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# Cell adhesion on phase-separated surface of block copolymer composed of poly-(2-methacryloyloxyethyl phosphorylcholine) and poly(dimethylsiloxane)

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ABSTRACT

We investigated the morphological effect of phase-separated block copolymer surfaces composed of poly(2-methacryloyloxyethyl phosphorylcholine (MPC)) (PMPC) and poly(dimethylsiloxane) (PDMS) on protein adsorption and cell adhesion behavior. We observed three different types of phase-separated surface morphologies by TEM and AFM. The elemental composition of phosphorus on the surface increases with the PMPC composition. Furthermore, the polymer surface formed by a block copolymer-containing a higher MPC unit composition shows a slightly lower static water contact angle. This result indicates that the elemental surface ratio of the surface depends on the MPC composition in the block copolymer. Protein adsorption tests revealed that only hydrophobic PDMS domains showed selective protein adsorption. Cell adhesion tests revealed that the number of adhered cells increased with increasing hydrophobic PDMS domain size of block copolymers in serum-containing media. In contrast, no cells adhered onto block copolymer surfaces in serum-free media, whereas a large amount of adhered cells were observed on the hydrophobic PDMS surface. This result indicates that segregated hydrophobic domains on a biocompatible PMPC surface strongly affect serum protein adsorption, thereby promoting considerable cell adhesion, although the surface is hydrophilic. Thus, both the composition of MPC units and the segregated hydrophobic surface morphology are important considerations in biomaterial surface design.



## 1. Introduction

It is well known that significant plasma protein adsorption initially occurs on artificial material surfaces in biological environments. Such adsorbed proteins are believed to mediate cell adhesion and activation, thus promoting platelet adhesion and even thrombosis [1], which are principal drawbacks to medical applications of artificial biomaterials. For that reason, investigation of surface-induced effects of polymer materials, such as roughness and hydrophilicity, on plasma protein adsorption and, ultimately, on cellular adhesion behavior has been a key area of biomaterial research. Over the past few years, several research groups have studied the quantitative relationship between cellular adhesion and surface adsorption of serum proteins [2]. These studies reported that even very low concentrations of cell adhesive serum proteins, such as fibrinogen or von Willebrand's factor adsorbed on hydrophilic material surface, have almost the same effect on platelet adhesion with those of adsorption levels on hydrophobic surfaces [3].

Recently, in addition to the quantitative relationships described above, the spatial relationship of heterogeneous material surfaces and serum protein adsorption has become a matter of concern in investigations of cellular responses on material surfaces. Sousa et al. reported that heterogeneous polymer domains obtained by phase separation of polymer blends induce preferential adsorption behavior of serum proteins [4]. Another study showed that heterogeneous morphologies developed by different monomer compositions of block copolymers had different cell adhesion morphologies with regard to serum proteins [5]. More recently, Arnold et al. reported that only a single cell-adhesive peptide, spatially existing on a poly(ethylene glycol) (PEG) passivated surface, influences cell adhesion behavior [6]. Although several important factors, such as roughness or stiffness of the materials surface, must be considered in order to gain an integral understanding of cellular interactions on surfaces, it is now clear that even a small amount of cell-adhesive serum proteins on heterogeneous material surfaces must be a primary consideration when designing any biomaterial.

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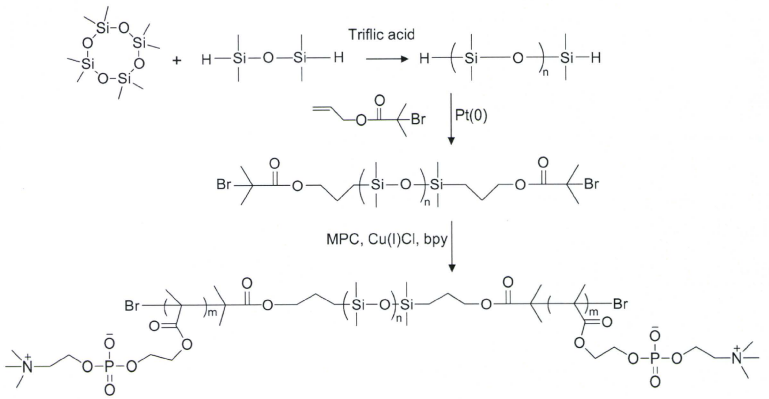



**Scheme 1.** Synthetic route and molecular structure of block copolymer.

**Table 1**
Characterization of synthesizes PDMS and block copolymers.

| | Monomer unit composition (%, NMR) | | Molecular weight ($M_n$, kDa) | | Polydispersity index (PDI) |
|---|---|---|---|---|---|
| | MPC | PDMS | GPC | NMR | |
| PDMS1 | – | – | 1.02 | – | 1.44 |
| PDMS2 | – | – | 4.21 | – | 1.90 |
| PDMS3 | – | – | 7.96 | – | 1.51 |
| PM1 | 88.0 | 12.0 | 36.7 | 31.9 | 1.27 |
| PM2 | 59.3 | 40.7 | 56.7 | 28.3 | 1.35 |
| PM3 | 44.6 | 55.4 | 34.2 | 31.7 | 1.43 |

2-Methacryloyloxytheyl phosphorylcholine (MPC) is a very well known biomaterial that exhibits excellent blood compatibility and biocompatibility by suppression of cellular responses on its surface [7–12]. Because the phosphorylcholine group possesses a large amount of free water, serum proteins can contact the MPC polymer surface in a reversible manner [13]. For that reason, many biomedical interfaces containing MPC polymers have been successfully developed by a wide range of materials preparation methods, such as random copolymerization of MPC with other alkyl methacrylates or surface grafting from various substrates [14–17]. As a result, homogeneously prepared material surfaces derived from MPC polymers have shown excellent antibiofouling and antithrombosis properties, even when they were in contact with whole blood [18].

In addition to homogeneously prepared biomaterials, MPC polymer-containing binary materials, such as polymer blends or block copolymers, have also been introduced for the development of biomedical applications [19–22]. These materials have distinctive physical properties, including flexibility and formability. Thus, a MPC polymer-containing binary material has found application as a safe blood contact material, with use as an artificial blood vessel [23].

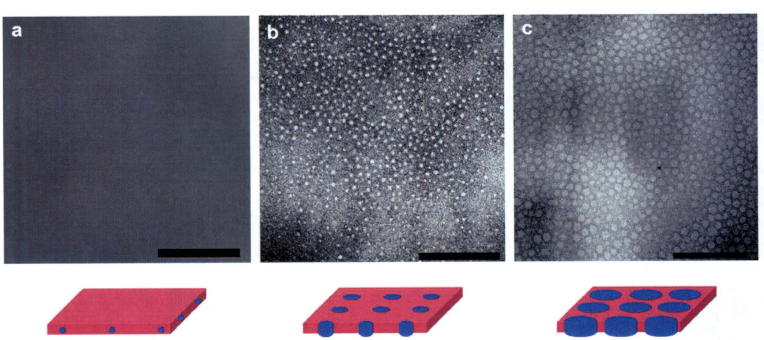



**Fig. 1.** Bright-field TEM images of block copolymers (a) PM1, (b) PM2, and (c) PM3 taken after staining with $OsO_4$. The dark region indicates $OsO_4$ stained PMPC domains and bright region indicates segregated PDMS domains. Scale bar = 300 nm. Red and blue region in the scheme indicate the PMPC and PDMS domains, respectively.

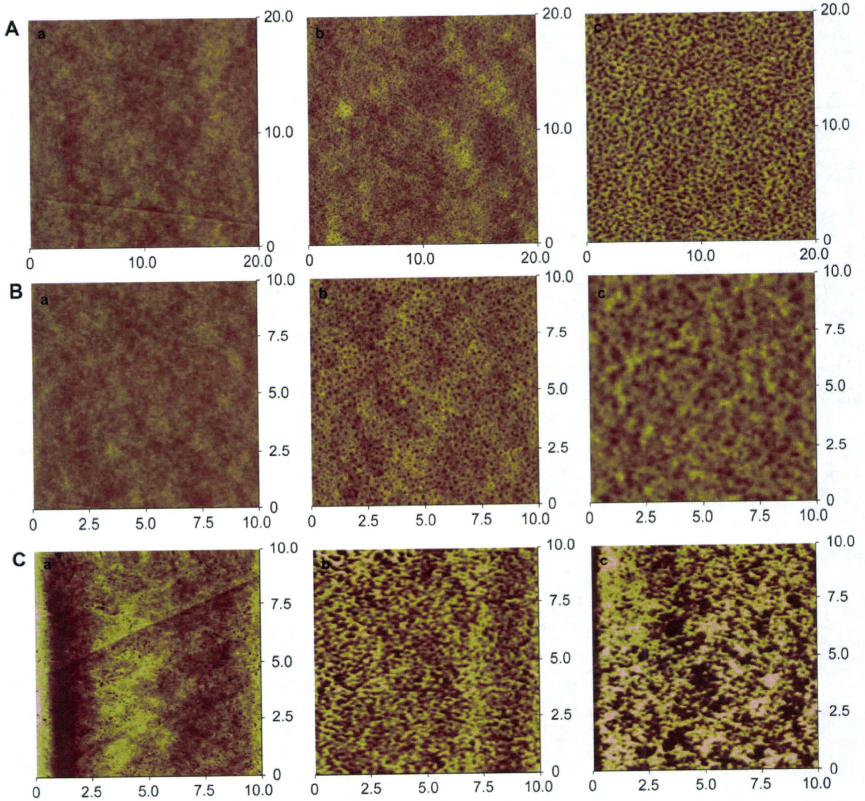



**Fig. 2.** A. AFM topological images of (a) PM1, (b) PM2, and (c) PM3 under dry conditions with 20 μm × 20 μm scan size. B. AFM topological images of (a) PM1, (b) PM2, and (c) PM3 under dry conditions with 10 μm × 10 μm scan size. C. AFM topological images of (a) PM1, (b) PM2, and (c) PM3 taken in PBS with 10 μm × 10 μm scan size.

Although these binary materials have been effective in suppressing thrombus formation or protein adsorption, their surface morphological effects have not been studied extensively. Because contact between each component in these binary materials is unfavorable from an enthalpy standpoint, heterogeneous morphologies induced by phase separation are inevitable in such binary material systems [24]. For that reason, interactions between heterogeneous MPC polymer surfaces and plasma proteins, which could promote cell adhesion behavior as above-mentioned references, have to be investigated for defining cellular responses on MPC polymer-containing binary materials surfaces.

