

the substrate (i.e., dipping, cross-linking, and ready-made polymers with reactive end groups reacting with the functional groups of the substrate) [3–5].

2-Methacryloyloxyethyl phosphorylcholine (MPC) polymers have attracted considerable attention as surface modifiable polymers for several medical devices [6–16]. MPC is a monomer for preparing novel polymer biomaterials and can undergo conventional radical copolymerization with other methacrylates, such as *n*-butyl methacrylate (BMA), *n*-dodecyl methacrylate (DMA), and 3-methacryloxypropyl trimethoxysilane (MPSI), to form poly(MPC-co-BMA), poly(MPC-co-DMA), and poly(MPC-co-MPSI), respectively [10–16]. They have potential applications in a variety of fields such as biomedical science, surface science, and bioengineering because they possess unique properties such as excellent anti-biofouling ability and low friction ability. Thus, surface modification with the MPC polymer on medical devices is effective for obtaining biocompatibility. In fact, several medical devices have already been developed by utilizing MPC polymers and used clinically; therefore, the efficacy and safety of MPC polymers as biomaterials are well established [14–16].

In this study, we hypothesize that the structure of surface modification layers might affect the long-term stability, hydration kinetics, articular cartilage retention, etc., and particularly that the poly(MPC) (PMPC)-grafted surface might assure the long-term performance of the artificial femoral head for partial hemiarthroplasty. Therefore, we investigated the surface properties of various surface modification layers with the MPC polymer and the effects of the surface properties on the friction of the artificial femoral head against articular cartilage. The results reveal that the structure of the PMPC-grafted layer on the Co–Cr–Mo surface plays an important role in the articular cartilage retention in the long term.

## 2. Materials and methods

### 2.1. Chemicals

MPC was industrially synthesized by using the method reported by Ishihara et al. and supplied by NOF Corp. (Tokyo, Japan) [17]. MPSI was purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Succinic acid and ethanol were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 2-Hydroxy-1,4-(hydroxyethoxy)phenyl-2-methylpropan-1-one (DAROCUR 2959; D2959), as a highly efficient radical photoinitiator for ultraviolet (UV) curing, was purchased from Ciba Specialty Chemicals Holding Inc. (Basel, Switzerland) Poly(MPC-co-BMA) (PMB30; MPC unit mole fraction = 0.3) [17], poly(MPC-co-MPSI) (PMSI90; MPC unit mole fraction = 0.9) [13], and PMPC (for lubricant additive in friction test) were synthesized in ethanol using 2,2'-azobisisobutyronitrile as an initiator by a conventional radical copolymerization method.

### 2.2. Co–Cr–Mo alloy substrate and pretreatments

The Co–Cr–Mo alloy was supplied by Yoneda Advanced Casting Co., Ltd. (Takaoka, Japan). This alloy was manufactured according to the ASTM F75 standard specification for the Co–28Cr–6Mo alloy. The Co–Cr–Mo samples were machined and polished so that the average surface roughness was approximately 0.01  $\mu\text{m}$ ; this surface was comparable to those of femoral ball products.

The polished Co–Cr–Mo samples were washed with acetone, and then immersed in 35 vol% nitric acid at room temperature for 35 min. This treatment aimed at passivation by surface oxidation; this would lead to the dissolution of certain foreign substance residues and the concentration of the Cr constituent by "resurfacing" [18]. After the nitric acid treatment, the Co–Cr–Mo samples were irradiated with  $\text{O}_2$  plasma at a 500-W high-frequency output and 150 mL/min  $\text{O}_2$  gas flow for 5 min by using an  $\text{O}_2$  plasma etcher (PRS00; Yamato Scientific Co., Ltd., Tokyo, Japan). The  $\text{O}_2$  plasma treatment increased the thickness of the surface oxide layer [18].

### 2.3. MPC polymer coating

The preparation of the MPC polymer-coated Co–Cr–Mo is schematically illustrated in Fig. 1. The physical adsorption of PMB30 was carried out by the solvent evaporation method, where the pretreated Co–Cr–Mo specimens were dipped into ethanol solution containing 0.2 mass% PMB30 ( $M_w = 6.0 \times 10^5$ ) for 10 s for coating

and then placed in an ethanol vapor atmosphere at room temperature for 1 h. The coated Co–Cr–Mo specimens were again dipped for 10 s and placed in the ethanol vapor atmosphere at room temperature for 1 h (PMB30-adsorbed Co–Cr–Mo).

The chemical immobilization of PMSI90 was also carried out by the solvent evaporation method. The pretreated Co–Cr–Mo specimens were dipped into ethanol solution containing 0.5 mass% PMSI90 ( $M_w = 9.8 \times 10^5$ ) and 0.063 mg/mL succinic acid for 12 h for the silanization of trimethoxysilane group of PMSI90 and placed in the ethanol vapor atmosphere at room temperature for 1 h. The coated Co–Cr–Mo specimens were annealed in air at 70 °C for 3 h for dehydration (PMSI90-immobilized Co–Cr–Mo).

### 2.4. MPSI silanization and MPC graft polymerization

The preparation of the PMPC-grafted Co–Cr–Mo is schematically shown in Fig. 1. The pretreated Co–Cr–Mo samples were immersed in an ethanol solution containing 5 mass% MPSI, 1 mass% succinic acid, and 0.1 mass% D2959 at room temperature for 12 h for silanization of the trimethoxysilane group. In this study, D2959 was used as a photoinitiator for surface-initiated polymerization so as to be included in the MPSI layer. They were then annealed at 70 °C for 3 h in air for dehydration. MPSI was dissolved in degassed pure water to obtain a concentration of 0.5 mol/L. Subsequently, the MPSI (containing D2959)-coated Co–Cr–Mo samples were immersed in MPC aqueous solutions. Photoinduced graft polymerization on the Co–Cr–Mo surface was performed using ultraviolet irradiation (UVL-400HA ultra-high pressure mercury lamp; Riko-Kagaku Sangyo Co., Ltd., Funabashi, Japan) with an intensity of 5 mW/cm<sup>2</sup> at 60 °C for 90 min; a filter (Model D-35; Toshiba Co., Tokyo, Japan) was used to restrict the passage of ultraviolet light to wavelengths of 350  $\pm$  50 nm (PMPC-grafted Co–Cr–Mo) [19]. After the polymerization, the PMPC-grafted Co–Cr–Mo samples were removed from the solution, washed with pure water and ethanol, and dried at room temperature. The molecular weight of the PMPC graft chain on the PMPC-grafted Co–Cr–Mo samples could not be determined due to the difficulty in separating the PMPC graft chain from the Co–Cr–Mo substrate. Additional efforts are needed in this aspect.

### 2.5. Articular cartilage from porcine ankle joint

Articular cartilage specimens were harvested from the flat part of the ankle joint of the fresh frozen porcine tibia (age 6–9 months) by using a surgical saw for the friction test. The pin-type (cylinder-shaped with a height of 5 mm and diameter of 9 mm) articular cartilage specimens had approximately a 1-mm cartilage layer and the subchondral bone used for mounting. Throughout the procedure, the articular cartilage surface was hydrated regularly with Dulbecco's phosphate-buffered saline (PBS, pH 7.4, ion strength = 0.15 M, Immuno-Biological Laboratories Co., Ltd., Takasaki, Japan).

### 2.6. Surface analysis of surface-modified Co–Cr–Mo with various MPC polymers

The functional group vibrations of the surface-modified Co–Cr–Mo samples were examined by Fourier-transform infrared (FT-IR) spectroscopy with attenuated total reflection (ATR) equipment. The FT-IR/ATR spectra were obtained using an FT-IR analyzer (FT/IR165; JASCO Co., Ltd., Tokyo, Japan) for 32 scans over the range from 800 to 2000 cm<sup>-1</sup> at a resolution of 4.0 cm<sup>-1</sup>.

The surface elemental conditions of the surface-modified Co–Cr–Mo samples were analyzed by X-ray photoelectron spectroscopy (XPS). The XPS spectra were obtained using an XPS spectrophotometer (AXIS-HS165; Kratos/Shimadzu Co., Kyoto, Japan) equipped with a 15-kV Mg-K $\alpha$  radiation source at the anode. The take-off angle of the photoelectrons was maintained at 90°. Five scans were taken for each sample.

The static-water contact angles on the surface-modified Co–Cr–Mo samples were measured by the sessile drop method using an optical bench-type contact angle goniometer (Model DM300, Kyowa Interface Science Co., Ltd., Saitama, Japan). Drops of purified water (1  $\mu\text{L}$ ) were deposited on the surface-modified Co–Cr–Mo surfaces, and the contact angles were directly measured with a microscope after 60 s of drooping. Measurements were repeated fifteen times for each sample, and the average values were regarded as the contact angles.

The surface-modified Co–Cr–Mo samples were stained with rhodamine 6G (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and observed by fluorescence microscopy (FM). According to previous literature, rhodamine 6G effectively stains the MPC polymer, which possesses great structural similarity to lipids [20]. This simple staining technique enables the evaluation of the surface-modified layer with MPC polymer by FM. An aqueous solution of 200 mass ppm rhodamine 6G was used for all the staining experiments. The samples were immersed in the rhodamine 6G solution for 30 s and then removed. Then, they were washed two times consecutively in distilled water for 30 s and then dried. A fluorescence microscope (Axioskop 2 Plus, Carl Zeiss AG, Oberkochen, Germany) was used for FM imaging and examination of all samples. Pseudo-color images were obtained using a charge coupled device (CCD) camera (VB-7010, Keyence Co., Osaka, Japan) and imaging software (VH analyzer 2.51). Lenses with  $\times 10$  magnification and appropriate exposure time (approximately 0.1 s) were employed to obtain the best image quality of the various samples.

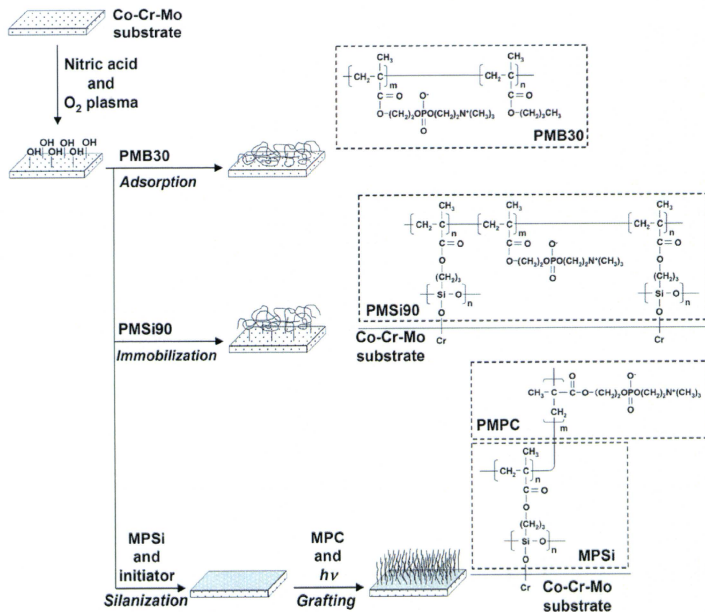


Fig. 1. Schematic illustration for the preparation of MPC polymer-coated and PMPC-grafted Co-Cr-Mo.

## 2.7. Cross-sectional observation by transmission electron microscopy

A cross-section of the surface-modified Co-Cr-Mo samples was observed using a transmission electron microscope (TEM) and by energy dispersive X-ray (EDX) spectroscopy. The specimens were precoated with an aluminum film; then, a thin film of the samples was prepared by the focused ion beam (FIB) technique using an FB-2000A (Hitachi High-Technologies Co., Tokyo, Japan) FIB system. The samples were thinned to electron transparency by a low gallium ion beam current. The thin film thus prepared was positioned onto a copper TEM mesh grid. TEM observations were then recorded using an HF-2000 electron microscope (Hitachi High-Technologies Co.) at an acceleration voltage of 200 kV. EDX spectra were analyzed on a cross-section of the samples using a Sigma EDX attachment (Kevex Instruments, Inc., Valencia, CA, USA) at an acceleration voltage of 200 kV.

## 2.8. Characterization of protein adsorption by micro bichromonic acid method

The amounts of protein adsorbed on the surface-modified Co-Cr-Mo samples were determined by the micro bichromonic acid (BCA) method. Each specimen was immersed in PBS for 1 h to equilibrate the surface modified by the MPC polymer. The specimens were immersed in bovine serum albumin (BSA, Mw =  $6.7 \times 10^5$ ; Sigma-Aldrich Corp., MO, USA), bovine blood  $\gamma$ -globulins (Mw =  $1.5 \times 10^5$ ; Sigma-Aldrich Co.), and bovine plasma fibrinogen (Mw =  $3.4 \times 10^5$ ; Sigma-Aldrich Co.) solutions at 37 °C for 1 h. The protein solutions were prepared in BSA,  $\gamma$ -globulins, and fibrinogen concentrations of 4.5, 1.6, and 0.3 g/L, respectively, i.e., 10% of the concentration of human plasma levels. Then, the specimens were rinsed five times with fresh PBS and immersed in 1 mass% sodium dodecyl sulfate (SDS) aqueous solution and shaken at room temperature for 1 h to completely detach the adsorbed BSA,  $\gamma$ -globulins, and fibrinogen on the surface modified by the MPC polymer. A protein analysis kit (micro BCA protein assay kit, #23235; Thermo Fisher Scientific Inc., IL, USA) based on the BCA method was used to determine the BSA concentration in the SDS solution, and the amount of BSA,  $\gamma$ -globulins, and fibrinogen adsorbed on the surface modified by the MPC polymer was calculated.

