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Well-Controlled Nanobiointerface Generated from Phosphorylcholine Block Copolymers Brushes via a "Grafting From" Process

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To better understand protein/material and cell/material interactions at the submolecular level, well-defined polymer brushes consisting of poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) and the block copolymer on silicon wafers were prepared by atom transfer radical polymerization (ATRP). The molecular weight and thickness of the PMPC brush layer on the silicon surface increased with an increase in the polymerization time. By selective decomposition of organosilanes acting as polymerization initiators, the PMPC brush region and sizes were well controlled resulting in the fabrication of micropatterns of the PMPC brushes. When the thickness of the PMPC brush layer was greater than 5.5 ± 1.0 nm fibroblast adhesion was effectively reduced, i.e., cells could recognize such thin polymer brushes on the surface. Furthermore, the block polymerization of glycidyl methacrylate (GMA) from the PMPC brushes was demonstrated and the brush surfaces were characterized.
Keywords: Polymer brush, Phosphorylcholine polymer, ATRP, Nonfouling, Biointerface

INTRODUCTION

There has been a considerable amount of theoretical and experimental interest in the micromanipulation of cell adhesion on solid surfaces, which makes use of the heterogeneous properties of the surface to control cell adhesion. In the presence of serum, adsorption of serum protein strongly influences cell adhesion. Control of serum protein adsorption is very important to form a well-defined pattern of adherent cells. It is generally difficult to control protein adsorption on solid surfaces because nonspecific protein adsorption is the first phenomenon that occurs when the surface is exposed to a physiological environment. The nonfouling properties of base materials might be important in the control of protein adsorption. To obtain a nonfouling polymer surface, we have been studying 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers synthesized as biomimetics in biomembrane structures[1]. The MPC polymers exhibit a property that resists nonspecific interaction with plasma proteins and cells. Further, it has been shown that the activation and inflammatory response of cells in contact with MPC polymers are not induced. While immobilization with MPC polymers is successful in obtaining nonfouling and biocompatible surfaces, the uses of polymers to manipulate proteins and cells on surfaces are rarely reported. Furthermore, the effects of the surface structures of MPC polymers on biofouling on a submolecular scale have not yet been studied.

To produce well-defined polymers, controlled "living" radical polymerization has been explored[2]. Atom transfer radical polymerization (ATRP) is one of the best methods for this process because it can be applied to a wide variety of monomers. An alternative process, pioneered by Wirth and Tsujii[3,4], to prepare well-defined polymer brushes on solid surfaces with ATRP is considerably theoretical and deals with experimental interests in the control of surface properties. Surface-initiated graft polymerization is known as the "grafting from" method and has the advantage of preparing dense polymer brushes. This can be compared with the adsorption of functionalized polymers to solid/liquid interfaces (i.e., the "grafting to" method) due to polymer steric hindrance[4].

ATRP is a robust method because of well-controlled molecular architecture[3]. A previous paper described the effectiveness of ATRP in preparing a well-defined graft polymer on a solid surface[5,6]. ATRP has also been applied to the fabrication of polymer brush micropatterns on solid surfaces. In addition, fabrication of block polymer brushes has been achieved using ATRP.

Control of the surface properties is very important in the production of bio-related materials that are used in biomedical and diagnostic applications. ATRP might be quite useful for this characteristic. However, only a few ATRP trials to optimize protein/material or

cell/material interfaces, that is, biointerfaces, have been reported.

Here, we report the preparation of well–defined PMPC and the block copolymer (PMPC-*b*-PGMA) brushes to optimize the surface structures of bio-related materials.

MATERIALS AND METHODS

Materials

MPC was synthesized by previously reported methods[7]. GMA was purified by distillation before use. 3-(2-Bromoisobutyryl)propyl dimethylchlorosilane (BDCS) was synthesized as previously described[5,6]. Other chemicals were obtained from Aldrich and used without further purification.

Monolayers of BDCS on silicon wafer

BDCS monolayers on silicon wafers were prepared by the method previously reported[6]. Briefly, freshly cleaned silicon wafers were placed in a dry flask to which dry toluene and BDCS were added under an argon gas atmosphere. The flask was allowed to stand for 18 h. The wafers were then removed from the solution, rinsed with toluene, acetone, and absolute ethanol and dried in a nitrogen or argon stream.

