Protein-Grafted Carboxylic Poly(ether sulfone) Membranes: Preparation and Characterization

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ABSTRACT: Carboxylic poly(ether sulfone) membranes were prepared by a controlled acetylating and surface-oxidating reaction followed by the grafting of bovine serum albumin (BSA) and bovine serum fibrinogen (BFG) onto the surfaces. Attenuated total reflection–Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy, and Micro BCA Protein Assay Kits confirmed that the proteins were successfully grafted onto the surfaces of the membranes. The protein grafting degrees were measured at different time intervals and under different conditions. The modified membranes showed higher hydrophilicity, lower protein (BSA and BFG) adsorption, and suppressed platelet adhesion values. Because of the binding of calcium ions in blood, the modified membranes showed longer plasma recalcification times, activated partial thromboplastin times, prothrombin times, and whole blood clotting times. The results indicate that the blood compatibility of the poly(ether sulfone) membranes could be improved after surface carboxylic modification and protein immobilization and that the modified membranes could be used in the blood purification field. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: biocompatibility; hydrophilic polymers; membranes; poly(ether sulfones)

INTRODUCTION

The surface modification of materials (especially membrane materials) has been studied extensively, with the goals of improving blood compatibility,[1–3] developing stimulus sensitivity,[4–6] and modifying the hydrophilicity and/or hydrophobicity.[7–10]

Because of their excellent oxidative, thermal, and hydrolytic stabilities and good mechanical properties, poly(ether sulfone) (PES) (BASF, Ludwigshafen, Germany) membranes have been used widely in many fields, including artificial organs,[11,12] hemodialysis,[13–15] stimuli-responsive membranes,[16,17] and hemodiafiltration.[18,19] However, when it is used in hemodialysis, the blood compatibility of the PES membrane is not adequate, and the injection of an anticoagulant is needed. As a result, the modification of PES membranes to improve blood compatibility is necessary.

With a goal of improving the blood compatibility of membrane materials, many surface-modification methods have been studied, among which protein grafting is a widely used one.[20–23] Recently, surface modification via protein grafting to improve the hydrophilicity and blood compatibility of PES membranes was reported. Liu et al.[24] modified PES membranes by blending PES with a copolymer of acrylic acid and *N*-vinyl pyrrolidone and then immobilizing bovine serum albumin (BSA) onto the surface. After grafting with BSA, the hydrophilicity and the blood compatibility of the membranes were increased. Wei et al.[20] coated polydopamine onto the surface of PES membranes, and then, BSA was immobilized onto the surface; the hydrophilicity and the blood compatibility of the modified membrane were increased.

In this study, carboxylic poly(ether sulfone) (CPES) membranes were prepared via a controlled acetylating and surface-oxidating reaction,[25] and then, proteins [BSA and bovine serum fibrinogen (BFG)] were grafted onto the surfaces of the membranes via the formation of amide linkages (—CO—NH—) between the proteins and the carboxyl groups on the membrane surfaces. Attenuated total reflection (ATR)–Fourier transform infrared (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), and water contact angle (WCA) analysis were used to characterize the surfaces of the modified membranes, and the protein grafting degrees were measured.

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Furthermore, to investigate the blood compatibility of the modified membranes, the protein (BSA and BFG) adsorption, platelet adhesion, plasma recalcification time (PRT), activated partial thromboplastin time (APTT), prothrombin time (PT), Ca^{2+} adsorption, and whole blood clotting time (WBCT) were investigated.

EXPERIMENTAL

Materials

PES (Ultrason E 6020P, CAS no. 25608-63-3) was purchased from BASF Chemical Co. (Germany). Acetyl chloride [C_2H_3ClO, analytical reagent (AR), Q/12HB 4195-2009] was purchased from Tianjin Kemel Chemical Reagent Co. $KMnO_4$ (AR, GB/T643-1988) was purchased from Chengdu Changlian Chemical Reagent Co. *N*-Methyl pyrrolidone (NMP; C_5H_9NO, AR, Q/HG 3248-99) was used as a solvent and was purchased from Chengdu Kelong Chemical Reagent Co. Sodium dodecyl sulfate (SDS; $C_{12}H_{25}O_4NaS$, AR, CAS no. 151-21-3) was purchased from Shanghai Aladdin Chemistry Co., Ltd. BSA (fraction V) and BFG were obtained from Sigma Chemical Co. 2-(*N*-Morpholino) ethanesulfonic acid (MES; $C_6H_{13}O_4NS{\cdot}H_2O$, 99%, CAS no. 145224-94-8) was obtained from Amresco, Ltd. *N*-Hydroxysuccinimide (NHS; 99%, CAS no. 6066-82-6), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; 99%, CAS no. 25952-53-8), and the Micro BCA Protein Assay Kits were the products of Pierce. Double-distilled water was used throughout the study. All of these chemical reagents were used without further purification unless otherwise described.

CPES membrane preparation

The CPES membranes were prepared by a controlled acetylating and surface-oxidating reaction. For the acetylating reaction, PES (10 g, 0.00025 mol) was dissolved in NMP with a concentration of 20 wt %. Acetyl chloride (80 g, 1.0 mol) and $AlCl_3$ (4 g, 0.03 mol) were mixed with NMP (41.6 g, 0.42 mol). Then, the mixed solution was poured into the PES solution slowly at a temperature of 90°C, and the reaction lasted 2 h with refluxing and stirring. Then, the product (PES–$COCH_3$) was washed with double-distilled water for several days, and the PES–$COCH_3$ was dried in an oven for over 24 h for the membrane-fabricating step.[25]

The PES–$COCH_3$ membranes were prepared by a liquid–liquid phase separation technique.[26–28] Dried PES–$COCH_3$ was dissolved in NMP with a concentration of 20 wt %, and then, the solution underwent vigorous stirring until a clear homogeneous solution was obtained. After degassing with a vacuum pump, the casting solutions were prepared into membranes by spin coating coupled with a liquid–liquid phase separation technique at room temperature. Then, the membranes were immersed in double-distilled water for several days to remove the residual solvent. Then, the membranes were cut into samples of 1×1 cm^2 for the surface-oxidating reaction step.

In the oxidation step, $KMnO_4$ (10 g, 0.06 mol, 4.54 wt %) and NaOH (10 g, 0.25 mol, 4.54 wt %) were dissolved in double-distilled water. Then, the PES–$COCH_3$ membranes (1×1 cm^2) were immersed in the solution at a temperature of 80°C, and the reaction lasted 2.5 h in a three-necked flask. Next, the CPES membranes were washed by an Na_2SO_3 aqueous solution (adjusted to pH 1 with HCl) several times and were then washed with double-distilled water. After the washing step, the membranes were immersed in double-distilled water for further study.

Protein grafting

As described in our previous publications,[29,30] the BSA grafting process onto the PES membrane is shown in Figure 1. As shown in the figure, MES was used as the conjugation buffer (0.1 mol/L, pH 4.5–5, adjusted with NaOH). NHS and EDC were added to the buffer solution at concentrations of 10 and 10 mg/mL, respectively. Then, the membranes were activated in the solution for 45 min at 4°C. Subsequently, the membranes were washed three times with a solution containing 0.1 mol/L MES and 10 mg/mL NHS. Then, the activated membranes were immersed in phosphate-buffered saline (PBS) solutions (pH 7.2, adjusted with HCl) containing BSA (concentrations of 1, 2, 5, 10, 20, and 40 mg/mL were investigated) for 1–24 h (i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 24 h) and subsequently rinsed slightly with PBS solutions and double-distilled water. The BFG-grafted CPES membranes were also prepared with the same methods.