In our current study, we examine the surface morphological effect of a heterogeneous MPC polymer on protein adsorption and cell adhesion behavior under existence of serum proteins. In order to investigate the size effect of segregated hydrophobic domains, we prepared three different compositions of ABA type triblock copolymers composed of poly(MPC) (PMPC) and poly(dimethylsiloxane) (PDMS) by the atom transfer radical polymerization (ATRP) method. As a result, three kinds of heterogeneous MPC polymer surfaces having different hydrophobic domain size were prepared by solvent cast method. Because PDMS is a very well known hydrophobic material that induces significant protein adsorption and cell adhesion on its surface, we expected heterogeneous surfaces developed from block copolymers to contain both biofouling and antibiofouling domains. We focus in this work on the effect of selective hydrophobic interactions between proteins and PDMS domains and the resulting cell adhesion behavior.

## 2. Materials and methods

### 2.1. Materials

MPC was synthesized by a previously reported method [25]. Octamethylcyclotetrasiloxane ($D_4$), 1,1,3,3-tetramethyldisiloxane (TMS), and trifluoromethanesulfonic acid (triflic acid) were purchased from Tokyo Chemical Industry Co., Ltd.

(Tokyo, Japan). Allyl 2-bromoisobutylate, Cu(I)Cl, 2,2′-bipyridyl, 10 nm diameters of gold colloid-labeled immunoglobulin G (IgG, whole molecules from goat), non-labeled IgG, fibronectin (from bovine plasma), and Karstedt's catalyst were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-Methyl-1,4-naphthoquinone, Triton X-100, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and all organic solvents (organic synthesis grade) were purchased from Wako Chemical Co. (Osaka, Japan) and used as-received. Dulbecco's phosphate buffered saline (×10) (PBS; pH 7.4, without calcium chloride and magnesium chloride), Alexa Fluor 488 phalloidin was purchased from Invitrogen Co. (Carlsbad, CA, USA). A micro-BCA™ protein assay reagent kit (#23235) was purchased from Pierce Chemical (Rockford, IL, USA) and a Cell Counting Kit-8 was purchased from Dojindo Laboratories Co. (Tokyo, Japan).

### 2.2. Synthesis of silylhydrated end functional PDMS

The representative synthesis process for hydrosilyl-terminated PDMS is as follows. 20 g of $D_4$ (0.068 mol) and 0.24 mL of 1,1,3,3-tetramethyldisiloxane (1.36 mmol) were placed in a round-bottomed flask, degassed by Ar bubbling for 10 min, and then sealed right after 0.13 mL of triflic acid was injected. The mixture was placed in a 55 °C oil bath for 3 days. Next, the white mixture was dissolved in ether and repeatedly washed with water until it was neutralized. The ether was isolated and stirred overnight with magnesium sulfate following filtration and vacuum treatment at 120 °C for 1 day. Three different molecular weights of PDMS were synthesized by controlling $D_4$/TMS ratios. Synthesis of PDMS macroinitiators and ATRP with MPC was carried out by means of a previously reported method [26]. The overall reaction scheme is illustrated in Scheme 1.

### 2.3. Preparation of block copolymer substrate

One weight percent of each block copolymer solution in ethanol was cast on poly(ethyleneterephthalate) (PET) substrate and naturally dried. Next, the substrates were heat treated at 60 °C under reduced pressure for 3 days. For the cell adhesion test, the same process was carried out in a 24-well tissue culture poly-(styrene) (TCPS) dish followed by ultraviolet treatment to ensure sterilization.

### 2.4. Surface characterization

#### 2.4.1. Morphological analysis of block copolymer surface

Block copolymers were cast on a copper grid (grid pitch 100 μm, carbon membrane supported) for observation by transmission electron microscopy (TEM). Cast membrane was then heat treated at 60 °C for 3 days *in vacuo*. For observation of protein adsorption behavior, commercial 10-nm gold colloid-labeled IgG solution was diluted one-third in phosphate buffered saline (PBS, pH 7.4), and placed in contact with a casting grid for 10 min at room temperature. After thorough washing with fresh PBS, the grid was dried under reduced pressure for 1 day and stained with 2% osmium (VIII) oxide solution (Wako Chemical, Osaka, Japan) by a dry staining method, after which TEM (Hitachi H-800, acceleration voltage: 100 kV, Tokyo, Japan) studies were conducted. Topological analyses of block copolymer templates were performed using atomic force microscopy (AFM; Nihon Veeco, Nanoscope IIIa, Tokyo, Japan). The excitation frequency range was 7.8–9.0 kHz, and the scan rate and scan scales were 0.5 Hz and 5 nm, respectively, with 20 μm × 20 μm scan sizes.

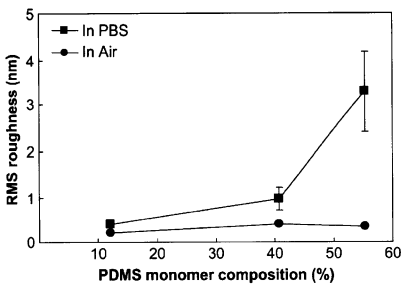



**Fig. 3.** Root mean square (RMS) value of surface roughness of block copolymer surfaces in PBS and under dry conditions as a function of PDMS compositions. Each point indicates PM1, PM2, and PM3 from left to right.

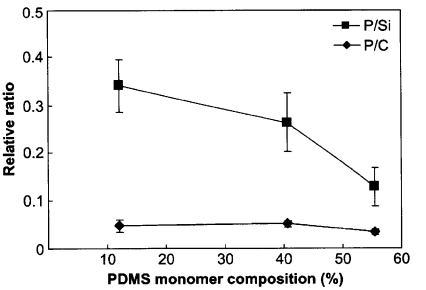



**Fig. 4.** P/C and P/Si values of block copolymer surfaces calculated by XPS analysis as a function of PDMS compositions. Each point indicates PM1, PM2, and PM3 from left to right.

Atomic force microscopy (AFM) under wet conditions was performed in PBS at room temperature after equilibration in PBS at 37 °C for 1 h.

#### 2.4.2. Surface characterization

Static water contact angles of the block copolymer surface were measured by using a goniometer (Kyowa Interface Science Co., Tokyo, Japan). 3 μL of water droplets were brought into contact with each sample for 10 s, and the contact angles were measured and recorded as photographic images. Elemental analyses of surfaces were performed by X-ray photoelectron spectroscopy (XPS) measurement (Kratos/Shimadzu, Kyoto, Japan), using magnesium Kα sources with 90° of photoelectron takeoff angle. Elements characterized included C, N, P, and Si. Binding energies were referenced to the C1s peak at 285.0 eV. P/C and P/Si values were calculated by integration of each peak area.

### 2.5. Biological responses on the block copolymer surfaces

#### 2.5.1. Proteins adsorption test

Block copolymer templates were prepared in 24-well plates (TCPS) for the protein adsorption test. 1 mL of IgG or fibronectin solution (0.45 mg/mL in PBS, pH 7.4) was poured into each well and incubated at 37 °C for 1 h. Next, the protein solutions were removed, and the wells were carefully washed with fresh PBS twice. After adding 0.5 mL of sodium dodecyl sulfate (SDS) (10 mg/mL), each plate was sonicated for 20 min at room temperature. Protein concentration in SDS solution was then determined using a micro-BCA™ protein assay reagent kit.

#### 2.5.2. Cell adhesion test

A cell adhesion test using L929 mouse fibroblast cells (RCB 0081, Cell Bank, Japan) on block copolymers was performed in 24-well plates. Approximately $3.0 \times 10^4$ cells were grown in 1 mL of minimum essential medium (Invitrogen Co.

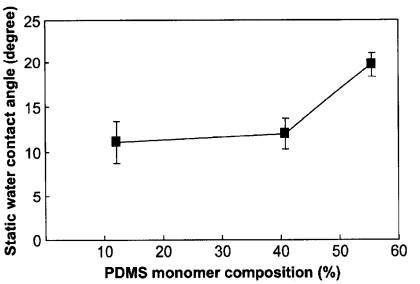



**Fig. 5.** Static contact angles of block copolymers as a function of PDMS compositions.

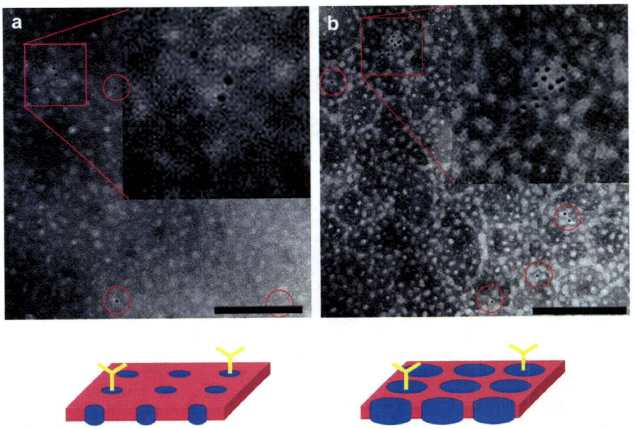



**Fig. 6.** Bright-field TEM images of block copolymer surfaces taken after contact with Au colloid-labeled IgG solution; (a) PM2 and (b) PM3. Small dark dots indicate Au colloid-labeled IgG molecules. Scale bar = 300 nm. The size of enlarged part is 170 nm × 170 nm and 200 nm × 200 nm, respectively. Red and blue region in the scheme indicate the PMPC and PDMS domains, respectively.

Carlsbad, CA, USA), supplemented (or not) by 10% fetal bovine serum (FBS). Plates were incubated in a 100% humidified incubator at 37 °C with 5% $CO_2$ for a maximum of 4 days. Cell morphologies were observed by using an optical microscope (Olympus Optical Co. LTD. IX71S1F-2, Tokyo, Japan). For fluorescent microscopic observation, cells on block copolymer templates were stained as follows: Each well was carefully washed with fresh PBS and fixed with 4.0% of paraformaldehyde for 10 min at room temperatures. After washing with fresh PBS, cells were permeabilized with 2.5% Triton X-100 for 10 min and rinsed again with PBS. Alexa Fluo 488 phalloidin (diluted 1:200) was then added, and the cells were incubated in the dark for 45 min at room temperature. After rinsing with fresh PBS, 1:1000 diluted DAPI solution (PBS, pH 7.4) was added, and the cells were incubated for another 15 min at room temperature. Next, the wells were washed with PBS, and block copolymer surfaces were kept in a wet condition with fresh PBS for the confocal laser microscopic observation (LSM 510, Carl Zeiss Japan, Tokyo, Japan). Number of cells was calculated by injecting 1/10 volume of Cell Counting Kit #8 into each well, followed by incubation at 37 °C for 2 h, using self-made calibration data.