Preparation of well-defined PMPC brushes on silicon-supported BDCS monolayer[8]

A mixed solvent of 4 parts methanol and 1 part water was used as a solvent for atom transfer radical polymerization of MPC. Argon gas was used in these solvents to purge any oxygen before the polymerization. Copper bromide (I) and 2,2'-dipyridyl were dissolved in methanol with stirring under argon at 0°C to which water was added. Then, ethyl-2-bromoisobutyrate was added as a sacrificial initiator. The BDCS-immobilized silicon wafers were then submerged into the flask. The MPC, which was separately dissolved in methanol, was added to the flask and polymerization occurred at room temperature with stirring under an argon gas atmosphere. The silicon wafers were periodically removed from the polymerization mixture and rinsed with methanol and water. Subsequently, they were extracted with a Soxhlet apparatus in methanol and dried in an argon stream. The number-averaged molecular weight of free polymer in solution was measured with a GPC system with a refractive index detector and size-exclusion columns with a poly(ethylene glycol) (PEG) standard in distilled water containing 10 mM LiBr.

Cell culture experiment

The PMPC patterned surface was prepared by decomposition of BDCS monolayer using UV-irradiation through a photomask and subsequent polymerization of MPC.

Mouse fibroblasts (L-929 cells) were maintained in a culture medium containing 10% FBS at 37°C in a humidified atmosphere of air containing 5% CO_2. For cell maintenance, the contents of the flasks were detached by treatment with trypsin. The concentration of the L-929 cells was adjusted to 5.0 x 10^4 cells ml/L. The L-929 cells were seeded on silicon wafers and cultured for 20 h in a CO_2 incubator at 95% humidity. After the medium was aspirated, the wafer was rinsed three times with PBS and stained with 8 μM Nile Red /PBS for a few seconds. The wafers were then rinsed with PBS and placed in the 2.5 vol% glutaraldehyde solution to fix the adherent cells on the wafer. The wafer was repeatedly rinsed with distilled water and observed with a scanning fluorescence microscope.

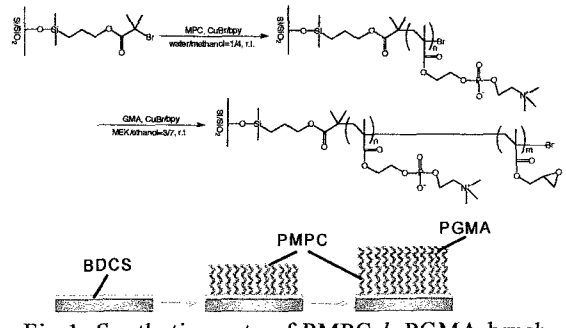


Fig.1. Synthetic route of PMPC-*b*-PGMA brush on silicon wafer via ATRP

Preparation of PMPC-*b*-PGMA brushes

GMA was polymerized from PMPC brushes prepared by 3 h-ATRP, using essentially the same protocol with ATRP of MPC. A mixed solvent of 7 parts methyl-ethyl-ketone (MEK) and 3 parts ethanol was used as the solvent. The silicon wafers were periodically removed from the polymerization mixture and rinsed with THF, acetone, and ethanol. Subsequently, they were extracted with a Soxhlet apparatus in THF for overnight and dried in an argon stream. Then, they were washed by sonication for 2 min in water, rinsed with ethanol, and dried in an argon stream. Fig.1 shows the synthetic route of a PMPC-*b*-PGMA brush on a silicon surface.

Surface analysis

The surface composition was measured by X-ray photoelectron spectroscopy (XPS) using a Scienta ESCA 200 spectrometer with Al Kα X-rays. The dynamic contact angles for the sample plates were recorded with an Erma G-1 contact angle goniometer using purified water as a probe fluid, . The advancing (θ_A) and receding (θ_R) contact angles were measured with addition to and withdrawal from the drop (0-20 μL), respectively. The thickness of the polymer brush was measured using ellipsometers operating with a 532-nm YAG laser at a 50° incident angle or a 632.8-nm He-Ne laser at a 70° incident angle.

RESULTS and DISCUSSION

Controlled radical polymerization techniques, such as nitroxide-mediated radical polymerization, atom-transfer radical polymerization, or reversible addition/fragmentation chain transfer, enable good control of molecular weight with narrow distribution[3]. Well-defined polymer brushes on a solid surface are important for clarifying protein/material and cell/material interactions at their interfaces.

