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A Simple Method to Fabricate Hemocompatible Coating by Crosslinkable Biomimetic Bipolymer†

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2-Methacryloyloxyethyl phosphorylcholine (MPC) and 3-(methacryloyloxy) propyltrimethoxysilane (TSMA) were used to synthesize the crosslinkable biomimetic copolymer PMT by the free radical copolymerization. The PMT82, the molar ratio 8:2 of MPC and TSMA in copolymer, was chosen as the coating material. It was dissolved in methanol and dip-coated on cover glass in order to obtain the biomimetic coating. The PMT coatings were fixed onto the glass substrate through heating in moisture atmosphere. The surface hydrophilicity, elemental composition, blood compatibility of the coatings were measured by dynamic contact angles, X-ray photoelectron spectroscopy, platelet adhesion and protein adsorption *in vitro*, respectively. The results indicated that the polymer coatings have been successfully immobilized onto the surface of cover glass, which improved the surface hydrophilicity of cover glass and granted it excellent blood compatibility. The biopolymer PMT will be a promising coating material, and the simple surface modification method will be useful in biomaterials field.

Keywords: Phosphorylcholine, Surface modification, Biomimetic coating, Hemocompatibility, Crosslinkable polymer.

INTRODUCTION

With the rapid development of modern medical science, various kinds of minimally invasive interventional medical devices, such as catheters, vessel stents, sensors, heart aids and the like, have been widely applied to medical application and have greatly enriched the treatment means of modern medicine clinics[1]. However, when these devices come into contact with living organisms, thrombus formation, infection and other unfavorable immune responses occur sooner or later, which cause discomfort to the patient and increase the cost and, in some cases, means that more surgery is required[2]. At present, the main methods on improving the blood compatibility of interventional medical materials, are grafting or coating with vascular endothelial cell, albumin and phosphorylcholine groups or polymers[3]. The hydrophilic zwitterionic polar phosphorylcholine (PC) group is the major lipid headgroup of the extracellular lipid bilayer, which is the cause of the extracellular surfaces of blood cell membranes thromboresist[4]. Thus, several researchers have used various approaches to bind PC groups on the surface of many biomaterials to improve the blood compatibility[1,5-8]. The substrate materials include glass[9,10], chitosan[11,12], stainless steel[13,14], carbon nanotube[15], polymers[16-18] and so on. Surface grafting can covalently bind PC groups on the surfaces firmly, but requires a complicated process and results in a lower grafting density[9,10,15,19]. Relatively, a coating procedure is simple, convenient and practical. In fact, a physically adsorbed polymer layer may dissolve or decompose upon implantation *in vivo*[20]. In addition, amphiphilic PC polymer coated on a biomaterial surface forms a film with hydrophobic groups on its surface, which means the morphology is similar to the reverse structure of the cell outer membrane[8]. These unstable factors affected the surface properties and applications of these kinds of surface modification materials. The researchers are searching for a technique that can modify the surface simply like the physical coating process and the binding strongly like grafting onto the surface.

Ishihara *et al.*[21] synthesized a mussel-inspired adhesive polymer bearing PC groups, which can adhere on the surface of titanium substrate quickly and firmly and improve the

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hemocompatibility of substrate. Gong *et al.*[22] prepared a doubly biomimetic polymer bearing PC groups and mussel adhesive protein catechol groups (DOPA). This polymer has excellent compatibility and versatile adhesivity on many substrate surfaces. However, the synthesis of the polymers bearing PC groups and DOPA groups is difficult. Inspired from the adhesion arise from the reactivity of DOPA, we have thought to bring the crosslinkable siloxane into the PC polymers to acquire the same effect. Our previous researches showed that the crosslinkable siloxane groups in PC polymers can be immobilized by synergistic grafting and crosslinking effects[11,18].

