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One-Pot Synthesized Poly(vinyl pyrrolidone-*co*-methyl methacrylate-*co*-acrylic acid) Blended with Poly(ether sulfone) to Prepare Blood-Compatible Membranes

Jie Li,[1] Shengqiang Nie,[2] Lingren Wang,[2] Shudong Sun,[2] Fen Ran,[1,2] Changsheng Zhao[2]

[1]State Key Laboratory of Gansu Advanced Non-Ferrous Metal Materials, Lanzhou University of Technology, Lanzhou 730050, People's Republic of China

[2]College of Polymer Science and Engineering, State Key Laboratory of Polymer Materials Engineering, Sichuan University, Chengdu 610065, People's Republic of China

Correspondence to: F. Ran (E-mail: ranfen@163.com) or C.S. Zhao (E-mail: zhaochsh70@163.com)

ABSTRACT: In this study, a random copolymer of poly(vinyl pyrrolidone-*co*-methyl methacrylate-*co*-acrylic acid) was synthesized via a one-pot reaction with the reversible addition–fragmentation chain-transfer method and was then blended with poly(ether sulfone) (PES) to prepare flat-sheet membranes that were expected to have anticoagulant and antifouling properties. The synthesized copolymer was characterized by Fourier transform infrared (FTIR) and NMR spectroscopy. The molecular weights and molecular weight distributions were determined by gel permeation chromatography. Elemental analysis was used to calculate the molar ratios of vinyl pyrrolidone (VP), methyl methacrylate (MMA), and acrylic acid (AA) in the copolymer. A liquid–liquid phase-inversion technique was used to prepare the copolymer-blended PES membranes. X-ray photoelectron spectroscopy and attenuated total reflectance–FTIR spectroscopy were used to investigate the copolymer on the membrane surfaces. Compared with the pristine PES membrane, the modified PES membranes showed improved hydrophilicity, low hemolysis ratios, decreased protein adsorption, and suppressed platelet adhesion. Furthermore, the thrombin time and activated partial thromboplastin time indicated that the blood compatibility of the modified PES membranes were improved. The results of the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the cell morphology suggested that the cytocompatibility increased. In addition, the modified membranes showed good protein antifouling properties. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 4284–4298, 2013

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INTRODUCTION

Poly(ether sulfone) (PES) is a polymeric material with thermal stability, chemical inertness, and outstanding mechanical and film-forming properties;[1] it has been widely used as a membrane matrix in separation fields, such as microfiltration (MF) and ultrafiltration (UF).[2] It has also been used in the field of medical devices for blood purification, artificial organs, and disposable clinical instruments, including in hemodialysis, hemodiafiltration, hemofiltration, plasmapheresis, and plasma collection.[3,4] However, the hydrophilic properties of PES need to be further improved when it is used as a blood-contacting material. The adsorption of serum protein on the PES membrane and further platelet adhesion, aggregation, and coagulation can lead to life-threatening complications.[5] For this reason, injections of anticoagulants are inevitable during the process of clinical application to prevent thrombus formation.

To solve this problem, many studies have focused on the modification of the blood compatibility of PES. In most cases, the approaches adopted to modify PES membranes include coating, surface physical treatment, surface grafting, and blending.[6–9] Among these approaches, blending is the simplest method and is thus widely used in industry. In recent years, many hydrophilic polymers or copolymers have been used as additives to modify PES membranes by the blending method; these include poly(vinyl pyrrolidone) (PVP),[10] poly(ethylene glycol) (PEG),[11] and poly(acrylic acid-*co*-vinyl pyrrolidone) P(AA-co-VP).[12] However, the elution of hydrophilic polymers from PES membranes is inevitable because of their water-soluble characteristics. Thus, many amphiphilic copolymers have been synthesized, including poly(acrylonitrile-*co*-acrylic acid-*co*-vinyl pyrrolidone),[13] poly(vinyl pyrrolidone-*co*-acrylonitrile-*co*-vinyl pyrrolidone),[14] PVP-*b*-poly(methyl methacrylate)-*b*-PVP[15] and poly(styrene-*co*-acrylic acid)-*b*-PVP-*b*-poly(acrylic acid-*co*-

styrene),[16] to improve the hydrophilicity and anticoagulant abilities of PES membranes. In addition, amphiphilic copolymers of poly(vinyl pyrrolidone-*co*-acrylonitrile),[17] poly(vinyl pyrrolidone-*co*-styrene),[18] sulfonated poly(ether sulfide sulfone),[19] POEM-b-PES-b-POEM[20] have been used to improve the hydrophilicity of polysulfone membranes by a blending method; poly(vinylidene fluoride)-*g*-POEM,[21] poly(methyl methacrylate) PMMA-g-POEM[22] have been used to modify poly(vinylidene fluoride) membranes by the blending method. These copolymers could be synthesized by many methods, such as atom-transfer radical polymerization (ATRP) polymerization, reversible addition–fragmentation chain-transfer (RAFT) polymerization, and free-radical polymerization. These amphiphilic block copolymers consisted of hydrophilic chains, which provided hydrophilicity and blood compatibility, and hydrophobic chains, which could prevent the elution of the polymers from the substrate material. Although the block copolymers showed obvious anticoagulant activity, the synthesis procedure was complicated and would thus be hard to industrialize. On the basis of the work of Ran et al.,[15] we try to find a convenient way to achieve equal effect.

The copolymer of poly(vinyl pyrrolidone-*co*-methyl methacrylate-*co*-acrylic acid) [poly(VP-*co*-MMA-*co*-AA)] was first synthesized by Zou et al.[23] via free-radical solution polymerization. The pH sensitivity of the copolymer was investigated. They advocated that the electroviscous effect of the acrylic acid (AA) chain played a dominant role in the pH sensitivity, whereas the vinyl pyrrolidone (VP) chain provided hydrophilicity.[24] The copolymer was blended with PES to prepare a hollow-fiber membrane. The modified membrane showed good pH sensitivity, pH reversibility, and antifouling properties. However, the molecular weight distribution (MWD) of the copolymer was relatively large, and the anticoagulant properties of the copolymer-blended membrane was not investigated.

RAFT polymerization is a living/controlled method that is used to achieve well-defined polymers with both controlled molecular weights and MWDs.[25] Herein, a series of poly(VP-*co*-MMA-*co*-AA) copolymers were synthesized via RAFT polymerization and blended with PES to prepare anticoagulant membranes. In the copolymer of poly(VP-*co*-MMA-*co*-AA), the monomeric unit of AA is a typical monomer that provides a negatively charged surface and was reported to be anticoagulative,[26] whereas VP is a water-soluble monomer that has excellent blood compatibility, hydrophilicity, and protein resistance and is always used as an additive to modify polymeric membranes.[27,28] The copolymer was water-insoluble because of the methyl methacrylate (MMA) chain and prevented elution during the blending process.[29] Through blending with PES, a hydrophilic and negatively charged surface was obtained. The water contact angle, hemolysis ratio, protein adsorption, platelet adhesion, clotting time, cell toxicity, and antifouling properties of the membranes were investigated.

