

MOLECULAR ENGINEERING STUDIES ON NONTHROMBOGENIC BIOMATERIALS —A NOVEL CLASS OF NONTHROMBOGENIC BIOMATERIALS WITH ZWITTERIONIC STRUCTURE OF CARBOXYBETAINES*

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Abstract *N,N*-dimethyl-*N*-methacryloyloxyethyl-*N*-carboxyethyl ammonium (DMMCA) was graft-copolymerized onto the surface of segmented poly(ether urethane) (SPEU) and PE film. The carboxybetaine structure on SPEU and PE film surfaces was confirmed by ATR-FTIR. XPS and water contact angle measurements. Through the experiments with platelet adhesion and protein adhesion assay *in vitro*, the two materials studied, including poly-DMMCA gel, all show excellent nonthrombogenicity. This confirms once again that the zwitterionic molecular structure on the surfaces of materials is essential for improving their nonthrombogenicity and biocompatibility.

Keywords: Nonthrombogenic biomaterials; Zwitterionic biomaterial; Carboxybetaine structure; Surface graft-copolymerization.

INTRODUCTION

Nonthrombogenic biomaterials have been extensively investigated since late 1940s^[1–3]. Biomaterials including PEO structure and polyphosphorylcholine methacrylate developed by Ishihara *et al.* based on the biomimic idea of biomembrane have shown considerably improved nonthrombogenicity^[2, 3, 4]. Based on the hypothesis of maintaining normal conformation for blood compatible biomaterials, it is logically inferred that in blood the zwitterionic interfacial molecular structure of biomaterials would be thermodynamically advantageous for maintaining the natural conformation of plasma proteins contacted and hence would have excellent nonthrombogenicity^[2, 3]. Betaines are the typical representatives of zwitterionic molecules. Mainly, there are three classes of betaines: sulfobetaines, phosphobetaines and carboxybetaines. In our previous papers^[5–20], we have reported that biomaterials bearing zwitterionic structure, either sulfobetaines or phosphobetaines, show excellent nonthrombogenicity. Then, how is the nonthrombogenicity of carboxybetaine molecular structure. In this study, poly-*N,N*-dimethyl-*N*-methacryloyloxyethyl-*N*-carboxyethyl ammonium (PDMMCA) and two kinds of biomaterials bearing zwitterionic structure of PDMMCA were synthesized and their nonthrombogenicity evaluated by protein adsorption and platelet adhesion *in vitro* were revealed.

EXPERIMENTAL

Materials

2-(Dimethylamino) ethyl methacrylate was purchased from Aldrich, β -propiolactone was obtained from Acros and 25% glutaraldehyde water solution was provided by Shanghai Chemical Reagent Co. Sodium persulfate was

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recrystallized before used. 4,4'-Diphenylmethanediisocyanate (MDI) and 1,4-butanediol (BD) were purified by vacuum distillation. Methyl ethyl ketone, methanol, dimethylsulfoxide (DMSO), methylisobutylketone (MIBK) and *N,N*-dimethylformamide (DMF) were dehydrated before use.

Synthesis of DMMCA Monomer

DMMCA monomer was synthesized through the reaction of 2-(Dimethylamino)ethyl methacrylate with β -propiolactone^[21, 22], as shown in Fig. 1. Its structure and purity were determined by IR, ¹H-NMR and elemental analysis.

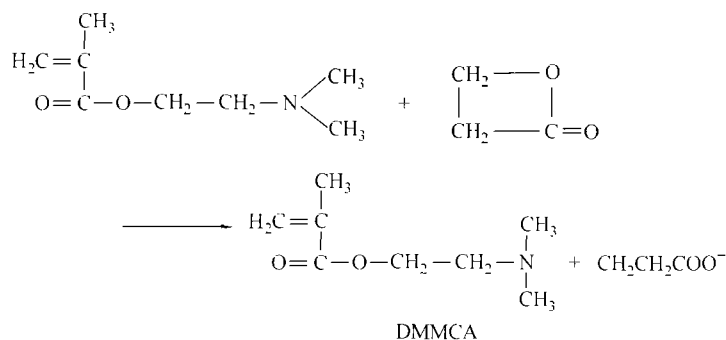


Fig. 1 Synthetic route for DMMCA

Polymerization of DMMCA

Monomer DMMCA, sodium persulfate and *N,N*-Methylenebisacrylamide was weighed according to the mole ratio of 100:0.5:3 and dissolved in distilled water. The concentration of the monomer was 1.0 mol/L. The solution was degassed under vacuum and then poured into a glass mold. Polymerization was carried out at 50°C for 72 h to produce transparent gel. In order to remove the residual monomers, the polymer film was immersed into distilled water for 1 week, during which distilled water was renewed every 24 h.