## 3. Results and discussion

### 3.1. Synthesis of end functional PDMS

Surface morphology of a block copolymer induced by phase separation is dominated by several physicochemical factors, including solubility, composition, degree of polymerization, and molecular weight [24]. In order to minimize other possible variables, compositions of block copolymers were carefully controlled to ensure similar molecular weights. Table 1 summarizes the synthesized PDMS and block copolymers used in this study. Three different molecular weights of PDMS were prepared by controlling $D_4$/TMS ratios. We have shown earlier that block copolymers composed of PDMS and PMPC can be synthesized in a very well controlled manner by the ATRP method in protic media, even though both components display extremely disparate solubilities [26]. As shown in Table 1, three different compositions of block copolymers with similar molecular weights (determined by NMR) were successfully synthesized by the ATRP method. Unfortunately, not all polydispersity indices (PDI) measured achieved the appropriate low level (<1.3) because of the solubility problems. However, because morphology of block copolymers is not that significantly affected by PDI, except in theoretical modeling [27], we considered compositions of block copolymers as variables only for the preparation of block copolymer templates.

### 3.2. Surface morphologies of block copolymer templates

The difference in solubility of each block plays a dominant role in the phase separation behavior of block copolymers. Because degree of immiscibility is determined by solubility parameters, block copolymers that contain two extremely different hydrophobic (PDMS) and hydrophilic (PMPC) parts are expected to form phase-separated structures easily.

Fig. 1 shows the bright-field TEM image of a block copolymer template stained with $OsO_4$. In PM1, no PDMS domains were

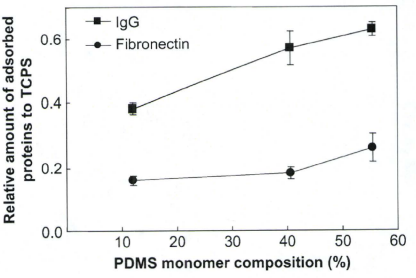



**Fig. 7.** Relative amount of adsorbed proteins on block copolymer surfaces as a function of PDMS compositions calculated by the micro-BCA™ experimental method. All values were normalized to the amount of adsorbed proteins on TCPS (=1).

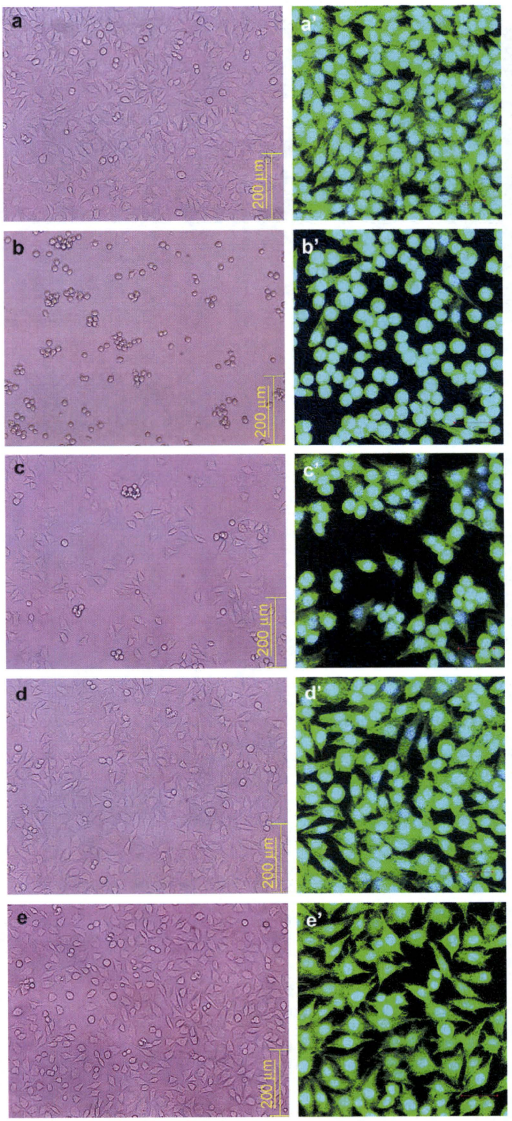



**Fig. 8.** Optical and confocal microscope images of L929 mouse fibroblasts after 2 days of cultivation on block copolymer surfaces in serum-containing medium. (a), (a') Noncoated TCPS; (b), (b') PM1; (c), (c') PM2; (d), (d') PM3; and (e), (e') PDMS (PDMS2-coated surface). Scale bar in optical image = 200 µm. Scan size of confocal images = 300 µm × 300 µm.

observed by TEM. This finding is probably because of the low level of the PDMS monomer composition, so that PDMS-core structure forms in a PMPC matrix, as illustrated. PM2 and PM3 exhibit cylindrical PDMS domain structures in a PMPC matrix with different domain sizes because of the high level of the PDMS monomer composition in PM3. Because cylindrical domain size can theoretically be controlled by PDMS monomer composition, this result agrees well with the molecular profile of the block copolymers.

Fig. 2A and B shows topological AFM images of block copolymers in a dry condition with different scan sizes. These AFM images agree well with the TEM images of Fig. 1 over a broad region, except for the individual domain sizes. Because measurement conditions for TEM and AFM are quite different, it is expected that these dimensions would not match exactly. However, the tendency shown in the AFM images that PDMS domain size increases, thereby allowing PDMS monomer compositions, agrees well with that of the TEM images. A similar tendency was also apparent in PBS.

Fig. 2C shows the AFM images taken in PBS. It is apparent that hydrophobic PDMS domain size increases as PDMS monomer composition increases. Morphological changes under wet conditions are possibly attributable to the swelled hydrophilic PMPC domain, which induces a significant increase in surface roughness (Fig. 3), which is an important variable for the determination of cellular responses. Difference in surface roughness between dry and wet conditions increases as PDMS monomer composition increased. This result indicates that the effect of a swelled hydrophilic PMPC domain on surface roughness increases when it coexists in nearly the same proportion as a non-swelled hydrophobic PDMS domain. This result is thought as schematically reasonable because the density of swelled/non-swelled domain is maximized when two domains coexist in nearly same proportion.

### 3.3. Surface characterization of block copolymer templates

Fig. 4 shows the quantitative influence of surface elements measured by XPS. In each block copolymer template, a strong phosphorus ($P_{2p}$) peak was detected at 134.0 eV and a strong silicone ($Si_{2p}$) peak was detected at 103.0 eV in different ratios. This P/Si ratio, calculated by peak integrals, continuously decreases as PDMS monomer composition increases. This finding indicates that the surface ratio of the PDMS domain increases as the PDMS monomer composition increases. This result corresponds well with the results from the earlier TEM and AFM observations. In contrast, the P/C ratios on the block copolymer surfaces have almost same value. This result is possibly due to the large amount of $C_{1s}$ which detected in both PMPC and PDMS domains.

Fig. 5 shows results of static water contact angle measurement. It is apparent that hydrophobicity of block copolymer templates increases continuously as PDMS domain ratio increases. Nevertheless, it is also clear that all block copolymer surfaces showed a significantly low value of contact angle with regard to the PDMS surface (100% PDMS monomer composition), which had an approximately 95-degree static water contact angle. That is, even though the block copolymer template prepared by PM3 exhibits the maximum hydrophobic tendency among all the samples examined, the static contact angle of PM3 is only around 20°, so that there are enough hydrophilic surfaces such that homogeneously prepared MPC polymer surfaces are nonbiofouling in nature [28].

### 3.4. Biological responses on the block copolymer surfaces

#### 3.4.1. Protein adsorption behavior

We investigated the effect of heterogeneous block copolymer surfaces on protein adsorption by TEM, using the micro-BCA™ experimental method with IgG and fibronectin as model proteins. We used the IgG molecules labeled with 10-nm diameters of gold colloid so that their adsorption behavior could be observed easily by TEM. Fig. 6 exhibits the resulting TEM images of each block copolymer template after contact with IgG solution. In all the block copolymer templates, IgG molecules were selectively adsorbed only onto the hydrophobic PDMS domains. This result indicates that with phase separation, each block copolymer domain could retain its own biofouling and antibiofouling properties.

Similar phenomena for other block copolymer templates have been reported previously. For example, in an early study, Okano et al. proposed an interesting effect of block copolymer surfaces on protein adsorption behavior. They reported that a heterogeneously prepared block copolymer surface composed of hydrophilic poly(hydroxyethyl methacrylate) (PHEMA) and hydrophobic PS induced preferential protein adsorption on each domain, thereby suppressing activation of adhered platelets [29]. Kumar et al. demonstrated selective protein adsorption on phase-separated block copolymer templates composed of PS and poly(methyl methacrylate) [30], and they also reported that such selective protein adsorption could also occur on more hydrophobic domains, even though the materials that comprise each block copolymer domain are all hydrophobic components [31]. However, all previously reported experiments were performed on surfaces that exhibit relatively high water contact angles (to the best of our knowledge, 50° as a minimum).

In our current study, we can confirm that heterogeneous MPC polymer surfaces derive selective protein adsorption even though these surfaces may have a very hydrophilic nature, i.e., a water contact angle of less than 20°. Because IgG is not a cell adhesive protein, we carried out a protein adsorption test by using fibronectin, another plasma protein that is involved in cellular adhesion.

Fig. 7 shows the amount of adsorbed proteins determined by the micro-BCA™ method. It is apparent that the adsorption tendency of fibronectin is similar to that of IgG; that is, the amount of adsorbed protein depends on the hydrophobicity of the surfaces. Unfortunately, because of technical problems, direct observation of fibronectin adsorption was not possible. However, we expect that adsorption behavior of fibronectin would resemble that of IgG, because selective fibronectin adsorption occurred only onto the PDMS domains.