In this study, Micro BCA Protein Assay Kits (Pierce) were used to determine the amount of the grafted proteins on the membrane surfaces. The membranes were immersed in an SDS washing solution (2 wt % SDS, 0.05 mol/L NaOH) at room temperature and shaken for over 24 h to remove the adsorbed proteins. The protein amount remaining on the membrane was deemed to be the grafted protein amount and was determined with the protein assay reagent. The protein amount was measured with an ultraviolet–visible spectrophotometer (model 752, Shanghai Spectrum Instruments Co., Ltd., Shanghai, China) at 562 nm.

The amount of the protein grafted onto the membrane was calculated as the grafting yield (GY; μg/cm^2) with the following equation:

$$\mathrm{GY} = \frac{W_g}{A} \tag{1}$$

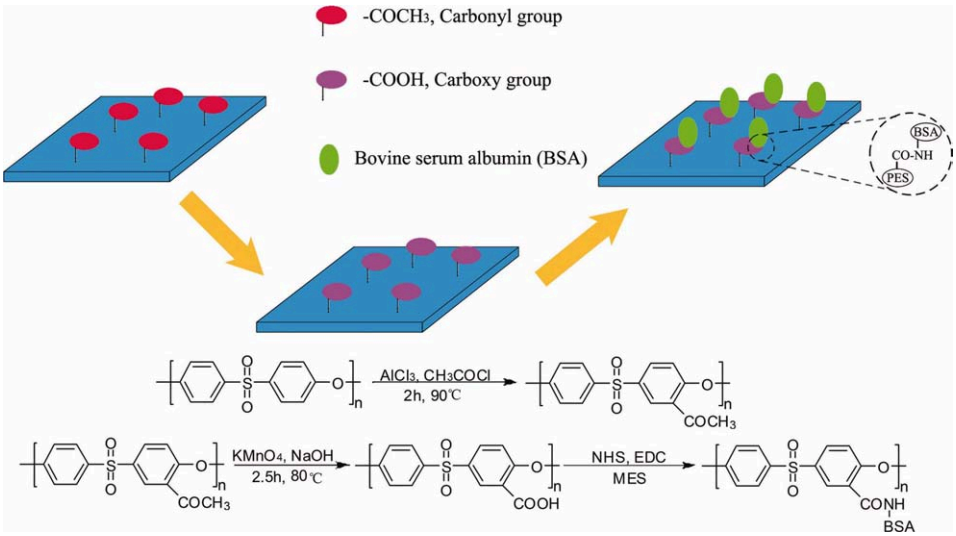

Figure 1 Process of BSA grafting. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

where W_g is the weight of the protein grafted onto the membrane (μg) and A is the surface area of the membrane (cm^2).

Characterization of the membrane surface

To analyze the surfaces of the membranes (PES, CPES, CPES–BSA, and CPES–BFG), ATR–FTIR spectroscopy (Thermo Nicolet, Franklin, Massachusetts, USA) was used to obtain the ATR–FTIR spectra.

Surface analysis of the membranes (PES, CPES, CPES–BSA, and CPES–BFG) by XPS was performed with a KRATOS XSAM800 Britain XPS (KRATOS, Manchester, Britain) instrument with Al Kα excitation radiation (1486.6 eV). The measurements were conducted at a take-off angle of 20°, and the XPS resource was run at a power of 180 W (12 kV × 15 mA). Binding energies were calibrated with the containment carbon (C1s = 284.7 eV). Survey spectra were run in the binding-energy range of 0–1000 eV, and the high-resolution spectra of C1s were collected.

With a contact angle goniometer (OCA20, Dataphysics, Stuttgart, Germany) equipped with video capture, the hydrophilicity of the membrane surface was characterized. A piece of 1 × 1 cm^2 membrane was stuck onto a glass slide. Then, a total of 3 μL of double-distilled water was dropped onto the air side of the membrane at room temperature, and the contact angle was measured after 10 s. At least five measurements were averaged to obtain a reliable value.

To investigate the permeation and rejection properties of the modified membranes, the water flux of the prepared membranes was measured in a dead-end ultrafiltration cell. The pressure was supplied by an air compressor, and the effective membrane area was 13.8 cm^2. The test membranes were precompacted at 0.10 MPa for 30 min by double-distilled water flow, and then, a 1 mg/mL BSA solution was applied to the membranes with a pressure of 0.08 MPa. The permeated solution was collected over 10 min after 5 min of equilibration. All of the flux measurements were conducted at room temperature, and the membranes of PES, CPES, P-B-3, and P-F-3 were investigated. The water flux was calculated by the following equation:[30]

$$\text{Flux}\,(\text{lm}^{-2}\,\text{h}^{-1}\,\text{mmHg}^{-1}) = \frac{V}{StP} \tag{2}$$

where V is the volume of the permeated solution (L), S is the effective membrane area (m^2), t is the time of the solution collection (h), and P is the pressure applied to the membrane (mmHg).

The BSA rejection ratio (R) was calculated by the following equation:[30]

$$R(\%) = \left(1 - \frac{C_p}{C_f}\right) \times 100 \tag{3}$$

where C_p and C_f are the protein concentrations of the permeate and feed solutions (g/L), respectively.

Blood compatibility

Plasma collection

Healthy human fresh blood (from a 22-year-old male) was collected with vacuum tubes (6 mL, Venoject II, Terumo Co., Tokyo, Japan) with a citrate/phosphate/dextrose/adenine-1 mixture solution as the anticoagulant (anticoagulant-to-blood ratio = 1 : 9). The fresh blood was centrifuged at 1000

TABLE I
Compositions of the Modified PES Membranes

CPES–BSA			
Membrane	P-B-1	P-B-2	P-B-3
Grafting degree	10.6 μg/cm^2	26.5 μg/cm^2	38.2 μg/cm^2
CPES–BFG			
Membrane	P-F-1	P-F-2	P-F-3
Grafting degree	9.5 μg/cm^2	23.8 μg/cm^2	27.6 μg/cm^2

rpm for 10 min to obtain platelet-rich plasma (PRP) or at 3000 rpm for 15 min to obtain platelet-poor plasma (PPP).

Protein adsorption

The protein adsorption experiments were carried out with BSA and BFG, which were dissolved in isotonic PBS solutions (pH 7.4) with concentrations of 1 mg/mL. For protein adsorption, membranes (PES, CPES, P-B-1, P-B-2, P-B-3, P-F-1, P-F-2, and P-F-3, as shown in Table I) with areas of 1 × 1 cm^2 were first immersed in PBS solution for 24 h. Then, the membranes were immersed in the protein solutions for 2 h at 37°C. After protein adsorption, the membranes were gently rinsed with PBS solutions and then immersed in the washing solutions (2 wt % SDS aqueous, 0.05*M* NaOH, 37°C), shaken for 2 h to remove the adsorbed proteins. The amounts of proteins eluted in the SDS solution were quantified by a Micro BCA Protein Assay Kit, and then, the adsorbed protein amounts were calculated. At least three measurements were averaged to obtain a reliable value.

Platelet adhesion

The 1 × 1 cm^2 membranes (PES, CPES, CPES–BSA, and CPES–BFG) were immersed in PBS solution and equilibrated at 37°C for 1 h. Then, the PBS solution was removed, and 500 μL of fresh PRP was introduced. The membranes were incubated at 37°C for 90 min. Then, the PRP was removed, and the membranes were rinsed with PBS solution three times. The membranes were finally treated with a 2.5 wt % glutaraldehyde–PBS solution at 4°C for 4 h. Then, the membranes were passed through a series of grade alcohol–PBS solutions (30, 50, 70, 80, 90, 95, and 100%) and then graded isoamyl acetate–alcohol solutions (30, 50, 70, 80, 90, 95, and 100%). The samples were then freeze-dried with FD-1C-50 lyophilizer (Boyikang, Beijing, China). The platelet adhesion was observed with an S-2500C microscope (Hitachi, Yokohama, Japan). The number of adhering platelets on the membranes was obtained from five 500× scanning electron microscopy (SEM) pictures taken at different places on the surface of the same membrane.