## 2.9. Friction test and histological observation of articular cartilage

The coefficients of dynamic friction between the pins fabricated from articular cartilage and the surface-modified Co-Cr-Mo plates were measured by using a pin-on-plate machine (Tribstation 32; Shinto Scientific Co. Ltd., Tokyo, Japan). The friction tests were performed at room temperature and 37 °C with various loads in the range from 0.49 to 9.80 N, sliding distance of 25 mm, and frequency of 1 Hz for a maximum of  $5 \times 10^3$  cycles [21]. Pure water, mixture of 25 vol% bovine serum (BS), 20 mM/L of ethylene diamine tetraacetic acid (EDTA), 0.1 mass% sodium azide, and the BS mixture containing 0.02 mass% MPC polymer (PMB30 (Mw =  $5.0 \times 10^4$ ), PMSi90 (Mw =  $9.8 \times 10^4$ ), and PMPC (Mw =  $1.0 \times 10^7$ )) were used as a lubricant each. Subsequently, five replicate measurements were performed for each sample, and the average values were regarded as the coefficients of dynamic friction. After 100 cycles of friction tests, the plate samples were FM-observed. Then, after  $5 \times 10^3$  cycles friction test, articular cartilage pins against the untreated Co-Cr-Mo and PMPC-grafted Co-Cr-Mo were fixed with 10% neutral buffered formalin for 3 d, then decalcified in KC-X solution (Falma Co., Tokyo, Japan) for 8 d, and then dehydrated in graded ethanol for histological observation. The decalcified cartilage specimens were embedded in paraffin (Tissue-prep; Fisher Scientific Corp., Fair Lawn, NJ, USA), and microsections were prepared and stained with hematoxylin (Real hematoxylin; Dako Co., Carpinteria, CA, USA) and eosin (Eosin yellowish; Kanto Chemical Co., Inc.) (H&E) as well as with Safranin O (Nacal Tesque, Inc., Kyoto, Japan) and observed with a microscope (Eclipse E600; Nikon Corp., Tokyo, Japan) equipped with a CCD camera (DP72; Olympus Co., Tokyo, Japan).

## 2.10. PBS soaking test

The surface-modified Co-Cr-Mo samples ( $10 \times 10 \times 1$  mm<sup>3</sup> in size) were soaked in 50 mL of PBS. After soaking with 120 rpm shaking at 37 °C for 1, 4, 8, and 12 weeks, the samples removed from the PBS and were characterized by XPS analysis, water-contact angle measurement, and FM observation.

**Table 1**  
Surface elemental composition ( $n = 5$ ) and static-water contact angle ( $n = 15$ ) of untreated, MPC polymer-coated and PMPC-grafted Co–Cr–Mo alloy.

Sample	Surface elemental composition (atom%)								Contact angle (deg)
	C <sub>1s</sub>	O <sub>1s</sub>	N <sub>1s</sub>	P <sub>2p</sub>	Si <sub>2p</sub>	Co <sub>2p</sub>	Cr <sub>2p</sub>	Mo <sub>3d</sub>	
Co–Cr–Mo (untreated)	14.6 (1.3) <sup>a</sup>	52.9 (2.7)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	26.7 (1.5)	5.4 (0.4)	0.4 (0.0)	81.6 (4.8)
PMB30-adsorbed Co–Cr–Mo	70.6 (1.4)	24.1 (1.3)	2.3 (0.4)	3.0 (0.3)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	95.8 <sup>b</sup> (3.5)
PMSi90-immobilized Co–Cr–Mo	61.4 (0.9)	29.5 (0.7)	3.6 (0.4)	4.2 (0.1)	1.3 (0.4)	0.1 (0.2)	0.0 (0.0)	0.0 (0.0)	16.6 <sup>b</sup> (2.4)
PMPC-grafted Co–Cr–Mo	61.7 (0.7)	28.0 (0.6)	5.0 (0.3)	5.3 (0.1)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	23.5 <sup>a</sup> (8.4)

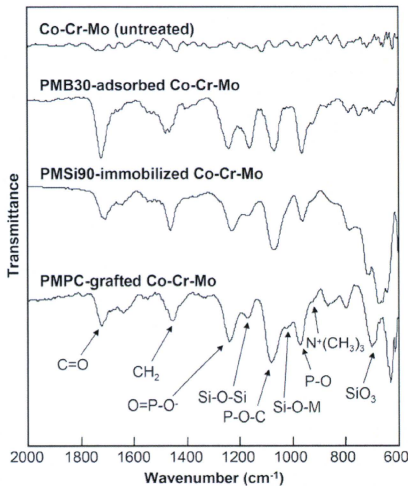
<sup>a</sup> The standard deviations are shown in parentheses.<sup>b</sup> Significant difference ( $p < 0.001$ ) as compared to the untreated Co–Cr–Mo.

### 2.11. Statistical analysis

The results derived from each measurement in the water-contact angle measurement, friction test, and protein adsorption test were expressed as mean values  $\pm$  standard deviation. The statistical significance ( $p < 0.05$ ) was estimated by Student's *t*-test.

## 3. Results

Fig. 2 shows the FT-IR/ATR spectra of the surface-modified Co–Cr–Mo samples with various MPC polymers. Absorption peaks were newly observed for the surface-modified Co–Cr–Mo with MPC polymers. The peaks at 1720, 1550, and 1460  $\text{cm}^{-1}$  are attributed to C=O and  $-\text{CH}_2-$  in the MPC polymer. The peaks at 1180, 1040, 700, and 630  $\text{cm}^{-1}$  are attributed to the trimethoxysilane group in the MPSi unit [19]. The peaks at 1240, 1080, 970, and 920  $\text{cm}^{-1}$  are attributed to the  $-\text{N}^+(\text{CH}_3)_3$  and phosphate groups in the MPC unit [22]. The absorption peak intensities of the phosphate groups of the

**Fig. 2.** FT-IR/ATR spectra of untreated Co–Cr–Mo, MPC polymer-coated Co–Cr–Mo, and PMPC grafted Co–Cr–Mo.

PMPC-grafted Co–Cr–Mo were the highest in the Co–Cr–Mo whose surface was modified by the MPC polymer.

Table 1 summarizes the surface elemental composition and static-water contact angle of the surface-modified Co–Cr–Mo samples with various MPC polymers. The nitrogen (N) and phosphorus (P) contents in all the Co–Cr–Mo samples whose surfaces were modified by the MPC polymer were observed. The elemental compositions of both N and P in the surface-modified Co–Cr–Mo increased with an increase in the MPC composition in the polymer for surface modification. In particular, these values of N and P in the PMPC-grafted Co–Cr–Mo surface were 5.0 and 5.3 atom%, respectively, and were almost equivalent to the theoretical elemental composition (N = 5.3, P = 5.3 atom%) of PMPC. The static-water contact angle of the untreated Co–Cr–Mo was 81.6°, and this decreased markedly to approximately 20° (i.e., 16.6°–23.5°,  $p < 0.001$ ) by the modifications with PMSi90 and PMPC.

Fig. 3 shows the cross-sectional TEM images of the surface-modified Co–Cr–Mo samples with various MPC polymers. For PMB30-adsorption, PMSi90-immobilization, and PMPC-grafting, a thickness of 50, 130, and 200 nm, respectively, of the MPC polymer layers was clearly observed on the surface of the Co–Cr–Mo substrate. No cracks due to poor adhesion and/or delamination were observed at the interface between the MPC polymer layer and Co–Cr–Mo substrate. These results indicate that each surface modification layer on the Co–Cr–Mo substrate is uniform and adheres closely, regardless of the binding conditions; the surface modification layers by PMB30-adsorption, PMSi90-immobilization, and PMPC-grafting combine with the substrate by physical adsorption and covalent bonds of Si–O–metal (M), respectively. However, in the PMB30-adsorbed Co–Cr–Mo, a bilayer structure for poor adhesion attributed to dipping twice was clearly observed on the surface modification layer. Further, in the PMB30-adsorbed and PMSi90-immobilized Co–Cr–Mo samples, a porous structure was clearly observed on the surface modification layer. This porous structure was also observed on the surface modification layer prepared by the slow-rate solvent evaporation method (approximately for 1 month at 4 °C, data not shown).

Fig. 4 shows the EDX spectra of the surface-modified Co–Cr–Mo samples with various MPC polymers. In spectra ( $b_{1-3}$ ), ( $c_{1-3}$ ), and ( $d_{1-3}$ ) of the MPC polymer layers, a significant peak attributed to the P atom was observed at 2.0 keV. This peak is mainly attributed to the MPC units. Interestingly, this peak was clearly observed in spectra ( $b_2$ ) and ( $c_2$ ) of the porous part of the MPC polymer layer. In spectra ( $c_1$ ) and ( $d_1$ ) of the interface of the PMSi90-immobilization layer and the intermediate layer of the PMPC-grafted Co–Cr–Mo, peaks were observed at 0.5 and 1.7 keV. These peaks are attributed to the O and Si atoms in the interface/intermediate layer between the silane of the MPSi and the metal oxide of the Co–Cr–Mo.

Fig. 5 shows the amounts of BSA,  $\gamma$ -globulins, and fibrinogen adsorbed on the surface-modified Co–Cr–Mo samples with various

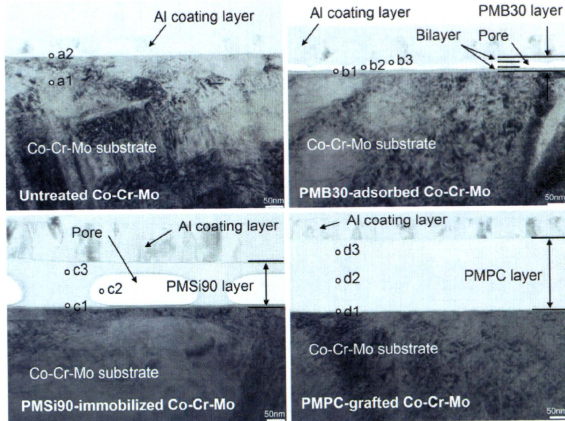


Fig. 3. Cross-sectional TEM images of the surface-modified Co–Cr–Mo with various MPC polymers. Aluminum coating layers (approximately 70–100 nm) for the preparation of TEM observation specimen are shown above the MPC polymer layer of the Co–Cr–Mo surface. Small open circles indicate EDX analysis points. Bar: 50 nm.

MPC polymers. The amount of each protein adsorbed on the Co–Cr–Mo surface modified by the MPC polymer was considerably lower ( $p < 0.001$ ) than that on the untreated Co–Cr–Mo. These results imply that the surface modification by the MPC polymer results in good biocompatibility.

Fig. 6 shows the coefficients of dynamic friction of the sliding couples and articular cartilage pins sliding against the surface-modified Co–Cr–Mo plates with various MPC polymers. The PMB30-adsorbed and PMSi90-immobilized Co–Cr–Mo samples showed a slightly higher friction coefficient than the untreated Co–Cr–Mo sample in water at room temperature (not significantly different); in contrast, the MPC polymer-coated Co–Cr–Mo showed a lower friction coefficient than the untreated Co–Cr–Mo in BS mixture at 37 °C ( $p < 0.05$ ). Further, the friction coefficient of the PMPC-grafted Co–Cr–Mo decreased drastically compared with untreated Co–Cr–Mo ( $p < 0.001$ ) and reached approximately  $< 0.010$  in both lubricant conditions, i.e., water at room temperature and the BS mixture at 37 °C; moreover, it remained almost steady. The friction coefficients of all MPC polymer-containing BS mixtures were drastically lower as compared with that of non-additive BS mixture (Fig. 6).

Fig. 7 shows the coefficients of dynamic friction of the untreated Co–Cr–Mo and PMPC-grafted Co–Cr–Mo samples as a function of the loads. At both 10 and 100 cycles, the PMPC-grafted Co–Cr–Mo sample showed a remarkably low friction coefficient of approximately 0.019 at a load of 0.49 N; this value decreased gradually and reached approximately  $< 0.010$  at a load of 9.80 N. Similarly, the friction coefficients of the untreated Co–Cr–Mo sample in the initial 10 cycles decreased gradually from 0.188 to 0.045. However, this value of the untreated Co–Cr–Mo at 100 cycles decreased to 0.082 up to loads of 1.96 N; it then gradually increased with an increase in the loads. Fig. 7B shows the friction coefficients of the untreated Co–Cr–Mo and PMPC-grafted Co–Cr–Mo samples as a function of the test durations. It was observed that the friction coefficient was significantly lower in the PMPC-grafted Co–Cr–Mo sample than in the untreated Co–Cr–Mo one. This value was almost constant throughout the  $5 \times 10^3$  cycles of the friction test.