EXPERIMENTAL

Materials

PES (Ultrason E 6020P, Chemical Abstracts Service (CAS) number 25608-63-3) was purchased from BASF Chemical Co. (Germany). VP (99.0% pure) was obtained from Alfa Aesar, and AA (99.5% pure) was purchased from Chengdu Kelong, Inc. (Chengdu, China). Both of these were pretreated with activated carbon before use. MMA (99.0% pure) was also purchased from Chengdu Kelong and was distilled before use. *N,N*-Dimethylacetamide (DMAC; analytical reagent, 99.0% pure) and *N,N*-dimethylformamide (DMF; 99.0% pure) were purchased from Chengdu Kelong and used as the solvents. Azobisisobutryonitrile (AIBN) was obtained from Chengdu Kelong and used as the initiator. Bovine serum albumin (BSA) and bovine serum fibrinogen (BFG) were obtained from Sigma Chemical Co. Micro BCA protein assay reagent kits were products of Pierce. All other chemicals (analytical grade) were obtained from Chengdu Kelong and were used without further purification.

Preparation of the RAFT Agent

According to the literature,[30] *S,S′*-bis(α,α-dimethyl-α″-acetic acid)–trithiocarbonate was synthesized as follows: carbon disulfide (27.4 g, 0.36 mol), acetone (52.3 g, 0.9 mol), chloroform (107.5 g, 0.9 mol), and tetrabutylammonium hydrogen sulfate (2.41 g, 7.1 mmol) were mixed with mineral spirits in a reactor cooled by water under a nitrogen atmosphere. Sodium hydroxide (50%) was added dropwise into the reactor over 90 min to keep the temperature below 25°C. The reaction was carried out overnight. Then, distilled water was added to dissolve the solid; concentrated HCl was then added to acidify the aqueous layer, and then, the mixture was stirred for 30 min with nitrogen purging. After filtering and rinsing with water, the solid was dried and a brown-colored product was collected.

Synthesis of the Poly(VP-*co*-MMA-*co*-AA) Copolymers

A series of poly(VP-*co*-MMA-*co*-AA) copolymers with different monomer ratios (the ratios of VP to MMA to AA were 50:40:10, 45:40:15, and 40:40:20 wt %, respectively) were synthesized via RAFT polymerization. The procedure was conducted as follows: 10 g of monomer (VP, MMA, or AA) was dissolved in 50 mL of DMF. The initiator, AIBN (0.0089 g) and the prepared RAFT agent (0.016 g) were introduced into the reaction solution.[31] Then, the mixture solution was placed under strong agitation continuously until all of the solutes were dissolved. After it was bubbled with nitrogen for 30 min, the reaction mixture was allowed to warm to 80°C under a nitrogen atmosphere, and the reaction lasted for 6 h. After precipitation in double-distilled water, the products were washed several times with double-distilled water to remove the residual monomers. The obtained copolymers were dried in a vacuum at 60°C overnight, and the copolymers with different monomer ratios were named S1, S2, and S3, respectively. In addition, a control sample (named S4) was synthesized via traditional radical solution polymerization (the monomer ratio was 50:40:10), and the procedure was the same as that described previously, only without the RAFT agent. In this study, the ratio of MMA was constant because when a lower proportion was used, the copolymer was water soluble.

Characterization of the Synthesized Copolymers

The compositions of the synthesized copolymers were determined by Fourier transform infrared (FTIR) spectroscopy and ^{1}H-NMR. The molecular weight and MWD were measured by gel permeation chromatography (GPC). To confirm the

monomer ratio of the macromolecule chain, elemental analysis was carried out.

FTIR samples were prepared as follows: the copolymer was dissolved in DMAC, cast onto a potassium bromide (KBr) disc with a thickness of about 0.8 mm, and then dried by an infrared light. The FTIR spectra were measured with an FTIR Nicolet 560 (Nicol America). ^{1}H-NMR spectra were obtained on a Varian Unity Plus 300/54 NMR spectrometer with hexadeuterated dimethyl sulfoxide (DMSO-d_6) as the solvent. GPC measurement, which is based on liquid chromatography analysis with an aqueous gel permeation column, was performed with a Waters 410 gel permeation chromatograph with DMF (1.0 mL/min) as the eluent. Elemental analysis, which is based on the determination of carbon (C), hydrogen (H) and nitrogen (N), was performed with a Carlo Erba 1106 elemental analysis instrument (Italy) with a carrier gas (He, at a flow rate of 100 mL/min) at a combustion temperature of 1000°C with the solid samples. The inverse proportions of C, H, and N were determined. The monomer ratios of the copolymer were calculated by a system of linear equations based on the elemental analysis data. The equations are listed below:

$$\begin{cases} xC_{MMA}+yC_{AA}+zC_{VP} = C_{copolymer} \\ xH_{MMA}+yH_{AA}+zH_{VP} = H_{copolymer} \\ xN_{MMA}+yN_{AA}+zN_{VP} = N_{copolymer} \end{cases} \quad (1)$$

where C_{MMA}, C_{AA}, C_{VP}, and $C_{copolymer}$ are the mass fractions of carbon in MMA, AA, VP, and the copolymer, respectively; H_{MMA}, H_{AA}, H_{VP}, and $H_{copolymer}$ are the mass fractions of hydrogen in MMA, AA, VP, and the copolymer, respectively; and N_{MMA}, N_{AA}, N_{VP}, and $N_{copolymer}$ are the mass fractions of nitrogen in MMA, AA, VP, and the copolymer, respectively. The unknown numbers x, y, and z represent the mass fractions of MMA, AA, and VP, respectively, in the copolymer, which could be obtained by the equations.

Preparation of Copolymer-Blended PES Membranes

The copolymer-blended PES membranes were prepared by a phase-inversion technique. PES and the synthesized copolymer were dissolved in the solvent DMAC by vigorous stirring until a clear homogeneous solution was obtained, and the concentration of PES was 16 wt %. In this study, different membranes were prepared with contents of the copolymer in the casting solutions of 0, 1, 3, and 5 wt %, respectively. After vacuum degassing, the casting solutions were prepared into membranes by spin coating coupled with a liquid–liquid phase-inversion technique at room temperature. The membranes were thoroughly rinsed with distilled water to remove the residual solvent. All of the prepared membranes had a uniform thickness of about 60–80 μm.

Characterization of the Modified PES Membranes

The structures and compositions of the membrane surfaces were characterized by X-ray photoelectron spectroscopy (XPS; Kratos XSAM800 Britain XPS instrument) and attenuated total reflectance (ATR)–FTIR spectroscopy (Nicolet 560).

The morphology of the membranes was observed by scanning electron microscopy (SEM). The membranes were dried for 2 days with a vacuum freeze dryer, and then quenched by liquid nitrogenous gas, attached to the sample supports and coated with a gold layer. A scanning electron microscope (S-2500C Hitachi, Japan) was used for the morphological observation of the membrane cross section.