Synthesis of Segmented Poly(ether urethane) (SPEU)

Segmented poly(ether urethane) (SPEU) was synthesized by the procedure described in Refs. [23] and [24]. The film was cut into circular pieces (10 mm in diameter), which were then purified by Soxhlet extraction with methanol for 24 h and dried in a vacuum oven at 40°C until constant weight.

Preparation of PE Film

The PE films were commercial products. They were cut into pieces (1 cm × 2 cm), extracted with methanol for 24 h in a Soxhlet extractor, and then dried in a vacuum oven at 40°C until constant weight.

Graft Polymerization

Graft polymerization of DMMCA onto SPEU film surface

The ozone-induced graft copolymerization was carried out using the method described in Refs. [25, 26], and was presented schematically in Fig. 2. The ozone pretreatment of films was carried out at 30°C in an ozone generator operating at 200 V for 30 min. The inlet flow rate of O₂ was 60 mL/min with the generative amount of O₃ = 2.45 × 10⁻⁴ mL/min. The ozone-activated films were degassed under vacuum at room temperature for 1 h, and then placed into ampoules. The deaerated aqueous solution of DMMCA of definite weight percent concentration (2%, 5%, 10%, 15% and 20%) containing 6 × 10⁻⁴ mol/L Fe²⁺ ion were added to the ampoules. The ampoules were then sealed and kept at 50°C for 24 h. Finally, the grafted films were first washed with 40–50°C saline water, and then washed by supersonic agitation for another hour to remove the homopolymer from the grafted films.

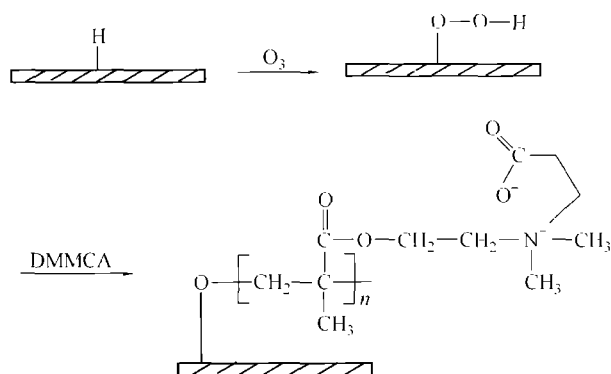


Fig. 2 Schematic representation of SPEU film surface modified with DMMCA

Surface graft copolymerization of PE film

The PE film was treated by a method similar to that used in the treatment of SPEU films. The difference in the treatment was that the films were ozone activated for a set of time (10, 20, 30 and 40 min) and then graft copolymerized with DMMCA of 10% weight percent concentration.

Determination of surface peroxide group

The concentration of surface peroxide group was determined by the method described in the Refs. [27] and [28]. The reported amounts are the averaged values of measurements on three specimens.

Surface Characterization

ATR-FTIR measurements were performed on a Nicolet 170 sx FITR equipped with an omni sampler over 32 scans. The spectra were recorded with a resolution of 4 cm^{-1} and with a 45° incident angle using a Ge crystal. The ESCA (ESCA LAB MK II, V. G. Scientific Co., UK) was equipped with Mg K α radiation source operated at 12 kV and 20 mA at the anode. The take-off angle of photoelectron was kept at 45° . The bonding energy was referenced by setting the C_{1s} hydrocarbon peak to be 285.0 eV. The water contact angle measurements were measured by a sessile drop method using an optical bench-type contact angle goniometer (Model RAMHAPT-100, USA). 0.5 μL purified water was dropped onto the film surface and direct microscopic measurement of the contact angles was performed with the goniometer.

Platelet Adhesion in vitro

Because platelet adhesion is the most pivotal step in blood coagulation, platelet adhesion study was always carried out to evaluate the blood compatibility of the materials. Freshly prepared platelet-rich plasma (PRP) was directly provided by Blood Center of Nanjing City Red Cross. The films were soaked in phosphate buffered saline (PBS, pH 7.4) for 2 h, and then incubated in PRP at 37°C for a given time. After that, the non-adherent platelets were removed by rinsing with PBS for 3 times. The films were then soaked in 2.5% glutaraldehyde for 30 min to fix the adhered platelets. After washing with PBS again, the platelets adhering to the films were dehydrated in a gradient ethanol solutions (50%, 60%, 70%, 80%, 90%, 95% and 100%) for 30 min each and allowed to dry at room temperature. The platelet-attached surfaces were gold deposited in vacuum and examined by SEM (LEO 1530VP Scanning Electronic Microscopy, Germany).