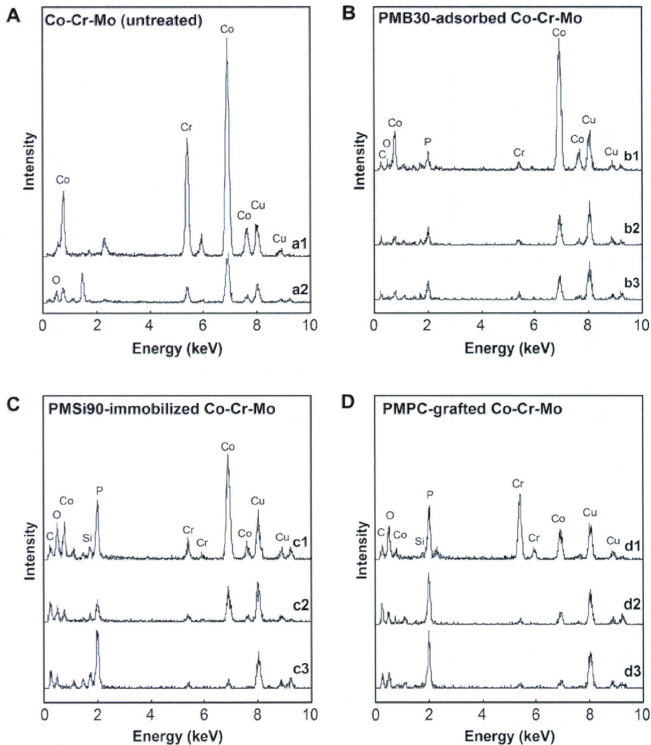
Fig. 8 shows the histological findings of the articular cartilage pins after  $5 \times 10^3$  cycles of friction tests. In the cartilage against the untreated Co–Cr–Mo, the cartilage layer of the worn surface became thicker as compared with the surrounding articular cartilage of the unworn surface. In contrast, in the cartilage against the PMPC-grafted Co–Cr–Mo, the layer of the worn surface did not differ considerably from that of the unworn surface.

Fig. 9 shows the time course of the surface modification layer of the untreated, MPC polymer-coated, and PMPC-grafted Co–Cr–Mo samples during PBS soaking. The elemental compositions of both N and P in the untreated, PMB30-adsorbed, and PMPC-grafted Co–Cr–Mo samples were almost constant throughout the 12 weeks of PBS soaking. In contrast, in the PMSi90-immobilized Co–Cr–Mo sample, these values decreased gradually with the PBS-soaking duration. Similarly, the static-water contact angle of untreated, PMB30-adsorbed, and PMPC-grafted Co–Cr–Mo samples were almost constant throughout PBS soaking, whereas the values in PMSi90-immobilized Co–Cr–Mo increased gradually.

Fig. 10 shows FM images of the surface-modified Co–Cr–Mo samples with various MPC polymers before and after friction tests/PBS-soaking tests. After 100 cycles of friction tests, the MPC polymer layer was removed from the PMB30-adsorbed and PMSi90-immobilized Co–Cr–Mo sliding surfaces; in contrast, most of the PMPC-grafted Co–Cr–Mo sliding surface was covered by the PMPC layer. After 12 weeks of PBS-soaking tests, most of the PMSi90 layer was removed from the Co–Cr–Mo surface, while most of the PMB30-adsorbed and PMPC-grafted Co–Cr–Mo surface was covered stably by the MPC polymer layer.

#### 4. Discussion

In the hemi-arthroplasty, the highly lubricious surface by a “mild treatment” with soft materials was requested with aim of preserving the degradation of the articular cartilage. In this study, we have prepared various surface modification layers formed on the Co–Cr–Mo surface by MPC polymer coating or photoinduced radical polymerization of MPC to form PMPC graft chains for



**Fig. 4.** EDX spectra of the untreated Co–Cr–Mo, MPC polymer-coated Co–Cr–Mo, and PMPC-grafted Co–Cr–Mo. The spectra were analyzed on the cross-section (small open circles in Fig. 3) of the untreated Co–Cr–Mo, MPC polymer-coated Co–Cr–Mo, and PMPC-grafted Co–Cr–Mo.

improving lubrication and preventing the degradation of articular cartilage. Here, we discuss the structures and stabilities of the surface modification layers of the MPC polymer and the effects of these characteristics on the retention of articular cartilage in hemiarthroplasty.

To ensure the *in vivo* long-term stability of the MPC modified layer on the Co–Cr–Mo surface, it is necessary to create strong covalent bonding between the Co–Cr–Mo substrate and the MPC polymer. Organosilanes have already been known as surface coupling agents that enhance bonding between a metal or a metal oxide surface and an organic resin such as dental resin; moreover, they can strongly bind metals to resins in dental implants [23]. Organic silanes or silane coupling agents comprise at least a hydrolyzable alkoxy silyl or chlorosilyl group and an organofunctional group. The agents are effective for introducing organofunctional groups into the siloxane network polymer. The organofunctional group in the silane could be useful for improving bonding with the organic overlayer. MPSi binds to the Co–Cr–Mo substrate by a condensation reaction in two steps; in the first step,

MPSi is hydrolyzed (activated) and in the second step, the hydrolyzed silane molecule binds to the surface by an Si–O–M bond, forming branched hydrophobic siloxane bonds, i.e., Si–O–Si. The hydrolyzed silane molecule has three –OH groups that can react with the –OH groups of the surface metallic oxide layer to form siloxane bonds covalently. The peaks at 1180 and 1040  $\text{cm}^{-1}$  in the FT-IR/ATR spectrum of the PMSi90-immobilized and PMPC-grafted Co–Cr–Mo surfaces were attributed to Si–O–Si and Si–O–M, respectively (Fig. 2).

However, several previous studies have reported that a silane coating has low water resistance due to hydrolysis of the siloxane bond and desorption of the physisorbed silane [24]. In fact, the PMSi90-immobilization layer was removed from the Co–Cr–Mo surface after 12 weeks of PBS soaking (Figs. 9 and 10). Zhang, et al. and others have reported that the water stability of Si–O–M could be improved by employing the following factors: (1) an induction of bridged silane coupling agents, when hydrolyzed, contain two or more –Si(OH)<sub>2</sub>, (2) the hydrophobic alkyl moieties that limit the contact with water, and (3) an increase in the thickness of the

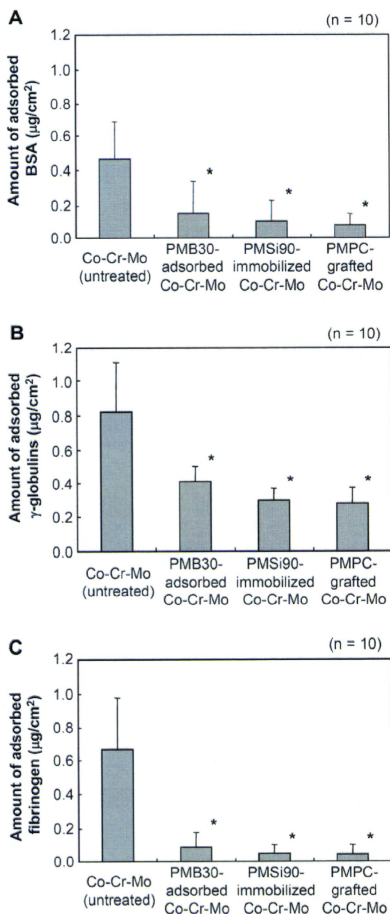


Fig. 5. Amounts of (A) BSA, (B)  $\gamma$ -globulins, and (C) fibrinogen adsorbed on the surfaces of the untreated Co-Cr-Mo, MPC polymer-coated Co-Cr-Mo, and PMPC-grafted Co-Cr-Mo. Bar: Standard deviations. \*: Significant difference ( $p < 0.001$ ) as compared to the untreated Co-Cr-Mo.

surface oxide layer [25]. Therefore, it was considered that the MPSi intermediate layer with a bridge of three methoxysilane groups with MPSi unit composition of 100% (MPSi unit of PMSi90 composition was 10% only) was essential in PMPC-grafted Co-Cr-Mo. Additionally, a functional methacrylate and pretreatment (nitric acid treatment and  $\text{O}_2$  plasma treatment) for the Co-Cr-Mo surface were used. As shown in Figs. 9 and 10, the high stability of

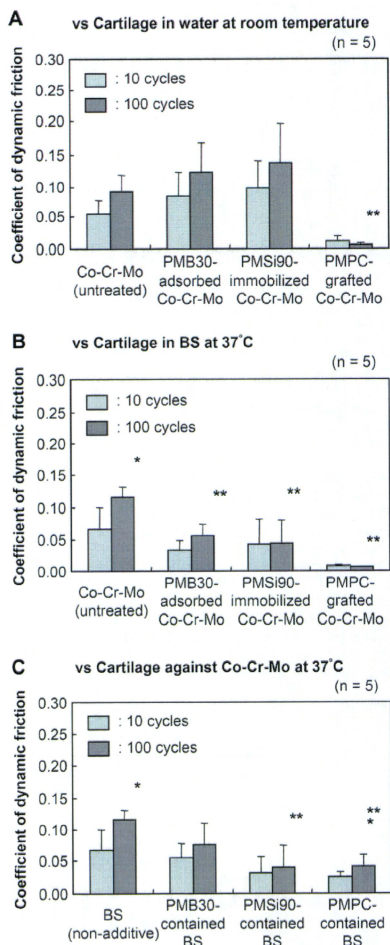
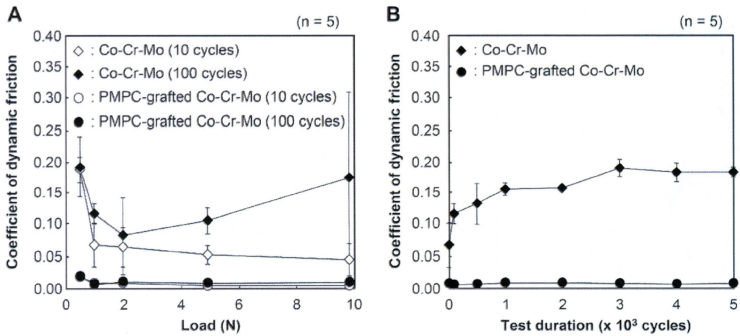


Fig. 6. Coefficients of dynamic friction of the untreated, MPC polymer-coated, and PMPC-grafted Co-Cr-Mo in the pin-on-plate friction test with various lubrication conditions. (A) Against cartilage pin with water at room temperature, (B) against cartilage pin with BS lubricant at 37°C, (C) untreated Co-Cr-Mo plate against cartilage pin with BS lubricant with MPC polymer as additive at 37°C. Bar: Standard deviations. \*:  $t$ -test, significant difference ( $p < 0.05$ ) as compared to the coefficients of dynamic friction at 10 cycles, and \*\*:  $t$ -test, significant difference ( $p < 0.05$ ) as compared to the untreated Co-Cr-Mo plate at 100 cycles.

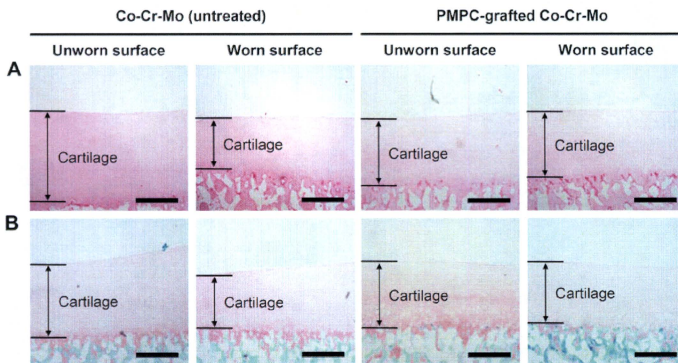


**Fig. 7.** Coefficients of dynamic friction of the untreated and PMPC-grafted Co-Cr-Mo in the pin-on-plate friction test. (A) Coefficients of dynamic friction of the untreated and PMPC-grafted Co-Cr-Mo against cartilage pin as a function of loads in the pin-on-plate friction test with BS lubricant at 37 °C. (B) Time course of coefficients of dynamic friction of the untreated and PMPC-grafted Co-Cr-Mo against the cartilage pin during  $5 \times 10^3$  cycles of loading with 0.98 N in the pin-on-plate friction test with BS lubricant at 37 °C. Bar: Standard deviations.

the PMPC-grafted layer was confirmed throughout 12 weeks of PBS soaking.

On the other hand, the high stability of the PMB30-adsorbed layer was also confirmed throughout 12 weeks of PBS soaking. As shown in Table 1, the static-water contact angle of the PMB30-adsorbed Co-Cr-Mo was 95.8°. Sibarani et al. reported that the PMB30-adsorbed polymer surfaces showed high advancing (approximately 100°) and low receding (approximately 20°) contact angles; PMB30 cannot be hydrated easily due to the low MPC unit composition of the copolymer [10]. However, as shown in Fig. 5, the PMB30-adsorbed Co-Cr-Mo surface, which could form a phosphorylcholine-enriched surface after equilibrating for 1 h, showed excellent biocompatibility as an anti-protein adsorption surface. Hence, the PMB30-adsorbed layer has been observed to be stable (insoluble and attachable *in vivo* condition) and useful on several medical devices [14,15].