The hydrophilicity of the membrane surfaces was investigated on the basis of contact angle measurements with a contact angle goniometer (OCA20, Dataphasics, Germany) equipped with video capture. A piece of membrane 2 × 2 cm^2 (dried overnight in a vacuum oven at 60°C) was attached to a glass slide and mounted on the goniometer. For the static contact angle measurements, 3 μL of double-distilled water was dropped onto the airside surface of the membrane at room temperature, and the contact angle was measured after 10 min to take out the effect of surface roughness. At least eight measurements were averaged to obtain a reliable value. The measurement error was ±3°.

Blood Compatibility

Sterilization. Before the biological tests [hemolysis test, thrombin time (TT)/activated partial thromboplastin time (APTT) test, protein adsorption test, and platelet adhesion test], the membranes were sterilized by immersion in medicinal alcohol for 1 h, moved into normal saline (NS), and stored at 4°C for the next tests.

Antihemolytic Activity. For hemolytic testing, sodium citrate stabilized human blood was first obtained from Huaxi Hospital. An amount of 20 mL of whole blood was centrifuged under 500 g for 15 min to get a concentrated red cell suspension. After that, the suspension was washed with NS five times. The procedure was as follows: the concentrated red cell suspension was diluted with NS and centrifuged under 500 g for 15 min, and the supernatant was removed. The suspension was diluted into NS with a final volume of 100 mL of red blood cell suspension (RBC). The sterilized membranes with a size of 1 × 1 cm^2 were immersed in NS for 24 h, moved into 1 mL of RBC, and incubated at 37°C for 3 h. Deionized water-dispersed RBC was used as the positive control, and the NS-dispersed RBC was used as the negative control. After the incubation, RBC was centrifuged under 500 g for 5 min. The absorbance of the released hemoglobin in the suspensions was measured by spectrophotometry at 546 nm. The hemolysis ratio was calculated with the follow formula:

$$\text{Hemolysis ratio}(\%) = \frac{A_s - A_n}{A_p - A_n} \times 100\% \quad (2)$$

where A_s, A_p, and A_n are the absorbances of the suspensions, positive control, and negative control, respectively. Two parallel tests were carried out to obtain a reliable value.

Protein Adsorption. Protein adsorption experiments[32] were carried out with BSA and BFG solutions [1 mg/mL in phosphate buffered saline (PBS), pH = 7.4]. The membrane, with an area of 1 × 1 cm^2, was incubated in PBS for 24 h and then immersed in the protein solution for 2 h at 37°C. After protein adsorption, the membrane was gently rinsed with PBS and then immersed in a 2 wt % aqueous sodium dodecyl sulfate solution and shaken for 2

h at 37°C to remove the protein adsorbed on the membrane. The amount of protein eluted into the sodium dodecyl sulfate solution was quantified with a Micro BCA protein assay reagent kit. Then, the amount of adsorbed protein was calculated.

Platelet Adhesion. For platelet adhesion,[33,34] healthy and fresh human blood was collected first with sodium citrate as an anticoagulant (anticoagulant-to-blood ratio = 1:9 v/v). Then, the blood was centrifuged at 1500 rpm for 15 min to obtain platelet-rich plasma (PRP). The PES and modified PES membranes were immersed in PBS at 37°C for 1 h. After the removal of PBS, 1 mL of fresh PRP was introduced. The membranes were incubated with PRP at 37°C for 2 h. Then, the membranes were rinsed three times with PBS. At last, the membranes were treated with 2.5 wt % glutaraldehyde in PBS at 4°C for 1 day. The membranes were washed with PBS and subjected to a drying process by their passage through a series of graded ethanol–PBS solutions (25, 50, 75, and 100%) and isoamyl acetate–ethanol solutions (25, 50, 75, and 100%). The critical point drying of the specimens was done with liquid CO_2. Platelet adhesion was observed by an S-2500C microscope (Hitachi, Japan).

Anticoagulant Activity. To investigate the antithrombogenicity of the copolymers and the modified membranes, the TT and APTT of the synthesized copolymers and the copolymer-modified membranes were measured with an automated blood coagulation analyzer CA-50 (Sysmex Corp., Kobe, Japan). The test method can be described as follows:[15,33] for the copolymers, 1, 2, and 3 mg of the copolymers were incubated in 0.1 mL of platelet-poor plasma (PPP) at 37°C for 30 min; then, 50 μL of the PPP was moved into a test cup. This was followed by the addition of 50 μL of the TT/APTT agent (warmed to 37°C for 10 min before use) and incubation at 37°C for 3 min. Then, the TT/APTT was measured. For the membranes, a 0.5 × 0.5 cm^2 membrane was immersed in 0.2 mL of PBS buffer (pH = 7.4) for 1 h. The PBS was removed, and then, 0.1 mL of PPP was introduced. After incubation at 37°C for 30 min, 50 μL of the incubated PPP was moved into a test cup, and the subsequent procedure was the same as the copolymer test.

Cytocompatibility

Cell Culture. Human embryonic hepatocytes (LO2) were cultured in R1640 nutrient medium, which was supplemented with 10% fetal bovine serum (Hyclone), 2 mmol of L-glutamine, and a 1 % v/v antibiotics mixture (10,000 U penicillin and 10 mg of streptomycin). The cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C (Queue Incubator, France). The culture medium was refreshed every day. Confluent cells were detached from the culture flask with sterilized PBS and a 0.05% trypsin/ethylene diamine tetraacetic acid solution.

The pristine and modified PES membranes were cut to 1 × 1 cm^2 to fit 24-well cell culture polystyrene plates and prewetted by immersion in the culture medium for 3 h in an incubator at 37°C. Then, the membranes were placed in the cell culture plates, rinsed with PBS, and sterilized by γ irradiation (25 kGy).

Cell Toxicity Test. The cell toxicity of the modified membranes was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after cell culture for 2, 4, and 6 days, respectively. The hepatocytes were seeded onto the membranes at a density of approximately 2.5 × 10^4 cells/cm^2. Cells cultured in the wells without membranes were chosen as controls in this study. After predetermined time intervals, 45 μL of MTT solution (1 mg/mL in the test medium) was added to each well and incubated at 37°C for 4 h. Mitochondrial dehydrogenases of viable cells selectively cleaved the tetrazolium ring, yielding blue/purple formazan crystals. Then, 400 μL of ethanol was added to dissolve the formazan crystals. Thus, the quantity of formazan dissolved in ethanol reflected the level of cell metabolism. The solution was shaken homogeneously for 15 min. After that, the sample solutions were aspirated into microtiter plates, and the optical density was read in a microplate reader (model 550, Bio-Rad) at 492 nm. Three parallel tests were carried out to obtain a reliable value, and the results were expressed as means plus or minus standard deviation. The statistical significance was assessed by a Student's t test, with the level of significance set at $p < 0.05$.