PRT

The 1 × 1 cm^2 membranes (PES, CPES, P-B-1, P-B-2, P-B-3, P-F-1, P-F-2, and P-F-3) were immersed in the PBS solution and equilibrated at 37°C for 1 h. Then, 100 μL of PPP was placed on the sample film attached to a watch glass and incubated statically at 37°C. Then, 100 μL of a 25 m*M* $CaCl_2$ aqueous solution was added to the PPP, and the plasma solution was monitored for clotting by manual dipping of a stainless steel hook coated with silicone into the solution to detect fibrin threads. The clotting times was recorded at the first sign of fibrin formation on the hook. The test was repeated four times for each sample to obtain a reliable result.

APTT and PT

To evaluate the antithrombogenicity of the membranes, APTT and PT were measured by an automatic blood coagulation analyzer CA-50 (Sysmex Corp., Kobe, Japan), for which citrate anticoagulated plasma was used. APTT and PT were measured as follows: 0.5 × 0.5 cm^2 membranes (PES, CPES, P-B-1, P-B-2, P-B-3, P-F-1, P-F-2, and P-F-3, with 16 pieces for every sample) were incubated with 200 μL of PPP in a transparent plastic tube at 37°C for 30 min, and then, APTT and PT were measured. The test was repeated three times for each sample to obtain a reliable value.

Adsorption of Ca^{2+} to the membranes

The adsorption of calcium ions onto the modified membranes was determined by an atomic absorption spectrophotometer (AA7000, Dongxi Electronic Technique Institute, Beijing, China). The membrane samples (PES, CPES, P-B-1, P-B-2, P-B-3, P-F-1, P-F-2, and P-F-3, about 10 mg in dry weight) were incubated in Ca^{2+} solutions (10 ppm, 2 mL) for 6 h, including an aqueous solution and serum sodium solution, respectively, and then, the residual calcium ion concentrations were determined by the atomic absorption spectrophotometer.

WBCT

Blood was collected from a healthy man (22 years old) by venipuncture and placed in sterile plastic disposable syringes. The blood donor had been screened for the study by a medical practitioner to ensure that he had not taken any medications for at least 1 week before the blood was collected. The

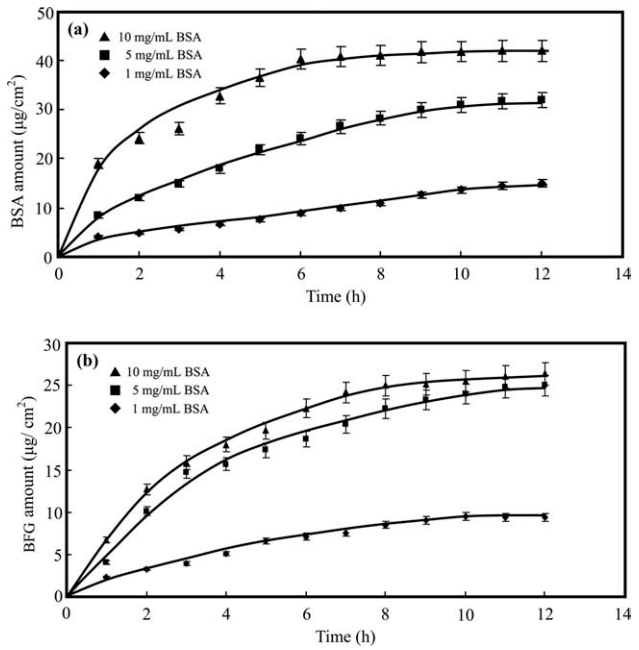

Figure 2 Immobilization of (a) BSA and (b) BFG onto the CPES membranes.

dried membranes with areas of 1 × 1 cm^2 (PES, CPES, P-B-1, P-B-2, P-B-3, P-F-1, P-F-2, and P-F-3) were attached to glass and subjected to the WBCT test. Fresh blood (50 μL) was placed on the membrane sample, and the stopwatch was started. The whole blood was monitored for clotting by the manual dipping of a stainless steel wire hook coated with silicone into the blood to detect fibrin threads. The clotting time was recorded at the first sign of any fibrin formation on the hook. The experiment was repeated three times for each sample, and a mean value was calculated.

RESULTS AND DISCUSSION

Time-dependent evolution of protein grafting

The time-dependent evolution of BSA grafting onto the CPES membrane is shown in Figure 2(a). The BSA immobilization rate was very fast in the beginning. During the first 3 h, with a BSA concentration of 1 mg/mL, about 30% of the equilibrium immobilization BSA amount was grafted onto the CPES membrane surface. These amounts were 35 and 45% for the BSA solutions with concentrations of 5 and 10 mg/mL, respectively. After 12 h, the immobilized amounts reached the equilibrium immobilization amounts, which were about 14.8, 31.9, and 42.0 μg/cm^2 for the BSA solutions with concentrations of 1, 5, and 10 mg/mL, respectively. The time-dependent evolution of the BFG grafting is shown in Figure 2(b). Similar to that of BSA grafting, the BFG immobilization rate was very fast in the beginning. During the first 3 h, about 25, 50, and 55% of the equilibrium immobilization BFG amounts were grafted onto the CPES membrane surfaces for BFG solutions with concentrations of 1, 5, and 10 mg/mL, respectively, and the equilibrium immobilization amounts were about 9.4, 25.0, and 26.4 μg/cm^2 for BFG solutions with concentrations of 1, 5, and 10 mg/mL, respectively.

Furthermore, the grafting rates for the protein solutions with different concentrations were different, and the following equation can be used to analyze the grafting kinetics:[31]

$$Q_t = Q_e e^{k/t} \tag{4}$$

where Q_t is the grafting degree, t is the time, Q_e is the equilibrium grafting degree, and k is the grafting coefficient. Table II shows the calculated Q_e and k for BSA and BFG solutions with different concentrations. It could be noticed that the Q_e was higher for the protein solution with higher concentration.

The grafting rate could be deduced from the derivation of the grafted degree; thus, from Eq. (4), the grafting rate could be written as follows:

$$v = \frac{dQ_t}{dt} = -\frac{kQ_e}{t^2} e^{\frac{k}{t}} \tag{5}$$

where v is the grafting rate. According to this equation, the grafting rate at any time t could be calculated and compared.

Figure 3 shows the calculated grafting rates for the membranes immersed in BSA and BFG solutions at different time intervals. As the figure shows, the grafting rate was high during the first 3 h and decreased to very low after 8 h for both BSA and BFG grafting. After 12 h, the protein grafting amounts almost reached the equilibrium immobilization amounts (as shown in Fig. 2), whereas the grafting rate was almost close to 0 μg cm^{-2} h^{-1} (as shown in Fig. 3). Both BSA and BFG showed similar results.