As glass based biomaterials are very important in clinic medicine[23,24], we use the common glass as the model substrate. This paper provides a simple technique to prepare biocompatible polymer coating. Blood compatible monomer 2-methacryloyloxyethyl phosphorylcholine (MPC) and crosslinkable monomer 3-(methacryloyloxy) propyltrimethoxysilane (TSMA) were applied to synthesize the polymer as coating material. The crosslinkable trimethoxysilyl groups in TSMA could covalently bond on the glass surface in certain conditions and the density of PC groups on the surface could be adjusted through the concentration of the copolymer solution and/or the thickness of coating layers.

EXPERIMENTAL

2-Methacryloyloxyethyl phosphorylcholine (MPC) was prepared according to the method reported by Ishihara *et al.*[25] 3-Trimethoxysilylpropyl methacrylate (TSMA) was purchased from Aldrich Chemical Co. and used without further refining. The initiator 2,2'-azoisobutyronitrile (AIBN) was recrystallized from methanol. Tetrahydrofuran (THF) and isopropanol (iPA) were both distilled over calcium hydride (CaH_2). Diethyl ether (Et_2O) was distilled over Na metal before use. Methanol was distilled over Mg metal. Bovine serum albumin (BSA) and bovine plasma fibrinogen (Fg) were purchased from Sigma-Aldrich. Platelet-rich plasma (PRP) was obtained by centrifugating the fresh blood, provided by a healthy volunteer, according at 1000 rpm for 10 min[26].

Synthesis and characterization of PMT: The copolymer PMT was synthesized with MPC and TSMA according to the method reported by Gong *et al.*[11], through the monomer-starved method of free-radical copolymerization (Fig. 1). The structure identification and composition of PMT were determined by ^{1}H NMR spectra with an INOVA400 NMR spectrometer (Varian, American, 400 MHz).

Surface modification of glass substrates: Cover glasses (24 mm × 24 mm × 0.18 mm) were cleaned according to a procedure reported by Gong *et al.*[8]. The clean and dry cover glasses were dip-coated with a PMT solution of 1-10 mg/mL in methanol in order to get the coatings with the thickness of 50-500 nm. The modified cover glasses were dried in vaccum at room temperature to make the coating adhere onto the surface. Then they were kept in a moisture atmosphere for 3-6 h at 110 ºC, in order to make the coatings crosslink and graft onto the surfaces of substrates.

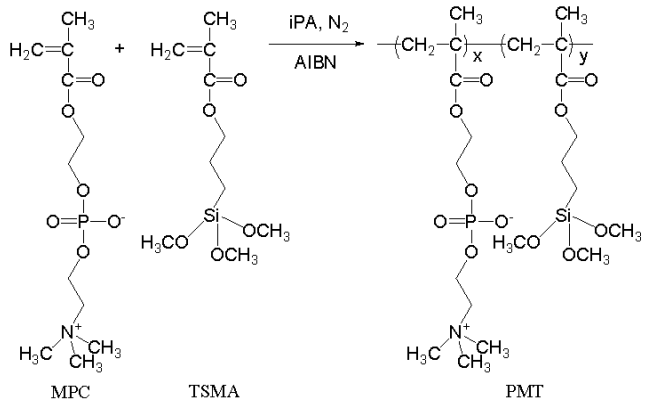

Fig. 1. Synthetic scheme of the polymer PMT

Dynamic contact angle: Water contact angle of the unmodified and modified cover glasses were both measured through the Wilhelmy plate technique using a DCAT21 tensiometer (DataPhysics Instrument, Germany). The measurement was performed successively for three cycles in ultrapure water at 20.0 ± 0.1 ºC, with a speed of 0.05 mm/s in advancing and receding. The surface tension of water was 72.75 mN/m and resistivity was 18.25 MΩ·cm at 20.0 ºC.

X-Ray photoelectron spectroscopy: The surface elemental composition was determined by X-ray photoelectron spectroscopy (XPS) using an Axis Ultra system (Kratos Analytical Ltd., England) with an Al K_α X-ray source (150 W, 15 kV, 1486.6 eV). All spectral data were collected at an electron take-off angle of 45º from the surface. The binding energy (BE) scale was corrected as a reference of C1s peak at 284.8 eV.