#### 3.4.2. Cell adhesion behavior

We studied cell adhesion behavior on block copolymer templates by using L929 mouse fibroblast cells. Fig. 8 depicts

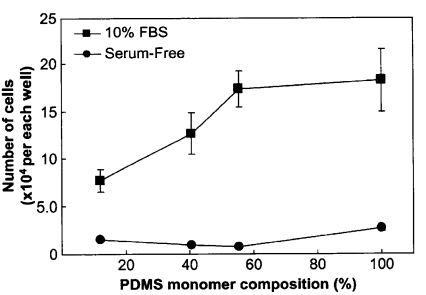



**Fig. 9.** Number of cells on block copolymer surfaces after 4 days of cultivation as a function of PDMS composition. One hundred percent indicates the PDMS2-coated surface as a negative control.

optical and confocal microscope images taken on block copolymer coated surfaces. A large number of cells adhered to and proliferated on the noncoated TCPS surface, because of the large amount of cell-adhesive serum proteins on the TCPS surface. On the other hand, only a limited number of cells were observed on the PM1 surface, which consisted of maximum PMPC monomer composition. Moreover, most of the observed cells were nonadhesive; i.e., they maintained a globular form thought of as a nonbiospecific physical adhesion. The number of adhered cells on the block copolymer surface increased as the number of PDMS monomer composition increased (from PM1 to PM3). Because the PDMS domain ratio increased as the PDMS monomer composition increased, this phenomenon is thought to occur because of the increased adsorption of serum proteins on hydrophobic PDMS domains.

Cell adhesion behavior on PM3 has a significant meaning in this work. Usually MPC polymer-containing materials exhibit an antibiofouling nature because of the thick hydrated layer that forms around the phosphorylcholine group [13]. As a result, homogeneously prepared MPC polymer surfaces normally have low water contact angles (0–20°), and they do not display a cell-adhesive surface nature, even though their MPC monomer composition is only around 30% [28]. For that reason, biocompatibility of MPC polymer-containing biomaterials has normally been determined by studying the hydrophilicity of the surfaces with a monomer composition of MPC. However, in our current study, we have been able to confirm that a heterogeneously prepared hydrophilic polymer surface (i.e., one with a water contact angle of less than 20°) induced a large number of cells to

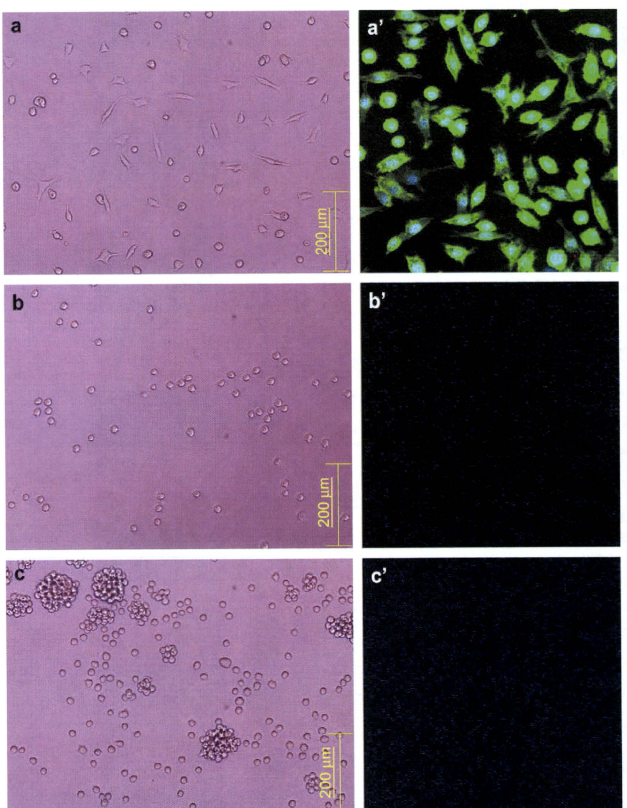



**Fig. 10.** Optical and confocal microscope images of block copolymer surfaces taken after two days of cultivation in serum-free medium. (a), (a') noncoated TCPS; (b), (b') PM1; (c), (c') PM2; (d), (d') PM3; and (e), (e') PDMS. Scale bar in optical image = 200 μm. Scan size of confocal images = 300 μm × 300 μm.

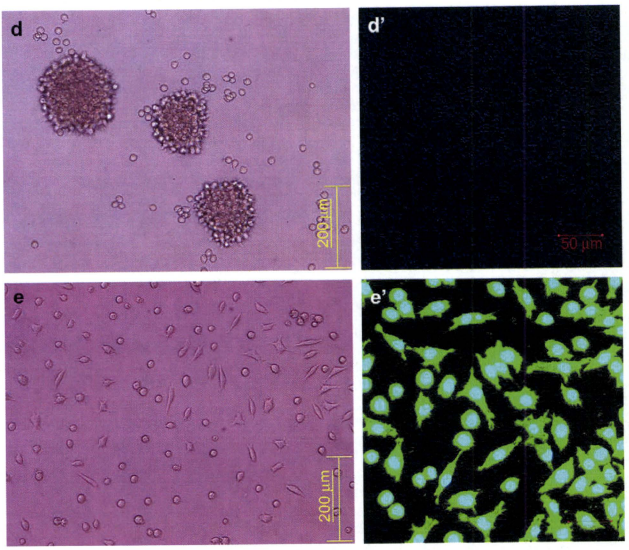
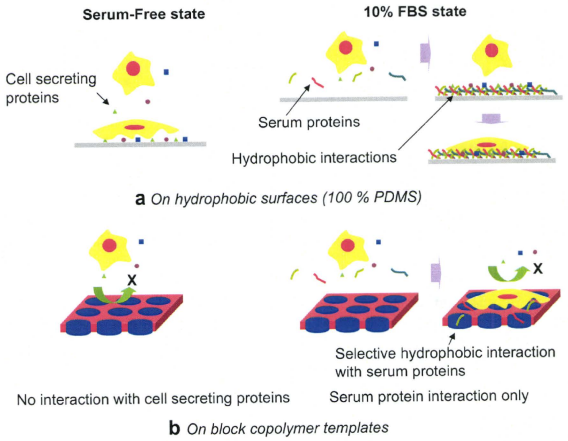



**Fig. 10.** (*continued*).

adhere onto its surface even though the MPC monomer composition was around 45% (PM3). This result emphasizes the importance of surface morphologies in designing a biomaterial. In other words, heterogeneously segregated hydrophobic domains can have a significant effect on cellular response, even with a hydrophilic MPC polymer surface. Cell proliferation on block copolymer templates at a 10% FBS level is summarized in Fig. 9.




**Scheme 2.** Schematic explanations of serum protein-block copolymer interaction and resulting cell adhesion behavior.

In order to investigate the effect of serum proteins on cell adhesion behavior, a cell adhesion test was carried out in a serum-free medium. As shown in Fig. 8, no significant cell proliferation was observed on any of the sample surfaces in serum-free medium, including the PDMS/noncoated TCPS control surfaces due to the lack of growth factors and cell adhesive serum proteins. However, cellular responses observed for PDMS/noncoated TCPS and block copolymer surfaces were clearly different.

Fig. 10 presents optical and confocal microscopy images of cells on sample surfaces in a serum-free medium. Although cells were not proliferated, strong cell attachments were observed on hydrophobic TCPS and PDMS surfaces. On the other hand, no cells adhered on any of the block copolymer surfaces. Cell adhesion on materials surfaces, especially for fibroblasts, is controlled mainly by the extracellular matrix (ECM), which is induced by plasma proteins and cell-secreting proteins [32]. In the serum-free state, a serum-induced ECM could not be generated to bring about cell adhesion; therefore, only cell-secreting proteins could affect cell adhesion behavior. On 100% hydrophobic surfaces such as TCPS or PDMS, these secreted proteins seemed to have an effect on cell adhesion behavior that was probably caused by strong hydrophobic interactions between the proteins and the surfaces. However, heterogeneously prepared MPC polymer surfaces suppressed the effect of these secreted proteins.

There are two possible explanations for this result. One possibility is the density problem of cell-adhesive proteins on block copolymer surfaces. Because hydrophobic domains exist only sparsely on the PMPC matrix, the adsorbed cell adhesion factors, "without serum proteins," may not be sufficient to induce cell adhesion. Of course, this hypothesis would have to be demonstrated with much more experimental evidence, such as quantitative analysis of surface-adsorbed proteins, and exact adsorption distribution of serum proteins on heterogeneous surfaces, which is out of the range of this current study. Another possible explanation is the size effect of cell-adhesive proteins and segregated PDMS domains. Fig. 7 makes clear that the overall amount of adsorbed proteins decreases as the molecular weight of the protein increases (e.g., IgG ~ 150 kDa, Fibronectin ~ 440 kDa). Because the hydrophobic PDMS domain exists only to a limited extent on PMPC domains with a nanoscale size, protein molecules with higher molecular weight might have a rare chance to adsorb onto it, as previously reported (size effect) [33]. Cell adhesion factors including laminin, secreted by the fibroblast itself, have a much higher molecular weight (~900 kDa) compared to other cell-adhesive proteins contained in serum. As a result, we speculate that this size effect could be a cause of the noncellular adhesive nature of the block copolymer surface in a serum-free condition, as shown in Scheme 2.

Further investigation of protein distribution on block copolymer surfaces is now underway. In any event, we wish to emphasize that segregated hydrophobic domains on MPC polymer surfaces have a significant effect on cellular response under serum conditions.

## 4. Conclusions

We investigated protein adsorption and cell adhesion behavior on heterogeneously prepared MPC polymer surfaces. Although MPC polymers are well known to be noncell adhesive, and, thus, do not promote cell proliferation, existing heterogeneous hydrophobic PDMS domains strongly affect serum protein adsorption and cell adhesion behavior. From our investigation, we conclude that segregated hydrophobic domains have to be taken into account when designing serum contact biomaterials that contain MPC polymers, even though the surface reveals a higher level of hydrophilicity, owing to the large amount of phosphorylcholine groups present. We expect that this suggestion could be a useful inspiration not only for understanding of cellular response on polymer surfaces, but also for designing cell attachable various biomaterial surfaces applied in cell and tissue engineering.

## Acknowledgement

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## Appendix

Figures with essential colour discrimination. Scheme 2 and many of the figures in this article 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.2009.06.031.