Protein Adhesion in vitro

Bovine fibrinogen (BFG, Sigma, F-8630) was obtained as lyophilized powders. The buffer used in the protein adsorption experiments was PBS, pH 7.4. Quantitation of adsorbed protein on the polymer surfaces was performed using ^{125}I -labelled protein, which was added to unlabelled protein solution in order to obtain a final activity of about 10^7 cpm/mg. The samples were immersed in 1 mL buffer solution at 37°C , and then 1 mL fibrinogen solution (0.2 mg/mL) was added and mixed. Adsorption tests were carried out at 37°C for 1 h. After protein adsorption, samples were rinsed three times with 2 mL buffer. The gamma activities of the samples placed in the radio-immunoassay tubes were counted by a Gamma Counter (FH-408, Beijing Nucleus Instrument

Factory). Four replicates were done. The counts from each sample were averaged and the surface concentration was calculated by the following equation:

$$\text{BFG} (\mu\text{g}/\text{cm}^2) = \text{Counts (cpm)} C_{\text{solution}} (\mu\text{g}/\text{mL}) / [A_{\text{solution}} (\text{cpm}/\text{mL}) S_{\text{sample}} (\text{cm}^2)] \quad (1)$$

Where count measures the radioactivity of the samples, S measures the surface area of the samples, C_{solution} and A_{solution} are the concentration and the specific activity of the protein solution, respectively.

RESULTS AND DISCUSSION

Influence of Ozone-Induced Time on the Concentration of Surface Hydroperoxide Group

The concentration of hydroperoxide group on the film surfaces after ozonization for different time results is shown in Fig. 3 and Fig. 4. The hydroperoxide group increases with the increase of ozone-induced time. The hydroperoxide group concentration on SPEU surface increases rapidly in the first 10 min, and then decreases. After 30 min, the rate was obviously slow. And the concentration changed a little from 30 min to 60 min. So we selected 30 min as ozone-induced time for SPEU with different monomer concentration (2%, 5%, 10%, 15% and 20% weight percent concentration). As for PE films, the hydroperoxide group also increased along with the increase of the ozone-induced time. But in the first 60 min the concentration increased step by step. And after 90 min the rate slowed down obviously. So we employed a set of time (10, 20, 30 and 40 min) as ozone-induced time with the same monomer concentration (10% weight percent concentration).

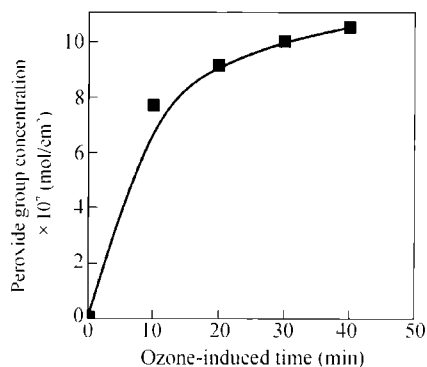


Fig. 3 Influence of ozone-induced time on the hydroperoxide group concentration on SPEU films

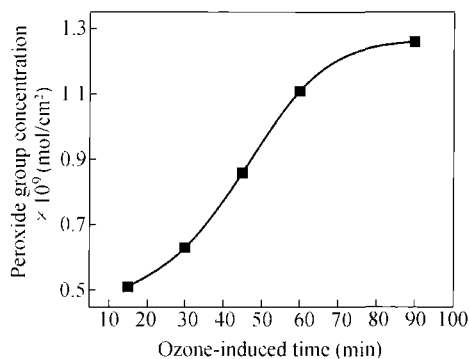


Fig. 4 Influence of ozone-induced time on the hydroperoxide group concentration on PE films