Protein adsorption: *In vitro* protein adsorption experiments were carried out in phosphate buffer saline (PBS, pH = 7.4, I = 0.16 mol/L). The surfaces were determined with two proteins BSA and Fg. The solution of proteins was prepared according to the reported protocol[27] with 4.5 mg/mL of BSA and 0.3 mg/mL of Fg. The surfaces were equilibrated in PBS for one night and then incubated in each protein solution at 37 ºC for 2 h[28,29]. After incubation, the surfaces were rinsed three times with PBS to remove the proteins not adsorbed. The amount of adsorbed proteins was determined by the micro bicinchoninic acid (BCA) method[30].

Platelet adhesion: The surfaces were immersed in PBS for 2 h and then rinsed three times with PBS before the platelet adhesion test. Then, 20 µL PRP was placed on the center of each sample, which was incubated at 37 ºC for 2 h in a humidified air. After that, the plates were rinsed carefully with PBS for three times. Subsequently, they were immersed into 2.5 wt % glutaraldehyde solution for 1h in order to fix the adhered platelets. At last, they were washed with PBS and water and dried at room temperature. The platelet adhesion on the surfaces were observed by scanning electron microscopy (JEOL JSM-6700F, Japan)[29,31-33].

RESULTS AND DISCUSSION

Synthesis and characterization of PMT copolymers: As being apt to crosslink under moisture, heat or UV light, the amount of TSMA in PMT copolymers was difficult to higher than 40 mol %. Monomer-starved method was used to produce

polymers which composition of monomer is more consistent with its feed[34]. The structure of PMT copolymers can be identified by ^{1}H NMR spectra (as solvent of $CDCl_3$:CD_3OD = 1:1 (v/v), Fig. 2). δ (ppm): 0.7(CH_2Si), 0.9(α-CH_3), 1.3 ($SiCH_2C\underline{H}_2$), 1.9 (CCH_2), 3.3 [$N^+(CH_3)_3$], 3.6 [$Si(OCH_3)_3$], 3.7 (NCH_2), 4.0-4.4 (OCH_2). It can be found that there was no peak at 5.58 or 6.15 ppm, which means there is no monomer residue in the copolymer. The molar fractions of TSMA and MPC units in PMTs were calculated from the peak at 0.7 and 3.3 ppm, respectively. Table-1 listed the relationship between monomers in feed and in copolymers. As the TSMA units in PMTs were lower than 10 mol %, the copolymer was difficult to crosslink or react with glass substrates; while as the TSMA units were higher than 30 mol %, the copolymer was apt to crosslink itself. Experiment showed that about 20 mol % of TSMA units in PMTs was a proper amount. Thus, PMT82, the molar ratio of MPC and TMSA units was 8/2, was used in the following.

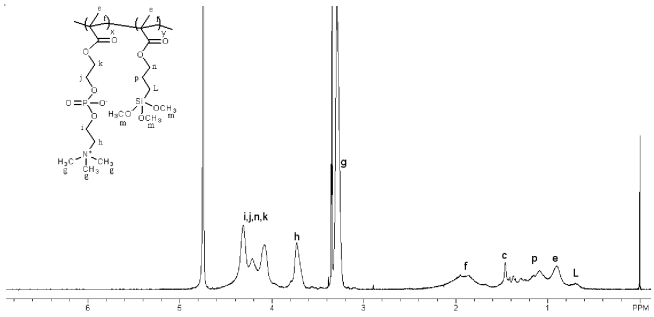

Fig. 2. ^{1}H NMR of PMT

TABLE-1
RELATIONSHIP BETWEEN COMPOSITION OF PMTS IN FEED AND IN COPOLYMER (^{1}H NMR)

MPC/TMSA (mol %)	PMT64	PMT73	PMT82	PMT91
In feed	60.0/40.0	70.0/30.0	80.0/20.0	90.0/10.0
In copolymer	66.7/33.3	76.6/23.4	80.0/20.0	95.0/5.0

*The number after the PMT represents the molar ratio of MPC and TSMA units in feed.