Cell Morphology. The morphology of the cells seeded on the membranes was observed by SEM. The density of hepatocytes was approximately 2.5 × 10^4 cells/cm^2. After 6 days, the seeded membranes were rinsed with PBS and fixed with 2.5 wt % glutaraldehyde in PBS at 4°C for 12 h. For morphological observation, the fixed samples were subjected to a drying process by their passage through a series of graded alcohol–PBS solutions (25, 50, 75, and 100%) and isoamyl acetate–alcohol solutions (25, 50, 75, and 100%). Critical point drying of the specimens was carried out with liquid CO_2. The specimens were sputter-coated with a gold layer and examined in an S-2500C microscope (Hitachi, Japan).

UF of the Protein Solution

Protein solution fluxes of the prepared membranes were measured with an apparatus as described in our previous works.[35,36] The pressure was supplied by an air compressor. A dead-end UF cell was used with an effective membrane area of 13.8 cm^2. BSA was dissolved in the PBS solution at a concentration of 1 mg/mL (pH = 7.4), and the filtration experiments were carried out at room temperature at a pressure of 0.08 MPa. The flux (F) was calculated with the following equation:

$$F = \frac{V}{SPt} \tag{3}$$

where V is the volume of the permeated solution (mL), S is the effective membrane area (m^2), P is the pressure applied to the membrane (mmHg), and t is the time (h). The protein rejection ratio (R) refers to the proportion of the protein that rejected by the membrane during the UF process and could be calculated by the following equation:

$$R(\%) = \left(1-\frac{Cp}{Cb}\right)\times 100 \tag{4}$$

where C_p and C_b are the concentrations in the permeated and bulk solutions (mg/mL), respectively. For blood purification applications, the protein rejection ratio is an important indicator in evaluating the properties of preventing the loss of plasma proteins during dialysis.

The flux recovery ratio (R_{FF}) is a parameter used to assess the protein antifouling properties of membranes. R_{FF} is obtained by

the comparison of the initial PBS flux and that after protein solution UF and can be calculated by the following equation:

$$R_{FF}(\%) = \left(\frac{F2}{F1}\right) \times 100 \quad (5)$$

where F_1 and F_2 are the initial PBS flux and the PBS flux after protein solution UF (mL/m^2 h mmHg), respectively. A high R_{FF} indicates a high efficiency and reusability of the membrane.

RESULTS AND DISCUSSION

Already reported amphiphilic block copolymers via RAFT were proven to be anticoagulative and stable in a PES substrate.[15,16] Because of the preparation method, a long fabrication period and unsatisfying yield were inevitable. The one-pot method is an easy method with facile reaction conditions and is thus more suitable for industrialization. To find a substitution for anticoagulative block copolymers by a one-pot method, an amphiphilic and negatively charged random copolymer of poly(VP-*co*-MMA-*co*-AA) was synthesized.

Synthesis and Characterization of Poly(VP-*co*-MMA-*co*-AA)

FTIR and ^{1}H-NMR Spectral Analysis of the Copolymers. PVP is a polymer that is widely used to modify PES membranes by the blending method because of its hydrophilicity. The AA chains in the copolymer provide an anion group, which is expected to improve the hydrophilicity and anticoagulant properties of the copolymer. To prevent the elution of the copolymer from the PES matrix, MMA chains were introduced because of the principle of the similar solubility parameters of PMMA and PES (δ_{PMMA} = 22.7 and δ_{PES} = 21.9).[29]

Figure 1 shows the FTIR and ^{1}H-NMR spectra of the poly(VP-*co*-MMA-*co*-AA) copolymers. As shown in Figure 1(a), the peaks at 1674–1681 cm^{-1} were the characteristic peaks of the —C=O in the acylamide group; this was the evidence for the existence of VP units. The peaks at 2951 and 1387 cm^{-1} were attributed to —CH_3 of the MMA unit in the copolymer. The peaks at 3436–3442, 1728–1731, and 1435–1437 cm^{-1} were the characteristic peaks of the —OH, —C—O—, and —OH groups, respectively, from the AA unit. The structure of the synthesized copolymer was also confirmed by ^{1}H-NMR (500 MHz, DMSO-d_6) characterization [Figure 1(b)]. The existence of the VP unit was verified by the signals at δ 3.55 (s, H, —CH—N), δ 3.11 (s, 2H, —CH_2—N), δ 2.15 (s, 2H, —CH_2—C=O), δ 1.95 (s, 2H, —CH_2—C—C=O), and δ 1.75 (s, 2H, —CH_2—C—N). The resonance signals located at δ 3.55 (s, 3H, CH_3—O—), δ 1.95 (s, 2H, —CH_2—C—CH_3), and δ 1.46 (s, 2H, CH_3—C—C=O) were found for the MMA unit. The signals for the AA unit were found at δ 12.26 (s, H, —COOH), δ 1.95 (s, H, —CH—C=O), and δ 1.79 (s, 2H, —CH_2—C—C=O). The FTIR and ^{1}H-NMR spectra indicated that the copolymer of poly(VP-*co*-MMA-*co*-AA) was successfully synthesized.

Furthermore, the NMR integration ratio was used to estimate the composition of the copolymers. In this study, the monomer of AA contained a carboxyl group. Because of the high reactivity of the proton in carboxyl groups, the integration of AA was unreliable. In addition, the dispersity of the copolymers brought about a broad peak shape (see NMR spectra), and the signals with similar chemical shifts overlapped each other. As a result, the proton of methylene could not be used to determine the AA proportion. Thus, the values of VP/MMA were calculated from NMR integrations, regardless of AA, as shown in Table I. From the table, the integral values were similar to those obtained from elemental analysis. The integral values could be seen as corroborative evidence for the compositions of the copolymers.

In this study, the same monomers were used to prepare the copolymers via different polymerization methods; thus, the compositions of the copolymers showed no significant difference. The main differences between S1, S2, S3, and S4 were the monomer ratios, sequence isomerism, weight-average molecular weights (M_w's), and MWDs, which could not be clearly observed from the FTIR and NMR spectra. We provided the FTIR and NMR spectra to confirm that the synthesized copolymers contained all the used monomers.

GPC Analysis of the Copolymers. One-pot RAFT polymerization was used to synthesize a poly(VP-*co*-MMA-*co*-AA) copolymer in this work. Because of the extremely low radical concentration, the copolymer had a narrow MWD. The literature[37,38] suggests that the dispersity of the target copolymer is negatively related to the monomer conversion for typical RAFT polymerization. The conversion of the monomer was approximate 50% because of the electron donor/accepter effect (mentioned later in the Elemental Analysis of the Copolymers section) in this study. Consequently, the dispersity of the copolymer was greater than that of a typical RAFT polymer. When we compared the feed ratio and the monomer ratio of S3, almost all of the AA monomers were consumed. This meant that the polymerization of S3 was terminated at high monomer conversion. Sample 3 had the highest monomer conversion; accordingly, its dispersity was the lowest.