In Figs. 6 and 7, the PMPC-grafted Co-Cr-Mo surface shows an extremely low friction coefficient as compared to that of the untreated Co-Cr-Mo surface. Since MPC is highly hydrophilic and PMPC is water soluble, the water contact angle of the PMPC-grafted Co-Cr-Mo surface was lower than that of the untreated Co-Cr-Mo surface, as shown in Table 1. Consequently, the PMPC-grafted layer successfully provided high lubricity in the form of “surface gel hydration lubrication” to the Co-Cr-Mo surface (Fig. 3). A previous study has reported that the hydrogel cartilage surface is assumed to have a brush-like structure: a part of the proteoglycan brush is bonded with the collagen network on the cartilage surface [26]. The bearing surface with PMPC is assumed to have a brush-like structure similar to that of articular cartilage. Cartilage/PMPC-grafted Co-Cr-Mo bearing couples can therefore be regarded to be mimicking natural joint cartilage *in vivo*. The friction coefficient of cartilage/cartilage was reported to be approximately 0.01–0.02



**Fig. 8.** Histological findings of the articular cartilage pins after  $5 \times 10^3$  cycles of friction tests. (A) H&E stained, and (B) safranin-O stained. Bar: 500  $\mu$ m.

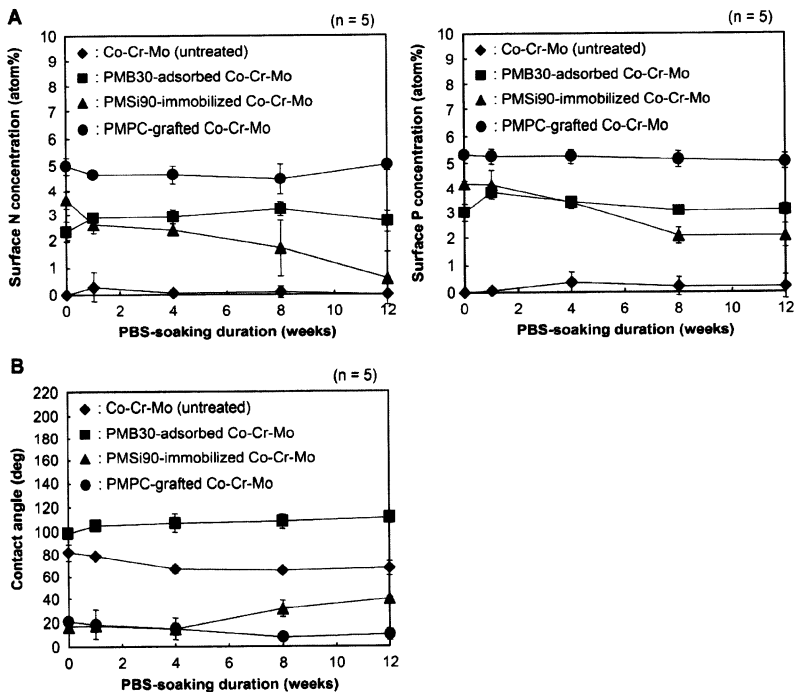


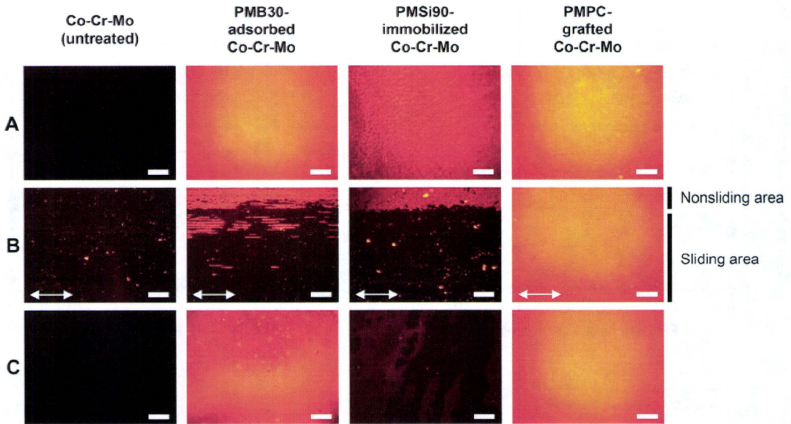
Fig. 9. Time course of the surface-modification layer of the untreated, MPC polymer-coated and PMPC-grafted Co–Cr–Mo during PBS soaking with 120 rpm shaking at 37 °C. (A) Surface N and P concentrations by XPS, and (B) static-water contact angle. Bar: Standard deviations.

[27,28]. In this study, it was found that the cartilage/PMPC-grafted Co–Cr–Mo interface mimicking a natural joint showed low friction (friction coefficient was  $<0.01$ ), i.e., as low as that of cartilage/cartilage interface. Hence, it was considered that the PMPC-grafted Co–Cr–Mo surface is well suited for application on the artificial femoral head that would chafe against articulating cartilage. We expect that hemi-arthroplasty with a PMPC-grafted Co–Cr–Mo femoral head will be a promising option that preserves acetabular cartilage and extends the duration before total hip arthroplasty (THA) in young patients. Moreover, we consider that these effects would occur continuously. In Fig. 7B, the friction coefficient shows a test duration-dependent response for articular cartilage against the untreated Co–Cr–Mo sample due to the continued loading of the cartilage tissue and the ensuing loss of fluid-film formation (or rehydration). It was thought that the thickness of the cartilage tissue atrophied due to the fairly poor access of the cartilage tissue to water (Fig. 8). Decreased water content often leads to the degradation of cartilage function. Although cartilage tissues are able to produce matrix components throughout life, i.e., carry out regeneration, their production cannot keep pace with the repair requirements after acute damage to articular cartilage; such damages limit the longevity of the artificial femoral head and its

stability after hemi-arthroplasty. In contrast, in articular cartilage against the PMPC-grafted Co–Cr–Mo sample, the friction coefficient remained at a steady low value due to the rehydration of the continuously loaded cartilage tissue, and the articular cartilage surface was preserved.

In Fig. 6A, the PMB30-adsorbed and PMSi90-immobilized Co–Cr–Mo samples show a slightly higher friction coefficient than the untreated Co–Cr–Mo sample in water at room temperature. Some pores in the PMB30 and PMSi90 layers on the Co–Cr–Mo surface could be observed (Fig. 3); these may have occurred due to the low density of the material because physical adsorption or chemical immobilization of the polymer was used as the surface modification method. Therefore, it is assumed that a sliding couple with cartilage and low-density MPC polymer layer may cause high friction by stick-slip motion with interpenetration [18]. Furthermore, the MPC polymer-coated Co–Cr–Mo showed a lower friction coefficient than the untreated Co–Cr–Mo in BS mixture at 37 °C (Fig. 6B). It was thought that the interpenetration of cartilage and low-density MPC polymer layer was blocked by the protein of BS presented between the interfaces. In contrast, the friction coefficient of the PMPC-grafted Co–Cr–Mo sample was drastically as compared with that of the untreated Co–Cr–Mo sample; the degree





**Fig. 10.** FM images of the untreated Co–Cr–Mo, MPC polymer-coated Co–Cr–Mo, and PMPC-grafted Co–Cr–Mo surfaces. FM images of the surface before tests (A), after 100 cycles of friction tests with BS lubricant (B), and after 12 weeks of PBS-soaking tests (C). Bar: 200  $\mu\text{m}$ . Arrow: Sliding direction of friction test.

of reduction in the coefficient was 93%. PMPC-grafted Co–Cr–Mo might have a high density because the polymerization method used was surface-initiated graft polymerization, termed as the “grafting from” method [19,29]. A sliding couple with cartilage tissue and high-density PMPC layer fabricated by the “grafting from” method may be responsible for low friction, such as that in the case of “super-lubricity,” because of resistance to interpenetration by volume effects resulting from the mobility of hydrophilic macromolecules of cartilage tissue and the PMPC-grafted layer [30–32].

As shown in Fig. 7A, the friction coefficients of the articular cartilage against the untreated and PMPC-grafted Co–Cr–Mo samples decreased with an increasing load in the initial 10 cycles. The elastic articular cartilage tissue and PMPC-grafted layer was slightly deformed by the loads; the low friction coefficient might occur in order to increase the contact area of the fluid film’s concave surface. However, the friction coefficient of the articular cartilage against the untreated Co–Cr–Mo at 100 cycles decreased to 0.082 up to loads of 1.96 N; a further increase in loads up to 9.8 N resulted in elevated friction coefficients. Under a high load, water exudes slowly from the articular cartilage with sliding [27]. As the result of water loss, the thickness of the surface layer and/or fluid film reduces, and the water content of the surface-hydration layer decreases. Consequently, the degree of adhesion of articular cartilage to the Co–Cr–Mo surface increases due to a lack of rehydration and because of the increase in frictional force. In contrast, the PMPC-grafted Co–Cr–Mo sample at 100 cycles showed a remarkably low friction coefficient that reached approximately <0.010 at a load of 9.80 N. We consider that this result implies that the rehydration and hydrodynamic lubrication mechanism of the articular cartilage is supported by the hydrated PMPC-grafted layer, similar to the interface between cartilage/cartilage of the natural joint.

The friction coefficients of cartilage/untreated Co–Cr–Mo interface with all MPC polymer-containing BS mixtures as a lubricant were drastically lower as compared with that with the non-additive BS mixture (Fig. 6C). Synovial fluid as a whole, and all

its components such as hyaluronic acid, glycoprotein (mainly lubricin), and surface-active phospholipids, have been proposed as lubricants responsible for boundary lubrication in the natural joint [33]. Similarly, it is considered that the additives of MPC polymer would play the role akin to synovial phospholipids for boundary lubrication, and the adsorption of the MPC polymer to the sliding surface could prevent direct contact between the cartilage and the untreated Co–Cr–Mo and hence decrease the frictional force between them. However, in the case of additives, the lubricity may change depending on the ambient *in vitro* and *in vivo* conditions, because the additives probably diffuse to synovial fluid *in vivo*.

The amounts of the representative protein, BSA,  $\gamma$ -globulins, and fibrinogen, adsorbed on the modified Co–Cr–Mo surface with the MPC polymer were significantly low, these reached to 7%–50% of that of the untreated surface, as shown in Fig. 5. It is hypothesized that the mechanism underlying protein adsorption resistivity of a surface modified by the MPC polymer is based on the water structure resulting from the interactions between water molecules and phosphorylcholine groups [34]. The large amount of free water around the phosphorylcholine group is considered to detach proteins easily and prevent conformational changes in the adsorbed proteins even when the proteins attached to the surface [3,34]. The reduction in protein adsorption is also considered to be caused by the presence of a hydrated layer around the phosphorylcholine group [35]. The latter consideration is consistent with the results of the water contact angle measurement, friction test, and TEM and FM observations of the Co–Cr–Mo samples whose surfaces were modified by the MPC polymer. It should be noted that the porous structure (low density) of the PMB30-adsorption and PMSi90-immobilization layer (in dry conditions) hardly affected the protein adsorption. Therefore, the Co–Cr–Mo sample whose surface is modified by the MPC polymer is expected to exhibit tissue and blood compatibility, i.e., biocompatibility, because previous studies have reported that the MPC polymer-modified surfaces exhibit *in vivo* biocompatibility [6–16].

## 5. Conclusions

In this study, we systematically investigated the surface properties of the various surface modification layers formed on the Co–Cr–Mo surface by the MPC polymer by dip coating or photoinduced radical grafting. We conclude that several important issues are involved in the long-term retention of the benefits of the MPC polymer used in artificial joints under variable and multidirectional loads, for example, strong bonding between the MPC polymer and the Co–Cr–Mo surface as also a high density of the MPC polymer. We suggest that the MPSi intermediate layer and photoinduced radical graft polymerization should be employed to create strong covalent bonding between the surface modification layer and Co–Cr–Mo substrate and to retain the high density of the polymer chains of that layer. The cartilage/PMPC-grafted Co–Cr–Mo interface, which mimicked a natural joint, showed an extremely low friction coefficient of <0.01, a value as low as that of a natural cartilage interface. We expect that the PMPC-grafted Co–Cr–Mo femoral head for hemi-arthroplasty will be a promising option for preserving acetabular cartilage and extending the duration before THA.

## Acknowledgements

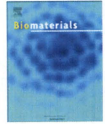
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## Appendix

Figures with essential color discrimination. Certain figures in this article, in particular Figures 8 and 10, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.09.083.