Surface Characterization

ATR-FTIR measurements

The ATR-FTIR spectra of SPEU film (a), SPEU-g-PMMCA film (b), PE film (c) and PE-g-PMMCA film (d) are shown in Fig. 5. In the spectrum (a) of SPEU film, the NH absorption peak appear at 3330 and 1533 cm^{-1} , the absorption peak of urethane $-\text{NHCOO}-$ carbonyl appears at 1700 cm^{-1} . The peak at 1110 cm^{-1} is assigned to the stretching vibration of $\text{C}-\text{O}-\text{C}$ band. Both the NCO absorption peak (2250 cm^{-1}) and OH absorption peak (3500 cm^{-1}) in polyether become disappeared indicating that the synthetic reaction of SPEU was completely achieved. In comparison with the spectrum (a), the spectrum (b) of SPEU-g-PDMMCA has an absorption peak of stretching vibration of $\text{C}=\text{O}$ at 1700 cm^{-1} , an asymmetric stretching vibration of COO^- at 1596 cm^{-1} and a symmetric stretching vibration of COO^- at 1375 cm^{-1} , which can be attributed to the respective groups of DMMCA grafted onto SPEU surface. In the spectrum (c), asymmetric and symmetric stretching vibration of $\text{C}-\text{H}$ appear at 2917 cm^{-1} and 2843 cm^{-1} , respectively. The deformation vibration of CH_2 and CH_3 is found at 1472 cm^{-1} . The peak at 718 cm^{-1} is the absorption peak of $(\text{CH}_2)_n$ (when $n > 4$). But in the spectrum (d), the presence of stretching vibration of $\text{C}=\text{O}$ at 1726 cm^{-1} , asymmetric stretching of COO^- at 1597 cm^{-1} and the symmetric stretching of COO^- at 1369 cm^{-1} indicate that the monomer has also been successfully grafted onto the PE films surface.

Figure 6 is the ATR-FTIR spectra of grafted PE films with different ozone-induced time. The intensities of three main peaks (1369 , 1597 and 1726 cm^{-1}) in the spectra are increased along with the increase of the ozonization time (10 , 20 , 30 and 40 min), which support the above conclusion that the concentration of hydroperoxide group increase along with the increase of ozonization time.

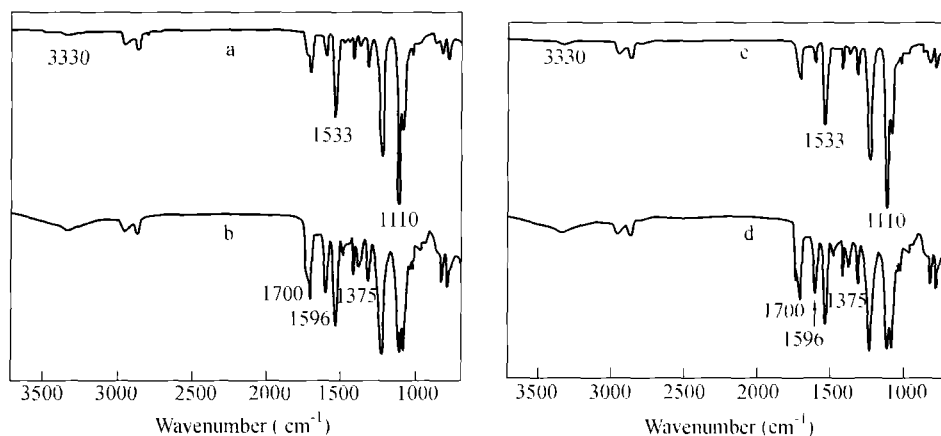


Fig. 5 The ATR-FTIR spectra of SPEU film (a), SPEU-g-PMMA film (b), PE film (c) and PE-g-PMMA film (d)

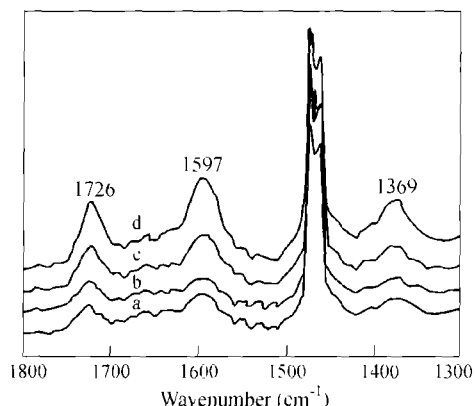


Fig. 6 the ATR-FTIR spectra of grafted PE films with different ozone-induced time (a) 10 min (b) 20 min (c) 30 min and (d) 40 min

X-ray photoelectron spectroscopy

The surface atomic composition of SPEU and SPEU-g-DMMCA (DMMCA 10% weight percent concentration) films are shown in Table 1. From the figures listed in Table 1, the content of N increases from 2.91% to 5.98% after grafting. It confirms that DMMCA has been grafted onto the SPEU surface. Moreover, the content of C and O changes a little after grafting.