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# Surface modification of a titanium alloy with a phospholipid polymer prepared by a plasma-induced grafting technique to improve surface thromboresistance

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ABSTRACT

To improve the thromboresistance of a titanium alloy ($TiAl_6V_4$) surface which is currently utilized in several ventricular assist devices (VADs), a plasma-induced graft polymerization of 2-methacryloyloxyethyl phosphorylcholine (MPC) was carried out and poly(MPC) (PMPC) chains were covalently attached onto a $TiAl_6V_4$ surface by a plasma induced technique. Cleaned $TiAl_6V_4$ surfaces were pretreated with $H_2O$-vapor-plasma and silanated with 3-methacryloylpropyltrimethoxysilane (MPS). Next, a plasma-induced graft polymerization with MPC was performed after the surfaces were pretreated with Ar plasma. Surface compositions were verified by X-ray photoelectron spectroscopy (XPS). In vitro blood biocompatibility was evaluated by contacting the modified surfaces with ovine blood under continuous mixing. Bulk phase platelet activation was quantified by flow cytometric analysis, and surfaces were observed with scanning electron microscopy after blood contact. XPS data demonstrated successful modification of the $TiAl_6V_4$ surfaces with PMPC as evidenced by increased N and P on modified surfaces. Platelet deposition was markedly reduced on the PMPC grafted surfaces and platelet activation in blood that contacted the PMPC-grafted samples was significantly reduced relative to the unmodified $TiAl_6V_4$ and polystyrene control surfaces. Durability studies under continuously mixed water suggested no change in surface modification over a 1-month period. This modification strategy shows promise for further investigation as a means to reduce the thromboembolic risk associated with the metallic blood-contacting surfaces of VADs and other cardiovascular devices under development.



## 1. Introduction

Suboptimal blood compatibility in many cardiovascular devices puts patients at increased risk for thromboembolism, often necessitating the use of chronic anti-coagulation and its accompanying increased risk for bleeding. The composition of the biomaterial surface, the nature of the blood flowing across the device surfaces and the bias of the patient's blood toward hemostatic reactions all combine to define thrombotic and thromboembolic risk. Thus much work has focused on utilizing computational fluid dynamics to improve flow characteristics over biomaterial surfaces in circulatory support devices [1] and similarly there has been great interest in developing chemical modifications for blood contacting surfaces [2].

Our recent interest has been in developing a rotary blood pump for pediatric applications in which aggressive anticoagulation may be problematic and biocompatibility is thus of primary concern. For design and machinability considerations the titanium alloy $TiAl_6V_4$ makes up the blood contacting surfaces of this pump as well as several other rotary blood pumps in clinical use and in pre-clinical development [1,3–9]. Although titanium and its alloys have exhibited generally acceptable biocompatibility in a variety of settings, its surface modification remains of interest for blood contact since platelet deposition still can occur in vitro and thrombosis and thromboembolism can occur in these devices in vivo [10–12].

Biomaterial surface modification by plasma based techniques has frequently been applied due to the high efficiency of this

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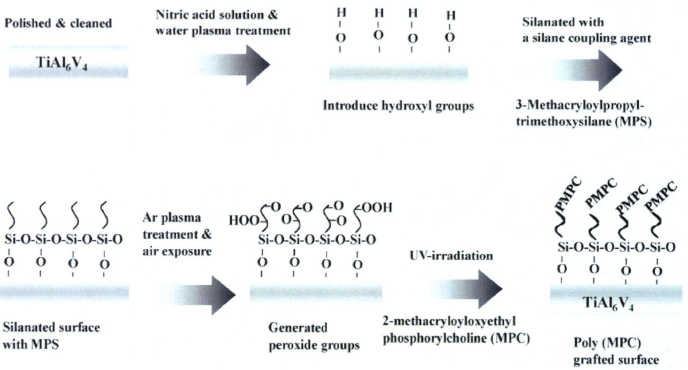


**Fig. 1.** Scheme of surface functionalization and modification of a $TiAl_6V_4$ surface with PMPC.

approach for a variety of substrates and geometry [13]. Active functional groups can be introduced by utilizing a specific atmosphere such as water, ammonia or argon (Ar) which allows further modification by covalent attachment of other polymers or biomolecules on the substrate [14–17]. Plasma induced graft polymerization after Ar plasma pretreatment is attractive for its high efficiency and others have previously reported on the effects of plasma treatment time, plasma power and storage time on surface modifications with this method [18,19]. Polytetrafluoroethylene, silicon, and stainless steel surfaces have successfully been modified by Ar plasma induced grafting copolymerization with a poly (ethylene glycol) containing macromonomer to improve their surface blood compatibility [20–22].

Synthetic phospholipid polymers (phosphorylcholine (PC) group-bearing polymers) have been extensively studied due to their excellent biological and blood compatibility [23]. One of the most representative phospholipid polymers, 2-methacryloyloxyethylphosphorylcholine (MPC) polymer has received considerable interest for medical applications [24–26]. Many researchers have shown previously that surface modification with the MPC polymers or the introduction of PC groups by blending, coating and graft modification techniques are effective in improving blood compatibility by resisting non-specific protein adsorption and platelet activation and adhesion on polymer biomaterial surfaces [27–31]. The MPC polymers have also been applied to some metallic biomaterial surfaces including ventricular assist devices (VADs) and demonstrated apparent improvements in blood compatibility [32–35]. However, to date most of the surface modification techniques with the MPC polymers involve physical adsorption onto metallic surfaces. We recently reported on the MPC polymer covalent immobilization approach for $TiAl_6V_4$ that showed promise in improving blood biocompatibility [36]. While effective, concerns with this technique were the lability of the amide bond linking the copolymer to the surface and the extent of surface coverage that might be achieved.

Our objective in this study was to introduce a new, potentially more robust method of polymerizing MPC off of a modified $TiAl_6V_4$ surface using a plasma-induced graft technique that might offer durability under high shear and for extended blood contacting periods. The modified $TiAl_6V_4$ surface was characterized in terms of its surface composition and acute platelet deposition and activation after contact with ovine blood in vitro.

## 2. Materials and methods

### 2.1. Materials

A titanium alloy ($TiAl_6V_4$) sheet was obtained from California Metal & Supply Inc., Gardena, CA. MPC was obtained from NOF Corp. (Tokyo, Japan), which was synthesized as previously described [24]. 3-Methacryloylpropyltrimethoxysilane (MPS, Aldrich, USA) was used as a silane coupling agent. Heparin (Pharmacia & Upjohn Co., Ann Arbor, MI) was used for blood anticoagulation.

### 2.2. Surface pretreatment and silanization of a $TiAl_6V_4$

The $TiAl_6V_4$ sheet was polished with 3.0, 1.0, 0.25, and 0.1 μ diamond pastes in sequence (Electron Microscopy Sciences, Washington, PA) and cleaned ultrasonically three times for 5 min each with ethanol and acetone. The surfaces were then passivated with a 35% nitric acid solution for 1 h and rinsed with distilled water. Next, the $TiAl_6V_4$ surfaces were pretreated under $H_2O$ plasma with radio frequency glow discharge (RFGD, MARCH GCM250, March Instrument Inc, CA). The RFGD power applied was 25 W at a frequency of 13.65 MHz. The titanium surface was subjected to RFGD for 5 min supplying $H_2O$ vapor at a vacuum pressure of 0.4 Torr. The $H_2O$ plasma pre-treated $TiAl_6V_4$ surfaces were silanated by immersion in an MPS solution for 3 h in a 90 °C oil bath. The MPS solution consisted of 2% MPS in ethanol that was hydrolyzed by adding water and stirring for 1 h. The pH of the MPS solution was adjusted to approximately 3–4 by adding 0.1 M HCl. The silanated samples were dried at 110 °C for 1 h, then rinsed repeatedly with ethanol and water, and stirred in deionized water for 1 h to remove adsorbed MPS. Samples treated in this manner were referred to as Ti-MPS (Fig. 1).

### 2.3. Plasma-induced surface graft modification with MPC

The silanated $TiAl_6V_4$ samples were treated with Ar plasma by using RFGD (25 W, 20 s, 0.6 Torr), and then the surface was exposed to the atmosphere for 10 min to create surface peroxide groups. The $TiAl_6V_4$ sample was then immersed in MPC solution (0.5 mM) which was placed in a transparent polystyrene round-bottom tube (BD Bioscience, San Jose, CA) The monomer solution was passed through argon gas for 1 min and 0.005 mM

riboflavin (Sigma–Aldrich, St. Louis, MO) was added to eliminate any oxygen [14]. Then, graft modification onto the $TiAl_6V_4$ surface was carried out under a high intensity UV lamp for 24 h. This decomposed the surface peroxide groups to free radicals and poly(MPC) (PMPC) was grafted from the surface. The modified samples were rinsed three times with ethanol and water and stirred in deionized water for 24 h to remove physically adsorbed PMPC. Samples treated in this manner were referred to as Ti–MPS–PMPC (Fig. 1).

### 2.4. Surface characterization

The surface composition of the titanium samples was analyzed by X-ray photoelectron spectroscopy (XPS) using a surface science instruments S-probe spectrometer and a take-off angle of photoelectron was 55°. The Service Physics ESCAVB Graphics Viewer program was used to determine peak area, calculate the elemental compositions from peak areas, and peak fit the high-resolution spectra. The surface composition on a given sample was averaged from three composition spots for each sample. The mean value for three different samples was determined.

The static contact angle of water on the surfaces of unmodified and modified titanium samples was measured at room temperature using a contact angle goniometer (VCA optima, AST Product Inc., Billerica, MA) by placing 1 μL of double distilled water on the surfaces. The droplet was imaged using a video camera coupled to a light microscope, and the contact angle was determined on the screen of a monitor employing imaging software. Five measurements were made on each sample to obtain the contact angle of the sample. The contact angle was also measured weekly in several of the modified samples that underwent continuous stirring under deionized water for 1 month to test the long term stability of the surface modification. XPS was also performed on the surfaces after 1 month of water contact.

### 2.5. Blood collection and blood contact test

Whole ovine blood was collected by jugular venipuncture directly into a syringe containing heparin (3.0 or 6.0 U/mL for 1 and 2.5 h blood contacting experiments, respectively) using an 18 gauge 1 ½″ needle, after discarding the first 3 mL. NIH guidelines for the care and use of laboratory animals were observed. Modified titanium and unmodified samples were placed into Vacutainer® blood collection tubes without additives (BD Biosciences, Franklin Lakes, NJ) filled with heparinized ovine blood and incubated for a specified time at 37 °C on a hematology mixer (Fisher Scientific, Pittsburgh, PA).

### 2.6. Observation and quantification of platelet deposition and activation

The $TiAl_6V_4$ surfaces were observed by scanning electron microscopy (SEM; JSM-6330F, JEOL USA, Inc., Peabody, MA) after 2.5 h contact with heparinized blood (6.0 U/mL). The $TiAl_6V_4$ surfaces were also observed with epi-fluorescence microscopy (ZEISS, Carl Zeiss, Inc. Thornwood, NY) after contact for 1 h with heparinized blood (3.0 U/mL) that was treated with quinacrine dihydrochloride (10 μM final concentration, Sigma) to fluorescently label the platelets. The number of platelets for each sample was also estimated by a lactate dehydrogenase (LDH) assay [37] with an LDH Cytotoxicity Detection Kit (Clonetech Laboratories Inc., CA). In this assay, the surfaces were rinsed thoroughly with 50 mL PBS following 2.5 h contact with blood (6.0 U/mL heparin) and then immersed in 1 mL of 2% Triton X-100 solution (Sigma) for 20 min to lyse deposited platelets. Calibration of spectrophotometer absorbance results to platelet numbers was accomplished using a calibration curve generated from known dilutions of ovine platelet rich plasma in the lysing solution.