In our previous study, copolymers of poly(VP-*co*-MMA-*co*-AA) and poly(vinyl pyrrolidone-*co*-acrylonitrile-*co*-acrylic acid) were synthesized via free-radical solution polymerization, and the MWDs were about 7.62 and 5.43.[13,23] Compared with S4 (via free-radical polymerization), as shown in Table II, the copolymers of S1 to S3, which were prepared by RAFT polymerization, had narrower MWDs. When an amphiphilic copolymer was used as an additive to modify the PES membrane, the low molecular weight and wide MWD were disadvantages because the low-molecular-weight amphiphilic polymer was easy to elute from the substrate during the liquid–liquid phase-inversion process.[39] According to these, S3 might have been the best choice for modifying the PES membrane.

Elemental Analysis of the Copolymers. Elemental analysis was used to investigate the monomer ratios in the copolymers. The proportions of carbon, hydrogen, and nitrogen in each sample were determined by elemental analysis. Simultaneous equations were used to calculate the monomer ratios in the copolymers.

Table I showed the elemental analysis data. As shown in the table, the ratios of VP in the prepared copolymers were lower than those in the feed ratios (50, 45, 40, and 50% for S1, S2, S3, and S4, respectively), whereas the ratios of AA in the prepared copolymers were higher compared with the feed ratios. The proportions of MMA in the copolymers were almost the

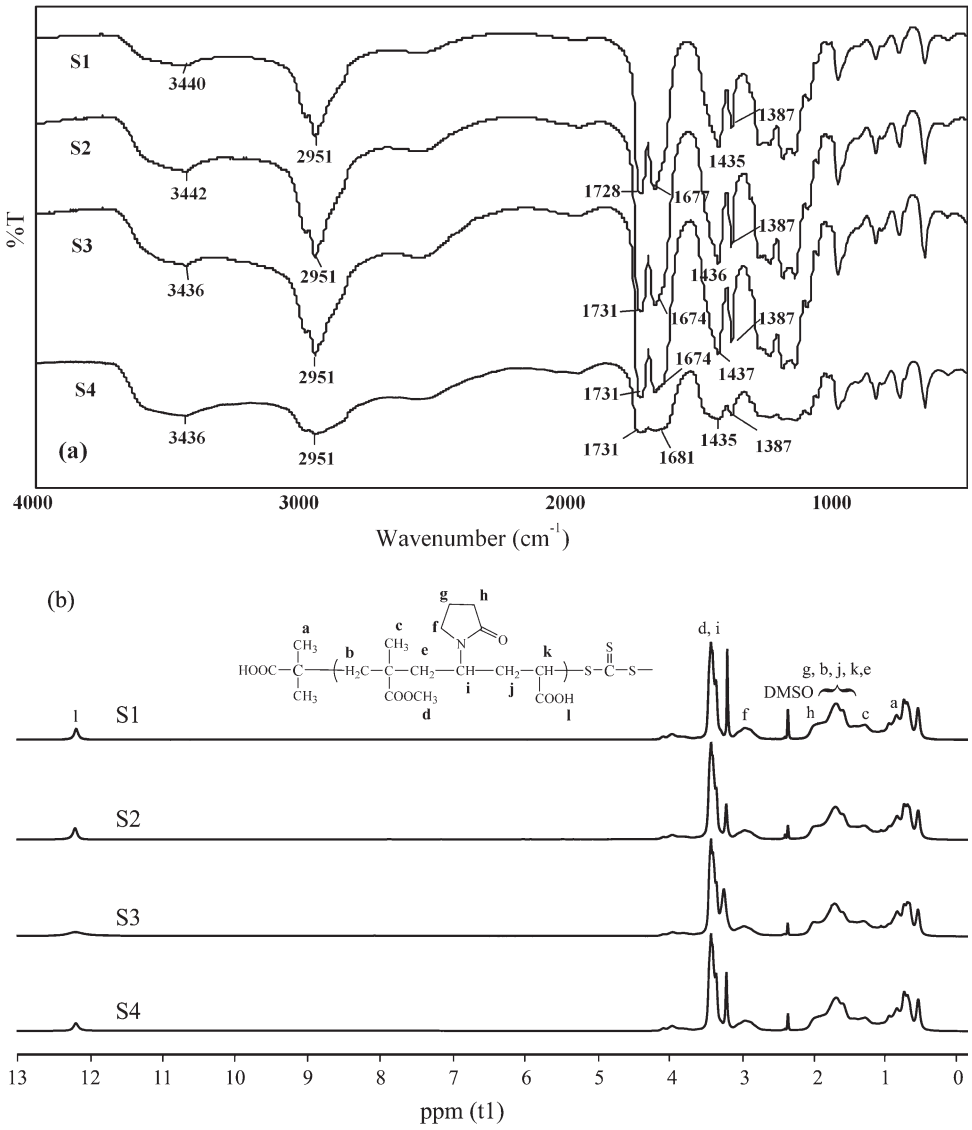

Figure 1. (a) FTIR and (b) ^{1}H-NMR spectra of the copolymers (S1, S2, S3, and S4).

same as the feed ratios. In addition, with increasing AA in the feed ratios (10, 15, and 20% for S1, S2, and S3, respectively), the AA proportions increased in the copolymers.

The distinction between the feed ratio and comonomer ratio was mainly caused by the different reactivity ratios of the monomers. As we know, in the system of binary copolymerization, when the reactivity ratio of monomer 1 (r_1) is less than 1 and the reactivity ratio of monomer 2 (r_2) is greater than 1, the comonomer ratio of the copolymer may differ from the feed ratio. Furthermore, the larger the gap between r_1 and r_2 is, the greater the difference is. Kavlak et al.[40] studied the mechanism of the ternary copolymerization of citraconic anhydride, styrene,

Table I. Elemental Analysis Data

Sample number	Carbon (%)	Hydrogen (%)	Nitrogen (%)	VP/MMA/AA[a]	VP/MMA[b]
S1	60.19	8.81	3.14	29.3:41.4:29.3	1:1.12
S2	60.24	9.06	2.89	27.0:39.4:33.6	1:1.33
S3	58.97	8.05	2.46	23.0:40.2:36.8	1:1.68
S4	59.82	8.83	2.98	27.9:41.4:30.7	1:1.10

[a] Calculated from elemental ratios (wt %).
[b] Estimated from the NMR spectra and converted into mass fraction (wt %).

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Table II. Conversion, Molecular Weight, and MWD Data for the Copolymers

Sample number	Conversion (%)[a]	M_n[b]	M_w[b]	M_w/M_n[b]
S1	43.2	7750	13146	1.70
S2	43.9	5143	8640	1.68
S3	42.6	23603	28334	1.20
S4	47.8	6267	13404	2.14

[a] Conversion was determined gravimetrically.
[b] Number-average molecular weight (M_n), M_w and MWD were determined by GPC.