Synthesis of PMPC brushes on silicon surface via ATRP

ATRP of MPC in protic solvents had been studied previously. It has been reported that MPC could be polymerized to high conversions in both water and methanol at ambient temperature. A mixed solvent of water and methanol was used as a polymerization media. We have demonstrated the adsorption of end-reactive PMPC on an organosilane monolayer[9]. The XPS phosphorus composition of the PMPC brush surface prepared by ATRP was much higher than that of the PMPC polymer-adsorbed surface. The water contact angles (θ_A/θ_R) of silicon-supported BDCS monolayers rapidly decreased with an increase in the polymerization periods of MPC and reached a plateau at 15-18/1° after 60 min. Surface wettability was not affected by the substrate after the reaction period. In contrast, equilibrated contact angles of the PMPC brush surface prepared by a "grafting to" method were 60/5°[9]. As mentioned in a previous paper, the surface density of the PMPC brushes prepared by a "grafting from" method would then be higher than that prepared by a "grafting to" method. Moreover, the hysteresis of the equilibrated contact angles (θ_A-θ_R) of the PMPC brush surface prepared by ATRP was <20°. This value is significantly lower than that of the PMPC brush surface prepared by the "grafting to" method or poly(MPC-co-*n*-butyl methacrylate) (PMB), which is mentioned in much of the literature as a nonfouling material[2,8]. From the measurement of the water contact angle, it has been shown that the mobility of the PMPC brushes prepared by ATRP is relatively low and the surface is homogeneous.

The polymer thickness increased with the polymerization time and was controlled from 0~15 nm. The molecular weight of a PMPC brush on a silicon wafer was determined by measuring the molecular weight of a free polymer because reports have described that these molecular weights have similar values. For 18-h polymerization, the molecular weight measured by PEG-calibrated GPC reached at 4.8 x 10^4. The conversion of the PMPC was 98%. In this reactive condition, the ratio of MPC and the free initiator is 200/1. The molecular weight of MPC is 295.3, giving the theoretical molecular weight of the bulk polymer as 5.8 x 10^4 at 98%-conversion. The molecular weight estimated by a PEG-calibrated GPC was almost same as the theoretical molecular weight. The polydispersity ranged from 1.1 to 1.4 were obtained.

From the data in Fig.2, a cross-sectional area per chain, Ax, can be determined from the molecular weight of the chain, M, and the corresponding film thickness, t, by

$$Ax=M/t\rho N_A$$

where ρ is the mass density as determined with an oscillation U-tube (1.30 g/cm^3 for PMPC) and N_A is Avogadro's number. Ax was estimated at ca. 600 Å^2. When the cross-sectional area of PMPC is calculated considering the C-C-C bond (0.25 nm) as the contour length per monomer unit and the bulk density assumed

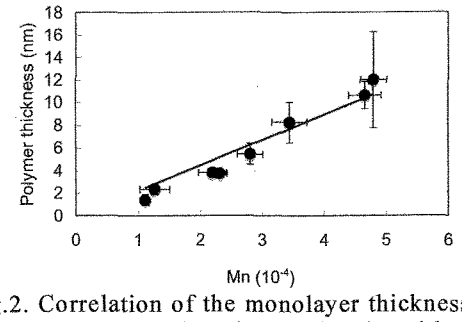
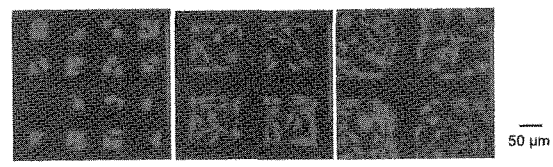


Fig.2. Correlation of the monolayer thickness of a silicon-supported polymer brush with the molecular weight of free PMPC produced by the sacrificial initiator in solution

to be unity[10], the value is ca. 150 Å^2. The experimental value was 4 times larger than theoretical value. The $L_d/L_{c,w}$ ratio was also calculated to be ca. 0.2 on average in this experiment, where L_d and $L_{c,w}$ are the dry thickness and the weight-average contour length of the chain[11], respectively.

Controlled cell adhesion

Fig.3 represents the fluorescence micrographs of fibroblasts that adhered to the pattern surface. Above a PMPC-brush thickness of about 5 nm, cell adhesion was remarkably reduced, i.e., the cells were able to recognize the thickness of the thin brush. Although the thickness of the cast film of PMB that we made has been normally controlled on the submicron scale, the thickness of the PMPC brush can be controlled on the scale of a few nanometers. This is a great advantage for surface modification in improving the nonfouling properties of micro- or nanodevices. Surface modification with well-defined MPC polymers would be considered as a robust method of optimizing biointerfaces on a molecular scale. Microfabrication with MPC polymers may prove to be important in separations, biosensors, and the development of biomedical materials.