Table 1. XPS surface elemental composition (%) of SPEU film and grafted SPEU film (DMMCA = 10% weight percent concentration)

Polymer	C _{1s}	O _{1s}	N _{1s}
SPEU	73.9	23.19	2.91
SPEU-g-DMMCA	69.86	24.16	5.98

The resolving of C_{1s}, N_{1s}, and O_{1s} peaks of SPEU (a) and SPEU-g-DMMCA (b) is shown in Fig. 7. The C_{1s} peak of SPEU (a) could be resolved into three peaks: $-\underline{\text{C}}-\text{C}-\text{C}-$ at 284.9 eV , $-\underline{\text{C}}-\text{O}-\text{C}-$ at 286.5 eV and $-\text{NH}-\underline{\text{C}}\text{OO}-$ at 289.0 eV . The C_{1s} of SPEU-g-DMMCA (b) could also be resolved into three peaks, but the peaks at 286.5 eV and at 289.0 eV becomes more intense, which are attributed to the grafting of DMMCA.

The N_{1s} peak of SPEU (a) has only one peak, $\underline{N}H-COO$ at 400.0 eV. But the N_{1s} peak of SPEU-g-DMMCA (b) could be resolved into two peaks, $\underline{N}H-COO$ at 400.0 eV and $\underline{N}^+(CH_3)_2CH_2$ at 402.7 eV. And the O_{1s} peak at 533.7 eV of SPEU-g-DMMCA (b) becomes broader, which was attributed to COO^- . All of those results demonstrate that DMMCA is grafted onto the SPEU films surface.

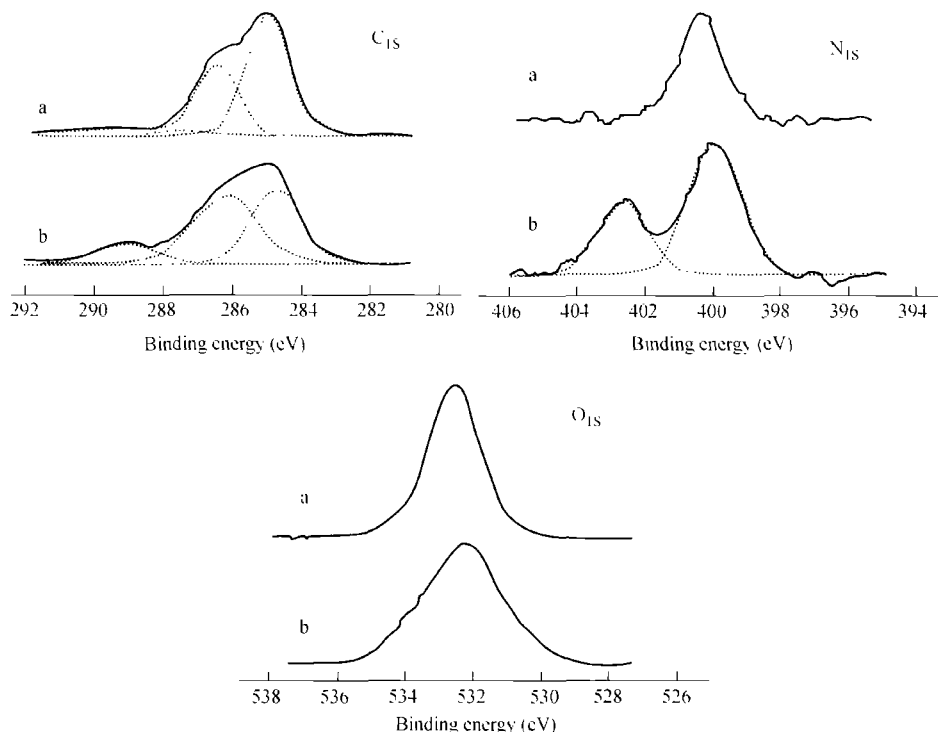


Fig. 7 XPS spectra of SPEU film (a) and SPEU-g-PDMMCA film (DMMCA 10 wt%) (b)

The surface atomic composition of PE and PE-g-DMMCA (ozonization time 30 min, DMMCA 10% weight percent concentration) films is shown in Table 2. A small amount of O (2.22%) and N (0.30%) of PE film perhaps comes from the contamination of additive. This composition changes obviously after grafting. The content of O and N reach 22.22% and 6.33%, respectively. This indicates that DMMCA has been grafted onto the PE films surface.