Table III. TT/APTT of the Copolymers

Sample number	TT (s)			APTT (s)		
	1 mg	2 mg	3 mg	1 mg	2 mg	3 mg
S1	29.2	46.3	56.7	317.7	465.2	600
S2	34.5	40.7	51.9	460.7	522.1	600
S3	49.1	53.2	62.1	477.5	588.3	600
S4	28.2	30.2	37.7	320.8	350.7	377.1

and vinyl phosphonic acid. They simplified the ternary system as a binary system based on the electron-donating/accepting effect of the monomer. This model was also suitable for the VP–MMA–AA system. VP has an electron-donating capacity because of the lone pair of electrons on the nitrogen nucleus, which can be considered as an electron donor. Simultaneously, AA has an electron-accepting capacity because of the electrophilicity of the hydrogen nucleus of the carboxyl, and it would be regarded as the electron acceptor. According to the model, VP···MMA and AA···MMA complexes are the major monomers. The reactivity ratio of the VP···MMA complex was slower than that of the AA···MMA complex. These led to the proportion of VP in the copolymer being lower than that in the feed ratio, whereas for AA, it was the opposite.

Random copolymers are large-scale concepts that include copolymers that tend to be alternating copolymers and others that tend to be block copolymers. Considering the impact of the reactivity ratios and the electron-donating/accepting effect, the structure of the synthesized copolymer can be described as follows: a few VP···MMA chains inset in primary AA···MMA chains.

Clotting Time of the Copolymers. To compare the anticoagulant properties of different copolymers, TT and APTT were measured. As shown in Table III, with the increase of the copolymer

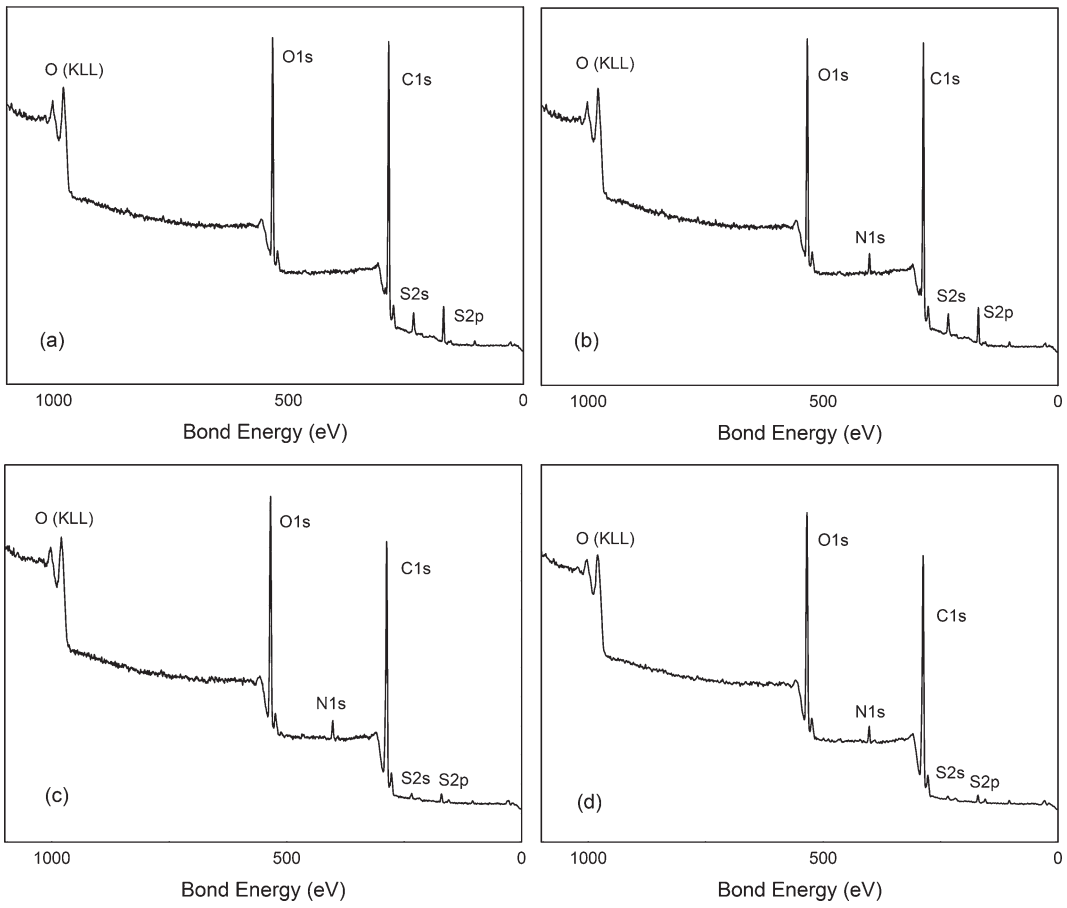

Figure 2. XPS spectra of the (a) pristine and (b) 1, (c) 3, and (d) 5% copolymer-blended PES membranes. O(KLL) presents the Auger electron of oxygen.

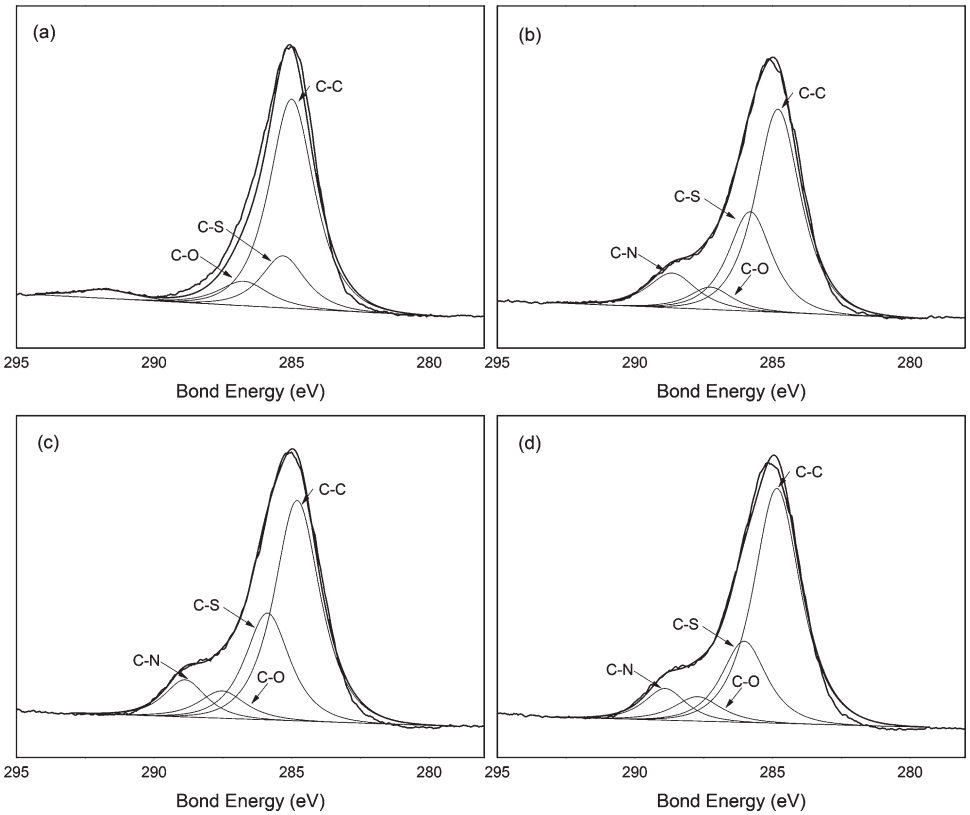

Figure 3. C1s peak magnified XPS spectra of the (a) pristine and (b) 1, (c) 3, and (d) 5% copolymer-blended PES membranes.

weight, TT and APTT increased for all the four kinds of samples. Furthermore, the copolymers prepared via RAFT polymerization (S1–S3) possessed higher TT and APTT values compared with the one prepared via free-radical polymerization (S4).