TABLE II
Calculated Q_e and k Values for the Grafting of BSA and BFG onto the Membranes

BSA			
Membrane	P-B-1	P-B-2	P-B-3
Q_e	13.35	34.18	46.3
K	−1.52	−1.66	−1.04
BFG			
Membrane	P-F-1	P-F-2	P-F-3
Q_e	9.54	27.83	28.73
k	−1.65	−1.97	−1.52

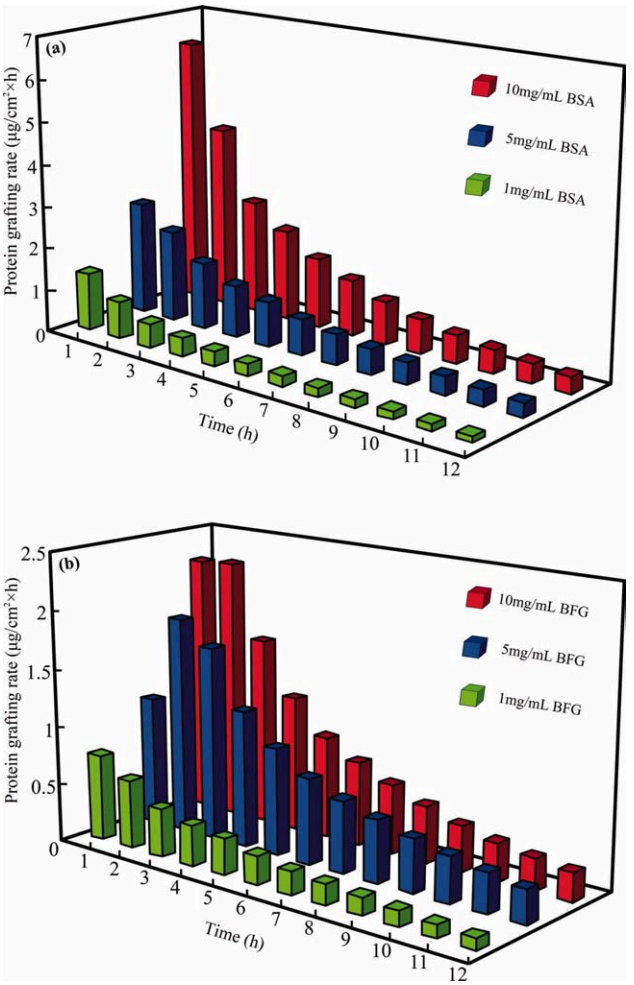

Figure 3 Grafting rates of (a) BSA and (b) BFG onto the membranes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

As Figure 3 shows, for BSA, the grafting rates in the 1st h were 1.35, 2.65, and 6.28 μg cm^{-2} h^{-1} for protein solutions with concentrations of 1, 5, and 10 mg/mL, respectively; whereas those in the 12th h were 0.14, 0.32, and 0.39 μg cm^{-2} h^{-1}, respectively. For BFG, the grafting rates in the 1st h were 0.74, 1.10 and 2.24 μg cm^{-2} h^{-1} for the protein solutions with concentrations of 1, 5, and 10 mg/mL, respectively, whereas those in the 12th h were 0.09, 0.29 and 0.25 μg cm^{-2} h^{-1}, respectively. Furthermore, it was found that with increasing protein concentration, the protein grafting rate increased obviously.

During the first grafting stage (from 1 to 3 h), the grafting rates for the membranes increased obviously with increasing protein concentration. During this stage, the membrane surface was blank, and there were many grafting sites on the membrane surface. The protein grafting rate was very fast, and about 30% of the equilibrium immobilization protein amount was grafted onto the membrane surface.

During the second grafting stage (from 4 to 8 h), the membrane surface was filled with a large amount of proteins, and the steric effect could be considered during this stage. Thus, the protein was first adsorbed onto the membrane surface and was then grafted onto the surface with the formation of amide linkages. After the membrane surface was filled with proteins, the protein adsorption step was inhibited, and the protein grafting rate decreased.

During the third grafting stage (from 9 to 12 h), the protein hardly grafted onto the membrane surface, and the grafting rate decreased to very low and showed no obvious difference because the membrane surface was full of the grafted proteins.

Protein grafting degree in different solutions

After 24 h of grafting, the membrane hardly conjugated BSA and BFG, and the equilibrium protein grafted amounts are shown in Figure 4. As Figure 4(a) shows, with the BSA concentrations increasing from 1 to 40 mg/mL, the amounts of grafted BSA increased from 18.3 to 62.3 μg/cm^2. As Figure 4(b) shows, with BFG concentrations increasing from 1 to 40 mg/mL, the amounts of grafted BFG increased form 15.6 to 32.3 μg/cm^2.

In fact, the protein grafting process could be described by the Langmuir monolayer adsorption theory. Before the grafting process, the protein was first adsorbed onto the CPES surface, and then, the amide linkage (—CONH—) between the protein and carboxyl groups was formed. After the membranes were washed by SDS solution, the adsorbed proteins

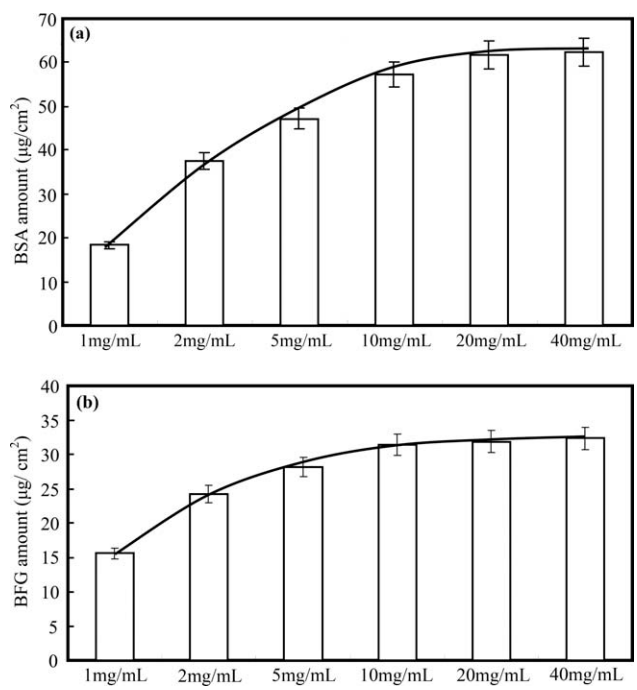

Figure 4 Immobilization of (a) BSA and (b) BFG under different conditions. The reaction lasted 24 h, and the protein concentrations were 1, 2, 5, 10, 20, and 40 mg/mL.

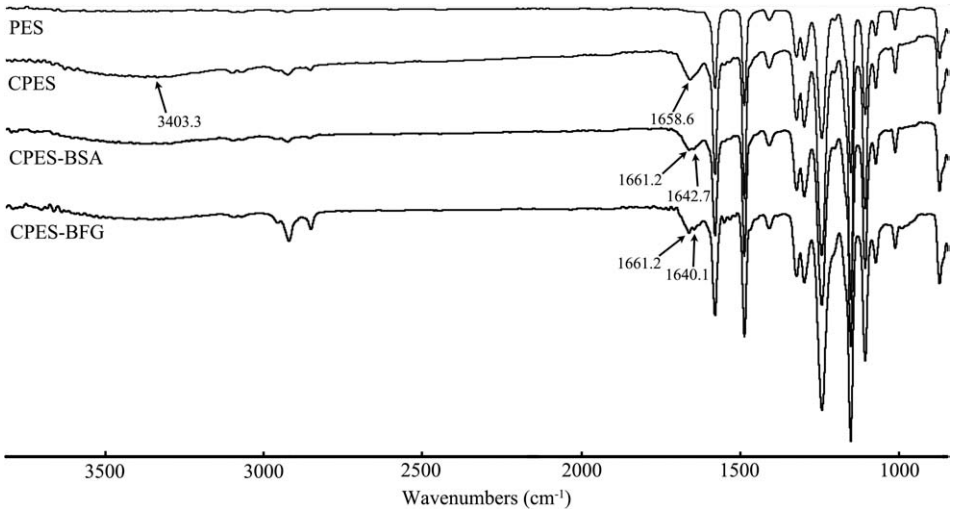

Figure 5 ATR–FTIR spectra of the membranes: PES, CPES, CPES–BSA, and CPES–BFG.

were eluted out, and the grafted proteins were retained on the surface of the membrane. In the grafting system, the protein was grafted onto the membrane surface, and no adsorbed protein was retained, so the grafted protein layer was considered a monolayer. An equation adapted from the Langmuir sorption isotherm was used for the grafting process. The protein grafting equation was as follows:[32,33]

$$\frac{[C_e]}{[Q]} = \frac{1}{k[Q]_{max}} + \frac{[C_e]}{[Q]_{max}} \quad (6)$$

where $[C_e]$ and $[Q]$ are the equilibrium concentration of protein (BSA and BFG) and the amount of protein grafted per square centimeter area ($\mu g/cm^2$) onto the CPES membrane, respectively, k is the grafting equilibrium constant related to the grafting energy, and $[Q]_{max}$ is the maximum amount of protein grafted onto per square centimeter area ($\mu g/cm^2$) of CPES membrane.