Table 2. XPS surface elemental composition (%) of PE film and PE-g-DMMCA film (ozone-induced time 30 min, DMMCA = 10% weight percent concentration)

Polymer	C_{1s}	O_{1s}	N_{1s}
PE	97.48	2.22	0.30
PE-g-PDMMCA	71.45	22.22	6.33

The resolving of C_{1s} , N_{1s} , and O_{1s} peaks of PE (a) and PE-g-DMMCA (b) are shown in Fig. 8. The C_{1s} peak of PE (a) is at 285.0 eV, which belongs to $\underline{C}-H$ and $\underline{C}-C$. But the C_{1s} peak of PE-g-DMMCA (b) could be resolved into three peaks: $\underline{C}-H$ and $\underline{C}-C$ at 284.6 eV, $C-O$ at 285.8 eV and COO^- at 288.6 eV. In addition, some new strong peaks of O_{1s} and N_{1s} appear too. All of these confirm that DMMCA has been grafted onto the PE film surface.

Water contact angle measurements

To compare the hydrophilicity of untreated films with that of grafted films, the water contact angles of the film surfaces were measured using the sessile droplet method and the results for SPEU, SPEU-g-DMMCA, PE and PE-g-DMMCA are shown in Table 3 and Table 4. After grafting, the hydrophilicity of the films are greatly

improved. The contact angle decreases with the increase of monomer concentration (cf. Table 3) and the ozonization time (cf. Table 4).

Table 3. Contact angle measurement of SPEU film and grafted SPEU films with different DMMCA concentration

DMMCA concentration (wt%)	0	2	5	10	15	20
Contact angle (°)	64±3	44±4	40±4	38±5	37±5	35±9

Table 4. Contact angle measurement of PE film and grafted PE films with different ozone-induced time

Ozone-induced time (min)	0	10	20	30	40
Contact angle (°)	97±3	69±7	62±6	55±5	32±4

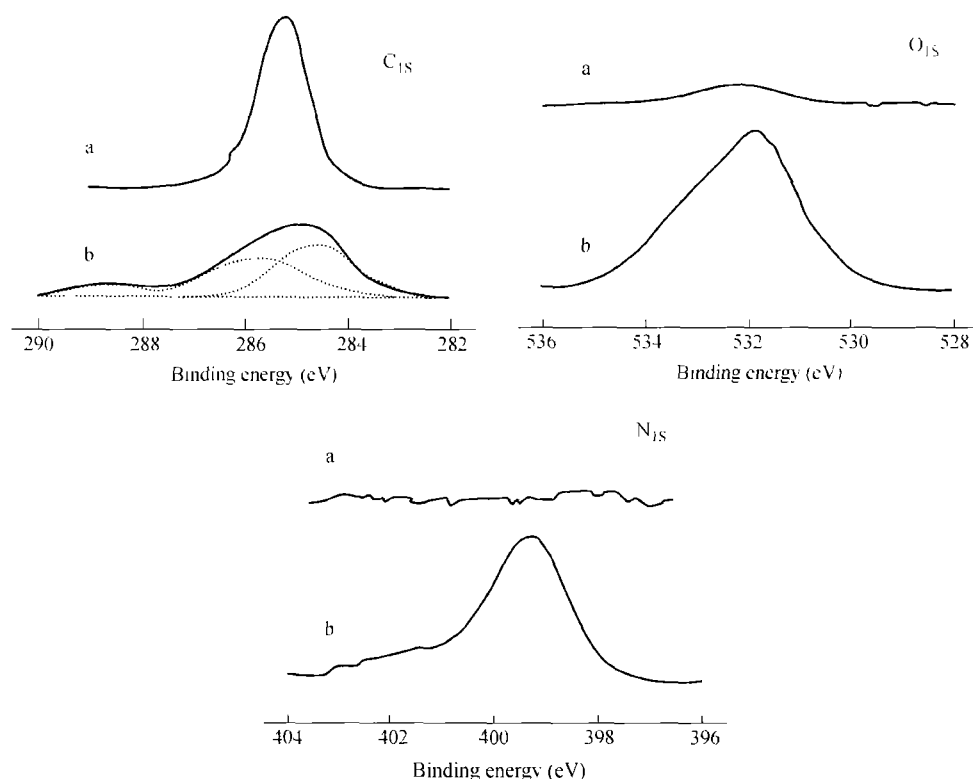


Fig. 8 XPS spectra of PE film (a) and PE-g-PDMMCA film (ozone-induced time 30 min, DMMCA 10% weight percent concentration) (b)

Blood Compatibility Test of SPEU-g-DMMCA and PE-g-DMMCA

Platelet adhesion *in vitro*

All the PDMMCA films were subjected to the platelet adhesion measurements with untreated films as the control. The degree and nature of blood platelet adhesion resulted from exposure to fresh human platelet rich plasma for a given time was regarded as a scale for evaluation. Blood platelet attachment *in vitro* was visualized by using SEM. The SEM microphotographs of treated PDMMCA using SPEU film as control are shown in Fig. 9. There is no adhered platelet on the PDMMCA surface after it was exposed to PRP for 3 h. The SEM photographs of SPEU and SPEU-g-DMMCA (DMMCA of 2%, 5%, 10%, 15% and 20% weight percent concentration) films after being exposed to fresh human platelet rich plasma for 3 h and 5 h are shown in Fig. 10 and Fig. 11, respectively.