The percentage of activated ovine platelets in the bulk phase of the blood during surface contact was determined using flow cytometric quantification of Annexin V binding as recently described [38]. Blood samples (10 μL) were taken during test surface contact experiments described above for LDH measurement of platelet deposition after 2 h. Activation levels from five independent samples were averaged for each surface type.

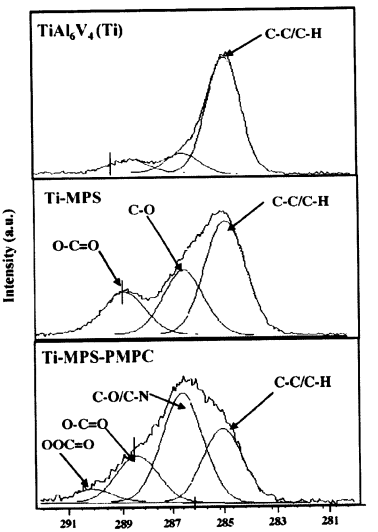


**Fig. 2.** XPS high resolution (C1s) spectra of the modified and unmodified $TiAl_6V_4$ samples.

### 2.7. Statistical analyses

Data are presented as means with standard deviation. Statistical significance between sample groups was determined using ANOVA followed by post-hoc Newman–Keuls testing and accepted at $p < 0.05$.

## 3. Results

The high-resolution spectra from XPS are shown in Fig. 2. The C1s data was calibrated to the hydrocarbon peak (C–C/C–H) at 285.0 eV. The MPS modified $TiAl_6V_4$ surface (Ti–MPS) showed an increase in the peak at 286.6 eV and 288.5 eV which is likely due to C–O and O–C=O type species. The PMPC modified $TiAl_6V_4$ surface showed a further increase in the peak at 286.6 eV by the addition of the C–N type species that are attributed to the MPC units. Furthermore, the Ti–MPS–PMPC also has a peak at 290.5 eV which is likely due to peroxide OOC=O type species that is attributed to the Ar plasma treatment. The surface atomic compositions of $TiAl_6V_4$ samples are shown in Table 1. The oxygen composition of the pre-treated $TiAl_6V_4$ surfaces (Ti–$H_2O$ plasma) rose significantly in comparison with the Ti which was not pre-treated ($p < 0.05$). The MPS mod-

**Table 1**
Atomic percentage by X-ray photoelectron spectroscopy.

| | C | O | Ti | Al | Si | N | P |
|---|---|---|---|---|---|---|---|
| $TiAl_6V_4$ (Ti) | 42.0 (±8.0) | 41.1 (±5.2) | 9.5 (±1.1) | 4.3 (±3.1) | 1.0 (±1.0) | 1.0 (±0.5) | 0.1 (±0.2) |
| Ti ($H_2O$ plasma) | 24.1 (±6.6) | 50.8 (±0.4) | 10.5 (±3.3) | 2.3 (±1.1) | 1.3 (±1.4) | 0.8 (±0.3) | 0.0 (±0.0) |
| Ti–MPS | 49.4 (±8.9) | 34.3 (±5.4) | 1.3 (±1.3) | 1.2 (±1.1) | 13.9 (±4.8) | 0.2 (±0.4) | 0.0 (±0.0) |
| Ti–MPS–PMPC | 42.8 (±12.6) | 40.0 (±7.8) | 1.4 (±1.6) | 0.0 (±0.0) | 14.1 (±3.8) | 1.8 (±0.3)* | 1.0 (±0.2)* |

$N = 7$, ± standard deviation for Ti. $N = 3$, ± standard deviation for Ti ($H_2O$ plasma). $N = 5$, ± standard deviation for Ti–MPS. $N = 4$, ± standard deviation for Ti–MPS–PMPC.
* $p < 0.05$ vs. other surfaces.

**Table 2**
Surface tension on the unmodified and modified titanium samples.

| | $TiAl_6V_4$ (Ti) | Ti ($H_2O$ plasma) | Ti–MPS | Ti–MPS–PMPC |
|---|---|---|---|---|
| Contact angle ( ) | 58.3 (±6.2) | 34.7 (±4.4) | 87.6 (±6.4) | 18.1 (±4.0)* |

$n = 3$, ± standard deviation.
* $p < 0.05$ vs. other surfaces.

ified surface showed an increase in Si which was attributed to the presence of MPS ($p < 0.05$). Furthermore, the XPS data from Ti–MPS–PMPC provide evidence for the successful modification with PMPC by reflecting increased nitrogen (N) and phosphorus (P) ($p < 0.05$).

The surface tension on the modified and unmodified titanium samples was shown in Table 2. The contact angle on $TiAl_6V_4$ surfaces was decreased from 58 ± 6° to 35 ± 4° after $H_2O$ plasma treatment. The Ti–MPS increased in surface tension to 88 ± 6° due to modification with hydrophobic MPS on the $TiAl_6V_4$ surface. However, the surface tension of the PMPC grafted surfaces decreased substantially (Ti–MPS–PMPC = 18 ± 4°) by modification with the hydrophilic PMPC on the surface in comparison with all of the other surfaces ($p < 0.05$). The surface contact angle on the PMPC grafted surface (Ti–MPS–PMPC) measured every 7 days during a 1-month period of mixing with water did not significantly change (Fig. 3). Additionally, the surface composition of several of the modified samples was also measured after mixing for 1 month in water. The XPS analysis results also showed no significant difference in the surface composition of phosphorus before ($P$: 1.1 ± 0.1%) and after ($P$: 1.0 ± 0.1%) the water contacting experiment.

The modified and unmodified $TiAl_6V_4$ surfaces after contact with anticoagulated ovine blood for 1 h at 37 °C were observed with an epi-fluorescence microscope (Fig. 4). Fluorescent platelets are seen to be adhered and aggregated on the surface. The unmodified titanium had relatively high numbers of deposited platelets, whereas the PMPC modified surfaces (Ti–MPS–PMPC) showed few adherent platelets. Platelet adhesion and morphology was also observed with SEM after contact with ovine blood for 2.5 h at 37 °C. The SEM images on the surfaces of the positive control polystyrene, unmodified and modified titanium samples are seen in Fig. 5. The polystyrene control surface (Fig. 5A) supported heavy platelet deposition with most of the deposited platelets exhibiting extended pseudopodia. The unmodified titanium surface (Fig. 5B) had a moderate amount of deposited platelets on its surface, with the number of platelets appearing to be less than for the polystyrene surface. The silanated titanium surface (Ti–MPS, Fig. 5C) also showed a moderate amount of deposited platelets, and these platelets exhibited extended pseudopodia. The quantity of adhered platelets on the Ti–MPS surface appeared to be slightly more than for the unmodified titanium (Fig. 5B). Platelet deposition was decreased dramatically on the Ti–MPS–PMPC surfaces, and the platelets that were adhered generally retained their discoid morphology with some pseudopodia extension (Fig. 5D). It is worth noting that it was difficult to identify adhered platelets on the Ti–MPS–PMPC surface. The number of deposited platelets as quantified by the lactate dehydrogenase (LDH) assay after blood contact (Fig. 6) was significantly less for Ti–MPS–PMPC surfaces than for all of the other surfaces ($p < 0.01$). Flow cytometric quantification of bulk phase platelet activation using the Annexin V assay (Fig. 7) was also significantly lower in blood contacting Ti–MPS–PMPC surfaces than for unmodified titanium and polystyrene samples ($p < 0.05$).

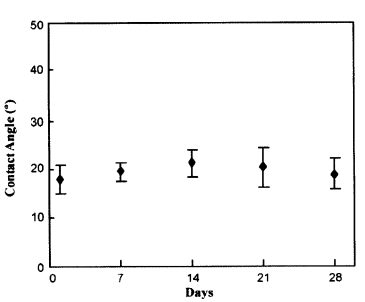


**Fig. 3.** Contact angle measurements of the PMPC modified $TiAl_6V_4$ surface (Ti–MPS–PMPC) after continuous mixing under deionized water.

## 4. Discussion

Improvement of surface blood compatibility is of keen interest to the cardiovascular device community given the morbidity and mortality associated with device-related thrombotic complications and with the anticoagulation applied to minimize these risks. In the case of VADs, biocompatibility concerns are a major reason why these devices are underutilized in heart failure patients [3,39–41]. PC groups and PC group-bearing polymer modified surfaces have previously demonstrated marked improvement in blood compatibility when compared to unmodified surfaces [24–31]. Moreover studies involving self-assembled monolayers (SAM) with well defined and highly ordered and oriented PC groups have shown that this type of surface is one of the most promising for anti-fouling since it strongly resists non-specific protein adsorption and cell adhesion [42–44]. There are several successful studies where a PC group-bearing polymer was physically adsorbed onto the metallic surfaces in vascular stent and VAD applications [9,33,34]. In the VAD studies the blood contacting surface containing physically adsorbed MPC polymer was shown to be superior in terms of blood compatibility when compared to the same device with a blood contacting surface modified with a diamond like carbon coating [9,34]. Despite the improved hemocompatibility in these studies, there was con-

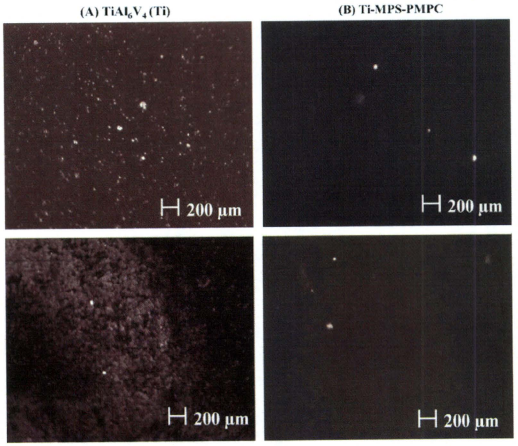


**Fig. 4.** Fluorescent micrograph images of unmodified and modified $TiAl_6V_4$ samples after contact with minimally anticoagulated (3.0 U/mL heparin) ovine blood for 60 min at 37 °C.

cern about the long term stability of the surface PMPC. Furthermore, in the report of Kihara et al, the physically adsorbed MPC polymer was demonstrated to elute over time during VAD operation [34].