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## Self-initiated surface grafting with poly(2-methacryloyloxyethyl phosphorylcholine) on poly(ether-ether-ketone)

Masayuki Kyomoto<sup>a,b,e</sup>, Toru Moro<sup>b,c</sup>, Yoshio Takatori<sup>b,c</sup>, Hiroshi Kawaguchi<sup>c</sup>, Koza Nakamura<sup>c</sup>, Kazuhiko Ishihara<sup>a,d,\*</sup>

<sup>a</sup> Department of Materials Engineering, School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

<sup>b</sup> Division of Science for Joint Reconstruction, Graduate School of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

<sup>c</sup> Sensory & Motor System Medicine, Faculty of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

<sup>d</sup> Center for NanoBio Integration, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

<sup>e</sup> Research Department, Japan Medical Materials Corporation, 3-3-31, Miyahara, Yodogawa-ku, Osaka 532-0003, Japan

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### ABSTRACT

Poly(ether-ether-ketone) (PEEK)s are a group of polymeric biomaterials with excellent mechanical properties and chemical stability. In the present study, we demonstrate the fabrication of an antibiofouling and highly hydrophilic high-density nanometer-scaled layer on the surface of PEEK by photo-induced graft polymerization of 2-methacryloyloxyethyl phosphorylcholine (MPC) without using any photo-initiators, i.e., “self-initiated surface graft polymerization.” Our results indicated that the diphenylketone moiety in the polymer backbone acted as a photo-initiator similar to benzophenone. The density and thickness of the poly(MPC) (PMPC)-grafted layer were controlled by the photo-irradiation time and monomer concentration during polymerization, respectively. Since MPC is a highly hydrophilic compound, the water wettability (contact angle <10°) and lubricity (coefficient of dynamic friction <0.01) of the PMPC-grafted PEEK surface were considerably lower than those of the untreated PEEK surface (90° and 0.20, respectively) due to the formation of a PMPC nanometer-scale layer. In addition, the amount (0.05 µg/cm<sup>2</sup>) of BSA adsorbed on the PMPC-grafted PEEK surface was considerably lower, that is more than 90% reduction, compared to that (0.55 µg/cm<sup>2</sup>) for untreated PEEK. This photo-induced polymerization process occurs only on the surface of the PEEK substrate; therefore, the desirable mechanical properties of PEEK would be maintained irrespective of the treatment used.

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### 1. Introduction

Poly(aryl-ether-ketone) (PAEK), including poly(ether-ether-ketone) (PEEK), is a new family of high performance thermoplastic polymers, consisting of an aromatic backbone molecular chain interconnected by ketone and ether functional groups, i.e., a benzophenone (BP) unit is included in its molecular structure. Polyaromatic ketones exhibit enhanced mechanical properties, and their chemical structure is stable, resistant to chemical and radiation damage, and compatible with several reinforcing agents (such as glass and carbon fibers); therefore, they are considered to be promising materials for not only industrial applications but also biomedical applications. In the 1980s, the *in vivo* stability of

various PAEK materials and the tissue response to those were investigated [1]. Recently, PEEK has emerged as the leading high-performance super-engineering plastic candidate for replacing metal implant components, especially in the field of orthopedics and spinal surgery [2]. In recent studies, the tribological and bioactive properties of PEEK, which is used as a bearing material and flexible implant in orthopedic and spinal surgeries, has been investigated [3–5]. However, conventional single-component PEEK cannot satisfy these requirements (e.g., antibiofouling, wear resistance, and fixation to a bone) for use as an artificial joint or intervertebral body fusion cage [2]. For further improving the capabilities of PEEK as an implant biomaterial, various studies have focused upon the lubricity and antibiofouling of the polymer, either via reinforcing agents or surface modifications [6,7]. Therefore, multicomponent polymer systems have been designed in order to synthesize new multifunctional biomaterials. In order to use PEEK and related composites in the implant applications, they can be engineered to have a wide range of physical, mechanical, and surface properties.

\* Corresponding author. Department of Materials Engineering, School of Engineering, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8656, Japan. Tel.: +81 3 5841 7124; fax: +81 3 5841 8647.

E-mail address: [ishihara@mpc.t.u-tokyo.ac.jp](mailto:ishihara@mpc.t.u-tokyo.ac.jp) (K. Ishihara).

Surface modification is one of the most important technologies for the preparation of new multifunctional biomaterials for satisfying several requirements; surface modifications used today include coating, blending, and grafting. In general, graft polymerization is performed most frequently using either of the following methods that utilize chemical and/or physical processes: (a) surface-initiated graft polymerization or “grafting from” methods in which the monomers are polymerized from initiators or comonomers, and (b) adsorption of the polymer to the substrate or “grafting to” methods, such as reaction of the end groups of the ready-made polymers with the functional groups of the substrate. The “grafting from” method has an advantage over the “grafting to” method wherein it forms a high-density polymer brush interface with a multifunctional polymer; this advantage proves to be functionally effective.

It is well known that when BP is exposed to photo-irradiation such as ultraviolet-ray (UV)-irradiation, a pinacolization reaction is induced; this results in the formation of semi-benzopinacol radicals (i.e., ketyl radicals) that act as photo-initiators. Therefore, in this study, we have focused upon a BP unit in PEEK and formulated a self-initiated surface-graft polymerization method that utilizes the BP unit in graft-from polymerization (Fig. 1) [8]. This polymerization reaction involving free radicals is photo-induced by UV-irradiation. Under UV-irradiation, a BP unit in PEEK can undergo the following reactions in the monomer aqueous solutions [9–15]: the pinacolization reaction (photo-reduction by the H-abstraction of a BP unit in

PEEK) results in the formation of a semi-benzopinacol radical, which can initiate the graft-from polymerization of the feed monomer as the main reaction, and the graft-to polymerization (the radical chain end of the active-polymer couples with the semi-benzopinacol radical of the PEEK surface) as a sub-reaction. In addition, a photo-scission reaction occurs as a sub-reaction, which may not need an hydrogen (H)-donor. The cleavage reaction induces recombination and the graft-from polymerization. When water polymerization is performed in the presence of an H-donor, a phenol unit may be sub-sequentially formed due to H-abstraction. This technique enables the direct grafting of the functional polymer onto the PEEK surface in the absence of a photo-initiator, thereby resulting in the formation of a C–C covalent bond between the functional polymer and PEEK substrate.

2-Methacryloyloxyethyl phosphorylcholine (MPC), a methacrylate monomer bearing a phosphorylcholine group, is used to synthesize polymer biomaterials having excellent biocompatibility [16–25]; the MPC polymers have potential applications in a variety of fields such as biomedical science, surface science, and bioengineering because they possess unique properties such as excellent antibiofouling, and low friction abilities. Thus, surface modification with the MPC polymer on medical devices is effective for obtaining biocompatibility. Several medical devices have already been developed by utilizing MPC polymers and have been used clinically; therefore, the efficacy and safety of MPC polymers as biomaterials are well-established [23–25].

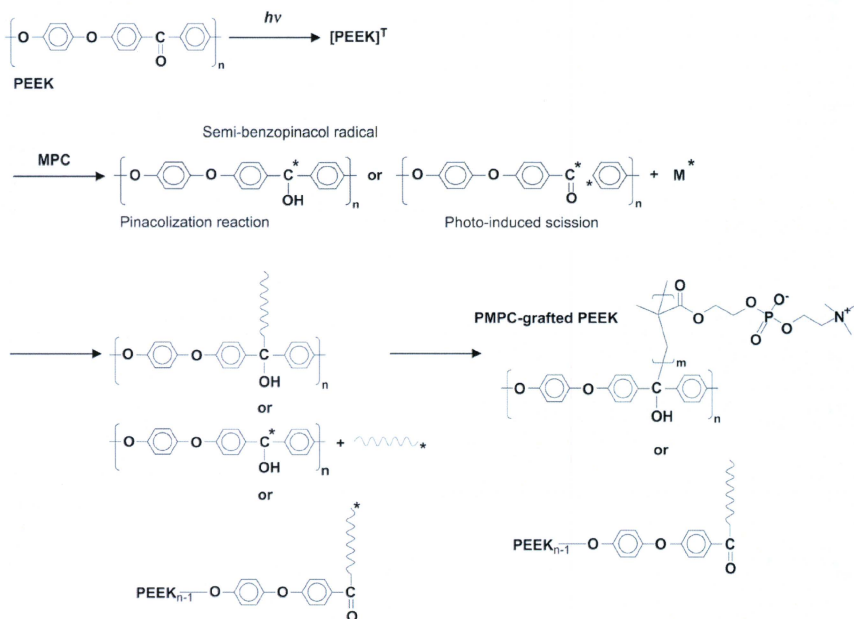


Fig. 1. Schematic illustration for the preparation of PMPC-grafted PEEK.

In this study, we have demonstrated the fabrication of a biocompatible and highly hydrophilic nanometer-scale-modified surface by poly(MPC) (PMPC)-grafting onto the self-initiated PEEK surface using a photo-induced pinacolization reaction; further, we also investigated the effects of photo-irradiation time and MPC concentration variability on PMPC-graft polymerization. The results revealed that it was possible to control the PMPC-graft layer in order to improve wettability, lubricity, and anti-protein adsorption for developing multifunctional PEEK biomaterials.

## 2. Materials and methods

### 2.1. Self-initiated graft polymerization of MPC

The preparation of PMPC-grafted PEEK is schematically illustrated in Fig. 1. PEEK specimens were machined from an extruded PEEK (450 G; Victrex P.L.C. Thornton-Cleveleys, UK) bar stock, which was fabricated without stabilizers or additives. The surfaces of the PEEK specimens were ultrasonically cleaned in ethanol for 20 min, and then dried in vacuum. MPC was industrially synthesized using the method reported by Ishihara et al. [26] and supplied by NOF Corp. (Tokyo, Japan). It was dissolved in degassed water to obtain 0.25– and 0.50-mol/L MPC aqueous solutions, and PEEK specimens were immersed in these solutions. Photo-induced graft polymerization was carried out at 60 °C for 5–90 min on the PEEK surface under UV-irradiation (UVL-400HA ultra-high pressure mercury lamp; Riko-Kagaku Sangyo Co. Ltd., Funabashi, Japan) with an intensity of 5 mW/cm<sup>2</sup>, a filter (model D-35; Toshiba Corp., Tokyo, Japan) was used to restrict the passage of UV-light to wavelengths of 350–50 nm. After polymerization, the PMPC-grafted PEEK specimens were removed from the reaction solution, washed with pure water and ethanol to remove non-reacted monomers and non-grafted polymers, and dried at room temperature.

### 2.2. Surface analysis of PMPC-grafted PEEK

The functional group vibrations of the PMPC-grafted PEEK surfaces were examined by Fourier-transform infrared (FT-IR) spectroscopy with attenuated total reflection (ATR) equipment. The FT-IR/ATR spectra were obtained using an FT-IR analyzer (FT/IR1615; JASCO Co. Ltd., Tokyo, Japan) for 32 scans over the range of 1000–1800 cm<sup>-1</sup> at a resolution of 4.0 cm<sup>-1</sup>. The surface elemental conditions of the PMPC-grafted PEEK surfaces were analyzed by X-ray photoelectron spectroscopy (XPS). The XPS spectra were obtained using an XPS spectrophotometer (AXIS-HSI165; Kratos/Shimadzu Co., Kyoto, Japan) equipped with a 15-kV Mg-K<sub>α</sub> radiation source at the anode. The take-off angle of the photoelectrons was maintained at 90°. Five scans were taken for each sample.

The static-water contact angles on the PMPC-grafted PEEK surfaces were measured by the sessile drop method using an optical bench-type contact angle goniometer (Model DM200; Kyowa Interface Science Co. Ltd., Saitama, Japan). Drops of purified water (10 μL) were deposited on the PMPC-grafted PEEK surfaces, and the contact angles were directly measured with a microscope after 60 s of drooping. Measurements were repeated fifteen times for each sample, and the average values were regarded as the contact angles.

### 2.3. Cross-sectional observation by transmission electron microscopy

A cross-section of the PMPC layer on the PMPC-grafted PEEK surface was observed using a transmission electron microscope (TEM). The specimens were first embedded in epoxy resin, stained with ruthenium oxide vapor at room temperature, and then sliced into ultra-thin films (approximately 100-nm thick) by using a Leica Ultra Cut UC microtome (Leica Microsystems, Ltd., Wetzlar, Germany). A JEM-1010 electron microscope (JEOL Ltd., Tokyo, Japan) was used for the TEM observation at an acceleration voltage of 100 kV. The thickness of the PMPC layer was determined by averaging ten points on the cross-sectional TEM image.

### 2.4. Gravimetric measurement of PMPC-grafted layer

The PMPC-grafted PEEK specimens were weighed on a microbalance (Sartorius Supremicro S4; Sartorius AG, Goettingen, Germany) to determine the physical properties of the PMPC-grafted layer. The physical properties were obtained by the following equations:

$$\text{PMPC-graft extent (g/cm}^2\text{)} = (W_g - W_0)/S \quad (1)$$

$$\text{PMPC-graft layer density (g/cm}^3\text{)} = (W_g - W_0)/S \times T \quad (2)$$

where  $W_0$  is the initial weight of the untreated PEEK substrate,  $W_g$  is the weight of the PMPC-grafted PEEK in dry condition,  $S$  is the grafted surface area of the PEEK substrate, and  $T$  is the thickness of the PMPC-graft layer determined by cross-sectional TEM observation. The weighing was repeated five times for each sample, and the average values were regarded as the weight of the samples.

