Introduction

According to the rapid advancement in developments of artificial organs, drug delivery systems, biochip-based diagnosis systems, and tissue engineering devices, the importance in the design of material surfaces that resist protein adsorption has been stressed [1–4]. Protein adsorption on material surfaces is the first phenomenon in contact with blood or tissues [5]. The adsorbed proteins are denatured, which is followed by platelet adhesions and cell adhesions for inducing thrombus formation and unfavorable immunoreactions. Thus, protein adsorption-resistant surfaces are essentially needed to obtain safe and stable medical treatment and diagnosis. Especially, cell-based tissue engineered devices and implantable artificial organs should have protein adsorption resistance surface for controlling cell/materials interactions for long period. To date, many protein adsorption-resistant surfaces have been designed. Recently, some research groups have achieved very low protein adsorption levels of $<10\ ng/cm^2$ by controlling the packing density and/or lengths of surface-tethered hydrophilic polymer chains [6–8]. On the other hand, the physico-chemical factors that determine the ability of the surfaces to resist protein adsorption have not been elucidated yet. A satisfactory understanding of such factors allows not only the systematic design of protein adsorption-resistant surfaces but also the elucidation of the mechanism of protein adsorption resistance.

Surface free energy on polymer materials has often been considered to be a key determinant of the extent of protein adsorption [9,10]. However, it has been demonstrated that there is no clear correlation between surface free energy and adsorbed amount of protein [11]. In addition, although the high conformational flexibility of surface-tethered chains has been experimentally and theoretically explained to make sterical inhibition of the access of proteins to surfaces by an excluded volume effect [12,13], it is not an essential requirement for protein adsorption resistance. In fact, self-assembled monolayers (SAMs) with polar functional end groups, such as short-chain poly(ethylene glycol) (PEG), i.e. oligo(ethylene glycol) (OEG), and an equimolar mixture of $-SO_3^-$ and $-N^+(CH_3)_3$ end groups, showed high resistance to protein adsorption [14,15].

The present discussion on the factors that determine the outcome of protein adsorption resistance is centered on the relationship between the hydration structures of material surfaces and protein adsorption [11,16]. The hydration structures of PEG chains have been intensively studied [17–19] because their utilization as surface modifiers is a well-known approach for rendering surfaces highly resistant to protein adsorption [6,9,11,14]. We have developed our original biocompatible polymer, poly(2-methacryloyloxyethyl phosphorylcholine) (poly(MPC)), which is inspired from the

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structure of phosphatidylcholines in cell membrane [4,20–22]. As a criterion of the hydration structures that provide the resistance of protein adsorption, the hydration structures of poly(MPC) chains provide us strong interest for understanding the biocompatibility. The surfaces grafted with the poly(MPC) chains resist platelet adhesion for longer periods than OEG-monomethacrylate polymer surfaces [23]. In addition, no conformation of albumin in poly(MPC) aqueous solutions changes during 72 h incubation, whereas albumin denatured by incubation in PEG aqueous solutions within 24 h [24]. Thus, it can be expected that the hydration of poly(MPC) chains may be different from that of PEG chains.

In this study, we investigated the hydration structures of poly(MPC) chains using a chemically cross-linked poly(MPC) hydrogel. The poly(MPC) hydrogels with six different equilibrium water contents (EWCs) were prepared in the range from 86.1 to 96.5 wt%. The different states of water absorbed in the hydrogels were classified and quantified by differential scanning calorimetry (DSC). They were compared with those in chemically cross-linked hydrogels composed of OEG-monomethacrylate polymer chains–poly(ω-methoxy tetra- or octa(ethylene glycol) monomethacrylate ($Me(EG)_nMA$) ($n=4$ or 8)) chains– with a similar EWC range. The origin of nonfreezable water around poly(MPC) chains was also discussed.

2. Experimental section

2.1. Materials

The detailed synthetic process of MPC (Fig. 1a) has been reported elsewhere [20]. $Me(EG)_nMA$ (Fig. 1b) with molecular weights of 286 ($n=4$) and 469 ($n=8$) was purchased from Sigma–Aldrich (St. Louis, MO) and used without further purification. Ammonium peroxodisulfate (APS) (>98.0%, Kanto Chemicals, Tokyo, Japan), triethylene glycol dimethacrylate (TEGDMA) (>95%, Tokyo Kasei Kogyo, Tokyo, Japan), and *N,N,N′,N′*-tetramethylethylenediamine (TMEDA) (>98.0%, Kanto Chemicals) were also used without further purification. Distilled water was used for all sample preparations.