In spite of widespread examination of grafting PC groups onto polymer or silica surfaces, there are few reports where a PC group-bearing polymer has been grafted onto metallic surfaces, and even fewer that have subsequently evaluated the hemocompatibility of the resulting surfaces [36,45,46]. In this study, we showed that a $TiAl_6V_4$ surface could be successfully modified with the MPC moiety by a plasma-induced grafting method after the $TiAl_6V_4$ surface was silanized, and then Ar plasma treated. Our XPS results demonstrated successful modification of the $TiAl_6V_4$ surface with PMPC and the surface showed significant decreases in platelet deposition and activation in comparison with unmodified surfaces. The higher platelet deposition observed on the control Ti–MPS surface relative to the Ti surface could generally be attributed to an increase in surface hydrophobicity as reflected by the higher contact angle of this surface relative to the unmodified Ti alloy. Such an increase in hydrophobicity may serve as a driving force for increased protein adsorption and in this process result in more fibrinogen adsorption in a state that would support platelet adhesion on the surface.

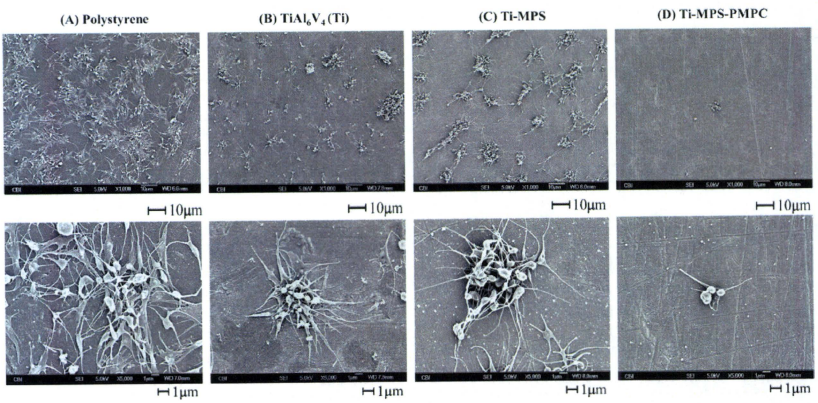


**Fig. 5.** SEM micrographs of polystyrene, unmodified and modified $TiAl_6V_4$ samples after contact with ovine blood (heparin 6U/mL) for 2.5 h at 37 °C. (A) polystyrene (B) Ti (C) Ti–MPS (D) Ti–MPS–PMPC.

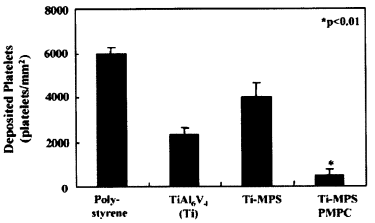


**Fig. 6.** Platelet deposition onto surfaces after contact with ovine blood for 2.5 h as determined by lactate dehydrogenase (LDH) assay ($n = 3$).

For the PMPC-grafted surface the contact angle and XPS results after a month of mixed water contact were not significantly different from our initial surface tension and XPS evaluation signifying the stability of the grafted PMPC and its sustained hydrophilicity. We previously reported on reduced thrombogenicity after immobilizing an MPC polymer poly(MPC-co-methacryl acid) (PMA) onto titanium surfaces. This surface modification strategy involved a condensation reaction between the carboxyl groups of the PMA and amino groups which were introduced on the titanium surface by a silane coupling agent [34]. However despite its covalent attachment, the long term stability of the PMA immobilized surface is a concern. The cause for concern is that over time the amide bonds between PMA and the titanium surface may be hydrolyzed under continuous blood contact. The PMPC grafting in this report is not linked to the titanium surface via an amide bond, potentially providing an advantage over the PMA immobilized surface in terms of longer-term stability under similar circumstances. Another advantage over the PMA immobilization strategy is that the PMPC grafting technique uses Ar plasma treatment which could be applied to other metallic surfaces, and also on many medically-relevant polymeric surfaces including polytetrafluoroethylene (PTFE).

The PMPC grafting method of this report might allow for more control of PMPC surface coverage and offer improved uniformity of surface PC groups when compared with the previous PMA immobilization method [34]. Immobilization of the long-chain PMA copolymer onto the titanium surface might have caused steric hindrance to further copolymer addition and this may have led to low and non-uniform areas of PMA coverage on the surface. Since the PMPC grafting method employs the addition of MPC rather than the long chain PMA copolymer there is less concern about steric hindrance. Less steric hindrance might lead to higher coverage of the MPC unit on the modified surface provided the surface has sufficient radical formation sites. However it is worth noting that in this grafting method it may be difficult to prepare a surface with 100% MPC unit coverage. This method also has some limitation in terms of the level of control achievable with respect to chain length of the modified PMPC. To obtain better control of the PMPC chain length on the surface, an alternative surface modification method such as surface initiated atom transfer radical polymerization could be considered. If a method were developed to prepare a covalently attached PMPC chain self assembled monolayer on a $TiAl_6V_4$ surface, it would ensure high coverage and uniformity of the PC group and might be even more advantageous in the manufacture of biocompatible blood contacting device surfaces.

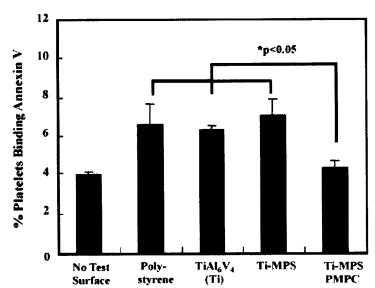


**Fig. 7.** Quantification of activated platelets in the bulk phase of ovine blood after surface contact under continuous rocking. No test surface indicates blood from a rocked tube into which no test surface was placed. Platelet activation was quantified by flow cytometric measurement of Annexin V binding onto platelets ($n = 3$).

In preliminary studies, we chose MPS as a pre-modification agent since it possesses a double bond in its structure and could provide a radical formation site in UV-initiated graft polymerization of MPC. This approach alone, without a UV catalyst, had minimal success (data not shown), so Ar plasma was employed to modify the Ti–MPS surface with the hypothesis that Ar plasma treatment would provide a greater number of radical formation sites, as well as peroxide, on the surface than with UV irradiation alone [18]. The application of both an Ar plasma pre-treatment followed by UV irradiation in the presence of a catalyst such as benzophenone [30,46] on the Ti–MPS surface might have led to even further surface grafting, however this was not investigated.

The graft modification approach using Ar plasma treatment with the PMPC on $TiAl_6V_4$ described in this study might be improved with additional study into ways to further control the surface coverage of the grafted PMPC polymer and therefore maximize the anti-thrombogenic properties of the surface. Several factors that should be considered to improve the surface modification process include control of the water plasma in the MPS silanization process, treatment time under Ar plasma and RFGD power settings. The latter two factors are important in this modification technique since they affect the amount of peroxide groups that are able to become radical formation sites by the decomposition of the peroxide groups [12]. In this study, the Ar plasma treatment time was set to 20 s under 25 W of plasma power at a pressure of 0.6 Torr. Several other Ar treatment time points were evaluated between 0 and 120 s in our preliminary studies. However, the PMPC grafted samples from the 20 s Ar plasma treatment showed the lowest surface contact angle ($18.2^{\circ} \pm 4.1$) and were used in our subsequent studies. The amount of peroxide concentration generated on the $TiAl_6V_4$ surfaces after the 20 s Ar plasma treatment was $3.4(\pm 1.6) \times 10^{-8}$ mol/cm$^2$, which was assessed with the plasma treated titanium samples in this study by a peroxide determination method using a 2,2-diphenyl-1-pricylhydrazyl (DPPH) solution [18,19]. However, further study may be necessary to better understand the relationship between the amount of generated peroxide groups and PMPC grafting rate under various Ar plasma treatment conditions in order to establish the best conditions and maximize PMPC incorporation onto the $TiAl_6V_4$ surface.

The MPS modified surface was prepared under hydrolytic condition which allows the methoxy groups in MPS to change to hydroxyl groups and activates the reaction between TiOH and MPS as well as MPS self interaction, thus producing bulk deposition on the surface. Furthermore, the surface roughness might be increased after PMPC grafting, because, the PMPC grafted layer might not be uniform [46]. However the surface roughness or thickness on the MPS and PMPC grafted surface could not measured. We polished the raw titanium alloy sheet by hand with diamond pastes of 3, 1, 0.25 and 0.1 μm size, thus the roughness of the original titanium surface was of a scale that additional texture added from the surface

modification was not likely to be detectable. We chose to use this surface polishing technique since this is the process used industrially to prepare the titanium alloy surfaces before use in blood pump assembly. The surface roughness of the unmodified $TiAl_6V_4$ sample and bulk layer deposition of MPS may have contributed to deviations observed in the atomic composition (Table 1) and contact angle (Table 2) data on the unmodified and modified surfaces. Further study is necessary to measure the surface roughness and thickness of the grafting layer depending on the reaction conditions with a highly polished $TiAl_6V_4$ sample. Fluorescence microscopy observation after staining the PMPC [46], atomic force microscopy and ellipsometry analysis data could be helpful to measure the thickness and roughness of PMPC grafting layer.

In this study, UV irradiation was used to decompose the generated peroxide group and initiate the graft polymerization of MPC. However, the peroxide decomposition also occurs by heating [12,13] and polymerization of MPC might be carried out at 70 °C without UV irradiation if UV irradiation is not appropriate due to complex device geometry. Another limitation to the study is that the blood biocompatibility tests were performed using an in vitro system with ovine blood and under flow conditions that, though mixed, do not replicate any specific application. A next step might be to apply this modification to the interior surfaces of a rotary VAD and assess platelet deposition onto these surfaces in a mock circulatory loop under appropriately high shear blood flow. Comparison studies with other types of modified surfaces such as poly(ethylene glycol)-based or other types of zwitterionic moieties might be of interest to compare the efficacy of this plasma induced grafting technique.

## 5. Conclusions

$TiAl_6V_4$ surfaces were successfully modified with PMPC chains by a plasma-induced grafting technique following surface silanization and Ar plasma treatment. The PMPC modified $TiAl_6V_4$ surfaces showed significantly decreased platelet deposition and bulk phase platelet activation in vitro relative to the unmodified Ti samples. This PMPC grafting on $TiAl_6V_4$ surfaces shows promise for further investigation as a means to reduce the thromboembolic risk associated with the blood-contacting surfaces of cardiovascular devices. In the setting of a rotary blood pump design, such a coating may allow reduction in anticoagulation levels. Further pre-clinical evaluation is warranted to investigate this potential.