### 2.5. Characterization of protein adsorption by micro-biochemical assay method

The amount of protein adsorbed on the PMPC-grafted PEEK surfaces was measured by the micro-biochemical assay (BCA) method. Each specimen was immersed in Dulbecco's phosphate-buffered saline (PBS, pH 7.4, ion strength = 0.15 M; Immuno-Biological Laboratories Co. Ltd., Takasaki, Japan) for 1 h to equilibrate the PMPC-grafted surface. The specimens were immersed in bovine serum albumin (BSA,  $M_w = 6.7 \times 10^5$ ; Sigma-Aldrich Corp., MO, USA) solution at 37 °C for 1 h. The protein solution was prepared in a BSA concentration of 4.5 g/L, i.e., 10% of the concentration of human plasma levels. Then, the specimens were rinsed five times with fresh PBS and immersed in 10.0 g/L sodium dodecyl sulfate (SDS) aqueous solution and shaken at room temperature for 1 h to completely detach the adsorbed BSA on the PMPC-grafted surface. A protein analysis kit (micro BCA protein assay kit, #23235; Thermo Fisher Scientific Inc., IL, USA) based on the BCA method was used to determine the BSA concentration in the SDS solution, and the amount of BSA adsorbed on the PMPC-grafted PEEK surface was calculated.

### 2.6. Friction test

The friction test was performed using a pin-on-plate machine (Tribotest 320; Shinto Scientific Co. Ltd., Tokyo, Japan). Each of the PMPC-grafted PEEK surfaces was used to prepare five sample pieces. A 9-mm-diameter pin with Co-Cr-Mo alloy was prepared. The surface roughness ( $R_a$ ) of the pin was <0.01, which was comparable to that of femoral head products. The friction tests were performed at room temperature with a load of 0.98 N, sliding distance of 25 mm, and a frequency of 1 Hz for a maximum of 100 cycles. Pure water was used as a lubricant. The mean coefficients of dynamic friction were determined by averaging five data points from the 100 (96–100) cycle measurements.

### 2.7. Mechanical test

The mechanical properties of untreated PEEK and PMPC-grafted PEEK with a 0.50-mol/L MPC concentration and 90-min photo-irradiation time were evaluated with tensile and flexural tests. Tensile testing was performed according to ISO527 standard using a type 1B tensile bar specimen and a crosshead speed of 50 mm/min. Flexural testing was performed according to ISO178 standard with a crosshead speed of 2 mm/min. The results derived from each measurement in the mechanical test were expressed as the mean values  $\pm$  standard deviation. The statistical significance ( $p < 0.05$ ) was estimated by Student's  $t$ -test.

## 3. Results

Fig. 2 shows the FT-IR/ATR and XPS spectra of untreated PEEK and PMPC-grafted PEEK with a 0.50-mol/L MPC concentration and 90-min photo-irradiation time. Transmission absorption peaks were observed at 1600, 1490, 1280, 1190, and 1160 cm<sup>-1</sup> for both untreated PEEK and PMPC-grafted PEEK (Fig. 2(A)). These peaks are chiefly attributed to the diphenyl ether group, phenyl rings, or aromatic hydrogens in the PEEK substrate [8,27]. However, absorption peaks at 1720 and 1080 cm<sup>-1</sup> (shoulder peak) were observed only for PMPC-grafted PEEK. These peaks corresponded to the carbonyl group (C=O) and phosphate group (P=O) in the MPC unit, respectively [8,25]. The XPS spectra of the binding energy region of the nitrogen (N) and phosphorus (P) elements showed peaks for untreated PEEK and PMPC-grafted PEEK, whereas, peaks were not observed in the case of untreated PEEK (Fig. 2(B)). The peaks at 403 and 134 eV were attributed to the -N<sup>+</sup>(CH<sub>3</sub>)<sub>3</sub> and phosphate groups, respectively. These peaks indicate the presence of phosphorylcholine in the MPC units. After PMPC-grafting, the peaks attributed to the MPC unit were clearly observed in both the FT-IR/ATR and XPS spectra of the PMPC-grafted PEEK. These results indicate that PMPC was successfully grafted on the PEEK surface [8,25].

Fig. 3 shows the N and P concentrations of the PMPC-grafted PEEK as a function of the photo-irradiation time during polymerization at 0.25- and 0.50-mol/L MPC concentrations. The N and P concentrations increased with the photo-irradiation time. When the photo-irradiation time was shorter than 45 min, the N and P concentrations of the PMPC-grafted PEEK surface with a 0.50-mol/L MPC concentration were higher as compared with those with a 0.25-mol/L MPC concentration. The N and P concentration in the PMPC-grafted PEEK with both 0.25- and 0.50-mol/L MPC

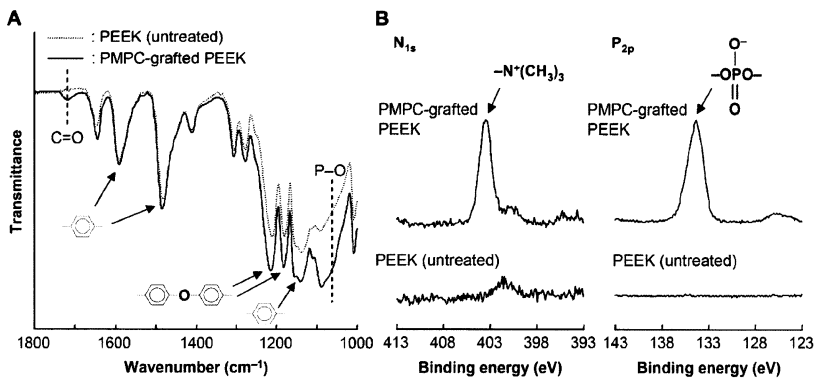


Fig. 2. FT-IR/ATR (A) and XPS (B) spectra of untreated and PMPC-grafted PEEK.

concentrations increased to 5.2 up to a photo-irradiation time of 90 min; those values were almost equivalent to the theoretical elemental composition (N = 5.3, P = 5.3) of PMPC. These results indicate that the PMPC layer fully covered the surface of the PEEK substrate.

Fig. 4 shows the cross-sectional TEM images of PMPC-grafted PEEK obtained with a 0.50-mol/L concentration and various photo-irradiation times as well as the PMPC-graft layer thickness determined by the TEM observations. With photo-irradiation times longer than 45 min, a uniform PMPC layer was clearly observed on the PEEK surface (Fig. 4(A)). The PMPC-graft layer thickness increased with the photo-irradiation time (Fig. 4(B)). In the case of both 0.25- and 0.50-mol/L MPC concentrations, when the photo-irradiation time was greater than 45 min, the PMPC-graft layer thicknesses became almost constant at 40 and 100, respectively.

Fig. 5 shows the physical properties of PMPC-graft layer on the PEEK surface as a function of the photo-irradiation time at MPC concentrations of 0.25 and 0.50 mol/L. The PMPC-graft extent of the PMPC-grafted PEEK with both 0.25- and 0.50-mol/L MPC concentration increased gradually with the photo-irradiation time (Fig. 5(A)). The PMPC-graft layer density of the PMPC-grafted PEEK with 0.25-mol/L MPC concentration increased proportionally to 2.3 g/cm<sup>3</sup> with the photo-irradiation time. In the case of PMPC-grafted PEEK obtained with 0.50-mol/L MPC concentration, the PMPC-graft layer density rapidly increased to 1.3 g/cm<sup>3</sup> up to a photo-irradiation time of 10 min; it then increased slowly to 2.2 g/cm<sup>3</sup> with an increase in the photo-irradiation time.

Fig. 6 shows the static-water contact angle of PMPC-grafted PEEK obtained with MPC concentrations of 0.25 and 0.50 mol/L as a function of the photo-irradiation time. The static-water contact angle of untreated PEEK was 90° and decreased markedly with an increase in the photo-irradiation time. When the photo-irradiation time was 90 min, the static-water contact angle of PMPC-grafted PEEK was the lowest value at <10°.

Fig. 7 shows the coefficient of dynamic friction of PMPC-grafted PEEK obtained with 0.25- and 0.50-mol/L MPC concentrations and various photo-irradiation times. For PMPC-grafted PEEK obtained with both 0.25- and 0.50-mol/L MPC concentrations, these coefficients of dynamic friction decreased markedly with an increase in photo-irradiation time. The PMPC-grafted PEEK obtained with

90 min photo-irradiation time was the lowest at <0.01, and exhibited approximately 95% reduction in their coefficients of dynamic friction when compared with the untreated PEEK.

Fig. 8 shows the amount of adsorbed BSA of PMPC-grafted PEEK as a function of the photo-irradiation time with MPC concentrations of 0.25 mol/L and 0.50 mol/L. The amount of adsorbed BSA of PMPC-grafted PEEK decreased remarkably with an increase in photo-irradiation time.

The mechanical properties of untreated PEEK and PMPC-grafted PEEK are summarized in Table 1. Tensile properties and flexural modulus did not differ significantly ( $p > 0.05$ ) between untreated PEEK and PMPC-grafted PEEK. In contrast, there was a small but significant difference ( $p < 0.05$ ) in the flexural strength and strain of untreated PEEK and PMPC-grafted PEEK examined in this study. However, both untreated PEEK and PMPC-grafted PEEK met the ASTM F2026 requirements [28].

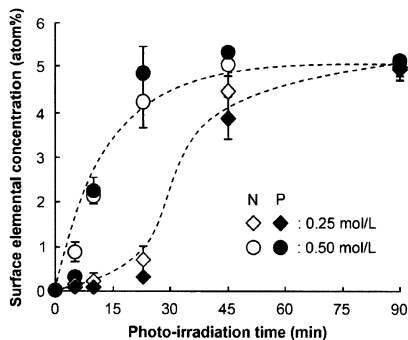
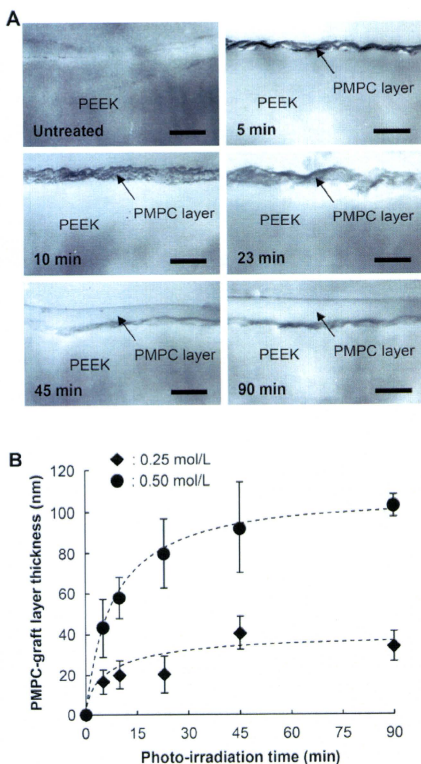


Fig. 3. Surface elemental concentration ( $n = 5$ ) of PMPC-grafted PEEK as a function of the photo-irradiation time with MPC concentrations of 0.25 and 0.50 mol/L. Bar: Standard deviation.



**Fig. 4.** Cross-section of the PMPC-grafted PEEK. (A) Cross-sectional TEM images of PMPC-grafted PEEK obtained with a MPC concentration of 0.5 mol/L and various photo-irradiation times. Bar: 100 nm. (B) PMPC-graft layer thickness ( $n = 10$ ) determined by TEM observation. Bar: Standard deviation.

#### 4. Discussion

In this study, we demonstrated the fabrication of a biocompatible and highly hydrophilic nanometer-scale-modified surface by PMPC-grafting onto the self-initiated PEEK surface using a photo-induced grafting from polymerization reaction, i.e., "self-initiated surface graft polymerization." The following methods were employed in our study: (a) grafting from polymerization for the formation of a high-density graft polymer layer, (b) photo-induced polymerization in the absence of photo-initiators, and (c) using biocompatible hydrophilic macromolecules, which exhibited photo-reduction by H-abstraction of a BP unit in PEEK from an H-donor; this induced surface-initiated graft polymerization of the feed methacrylate-type monomer (i.e., MPC) on the PEEK surface, even in the absence of a photo-initiator such as BP. This report discusses the structure of the PMPC layer of PMPC-grafted PEEK in

terms of the condition variability of self-initiated surface graft polymerization and its effects on antifouling and hydrophilicity.