Polymerization time: 18 h
Polymer thickness: 12 nm

Fig.3. Fluorescence micrographs of fibroblast adhesion to patterned PMPC brush surface after incubation for 20h. [Fibroblast] = 5.0 X 10^4 cells/mL.

Synthesis of PMPC-*b*-PGMA brushes on silicon surfaces via ATRP

To demonstrate the living characteristic of the surface-initiated polymerization, copolymerization with GMA were performed using the already grown PMPC brush as a macroinitiator for ATRP.

The PGMA brush thickness linearly increased with an increase in polymerization periods and reached about 8 nm after polymerization for 18 h (Fig. 4). Table I

shows XPS elemental composition and water contact angle data for polymer brushes. The C/O ratio was determined by XPS for these polymer brushes. The ratio of the PMPC-*b*-PGMA block polymer brush surfaces became similar to that of the GMA molecule (C/O=7/3) with an increase in the polymerization periods of GMA. These results indicated that GMA could be polymerized from PMPC brushes. PGMA brushes were also directly prepared from the BDCS monolayer without the PMPC brush layer. The water contact angle (θ_A/θ_R) decreased from 72/62° to 48/19° after polymerization for 17.5 h. In contrast, the water contact angle of the PMPC brush changed from 20/1° to 36/1° after GMA polymerization for 18 h. Although the PGMA-brush thicknesses of PMPC-*b*-PGMA and PGMA were nearly equal, the water contact angle of the block copolymer brush was significantly lower than that of PGMA. This difference is considered due to the substrate under the PGMA brush and the density of the brush. Detailed studies are currently underway.

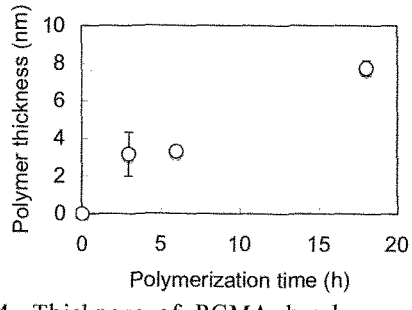


Fig.4. Thickness of PGMA brushes grafted from PMPC brushes as a function of polymerization time.

Table I . The surface composition determined by XPS and the water contact angles of the polymer brushes.

Abb.	Polymerization time of GMA (h)	XPS data (Take off angle 15°) (%)						Water contact angle (°)	
		C	O	Si	Br	N	P	θ_A	θ_R
PMPC7.2[a]	-	53.6	30.4	8.3	0.2	2.3	5.2	18	<1
Theoretical composition of PMPC	-	57.9	31.6	-	-	5.3	5.3	-	-
PMPC5.9[a]-*b*-PGMA4.1[a]	3	61.1	29.2	1.2	0.2	2.8	5.4	28	<1
PMPC5.9[a]-*b*-PGMA8.0[a]	18	63.3	30.4	1.1	0.3	1.5	3.3	36	<1
PGMA9.1[a]	17.5	71.2	27.7	0.9	0.2	-	-	48	19
Theoretical composition of PGMA	-	70.0	30.0	-	-	-	-	-	-

[a] Polymer thickness (nm)

CONCLUSION

A well-defined dense PMPC brush on a silicon wafer was prepared by the "grafting from" method with ATRP. The surface properties could easily be controlled, even on a nanoscale. The dense brush layer was able to reduce fibroblast adhesion with a polymer brush about 5 nm thick. Because the microscale pattern of the brush layer was fabricated by decomposition of the initiating monolayer with UV irradiation, cell adhesion was easily manipulated. Surface modification with dense PMPC prepared by ATRP might be important in creating optimal biointerfaces because cell/material interactions could be controlled with thin polymer brushes on a molecular scale. PMPC immobilization with ATRP is also a robust process for improving the nonfouling properties in the micro- and nanofabrication of biomedical materials. MPC block copolymer brushes with GMA were also demonstrated. Epoxy groups in GMA molecules have the potential for the immobilization of biomolecules. Well-defined block copolymer brush surface may be then useful to produce nanobiointerfaces.

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