2.2. Preparation of chemically cross-linked poly(MPC) and poly($Me(EG)_nMA$) hydrogels

The procedure for preparing chemically cross-linked poly(MPC) hydrogels has already been described [25]. The poly($Me(EG)_4MA$) and poly($Me(EG)_8MA$) hydrogels were prepared by the same procedure. In brief, the hydrogels were prepared in an aqueous medium by free radical polymerization. An aqueous monomer solution, TEGDMA (1.0 mol% to a monomer) as a cross-linker, and a 0.22 mol/L APS aqueous solution (0.53 mol% to a monomer) as an initiator were placed in a Petri dish. The concentrations of the monomer solutions were 1.5, 1.75, 2.0, 2.25, 2.5, and 3.0 mol/L for MPC and 0.75, 1.0, 1.25, and 1.5 mol/L for $Me(EG)_4MA$ and $Me(EG)_8MA$. The solution in the Petri dish was stirred for 30 min to allow complete mixing. The solution began to make gelation 1 min after the injection of TMEDA (5.3 mol% to a monomer) as a catalyst. After its complete gelation, the obtained hydrogel was removed from the Petri dish, and subsequently, it was immersed in excess distilled water for 48 h to remove any unreacted compounds and to allow complete swelling. The water in the dish was replaced several times. All the fully swollen hydrogels were transparent. All these processes were carried out at room temperature. The fully swollen hydrogels were used for the following EWC and DSC measurements.

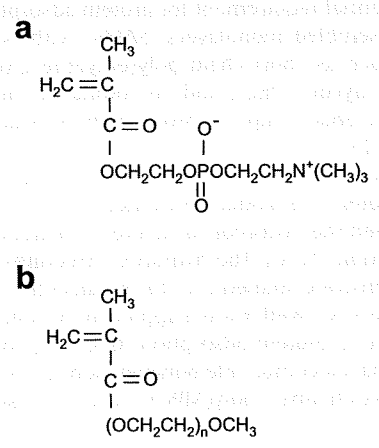

Fig. 1. Chemical structures of (a) MPC and (b) $Me(EG)_nMA$.

2.3. Determination of EWC

Each fully swollen hydrogel was freeze-dried for 24 h to remove the absorbed water. The weight of the freeze-dried hydrogel was recorded as W_d. The freeze-dried hydrogel was fully swollen again for 48 h. The excess water on the surface of the swollen hydrogel was gently removed with a filter paper before the measurement of its weight, W_s. The EWC of the hydrogel can be calculated by using the following equation.

$$\mathrm{EWC} = \frac{W_s - W_d}{W_s} \times 100 \quad (1)$$

2.4. DSC measurements

A 4–6 mg hydrogel was placed in an aluminum pan after gently wiping off the excess water on its surface, and then the pan was hermetically sealed. An empty aluminum pan was used as the control. Measurements were performed using an SII NanoTechnology (Chiba, Japan) model DSC6100 differential scanning calorimeter interfaced to an EXSTAR 6000 thermal analysis system version 5.8 (SII NanoTechnology). During the cooling and heating experiments, the sample cell was purged with nitrogen gas at a flow rate of 50 mL/min. The melting point peak of indium calibrated the temperature and heat flow of the equipment. The samples were initially cooled from room temperature to −70 °C at a rate of 5 °C/min and then heated to 40 °C at the same rate.

3. Results and discussion

Since the concentrations of water and polymer chains strongly influence their hydration properties, the poly(MPC) hydrogels with different EWCs were prepared. As shown in Fig. 2, the EWC of the poly(MPC) hydrogels could be controlled within the range of 86.1–96.5 wt%. To understand the effect of chemical structures on hydration, poly($Me(EG)_4MA$) and poly($Me(EG)_8MA$) hydrogels with a similar EWC range were prepared. The EWC of the poly($Me(EG)_4MA$) hydrogels and poly($Me(EG)_8MA$) hydrogels ranged from 87.5 to 95.9 wt% and 89.7 to 95.7 wt%, respectively. In the case of the poly($Me(EG)_8MA$) hydrogels, the EWC could not control below 89 wt% even when monomer solutions with concentrations higher than 1.5 mol/L were used.

Figs. 3–5 show the typical DSC heating thermograms of the poly(MPC), poly($Me(EG)_4MA$), and poly($Me(EG)_8MA$) hydrogels swollen with different EWCs, respectively. For comparison, the thermogram of bulk water is also presented in the respective figures. In the poly(MPC) hydrogels, a single endothermic peak was observed, and the transition occurred over a temperature range similar to that of the ice-to-water transition for bulk water. We