It is important to control the graft layer thickness and density for optimizing several areas of applications, e.g., in adhesion, colloid stabilization, and lubrication. In Fig. 4, when the photo-irradiation time was greater than 45 min, the PMPC-graft layer thicknesses became almost constant. Moreover, at the same photo-irradiation time of 90 min, it was shown that the higher the monomer concentration, the thicker the graft layer obtained: the PMPC-graft layer thickness (approximately 100 nm) of the PMPC-grafted PEEK obtained with 0.50-mol/L MPC concentration was thicker than that (approximately 40 nm) with 0.25-mol/L MPC concentration. The phenomenon can be attributed to the fact that the graft layer thickness increases with monomer concentration. When the PMPC layer has a brush-like structure, the graft layer thickness may correlate with the molecular weight of the grafted PMPC. The high-density PMPC-graft layer on the PEEK surface is assumed to exhibit a brush-like structure [29,30]. It is generally well known that the reaction rate of radical polymerization is extremely high [31]. It was observed that the graft layer thickness (i.e., molecular weight of the graft polymer) was greatly dependent on the monomer concentration, but virtually independent of the photo-irradiation time. Thus, in this study, the length (molecular weight) of the PMPC-graft chains was assumed to be successfully controlled by the MPC concentration used for polymerization. This indicates that the length of the PMPC chain grafted on the PEEK surface increased with the MPC concentration during polymerization [32]. Additionally, a uniform PMPC layer was clearly observed on the surface of the PEEK substrate, and no cracks or delamination were observed at the PEEK substrate or the interface between the PMPC layer and PEEK substrate. These results indicate that the PMPC layer formed on the PEEK substrate is uniformly distributed over the substrate and is bound to the substrate by covalent C–C bonds.

On the other hand, the PMPC-graft layer density on the PEEK surface almost linearly increased to  $>2.2 \text{ g/cm}^3$  with the photo-irradiation time, suggesting that the graft chain propagates steadily with increasing photo-irradiation time (Fig. 5(B)). In order to obtain the high-density PMPC-graft layer, the photo-irradiation time must be controlled. Further, interestingly, while using an MPC concentration of 0.50 mol/L, the rate of the increase in the PMPC-graft layer density was low with a photo-irradiation time above 10 min. The present self-initiated surface graft polymerization method is photo-induced by UV-irradiation onto the BP unit of PEEK surface. In this study, it is assumed that the UV-irradiation directly produces a high-concentration free radical, because this PEEK is a semi-crystalline structure (crystallinity, 30–40%) with a high-density BP unit in the surface. When the MPC concentration in a feed is also high, the self-initiated surface graft polymerization between the radicals on the PEEK surface and the MPC monomer occurs extremely rapidly in the reaction system, forming the high-density graft chain on the surface. Hence, diffusion of the MPC monomer onto the PEEK surface might be interfered by the high-density graft chain because of its high viscosity. When the monomers attached to the PEEK surface were subjected to UV-irradiation, radicals were freely formed on the PEEK surface in the early stage but not in the late stage of polymerization, probably because the high-density grafted polymer chains formed by then blocked the diffusion of the monomer to the PEEK surface. Therefore, it is supposed that the rate of increase in the PMPC-graft layer density changed due to the high concentration of free radicals and monomers.

The water-wettability of the PMPC-grafted PEEK surface is considerably greater than that of the untreated PEEK surface (Fig. 6), because of the presence of a nanometer-scaled PMPC layer (Fig. 4(A)): MPC is a highly hydrophilic compound, while PMPC is water-soluble. In Figs. 4–7, we observe that the dynamic coefficient

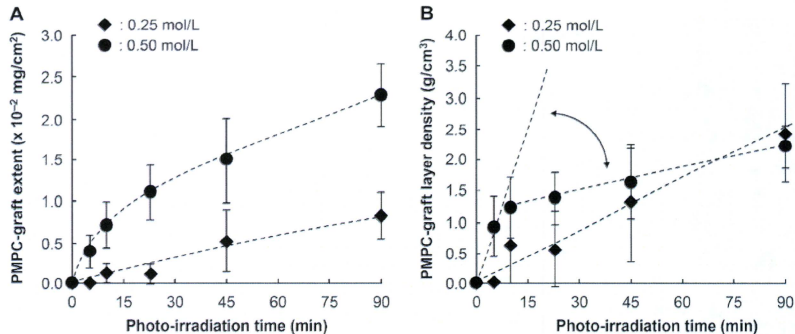


Fig. 5. Physical properties ( $n = 5$ ) of PMPC-graft layer on PEEK surface as a function of the photo-irradiation time with MPC concentrations of 0.25 and 0.50 mol/L. (A) PMPC-graft extent by weighing, and (B) density calculated by (A) and mean PMPC-graft layer thickness of Fig. 4(B). Bar: Standard deviation.

of friction was greatly dependent on the water-wettability (static-water contact angle), but virtually independent of the structure (thickness and density) of the graft layer. A significant reduction in the static-water contact angle of the PMPC-grafted surface resulted in a substantial improvement in friction property. Fluid-film lubrication (or hydration lubrication) with the PMPC-grafted surface was achieved by the intermediate hydrated layer. It can be affirmed that this highly lubricated surface utilizing PMPC mimics the natural cartilage structure [33]. When the PEEK surface is modified by PMPC-grafting, the grafted PMPC causes a significant reduction in sliding friction between the graft surfaces because the thin water films that are formed act as extremely efficient lubricants. The water-lubrication systems utilizing PMPC suppress direct contact of the counter-bearing face with the PEEK substrate in order to reduce the frictional force. Thus, the PMPC-graft layer is expected to significantly increase the durability of the bearing biomaterials. On the other hand, when the photo-irradiation time was shorter than 23 min, the decrease in the static-water contact

angle of the PMPC-grafted PEEK surface with a 0.25-mol/L MPC concentration was only slight. It was thought that the surface phenomenon was caused by the pinacolization reaction, because the static-water contact angle of the UV-irradiated PEEK surface without monomer was decreased to approximately 80° (data not shown).

The fabrication of surfaces that exhibit anti-protein adsorption and/or cell adhesion has been one of goals of surface engineering for medical devices. The adsorption of the BSA on the PMPC-grafted PEEK surface decreased to 10% ( $p < 0.001$ ) with an increase in photo-irradiation time, as shown in Fig. 8, as compared to that in untreated PEEK. It is shown that the extent of protein rejection is related to the PMPC-graft extent (Figs. 5(A) and 8). Extensive grafting gives rise not only to an increase in the thickness of graft layer but also to an increase in the volume fraction (i.e., density) of graft segments in the layer. Therefore, it was thought that these characteristics of thickness and density of the PMPC-graft layer had a significant influence on protein adsorption. The mechanism of protein adsorption resistance on the PMPC-grafted surface is

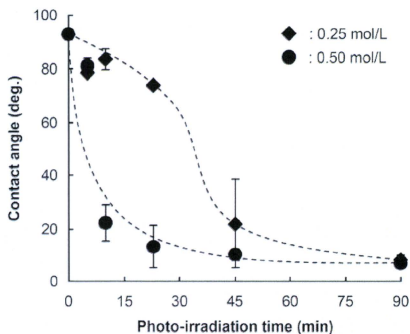


Fig. 6. Static-water contact angle ( $n = 15$ ) of PMPC-grafted PEEK as a function of the photo-irradiation time with MPC concentrations of 0.25 and 0.50 mol/L. Bar: Standard deviation.

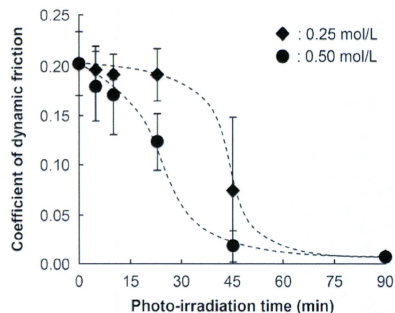


Fig. 7. Coefficient of dynamic friction ( $n = 5$ ) of PMPC-grafted PEEK as a function of the photo-irradiation time with MPC concentrations of 0.25 and 0.50 mol/L. Bar: Standard deviation.



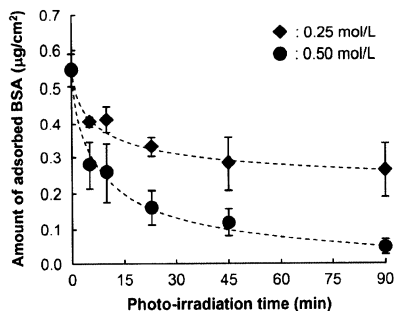


Fig. 8. Amount of adsorbed BSA ( $n = 3$ ) of PMPC-grafted PEEK as a function of the photo-irradiation time with MPC concentrations of 0.25 and 0.50 mol/L. Bar: Standard deviation.

hypothesized as follows: the protein adsorption resistance attributed to the water-fluid film and hydration layer was due to water and hydrophilic macromolecules with volume exclusion effects. The presence of the water-fluid film and hydration layer is responsible for the easy detachment of proteins and the prevention of conformational changes in the adsorbed proteins [34]. These observations are consistent with the results of the static-water-contact angle measurements, cross-sectional TEM observations, and PMPC-graft layer weighting of the PEEK whose surface was modified by PMPC-grafting. These results imply that the PMPC-grafted PEEK surface is biocompatible in terms of tissue and blood compatibility, because MPC polymer-modified surfaces are known to exhibit *in vivo* antibiofouling [16,20,23,24].

As shown in Table 1, the mechanical properties of the PEEK are unchanged even after PMPC-grafting. This indicates that the photo-induced radical graft polymerization proceeds only on the surface of the PEEK substrate, while the properties of the substrate remain unchanged. Retention of the properties of the PEEK substrate is very important in clinical use, because the biomaterials used in implants act not only as surface-functional materials but also as structural materials *in vivo*.

The design of a well-characterized surface is a considerably important and difficult task. The photo-induced graft polymerization in the absence of photo-initiators of this study, i.e., “self-initiated surface graft polymerization” successfully prepared the surface with controlled graft layer thickness and density. This simple self-initiated surface graft polymerization would be highly suitable for industrial applications [35,36] as well as the development of medical devices [2–7]. The synthesis of a self-initiated biocompatible polymer having unique properties such as anti-

protein adsorption and wettability by the photo-induced grafting-from polymerization reaction is a novel phenomenon in the field of biomaterials and bioengineering sciences, and the fabrication of the PMPC-grafted PEEK surface can result in the development of next-generation multifunctional biomaterials.

## 5. Conclusions

A biocompatible and highly hydrophilic nanometer-scale-modified surface was successfully fabricated on the PEEK substrate by the photo-induced graft polymerization of PMPC in the absence of photo-initiators, i.e., “self-initiated surface graft polymerization.” Since MPC is a highly hydrophilic compound, the water wettability and lubricity of the PMPC-grafted PEEK surface were greater than that of the untreated PEEK surface due to the formation of a PMPC nanometer-scale layer. In addition, the amount of BSA adsorbed on the PMPC-grafted PEEK surface considerably decreased compared to that in the case of untreated PEEK. The density and thickness of the grafting layer could be controlled by the photo-irradiation time and monomer concentration.

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Table 1  
Mechanical properties ( $n = 10$ ) of untreated and PMPC-grafted PEEK.

Test method	Property	PEEK (untreated)	PMPC-grafted PEEK	t-Test
Tensile test	Yield strength (MPa)	109.4 (0.4) <sup>a</sup>	109.9 (0.6)	N.S.
	Ultimate strength (MPa)	71.5 (1.5)	71.7 (1.7)	N.S.
	Elongation (%)	24.0 (9.5)	24.4 (9.0)	N.S.
Flexural test	Ultimate strength (MPa)	168.9 (0.6)	173.7 (1.8)	<0.05
	Ultimate strain (%)	6.4 (0.1)	6.7 (0.2)	<0.05
	Modulus (GPa)	3.9 (0.0)	3.9 (0.0)	N.S.

<sup>a</sup> The standard deviations are shown in parenthesis.

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## Cell-penetrating macromolecules: Direct penetration of amphipathic phospholipid polymers across plasma membrane of living cells

Tatsuro Goda<sup>a,c,1</sup>, Yusuke Goto<sup>a,c</sup>, Kazuhiko Ishihara<sup>a,b,c,\*</sup>

<sup>a</sup> Department of Materials Engineering, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan

<sup>b</sup> Department of Bioengineering, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan

<sup>c</sup> Center for NanoBio Integration, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan

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### ABSTRACT

Nanoscaled materials are normally engulfed in endosomes by energy-dependent endocytosis and fail to access the cytosolic cell machinery. Although some biomolecules may penetrate non-endocytically or fuse with plasma membranes without overt membrane disruption, to date no synthetic macromolecule of comparable size has been shown to exhibit this property. Here, we discovered mechanism of direct cell membrane penetration using synthetic phospholipid polymers. These water-soluble amphiphilic phospholipid polymers enter the cytoplasm of living mammalian cells in vitro within a few minutes without overt bilayer disruption even under conditions where energy-dependent endocytic uptakes are blocked. Furthermore, targeted cytosolic distribution to cell organelles was achieved by selecting specific fluorescent tags to the polymers. Thus, the phospholipid polymers can provide a new way of thinking about access to the cellular interior, namely direct membrane penetration.