The data shown in Figure 4(a,b) were fitted to the linear form of the protein grafting equation. With the increase in the protein (BSA and BFG) concentrations, the equilibrium concentrations increased. Thus, the grafting amounts increased both for BSA and BFG.

For the BSA grafting

$$\frac{[C_e]}{[Q]} = 0.0225 + 0.0154[C_e], k = 0.6844$$

$$[Q]_{max} = 64.94\,\mu g/cm^2 r^2 = 0.9996$$

For the BFG grafting

$$\frac{[C_e]}{[Q]} = 0.0207 + 0.0304[C_e], k = 1.4688$$

$$[Q]_{max} = 32.89\,\mu g/cm^2 r^2 = 0.9999$$

The maximum grafting amount for BSA was larger than that for BFG, which could be explained by the steric effect: the molecular weight of BSA was 68,000 Da, whereas that of BFG was about 340,000 Da. The membrane carrying capacity for BSA was much larger than that for BFG, and the maximum grafting amount for BSA was larger than that for BFG.

Characterization of the modified membrane surface

ATR–FTIR spectroscopy

The ATR–FTIR spectra of the PES, CPES, CPES–BSA, and CPES–BFG membranes are shown in Figure 5. After the carboxylic reaction, peaks at 3403.3 and 1658.6 cm^{-1} were observed, which indicated the presence of —COOH in CPES. For the spectrum of CPES–BSA, the peaks at 1661.2 and 1642.7 cm^{-1} indicated the presence of CO—NH, which suggested that the BSA was grafted onto the surface of the CPES membrane. Peaks at 1661.2 and 1640.1 cm^{-1} were found in the spectra of CPES–BFG, and the appearance of these two peaks indicated the presence of CO—NH, which also suggested the successful grafting of the BFG. The ATR–FTIR spectra indicated that the CPES, CPES–BSA, and CPES–BFG membranes were successfully prepared.

XPS analysis

To confirm the actual residence of BSA and BFG, XPS was employed to characterize the surface compositions of the PES, CPES, CPES–BSA, and CPES–BFG membranes.

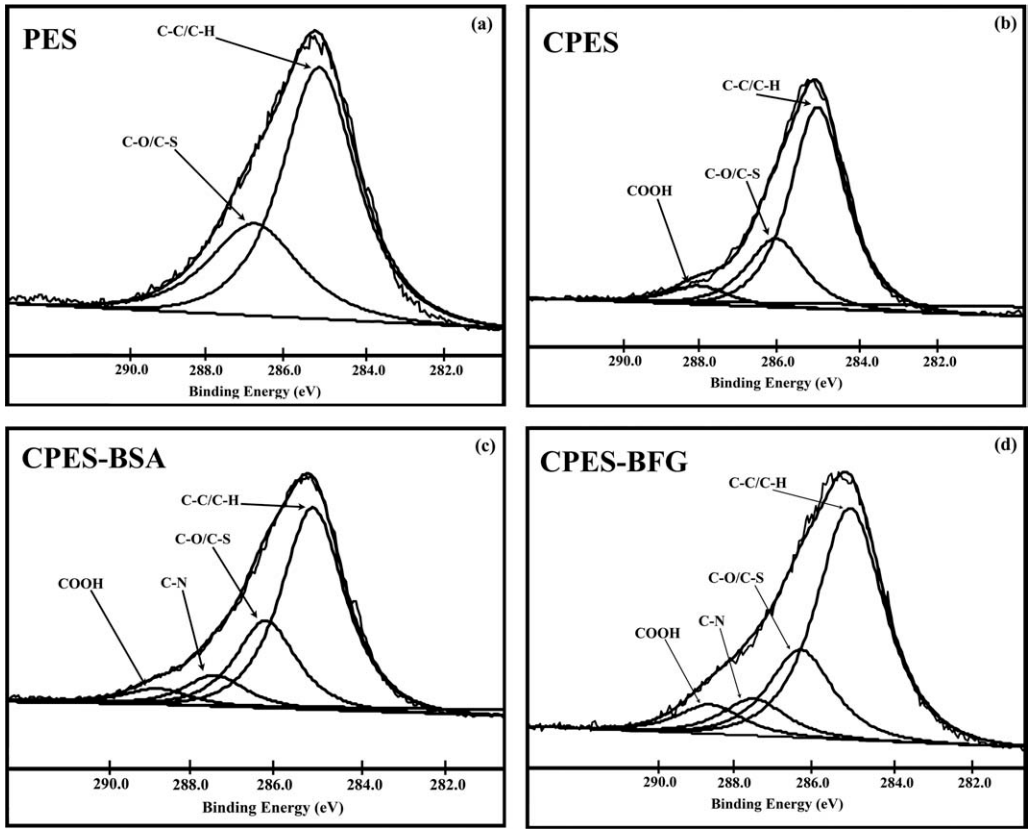

Figure 6 XPS for the membranes: (a) PES, (b) CPES, (c) CPES–BSA, and (d) CPES–BFG.

The high-resolution XPS spectra of C1s region are shown in Figure 6. As shown in Figure 6(a), the C1s peak was fitted by two unique carbon moieties: one was for C—C and C—H (284.76 eV), and the other was for C—O and C—S (286.00 eV).[34,35] However, in Figure 6(b), a new signal at 288.20 eV was observed, which was attributed to the appearance of C=O groups of the carboxyl group, due to the grafted carboxyl groups on the membrane surface. As shown in Figure 6(c,d), new signals were found at 287.31 and 287.40 eV, respectively, which were attributed to the appearance of C—N of the amide group (CO—NH), due to the grafted BSA and BFG on the membrane surfaces. Moreover, the signals for C=O and C—O shown in Figure 6(c,d) were much stronger than those shown in Figure 6(a,b); this was attributed to the appearance of carboxyl groups in the grafted BSA and BFG.

WCA analysis

WCA analysis is a convenient way to assess the hydrophobic/hydrophilic properties of the membrane surface and provides information about the interaction energy between the surface and water.[25] Figure 7 shows the WCAs of the PES and modified PES membranes.