According to the GPC and TT/APTT results, copolymer S3 had the highest molecular weight, most narrow MWD, and best anticoagulant properties and thus was chosen as the additive to modify the PES membranes.

Compositions and Structures of the Membranes

XPS and ATR–FTIR were used to investigate the compositions and structures of the pristine and modified PES membranes. To illustrate the relation between the blending amount and surface composition of the membranes, the magnifying C peaks were provided.

Figure 2 shows the XPS spectra of the pristine and modified PES membranes. The most significant difference between the pristine and modified PES membranes was the N1s signal located at 402 eV; this was attributed to the VP unit in poly(VP-*co*-MMA-*co*-AA). With increasing copolymer blending amount, the signal strength of N1s was enhanced. Furthermore, the S2s and S2p signals decreased continually with increasing copolymer blending amount. The results confirmed the existence of poly(VP-*co*-MMA-*co*-AA) on the membrane surface and the enhancement of the copolymer content with increasing blending amount.

Figure 3 shows the XPS spectra of the C1s region. As shown in the figure, the C1s peak was fitted by three carbon moieties: C—C (284.70 eV), C—S (285.31 eV), and C—O (286.34 eV). In the spectra shown in parts b, c, and d of Figure 3, the C—N signal at 287.79 eV appeared. The C—N signal was attributed to the VP unit in poly(VP-*co*-MMA-*co*-AA).

Figure 4 shows the ATR–FTIR spectra of the surfaces for the PES and modified PES membranes. The most significant changes were a characteristic peak for the MMA unit at 2918 cm^{-1}, a peak for the AA unit at 1726 cm^{-1}, and a weak peak at 1653 cm^{-1}, which was the characteristic peak for the VP unit. These results indicate that the copolymer existed on the modified membrane surfaces.

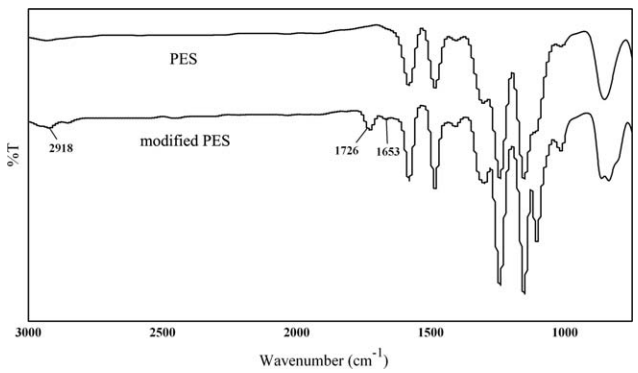

Figure 4. ATR–FTIR spectra of the 5% poly(VP-*co*-MMA-*co*-AA) blended PES membranes.

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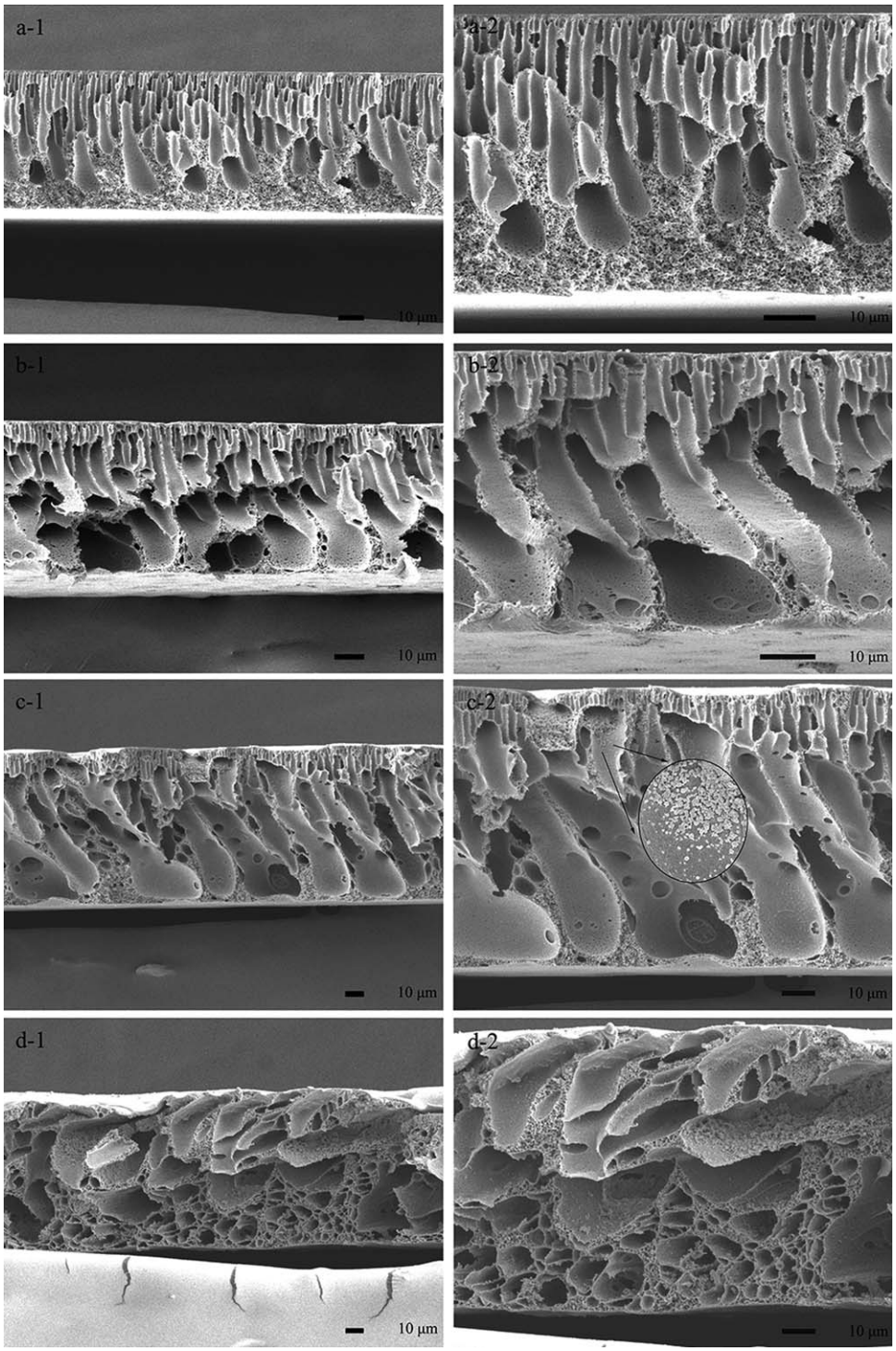

Figure 5. SEM images of the cross-sectional views of PES and copolymer-blended membranes: (a-1,a-2) PES, (b-1,b-2) 1% copolymer-blended, (c-1,c-2) 3% copolymer-blended, and (d-1,d-2) 5% copolymer-blended membranes.