Similarly, the SEM photographs of PE and PE-g-DMMCA films (ozonization times were 10, 20 30 and 40 min) for 3 h are shown in Fig. 12. It shows that there are no platelet attachments on the grafted film surfaces.

Compared with grafted films, not only there appear a large number of adhered platelets on the surfaces of the untreated films but also the shapes of the adhered platelets are greatly altered.

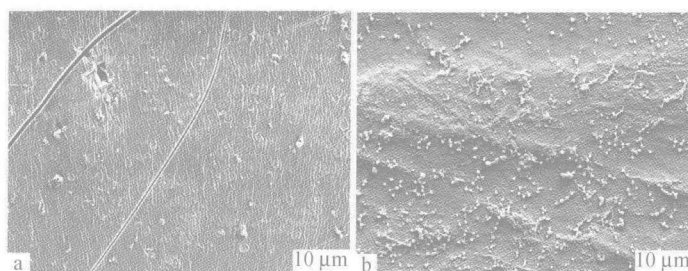


Fig. 9 The SEM microphotographs of PDMMCA (a) and SPEU (b) exposed to fresh human platelet rich plasma for 3 h ($\times 600$)

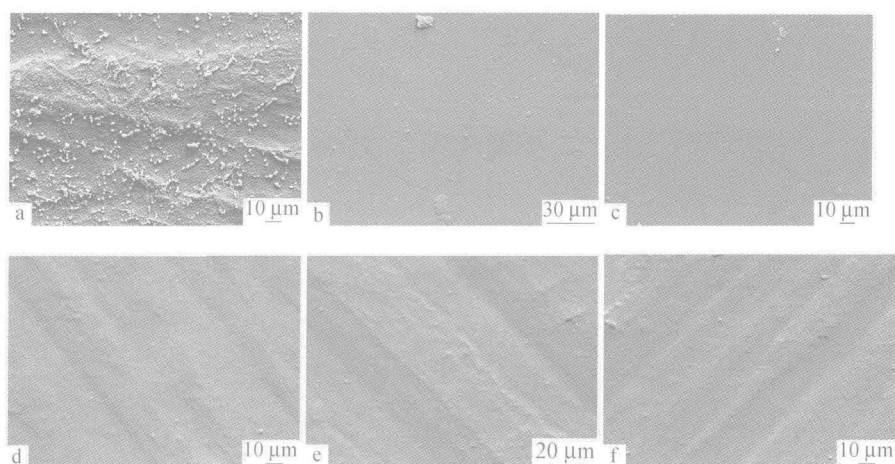


Fig. 10 The SEM microphotographs of SPEU and SPEU-g-DMMCA (DMMCA 2%, 5%, 10%, 15% and 20% weight percent concentration) films exposed to fresh human platelet rich plasma for 3 h ($\times 600$)

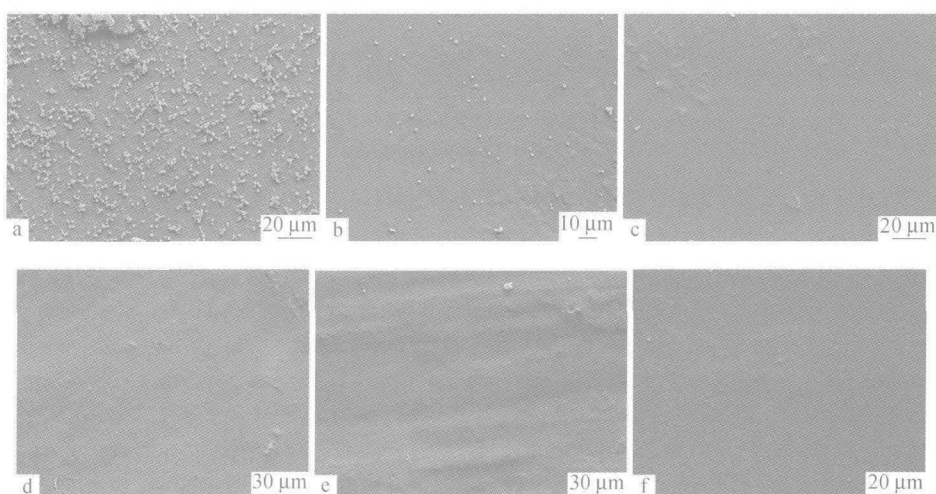


Fig. 11 The SEM microphotographs of SPEU and SPEU-g-DMMCA (DMMCA 2%, 5%, 10%, 15% and 20% weight percent concentration) films exposed to fresh human platelet rich plasma for 5 h ($\times 600$)