As shown in Figure 7, the WCA of the PES membrane was about 76.8° after the carboxylic reaction; the WCA decreased to about 72.2° (CPES). After BSA was grafted onto the surface of the CPES membrane, the WCAs decreased to about 60.8° (P-B-1), 51.6° (P-B-2), and 38.1° (P-B-3), respectively. After BFG was grafted onto the surface of the CPES membrane, the WCAs decreased to about 66.8° (P-F-1), 57.0° (P-F-2), and 50.4° (P-F-3), respectively. These indicated that the hydrophilicity of the membranes increased after the carboxyl groups were introduced; after grafting with BSA and BFG, the

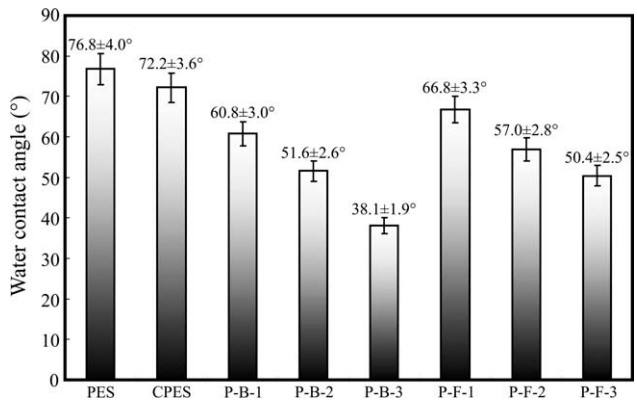

Figure 7 WCAs for the PES and modified PES membranes ($n = 5$).

hydrophilicities of the membranes increased further. Moreover, the hydrophilicity of the membrane had some relationship with the amount of protein grafted onto the membrane surface. The larger the amount of protein grafted was, the lower the contact angle was. The amount of grafted protein was a significant factor influencing the hydrophilicity of the membrane.

Water flux

The water flux of the PES membrane was very small (ca. 0.05 L m^{-2} h^{-1} $mmHg^{-1}$); after surface carboxyl group grafting, the CPES membrane showed a larger water flux (ca. 0.28 L m^{-2} h^{-1} $mmHg^{-1}$) because of the increased hydrophilicity of the membrane surface. After surface protein immobilization, the water fluxes for P-B-3 and P-F-3 increased sharply by geometric series (ca. 45.27 and 39.42 L m^{-2} h^{-1} $mmHg^{-1}$ for P-B-3 and P-F-3, respectively.), and this was attributed to the enhanced hydrophilicity of the membrane surface.

Furthermore, the BSA rejection ratios were 100, 100, 99.4, and 99.5% for the PES, CPES, P-B-3, and P-F-3 membranes, respectively. The water fluxes significantly increased for the BSA- and BFG-modified membranes; however, depressed protein fouling was still observed after the protein modification.

Blood compatibility

Protein adsorption

Protein adsorption onto the membrane was measured to evaluate the blood compatibility of the modified membranes. Protein adsorption has some relationship with blood compatibility. Protein adsorption is the first event in blood–material interactions, and the adsorption of contact-phase proteins may result in the activation of the intrinsic cascade.[36] There are many factors that affect the interaction between the membrane surface and proteins, such as the surface charge characteristics, surface free energy, surface roughness, topological structure, solution environment (e.g., pH, ionic strength, solution temperature, salt concentration), and protein characteristics.[37–39] The hydrophilic/hydrophobic of the membrane plays a very important role in the interaction between proteins and the membrane surface. The idea of increasing the hydrophilicity of the membrane material with the goal of reducing protein adsorption has been widely followed by many researchers.[40,41] In this study, the modified membranes were studied in relation to the adsorption of BSA and BFG, and the data are shown in Figure 8.

As shown in the figure, the protein adsorption amounts were higher for the PES membranes (ca. 18.5 and 11.4 μg/cm^2 for BSA and BFG, respectively), were lower for the modified membranes, and were lowest for P-B-3 (ca. 0.8 and 2.4 μg/cm^2 for BSA and BFG, respectively). After they were grafted with BSA and BFG, the modified membranes showed lower protein adsorption amounts than the PES and CPES membranes, and with the increase of the BSA and BFG grafting degrees, lower protein adsorption amounts were observed for the modified membranes. Protein adsorption on a surface includes two modes: (1) the protein covers the material surface aggressively, and (2) the protein interacts with the initially deposited layer to prevent further adsorption (surface passivation). The low protein adsorption amount suggested that the protein-grafted membranes might have had good blood compatibility and that they should be examined by further studies.

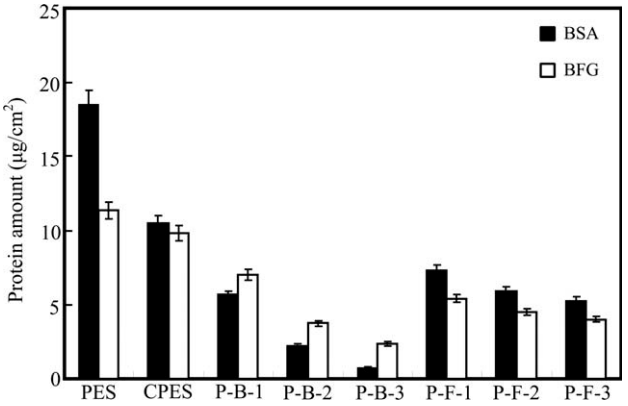

Figure 8 BSA and BFG adsorption onto the PES and modified membrane surfaces ($n = 3$).

It was noticed that after grafting with BSA, the protein adsorption amounts for the modified membranes decreased more than those for the BFG-modified membranes. For the BSA-modified membranes, the BSA adsorption was more effectively reduced than the BFG adsorption. For the BFG-modified membranes, the BFG adsorption was more effectively reduced than the BSA adsorption. However, whether a protein layer could effectively prevent the sediment of the same protein should be further studied.

Albumin and fibrinogen are two of the most important proteins in plasma. Albumin, which has the highest concentration in the plasma, transports many small molecules in the blood (e.g., bilirubin, calcium, progesterone, drugs), and it is of prime importance in maintaining the osmotic pressure of the blood.[20] Fibrinogen plays leading roles in blood coagulation and in mediating platelet adhesion to biomaterials. In the blood coagulation cascade, fibrinogen is a key protein; when treated with thrombin and Factor XIII (FXIII), fibrinogen is crosslinked and stabilized in the insoluble fibrin gel, and the blood is coagulated.[36] The adsorbed amounts of the protein