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# Nanoscale evaluation of lubricity on well-defined polymer brush surfaces using QCM-D and AFM

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ABSTRACT

For preparing a "highly lubricated biointerface", which has both excellent lubricity and biocompatibility, we investigated the factors responsible for resistance to friction during polymer grafting. We prepared poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC), poly(2-hydroxyethyl methacrylate) (PHEMA), and poly(methyl methacrylate) (PMMA) brush layers with high graft density and well-controlled thickness using atom transfer radical polymerization (ATRP). We measured the water absorptivity in the polymer brush layers and the viscoelasticity of the polymer-hydrated layers using a quartz crystal microbalance with dissipation monitoring (QCM-D) measurements. The PMPC brush layer had the highest water absorptivity, while the PMPC-hydrated layer had the highest fluidity. The friction properties of the polymer brush layers were determined in air, water, and toluene by atomic force microscopy (AFM). The friction on each polymer brush decreased only when a good solvent was chosen for each polymer. In conclusion, the brush layer possessing high water absorptivity and fluidity in water contributes to reduce friction. PMPC grafting is an effective and promising method for obtaining highly lubricated biointerfaces.


## 1. Introduction

In recent years, there has been increasing interest for the surface modification of biomaterials in order to improve their surface properties. All biomaterial surfaces require the ability to suppress biological reactions when they are in contact with living organisms, and we call such contact surfaces as "biointerfaces". Lubricity is also essential for biomaterials such as artificial joints, blood pump bearings, endoscope surfaces, and catheters. The loosening of artificial joints caused by wear between the articulating surfaces is the most serious problem that limits their life and clinical success [1–3]. Biomimetic molecular design of materials is one of the promising approaches to prepare biointerfaces. The cell membrane inspired surface based on phosphorylcholine-group-bearing polymers has shown an excellent resistance to protein adsorption and cell adhesion [4–13]. And also they showed prevention of cell response when they were implanted into tissues. These polymers include 2-methacryloyloxyethyl phosphorylcholine (MPC) units [14]. Poly(MPC) (PMPC) has the ability to resist protein adsorption and also stabilizes functional proteins such as enzymes and antibodies, even when the proteins are immobilized artificially on the surface [15,16]. PMPC is also expected to improve the lubricity of material surfaces since the same phospholipid polar groups are present on the surface of the human articular cartilage. In fact, it has been reported that PMPC grafting onto the polyethylene liner of an artificial hip joint clearly reduces the wear between the articulating surfaces occurring in the long run [17,18]. In this manner, PMPC grafting onto a material surface has already been proved to be an effective method for improving surface lubricity and biocompatibility. PMPC is known to possess a high free water fraction around the chain, which is one of the factors responsible for reducing protein adsorption [15,19]. In this study, we focused on the hydration of grafted polymers and carried out an in-depth investigation of the factors providing both lubricity and biocompatibility on a nanoscale.

We prepared high-density polymer brush layers on a silicon (Si) wafer. Recently, living polymerization techniques have been extensively investigated in order to grow high-density polymer brushes with a controlled length and narrow molecular weight distribution. Atom transfer radical polymerization (ATRP) is one of the best surface-initiated living radical polymerization methods because of its versatility in the choice of monomer types, tolerance to impurities, and mild reaction conditions [20,21]. In this research, we prepared nanoscaled PMPC, poly(2-hydroxyethyl methacrylate)

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(PHEMA), and poly(methyl methacrylate) (PMMA) brush layers by ATRP. We investigated the effects of the hydration of the polymer brush layers on resistance to friction on a nanoscale using a quartz crystal microbalance with dissipation monitoring (QCM-D) and an atomic force microscope (AFM). QCM-D allows the simultaneous measurements of mass and viscoelasticity on a material surface through changes in the frequency ($F$) and energy dissipation ($D$) in a noninvasive manner. The sensitivity of measurements in the ng/cm$^2$ range in liquid phase enables us to clarify the dynamic behavior of the polymer brush layer on a nanoscale [22,23]. Using the QCM-D technique, we evaluated the water absorptivity in the polymer brush layers and the viscoelasticity of the polymer-hydrated layers in water. Subsequently, many AFM or lateral force microscope (LFM) studies have been devoted to the understanding of the influence of thin film structure on friction [24–28]. By evaluating the combined results of the QCM-D and AFM measurements, we identified the key factors responsible for obtaining a highly lubricated biointerface.

## 2. Experimental section

### 2.1. Materials

MPC was synthesized and purified by the previously reported method [14]. The Si wafers were purchased from Matsushita Electric Industrial Co. (Osaka, Japan). The surfaces of the Si wafers were covered with approximately 100-nm-thick $SiO_2$ layers. HEMA and MMA were purchased from Kanto Chemical Co. (Tokyo, Japan) and used as received. Copper (I) bromide (CuBr), 2,2′-dipyridyl, and ethyl-2-bromoisobutyrate were purchased from Sigma–Aldrich Co. (St. Louis, USA) and used as received. All the other reagents and solvents were commercially available in extra-pure grade and were used as purchased. Oxygen and argon gases used were of high-purity grade.

### 2.2. Preparation of the polymer brush layers on the Si wafers

The $SiO_2$-coated Si wafers were cut into 1.0 cm × 2.0 cm chips, rinsed sufficiently with ethanol and acetone, and etched by oxygen plasma for 20 min (300 W, 100 mL/min gas flow, PR500, Yamato Scientific Co. Ltd., Tokyo, Japan). We used a monochlorosilane, 3-(2-bromoisobutyryl)propyl dimethylchlorosilane (BDCS) as the surface initiator for obtaining a homogeneous monolayer of the initiator on the Si wafers. We synthesized BDCS by the previously described method [29]. The cleaned substrates were immersed in a 5 mmol/L toluene solution of BDCS for 24 h. The wafers were removed from the solution, rinsed with methanol, and dried in an argon stream before being used for graft polymerization. The graft polymerization of MPC, HEMA, and MMA on the Si wafers was performed using ATRP. MPC was dissolved in 10 mL of dehydrated methanol, and HEMA and MMA were dissolved in 10 mL of a mixed solvent comprising 4 parts of methanol and 1 part of water. We used dehydrated methanol as the solvent for MPC since it underwent rapid, uncontrolled polymerization in water [30]. Copper (I) bromide (20 mg, 0.135 mmol) and 2,2′-bipyridyl (43 mg, 0.27 mmol) were added to the solutions of MPC, HEMA, and MMA with stirring under argon at room temperature. The molar ratio of the monomer to the free initiator, [Monomer]/[Initiator], was changed in order to change the polymerization degree, by which we controlled the thickness of the polymer brush layers. After the solution was stirred for 30 min under an argon atmosphere, the BDCS-immobilized Si wafers were immersed into the solution, and simultaneously, ethyl-2-bromoisobutyrate (20 μL, 0.135 mmol) was added as the free initiator. The polymerization was carried out at room temperature with stirring under an argon atmosphere. The Si wafers were removed from the polymerization mixture after the desired time period required for the monomer to completely convert into the corresponding polymer. Subsequently, they were placed in a Soxhlet apparatus, extracted with methanol for 20 h, and dried in vacuo at room temperature. The scheme for the preparation of the PMPC, PHEMA, and PMMA brush layers is shown in Fig. 1. The rates of conversion to the free polymer were confirmed by $^1$H NMR (JEOL JNM-NR30, Tokyo, Japan). We prepared PMPC with polymerization degrees of 20, 40, 50, 60, 80, 100, 150, and 200 (henceforth each sample will be referred to as PMPC20, PMPC50, . . ., PMPC200), and PHEMA and PMMA with polymerization degrees of 50, 100, and 150 (PHEMA/PMMA50, PHEMA/PMMA100, PHEMA/PMMA150). The number after abbreviation represents polymerization degree, that is, PMPC20 is PMPC graft chain consisting of 20 monomer units.

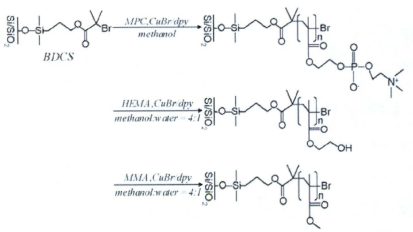


**Fig. 1.** Synthetic scheme for the fabrication of the PMPC, PHEMA, and PMMA brush layers on a Si wafer surface.

### 2.3. Surface characterization

The surface elemental composition was determined using X-ray photoelectron spectroscopy (XPS) (AXIS-Hsi, Shimadzu/Kratos, Kyoto, Japan) with a magnesium anode non-monochromatic source. All the samples were completely dried in vacuo before use. High-resolution scans for $C_{1s}$, $O_{1s}$, $N_{1s}$, and $P_{2p}$ were performed at takeoff angles of 90°. All the binding energies were referred to the $C_{1s}$ peak at 285.0 eV. The static water contact angles were measured using a goniometer (CA-W, Kyowa Interface Science Co., Tokyo, Japan) at room temperature. The samples were completely dried in vacuo before the measurements. Water droplets of 6 μL were contacted onto the substrates and the contact angles at 10 s were directly measured by photographic images. The data were collected at three positions on each sample. The thickness of the PMPC, PHEMA and PMMA brush layers on the Si wafers under dry conditions was determined by ellipsometry (DVA-36L3, Mizojiri Optical Co., Tokyo, Japan). Irradiation with a He–Ne laser (632.8 nm) was performed at an incident angle of 70°. The refractive indices ($n_r$) of Si, PMPC, PHEMA, and PMMA were applied 1.623, 1.488, 1.500, and 1.500, and the extinction coefficients ($k_e$) were 1.604, 0.000, 0.000, and 0.000, respectively [31]. All the measurements were recorded in air at room temperature. The data were collected at nine locations for each sample. The surface morphologies of the PMPC, PHEMA, and PMMA brush layers were observed with a Nanoscope IIIa AFM (Nihon Veeco, Tokyo, Japan) operated in tapping mode. The measurements were performed under ambient conditions using a standard cantilever at a scan rate of 1.0 Hz. Immediately prior to the measurements, the samples were rinsed by sonication in ethanol and dried in an air stream. The root-mean-square (RMS) surface roughness was calculated from the roughness profiles.