TABLE-2
ELEMENTAL COMPOSITION OF THE PMTS MODIFIED SURFACES MEASURED BY XPS

Surface	Atomic concentration (%)				
	C_{1s}	O_{1s}	N_{1s}	P_{2p}	Si_{2p}
Before DCA	67.87	21.35	3.18	6.21	1.38
After DCA	37.18	38.72	1.73	3.11	19.26
*PMT82	58.70	31.52	4.35	4.35	1.08

*Theoratical elemental composition calculated from the PMT82.

Surface properties of PMT coatings

Surface hydrophilicity: The surface hydrophilicity was investigated by dynamic contact angle (DCA) measurement. This technique can reflect the small changes on the surface sensitively[35]. Fig. 3 showed the contact angle cycles of cover glass and PMT82 modified surface. The advancing contact angle (θ_A) and receding contact angle (θ_R) of PMT82 modified surface were 42º and 5º, respectively; while the θ_A and θ_R of virgin cover glass treated at the same conditions were 62º and 30º, respectively. The lower θ_A and θ_R of the modified surface

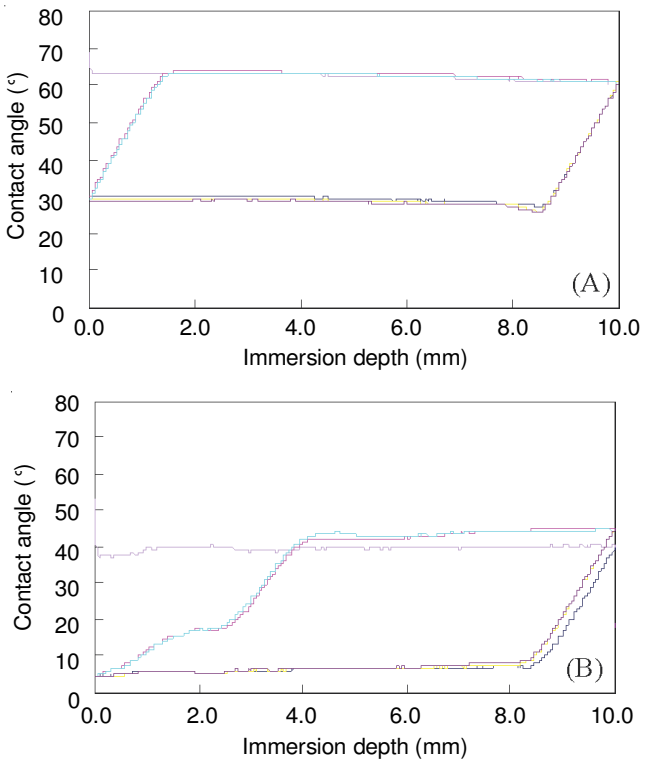

Fig. 3. Dynamic contact angle cycles of (A) cover glass and (B) PMT82 modified surfaces

showed that the dip-coating and heating treatment can make the PMT82 graft onto the surface of glass substrates and improve its hydrophilicity. If the coatings were placed in a 5 % triethylamine atmosphere for 10 days before keeping in the moisture and heating atmosphere, the θ_A would be reduced to about 30º. This result showed that the weak alkaline atmosphere is favorable for crosslink and fixing the MPC units of PMT coatings at outermost layer.