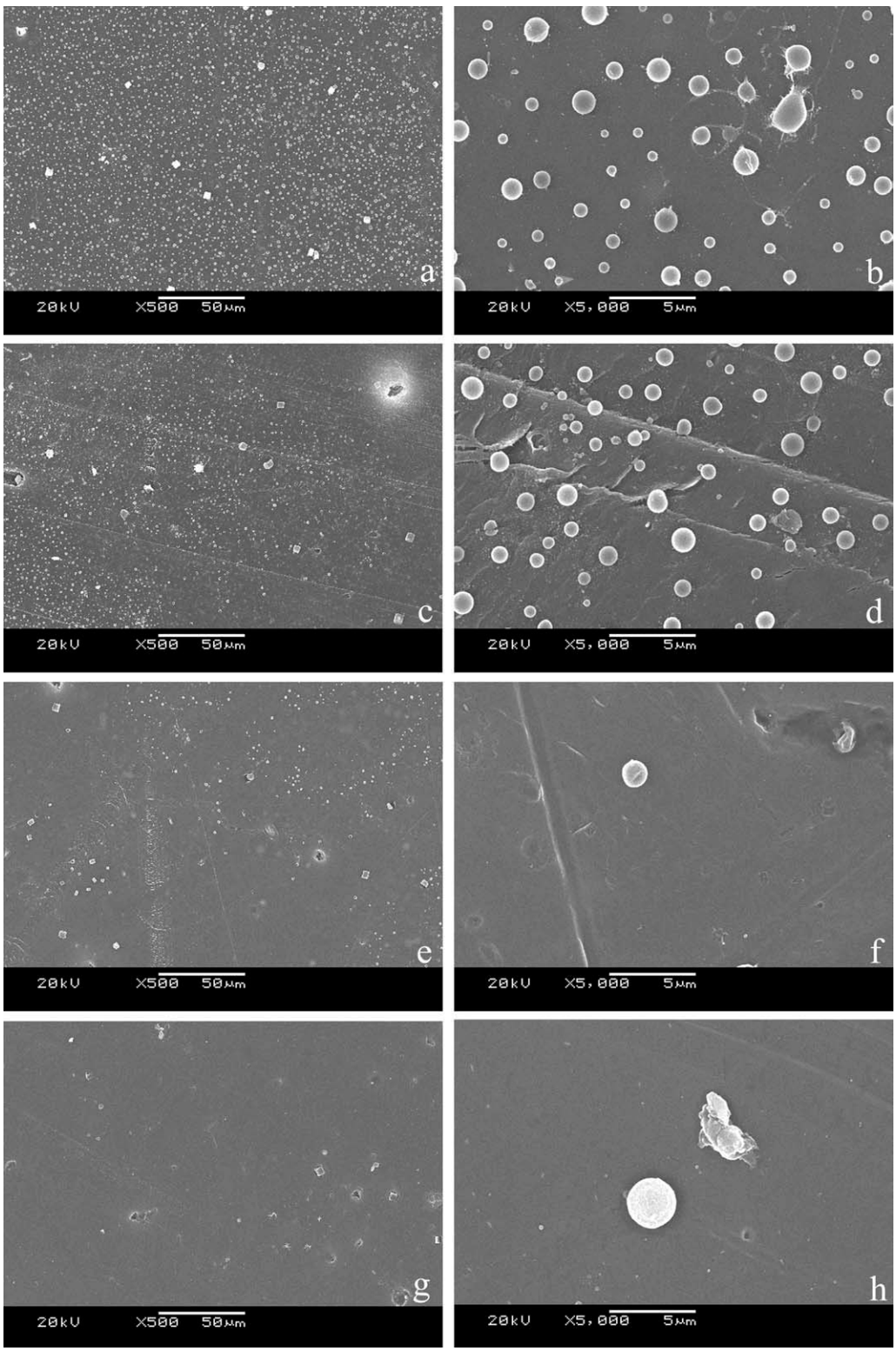

Figure 9 SEM images for the membranes (PES, CPES, CPES–BSA, and CPES–BFG) after platelet adhesion at magnifications of 500 and 5000×.

on the CPES membranes were smaller than that on the pure PES membranes. The adsorbed amounts on the BSA-modified membranes and BFG-modified membranes decreased compared with those on both the PES and CPES membranes.

The results demonstrate that the grafting of BSA and BFG onto the CPES membranes depressed the protein adsorption. This could be explained as follows: the chain movement and the negative charge of the grafted protein onto the surfaces of the

TABLE III
Number of Platelets Adhered onto the Surfaces of the Membranes (PES, CPES, CPES–BSA, and CPES–BFG)

	Sample			
	PES	CPES	CPES–BSA	CPES–BFG
Adhered platelets (per 1 × 1 cm^2 area)	1.48×10^7	2.37×10^6	2.37×10^4	1.14×10^4

membranes might have affected the surrounding area and, thus, prevented more protein from being deposited onto the surfaces of the membranes.[42,43]

Furthermore, the protein–surface interactions were accompanied by adsorbed protein conformation changes;[44] these could be a loss of biological activity in immunoassay or biosensor applications, the activation of coagulation and immune response upon blood contact with artificial implant materials, and so forth.[45]

Platelet adhesion

The adhesion and activation of platelets on a material's surface can lead to blood coagulation, and the activated platelets can further activate many coagulation factors;[36] this results in thrombus formation on the material's surface. The platelet adhesion test can be carried out to investigate the blood compatibility of the material's surface. The shape of the adhered platelet should be carefully observed by SEM. When the platelets are activated during the adhering process, the pseudopods can be observed and are accompanied by the aggregation of the platelets.

Figure 9(a–h) shows the SEM images of the adhering platelets on the PES and modified PES membranes from PRP. For the PES membrane, almost circular platelets were observed, and no aggregation of the adhered platelets was found. As Figure 9(b) (SEM image for human blood platelet adhesion with a magnification of 5000×) shows, pseudopods were observed on some of the adhering platelets. For the CPES, CPES–BSA, and CPES–BFG membranes, almost no pseudopods were found, and the adhered platelets retained their shape. For the CPES–BSA and CPES–BFG membranes, almost no substantial platelet attachment was found. Compared to the PES membrane surface, the CPES, CPES–BSA, and CPES–BFG membrane surfaces showed lower adhering platelet numbers. Table III shows the number of platelets adhered onto the PES and modified PES membrane surfaces. The CPES–BSA and CPES–BFG membrane surfaces showed similar platelet adhering numbers, which were much lower than those for the PES and CPES membrane surfaces. The results indicate that the modified membranes had lower platelet activation, and through the introduction of carboxyl groups and grafting proteins onto the membrane surface, the blood compatibility of the membrane could be improved.

Fang et al.[30] investigated the platelet adhesion for BSA-modified PES membranes, and similar results were obtained. For the PES membrane, a large number of adhered platelets were observed, and the adhered platelets spread, flattened, and extended into irregular shapes.

PRT test for the modified membranes

PRT is an indicator of intrinsic coagulation cascade activation.[46] The measured results are shown in Figure 10. As the figure shows, the modified membranes had significantly longer PRTs than the glass (381.5 s) and the PES (503.7 s) membrane. Furthermore, for the BSA-and BFG-grafted membranes, the PRTs were gradually prolonged with increased amounts of proteins grafted onto the membrane surfaces. For the BSA-modified membranes, the PRTs increased from 561.5 to 767.3 s for the P-B-1 and P-B-3 membranes. For the BFG-modified membranes, the PRTs increased from 630.4 to 790.8 s for the membranes of P-F-1 and P-F-3. The results indicate that after they were grafted with carboxyl groups and proteins, the anticoagulant properties of the membranes could be improved. Also, these improvements might have been due to the improved hydrophilicity and lower protein adsorption.

APTT and PT

To evaluate the antithrombogenicity of the membranes, APTT and PT were measured, and the results are shown in Figure 11. APTT and PT are usually used mainly to examine the intrinsic

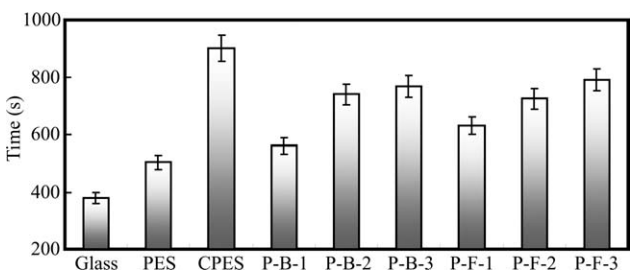

Figure 10 PRTs for the membranes ($n = 4$).

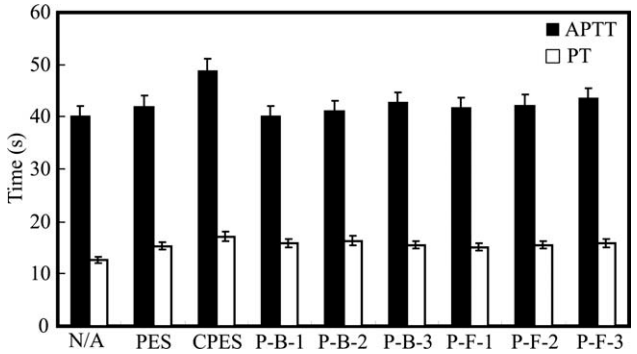

Figure 11 APTTs and PTs for the membranes ($n = 3$). N/A, pure plasma.

pathway and exhibit the bioactivity of intrinsic blood coagulation factors.[47] As the figure shows, compared to that of the PES membrane, APTT of the CPES membrane was slightly prolonged, and the prolonged clotting time might have been due to the increased hydrophilicity, lower protein adsorption, and depressed platelet adhesion and activation. However, compared to those of the PES membrane, the APTTs and PTs of the modified membranes were only slightly increased. Also, it was reported by Kang et al.[48] that APTT increased slightly after carboxyl groups were grafted onto the membrane surfaces of polyurethanes (PUs) and poly(ethylene terephthalate) (PET).