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### 1. Introduction

Nanomaterials are of great interest for their potential biomedical applications as imaging tools [1,2] therapeutic agents [3], and drug/gene delivery carriers [4,5]. However, the plasma membrane of eukaryotic cells constitutes a highly selective permeability barrier that prevents the passage of nanomaterials, allowing only small, uncharged molecules to gain access to the cell interior. Thus, translocation of nanomaterials across plasma membranes is currently one of the most actively researched topics in nanobiology. The most common mechanism of cellular uptake for nanomaterials is endocytosis, an energy-dependent process in which the nanomaterial is trapped within endosomes that carry ingested material into the cellular interior while the engulfed materials do not gain access to the cytosol [6]. Cationically charged nanomaterials, such as dendrimers [7], quantum dots [8], and polymeric micelles [9], can pass through anionic plasma membranes by causing transient pore formation, a process that is also associated with cytotoxicity. Alternatively, nanoparticles coupled with exogenous cell-

penetrating peptides (CPPs) have been designed to explicitly disrupt the endolysosomal membrane, thereby facilitating entry into the cytosol [10,11]. CPP-conjugated nanomaterials may be capable of directly penetrating the membrane without overt lipid bilayer disruption. To the best of our knowledge, however, no synthetic macromolecules larger than a few nanometers in size can penetrate directly through the plasma membrane without disturbing the integrity of the biological barriers.

Phospholipid molecules play a crucial role in determining the composition of the cytosol by forming a bilayer structure with barrier properties and mass transport function. In addition, they form a bio-inert surface in the cell membrane that facilitates highly efficient, selective, and specific biological reactions. By mimicking the chemical functionality as well as the molecular structure of phospholipids in the plasma membrane, we have synthesized phospholipid polymers comprising 2-methacryloyloxyethyl phosphorylcholine (MPC) as a polar group and *n*-butyl methacrylate (BMA) as a hydrophobic part [12]. The poly(MPC-co-BMA) (PMB), which forms nanoassemblies (polymeric aggregates) or nanoparticles in aqueous media depending on the degree of hydrophobicity and molecular weight of the polymers, has considerable potential for therapeutic nanomaterials [13]. Previously, a PMB carrying an antineoplastic drug was demonstrated to exhibit antitumor effects on cancer cells without cytotoxicity both *in vitro* and *in vivo* [14–16]; however, the mechanism whereby the drug in the PMB was delivered across the plasma membrane of cells remains

\* Corresponding author. Department of Materials Engineering, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan. Tel.: +81 3 5841 7124; fax: +81 3 5841 8647.

E-mail address: [ishihara@mpec.t.u-tokyo.ac.jp](mailto:ishihara@mpec.t.u-tokyo.ac.jp) (K. Ishihara).

<sup>1</sup> Current address: Biomaterials Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan.

poorly understood. In this study, we discovered direct penetration mechanism across the plasma membrane of living mammalian cells using fluorescence-tagged PMBs.

## 2. Materials and methods

### 2.1. Materials

MPC, which was synthesized as previously described [17], was purchased from NOF (Tokyo, Japan). The commercially available reagents *n*-butyl methacrylate (BMA; Nacal Tesque, Tokyo, Japan), methacryloxyethyl thiocarbonyl rhodamine B (MTR; Polysciences, PA, USA), *o*-methacryloyl Hoechst 33258 (Polysciences), fluorescein *o*-methacrylate (Polysciences), 1-pyrenylmethyl methacrylate (Polysciences), *t*-butyl peroxydecanoate (Perbutyl-ND; NOF), and 2,2'-azobisisobutyronitrile (AIBN; Kanto Chemical Co., Tokyo, Japan) were of extra pure grade and used without further purification. Unless stated otherwise, all the other materials were purchased from commercial sources and used as received.

### 2.2. Synthesis of fluorescent polymers

The poly(MPC-co-BMA) with covalent labeling of rhodamine B (rhoPMB) was synthesized using a free radical polymerization technique with Perbutyl-ND as an initiator. For rhoPMB30 synthesis, MPC (2.66 g, 9.00 mmol), BMA (2.98 g, 21.0 mmol), and MTR (20.5 mg, 30.0 μmol) were dissolved in ethanol (30.0 mL) and argon gas bubbled through the resulting solution for 30 min in order to remove dissolved oxygen. Perbutyl-ND (70 wt% in hydrocarbons, 0.367 g, 1.05 mmol) was then added to the solution and the temperature was elevated to 60 °C for 12 h to induce polymerization. Thereafter, the polymer was reprecipitated twice in diethylether/chloroform (7/3 v/v). The polymer was then dialyzed against water through a regenerative cellulose membrane (MWCO, 3.5 kDa) for 7 days, and subsequently ultrafiltered in methanol/water (7/3 v/v) for 7 days (MWCO, 5.0 kDa) to remove unreacted monomers and absorbed dyes. rhoPMB50, rhoPMB80 and rhodamine B-labeled poly(MPC) (rhoMPMC; MTR = 100/0.1 mol/mol) were synthesized using the same method. Hoechst 33258-tagged PMB30 (hoehchstPMB30) and FITC-tagged PMB30 (fitcPMB30) were synthesized using the same method used to synthesize rhoPMB30, but using *o*-methacryloyl Hoechst 33258 and fluorescein *o*-methacrylate, respectively, instead of MTR. High molecular weight polymers were synthesized using a free radical polymerization technique with AIBN (4.90 mg, 30.0 μmol) instead of perbutyl-ND as an initiator, and were purified using the same method described above. The high molecular weight polymers were dialyzed against water through a regenerative cellulose membrane (MWCO, 50 kDa).

### 2.3. Cell culture

The human Caucasian hepatocyte carcinoma (HepG2) cell line (RIKEN BioResource Center, Ibaraki, Japan) was seeded on tissue culture polystyrene ( $1.0 \times 10^6$  cells/cm<sup>2</sup>) in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco), and penicillin/streptomycin (100 μg/mL, Gibco) at 37 °C in 5% CO<sub>2</sub>. Sub-confluent cultures (70–80%) were passaged using 0.25% trypsin/EDTA at 37 °C in 5% CO<sub>2</sub>. HeLa cells (RIKEN BioResource Center) were passaged at sub-confluency in DMEM containing 10% FBS.

### 2.4. Cellular uptake of fluorescent-labeled polymers

Cells were seeded into glass-bottom dishes (0.12–0.17 mm thick; Matsunami, Osaka, Japan) at  $5.0\text{--}6.0 \times 10^4$  cells/mL in 1.0 mL of culture medium and grown for 1 day before incubation with the fluorescent polymers. The cells were then rinsed 3 times with DPBS. The medium was subsequently exchanged with fresh warm serum-free or serum-containing (10% FBS) DMEM and fluorescent polymers were added from a concentrated stock solution in DMEM at a final concentration of 1.0 mg mL<sup>-1</sup>. Cells were incubated with fluorescence-tagged polymer at 37 °C in 5% CO<sub>2</sub> for the desired length of time. After incubation with the polymer, the cells were rinsed 3 times with DPBS and placed in 1.0 mL of DMEM for live cell imaging. Staining of cells under conditions of energy depletion was performed by incubating the cells with 3.0 mM sodium azide and 50 mM 2-deoxy- $\alpha$ -glucose in DPBS (ATP depletion solution). Cells were pre-incubated in ATP depletion solution at 37 °C for 30 min and then incubated with 1.0 mg mL<sup>-1</sup> fluorescent polymers in ATP depletion medium at 37 °C in 5% CO<sub>2</sub>. The cells were then washed 3 times with DPBS and placed in 1.0 mL of DMEM. Staining under conditions inhibitory to the mechanism of active uptake by cells was performed by incubating at 4 °C. Cultured cells were washed 3 times with DPBS and precolored in serum-free DMEM at 4 °C for 30 min. The cells were then incubated in serum-free DMEM with 1.0 mg mL<sup>-1</sup> fluorescence-tagged polymer at 4 °C. Thereafter, the cells were rinsed 3 times with DPBS and placed in 1.0 mL of serum-free DMEM.

### 2.5. Confocal fluorescence microscopy studies

The polymers in living cells were imaged using an LSM-510 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 63 $\times$  oil-immersion objective lens with a numerical aperture of 1.4 with respect to the beam waist. Confocal-DIC images were obtained by excitation with a 633-nm HeNe laser line. Fluorescent probes in Hoechst 33258-, FITC-, and rhodamine B-tagged polymers in the cells were excited with a 405-nm line (selected by an extra 405  $\pm$  10 nm interference-based laser cleanup filter) of a blue diode laser, a 488-nm line of an Ar laser, and a 543-nm line of a HeNe laser, respectively. The excitation light was directed to the sample by a dichroic mirror (DC; HFT 405, 488, and 543, respectively). Each fluorescent light was sent through the DC and a long-pass LP 420 nm, band-pass BP 520–550 nm, and LP 560 nm emission filter to an analog photomultiplier tube used for detection. The detector gain was set to 650–850 units in the Zeiss software. Dark signal contributions to the images were effectively zeroed out by setting the proper detection offset value as provided in the Zeiss software. The image size was typically set to 512  $\times$  512 or 1024  $\times$  1024 pixels. Multi-stained cells were imaged using multi-track mode for the prevention of cross-talk. The full cellular thickness (approximately 11 μm) was scanned with the confocal microscope in 1.4-μm steps. Confocal Z-section scanning for imaging of the nucleus was conducted in 0.4-μm steps. The internalization of rhodamine B-tagged polymers into living cells was monitored by time-lapse imaging every 30 s up to 30 min at 37 °C. After rinsing 3 times with DPBS, cultured cells were placed in fresh warm serum-free DMEM and the glass-bottom dish was placed on a temperature control module under a 63 $\times$  oil lens. Time-lapse imaging was started just after the fluorescent polymer was added from a concentrated stock solution in DMEM at a final concentration of 1.0 mg mL<sup>-1</sup>.

### 2.6. Fluorescence correlation spectroscopy (FCS) studies

Scanning measurements were performed using an LSM 510 Meta microscope equipped with a Confocor 3 unit for FCS (Carl Zeiss). MTR and rhodamine B-tagged polymer were excited at 543 nm by the built-in HeNe laser, focused onto the sample by a 40 $\times$  water-immersion objective lens (C-Apochromat) with a numerical aperture of 1.2 relative to the beam waist with  $1/e^2$  radius  $\omega_{0.2} = 0.2$  μm. The pinhole in front of the avalanche photodiode was set to 78 μm. Fluorescence was detected by an avalanche photodiode in the Confocor unit. To select the detected spectral range, an LP 560-nm filter was used for the measurements. The fluorescence temporal signal was recorded, and the autocorrelation function  $G(t)$  was calculated using the internal correlator. Curve fittings were carried out using Confocor, with a triplet state identified in the fitting, and with two-dimensional fitting. The waist radius ( $\omega_0$ ) of the 543-nm laser lines was set at 0.20 μm on the basis of the known diffusion coefficient of rhodamine B in solution ( $D = 28 \times 10^{-6}$  m<sup>2</sup>/s). The effective diffusion coefficients of the fluorescent-tagged polymer ( $D$ ) were determined using the calibrated beam waist and the diffusion time obtained from the autocorrelation curve via the equation  $D = \omega_0^2/4t_D$ , where  $t_D$  is the diffusion time. The temperature dependence of the diffusion coefficient can be written in the form of the Stokes-Einstein equation,  $R_H = k_B/6\pi\eta D$ , where  $R_H$  denotes the hydrodynamic radius,  $k_B$  Boltzmann's constant,  $T$  the absolute temperature, and  $\eta$  the solution viscosity. The autocorrelation curve was generated from 30  $\times$  15-s data collections.

### 2.7. Subcellular distribution of the fluorescent polymers

HepG2 cells were pre-incubated with 100 nM of MitoTracker Green FM (Molecular Probes), which is a specific fluorescence dye for mitochondria, in DMEM containing 10% FBS for 24 h at 37 °C in 5% CO<sub>2</sub>. After rinsing 3 times with DPBS, the stained cells were incubated in serum-free DMEM containing rhoPMB (1.0 mg mL<sup>-1</sup>) at 37 °C in 5% CO<sub>2</sub> for 30 min. The cells were then rinsed 3 times with DPBS and placed in 1.0 mL of DMEM for imaging using an LSM 510 Meta confocal microscope operating in multi-track mode. MitoTracker was excited with a 488-nm line and imaged through band-pass BP 520–550-nm emission filter to the PMT.

### 2.8. Flow cytometry studies

HepG2 cells ( $5.0 \times 10^5$ ) were seeded in 6-well culture plates and grown overnight in DMEM containing 10% FBS at 37 °C in 5% CO<sub>2</sub>. After rinsing 3 times with DPBS, the cells were incubated with fluorescence-tagged polymers under the desired conditions. Thereafter, the cells were rinsed 3 times with DPBS and treated with trypsin/EDTA. The cells were then suspended in DMEM containing 10% FBS and analyzed immediately using an EPICS XL flow cytometer (Beckman Coulter, CA, USA) operating at 488-nm excitation and with either FL-2 or FL-3 emission filters. For the measurement of release of the fluorescence-tagged polymer from the cells, the suspension was incubated for 0–180 min at room temperature before flow cytometric analysis. A minimum of  $1.0 \times 10^4$  cells were analyzed from each sample with fluorescence intensity.

### 2.9. Calcine-AM staining

Calcine-AM (10 μM in DPBS, Invitrogen) was pre-incubated with cells for 30 min in serum-containing DMEM (final concentration: 100 μM). Cells were then rinsed 3