Surface elemental composition of the PMT82 coatings: The XPS spectra of PMT82 coatings before and after DCA measurement were given in Fig. 4 and the surface elemental composition results were listed in Table-1. Before DCA, the coating was 50 nm in thickness. The ratio of N/P near to 1:2, lower than the theoretical value 1:1, showed that about a half of MPC units have cracked and lost the section of $-OCH_2CH_2N^+(CH_3)_3$ during heating treatment. Thus, the contents of elements C, P and Si were all higher than the theoretical value. After DCA, the content of element Si increased much with the decrease of other elements, which showed that PMT82 coatings had partial loss during DCA determination. Hence, the above-mentioned heating treatment can't make the PMT82 coatings crosslink and graft onto the glass substrate thoroughly. This problem was incurred by the treatment for a long time at higher temperature. Gong *et al.*[11] reported that the PMT coating can be immobilized on chitosan surface after keep in a weakly alkaline atmosphere and heating treatment. However, the same treatment was not effective for the glass substrate. The glass substrate has less -OH radicals than chitosan and its surface is so rigid that can't change its structure during treatment. Hence, its reactivity and grafting degree are not high. Shorten treating time and/or decrease the temperature properly may be helpful for decreasing the crack of MPC units,

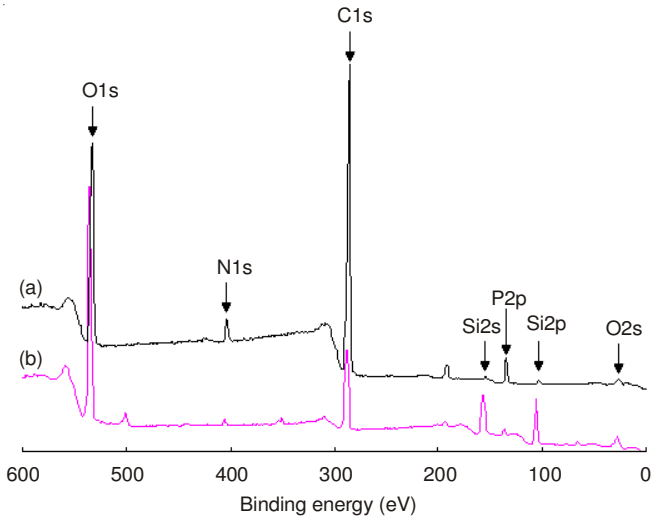

Fig. 4. XPS spectra of PMT82 coatings (a) before and (b) after DCA

while the dissolving loss in DCA determination should be solved through other approaches.

Blood compatibility evaluation: The blood compatibility plays a vital role in the application of biomaterials[11], which can be tested by the non-specific protein adsorption and platelet adhesion *in vitro*.

Protein adsorption: Protein adsorption result was presented in Fig. 5. Although the virgin glass surface exhibits certain hydrophilicity, it absorbed proteins both BSA and Fg significantly, respectively 0.76 and 1.11 μg/cm^2. However, the amount of protein adsorption on the PMT82 modified glass surfaces reduced sharply, respectively just 0.09 and 0.04 μg/cm^2, as the result of the surface PC groups. PC group was strong hydrophilic and could bond large of free waters, thus the PC polymer modified surface could suppress protein adsorption and inhibit the conformational change of protein adsorbed[36].

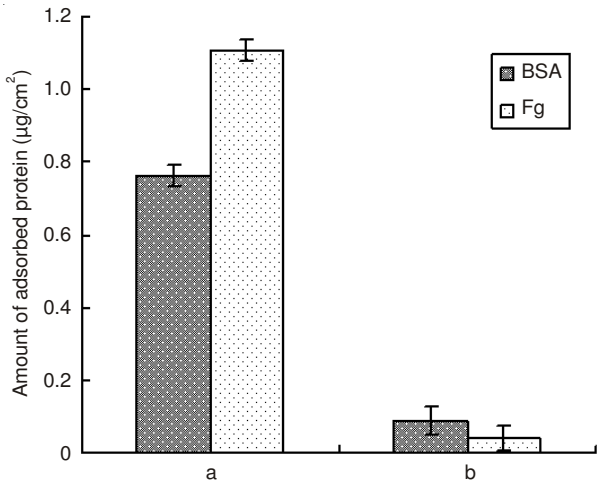

Fig. 5. Protein adsorption on the (a) virgin cover glass and (b) PMT82 modified surfaces. The bars represent the standard deviation (n=3)