The increased APTTs and PTs for the CPES membrane might have been caused by the Ca^{2+} adsorption of the carboxyl groups; this is discussed in the next section.

Adsorption of Ca^{2+} to the membranes

Calcium plays a very important role in the processes of the coagulation cascade. Calcium and phospholipid (a platelet membrane constituent) are required for the tenase and prothrombinase complexes to function. Calcium mediates the binding of the complexes via the terminal γ-carboxyl residues on Factor Xa (FXa) and Factor IXa (FIXa) to the phospholipid surfaces expressed by platelets and to the procoagulant microparticles or microvesicles shed from them.[36] Calcium is also required at other points in the coagulation process.

It is well known that grafted carboxyl groups can bind Ca^{2+} and, thus, act as anticoagulants. To prove the relationship between blood compatibility and the adsorption of Ca^{2+} to the membranes, Ca^{2+} adsorption onto the membrane surface was measured, and the results are shown in Figure 12.

As the figure shows, for the Ca^{2+} aqueous solution, after 6 h of adsorption, the adsorbed Ca^{2+} amount for the PES membrane was about 0.76 mg/g; this was much lower than those of for the CPES, P-B-1, P-B-2, P-B-3, P-F-1, P-F-2, and P-F-3 membranes. The adsorption of Ca^{2+} to the PES membrane might have been caused by the porosity of the membrane. The adsorbed Ca^{2+} amount for the CPES membrane was the highest, at about 1.44 mg/g. After grafting with BSA and BFG, the adsorbed Ca^{2+} amounts were decreased and were about 1.35 and 1.37 mg/g for the P-B-1 and P-F-1 membranes, respectively. With increasing grafted protein amounts, the modified membranes showed decreased Ca^{2+} adsorption amounts. The Ca^{2+} was bound to the grafted carboxyl groups onto the membrane surface; after the proteins were grafted, some carboxyl groups were changed to amide groups, and thus, the Ca^{2+} adsorption amount decreased.

When a Ca^{2+} serum sodium solution was used as the adsorption solution, similar results were observed; however, the adsorbed Ca^{2+} amounts were a little lower.

WBCT

To further investigate the anticoagulant properties of the modified membrane and for practice application, WBCT was determined with 50 μL of whole blood without the use of any coagulants, and the results are shown in Figure 13. As the figure shows, the mean value for the PES membrane was 52.2 s, and the values were 259.3, 156.7, and 135.4 s for the CPES, P-B-1, and P-F-1 membranes, respectively. Obviously, the WBCTs increased significantly for the modified membranes. Also, for the BSA- and BFG-modified membranes, with the increased amount of proteins grafted onto the CPES membrane surface, the WBCTs increased. For the BSA-modified membranes (P-B-1 and P-B-3), the WBCTs increased from 156.7 to 225.5 s. For the BFG-modified membranes (P-F-1 and P-F-3), the WBCTs increased from 135.4 to 217.9 s. The increased clotting time might have been due to the increased hydrophilicity, lower protein adsorption, and depressed platelet adhesion and activation.

As mentioned earlier, the platelet adhesion for the CPES membrane was higher than those of the

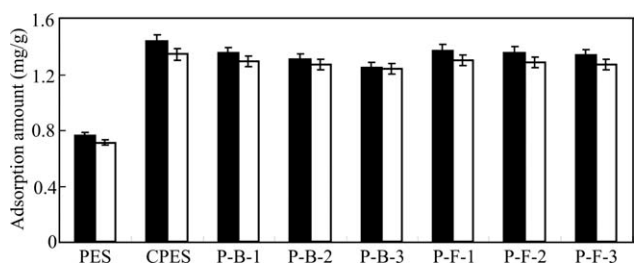

Figure 12 Accumulation of Ca^{2+} for the membranes and the adsorption solutions: (■) Ca^{2+} aqueous and (□) Ca^{2+} serum sodium solution.

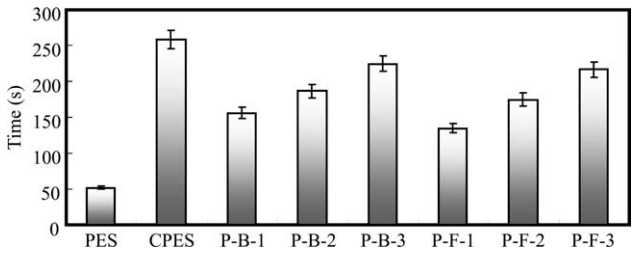

Figure 13 WBCTs for the membranes ($n = 3$).

CPES–BSA and CPES–BFG membranes. It is well known that the platelet adhesion and blood clotting time have some relationship. However, the CPES membrane showed the longest WBCT; one reason might have been the larger adsorption amount of Ca^{2+} ions.

The results of the PRT, APTT, and PT tests may have been affected by the adsorption of Ca^{2+} ions onto the membrane surfaces. As a blood coagulation factor, the concentration of Ca^{2+} ions in the blood is hypersensitive to the blood clotting time.[36,49] During the APTT experiment, the addition of Ca^{2+} (0.025 mol/L of PPP) caused the blood to coagulate in 40 s; however, without this addition, no blood coagulation was observed. The relation between the Ca^{2+} concentration and the blood clotting time will be investigated in detail in our future studies. Furthermore, blood coagulation is a very complicated process; Ca^{2+} is only one of the factors that caused the CPES membrane to have the longest clotting time. After protein grafting, the modified membranes showed improved hydrophilicity and decreased protein adsorption; this was beneficial for prolonging the blood clotting time.

Moreover, after BFG grafting, the modified membranes showed suppressed platelet adhesion and prolonged clotting times because of the decreased BFG adsorption amount. It is well known that the specific fibrinogen conformational changes upon adsorption may influence the propensity for platelet adhesion;[50] therefore, the suppressed platelet adhesion for the BFG-modified membranes might have been due to the decreased BFG adsorption amount. Fibrinogen is the key protein in the blood coagulation cascade; treated with thrombin and FXIII, the fibrinogen is crosslinked and stabilized in the insoluble fibrin gel, and the blood is coagulated.[36] After BFG grafting onto the membrane surfaces, lower BFG adsorption onto the membrane surfaces was observed, and the fibrinogen crosslinking process was restrained; this caused the prolonged clotting times.

CONCLUSIONS

In this study, CPES membranes were prepared by a controlled acetylating and surface-oxidating reaction; this was followed by the grafting BSA and BFG onto the membrane surface. ATR–FTIR spectroscopy, XPS, and protein assays confirmed that the protein was successfully grafted onto the surfaces of the membranes. The WCA of PES membrane was about 76.8° and decreased to about 72.2, 38.1, and 50.4° for the modified CPES, P-B-3, and P-F-3 membranes, respectively. The protein adsorption amounts were higher for the PES membranes (ca. 18.5 and 11.4 $\mu g/cm^2$ for BSA and BFG, respectively), whereas they were lower for the modified membranes and were the lowest for P-B-3 (ca. 0.8 and 2.4 $\mu g/cm^2$ for BSA and BFG, respectively). After the surface carboxylic modification and protein immobilization, the modified membranes showed suppressed platelet adhesion. Because of the Ca^{2+} adsorption, the modified membranes showed longer blood clotting times, and the CPES membranes showed the longest clotting times (902.2 s PRT, 48.8 s APTT, 17.2 s PT, and 259.3 s WBCT). Furthermore, the protein-modified membranes showed increased water fluxes and depressed protein fouling abilities. These results indicate that the modified PES membranes could be used in biomedical fields for real applications in hemodialysis and bioartificial liver supports.

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