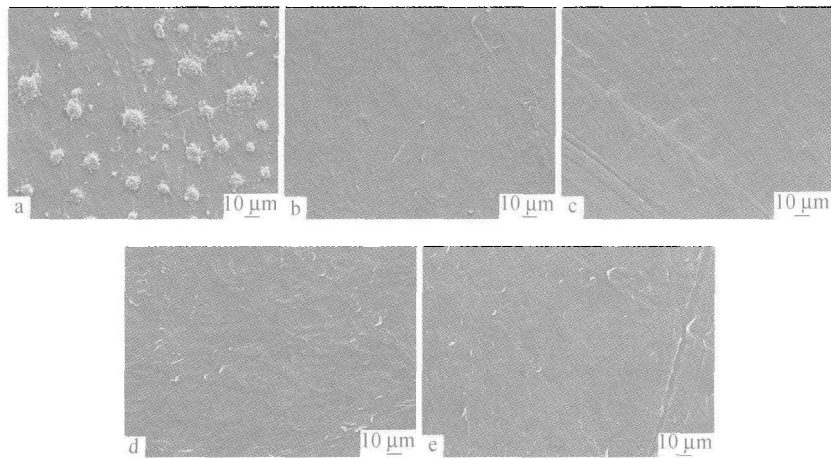


Fig. 12 The SEM photographs of PE and PE-g-DMMCA films (ozone-induced time 10, 20 30 and 40 min)exposed to fresh humanplatelet rich plasma for 3 h (× 600)

Protein adhesion in vitro

The protein adhesion results of SPEU, SPEU-g-DMMCA and PE, PE-g-DMMCA, PDMMCA are shown in the Tables 5, 6 and 7, respectively. It could be obviously seen that the content of protein adhered onto the film surfaces is decreased greatly after grafting. And the adhered protein onto PDMMCA surface are less than half of that onto SPEU and PE films surface.

Table 5. The protein adhesion results of SPEU, SPEU-g-DMMCA films

DMMCA concentration (wt%)	0	2	5	10	15	20
BFG adsorption (μg/cm ²)	2.54±	1.755±	1.194±	1.053±	1.152±	1.286±
	0.177	0.059	0.158	0.082	0.041	0.092
Decreased adsorption (%)	0	31	53	59	55	49

Table 6. The protein adhesion results of PE, PE-g-DMMCA films

Ozone-induced time (min)	0	10	20	30	40
BFG adsorption (μg/cm ²)	2.230±0.078	1.353±0.092	1.297±0.066	1.259±0.113	1.288±0.077
Decreased adsorption (%)	0	39	42	44	42

Table 7. The protein adhesion results of PDMMCA, SPEU and PE

Polymer	PDMMCA	SPEU	PE
BFG adsorption (μg/cm ²)	1.028±0.056	2.544±0.177	2.230±0.078

Based on these results, we may conclude that the blood compatibility of materials studied was greatly improved by grafting DMMCA onto their surface. It is considered that the improved antithrombogenicity could be attributed to the zwitterionic structure of carboxybetaine. In aqueous medium (*i.e.* blood), the zwitterionic interfacial molecular structure of biomaterials, thermodynamically, is not only unfavourable to penetrate into the interior of protein conformations, but also minimize the affect with the exterior ionic groups of protein conformations, and therefore, it is favorable to maintain the normal conformation of contacted plasma proteins and cells. As a result, the nonthrombogenicity and blood compatibility of biomaterials are improved.

CONCLUSION

PDMMCA was successfully constructed onto the surfaces of SPEU and PE films by means of ozone induced graft copolymerization. It is proved experimentally that both the crosslinked PDMMCA and the biomaterials

bearing PDMMCA all show excellent nonthrombogenicity evaluated by platelet adhesion resistance. Combining with the results reported before, it is reasonable to conclude that zwitterionic interfacial molecular structure is significant for improving the nonthrombogenicity, blood compatibility and biocompatibility of biomaterials.

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