Platelet adhesion: Platelets have disc-shaped appearance, 2-4 μm in diameter. They are indispensable in haemostasis following injury, while the inappropriate platelet activation can lead to thrombosis[37]. Fig. 6 presented that many platelets adhered on the virgin glass surface and most of them had

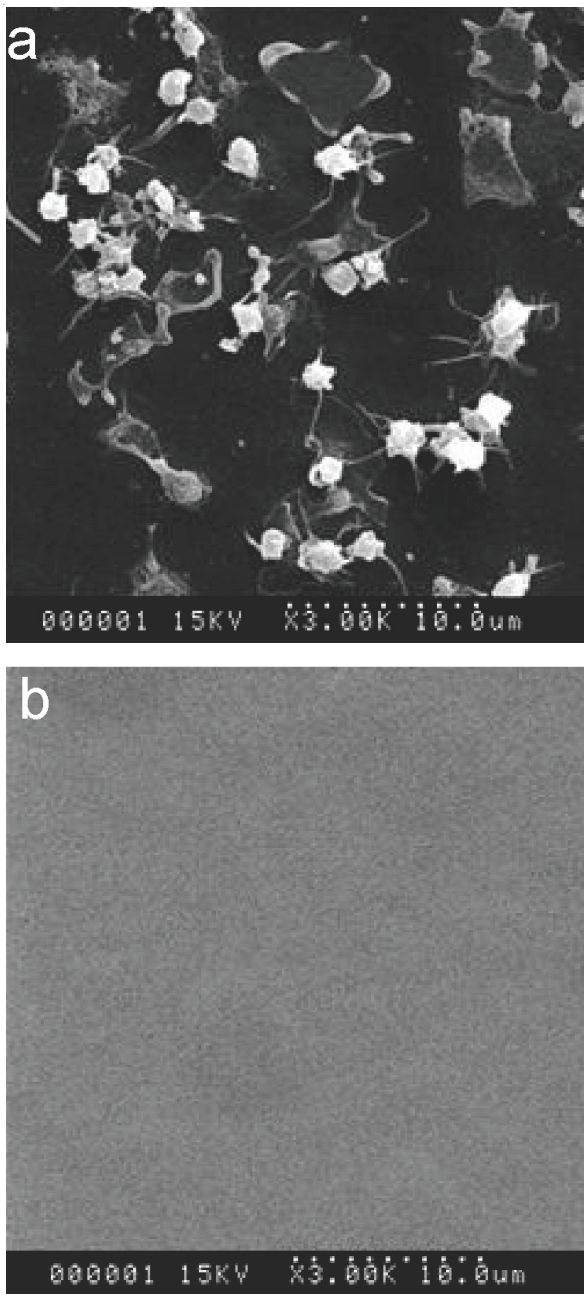

Fig. 6. SEM photographs of platelet adhesion on the (a) virgin cover glass and (b) PMT82 modified surfaces

pseudopodia and aggregated together, while no platelet was detected on the PMT82 modified surface. This showed that the hydrophilic PC groups on the surface can effectively inhibit the platelet adhesion, activation and coagulation.

Conclusion

This report has provided a simple surface modification method with a PC copolymer bearing crosslinkable units of TSMA as coating material and common glass as model substrate. The modified surface was verified to have fine hydrophilicity as a result of the cell outer membrane hydrophilic PC groups in the coating. Although the surface grafting ratio of PC groups was not as high as predicted, the excellent blood compatibility of the coating makes the PMT82 polymer to be a promising coating material in biomaterials field. The surface

modification method by dip-coating and heating treatment in moisture atmosphere can be applied to various hydrophilic surfaces of biomaterials.

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