The cross sections of the PES membrane and poly(VP-*co*-MMA-*co*-AA) modified PES membranes were observed by SEM, as shown in Figure 5. The PES membrane [Figure 5(a-1,a-2)] exhibited the characteristic morphology of an asymmetric membrane, consisting of a dense top layer and a porous sublayer with a fingerlike structure. For the copolymer-modified membranes, the pore size of the fingerlike structure was enlarged, and the morphology of the fingerlike structure was irregular with the copolymer increment. This might have been caused by the existence of the VP chain because PVP is always used as a pore-forming agent in the UF membrane preparation procedure.[41] Furthermore, for the modified PES membranes, a

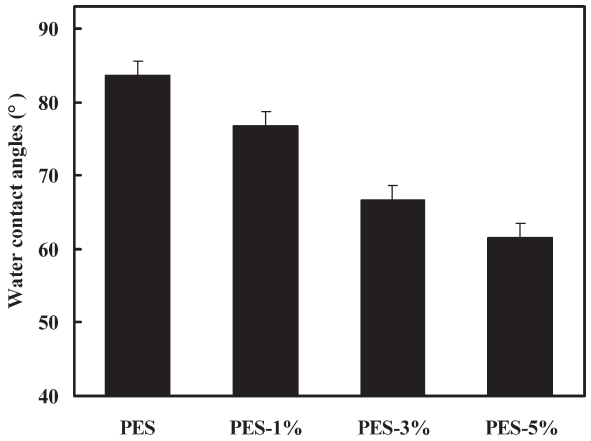

Figure 6. Static contact angles for the PES and modified PES membranes ($n = 8$).

number of self-assembled microspheres were observed and were embedded in the pores [see Figure 5(c-2,d-2)], especially in the 3 and 5% copolymer-blended PES membranes. The formation of self-assembled microspheres occurred during a rapid solvent-exchange process. A part of the amphiphilic copolymer shifted to the solvent–solute interface and self-assembled to decrease the surface energy. The SEM images indicated that the structure of the modified membranes were altered through the addition of the synthesized copolymer as an additive.

Water Contact Angles

The water contact angle, which is an indicator of the hydrophilicity of the membrane surfaces, is widely used to determine the hydrophilic/hydrophobic properties of a material surface. In this study, the water contact angles for the PES and modified PES membranes were measured. The angles were measured through the aqueous phase, so angles of 90° and greater corresponded to hydrophobic surfaces, and angles less than 90° corresponded to hydrophilic surfaces.[42] As shown in Figure 6, all of the water contact angles were less than 90°; the pure PES membrane possessed the highest contact angle of 83.6°. When the copolymer blended into the membrane, the contact angle decreased. Moreover, with increasing copolymer amount, the contact angles decreased further. These results demonstrated that the hydrophilicity of the modified membranes was improved after blending with poly(VP-*co*-MMA-*co*-AA).

Furthermore, the water uptake was not examined in this study. Because the PES membrane had a porous structure, as shown in the SEM pictures, most of the water that was taken up was located in the pores of the membrane rather than in the material itself. For this reason, the mechanical properties of the membranes would not decrease obviously because of the water that was absorbed.

Blood Compatibility

Hemolysis Ratio. The hemolysis ratio is one of the key indicators used to evaluate the blood compatibility of blood-contacting material. The hemolysis results are shown in Figure 7. All of the modified membranes presented low hemolytic activity, ranging from 1.1 to 1.25%. The literature[43] suggests that AA-containing copolymers have the potential to be hemolytic; a high surface density of carboxyls leads to serious hemolysis. In this work, the copolymer used as the additive had 36.8 wt % AA. After blending with PES to prepare membranes, the overall content of AA in the membrane was reduced to a low level; accordingly, the surface density of carboxyls was decreased. The hemolysis data indicated that copolymer blending amounts of 5% and lower were not sufficient to make the membranes hemolytic. We concluded that the modified membranes had antihemolytic activity.

Protein Adsorption. Protein adsorption on the material surface is a common phenomenon during thrombogenisis.[44] When a membrane is used for blood purification, protein adsorption is the first stage of the interactions between the membrane and the blood; this may lead to further undesirable results. The amount of protein adsorbed on the membrane is considered to be one of the important factors in the evaluation of the blood

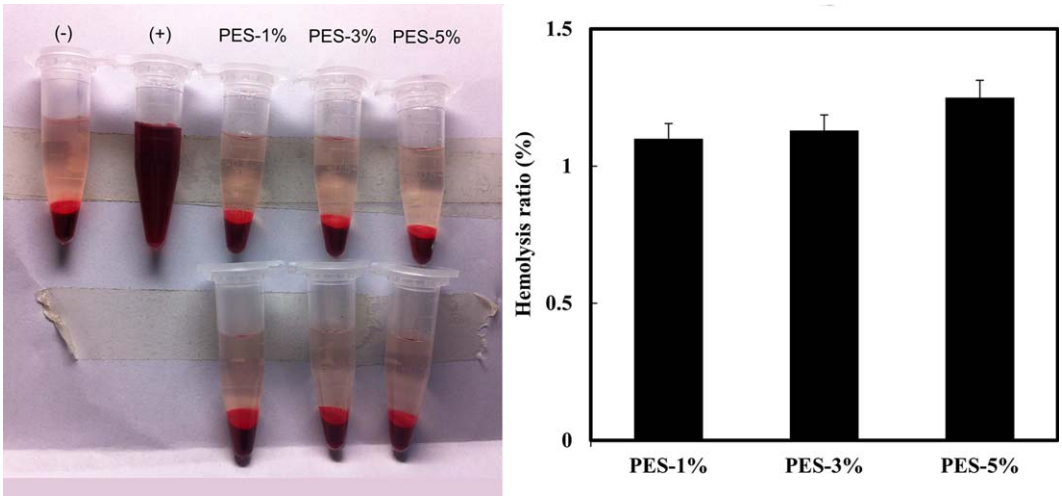

Figure 7. Photographs of the centrifuged RBCs after incubation with modified membranes for 3 h (left; where + represents the positive control, and − represents the negative control) and hemolysis ratios of the RBCs incubated with different modified membranes (right). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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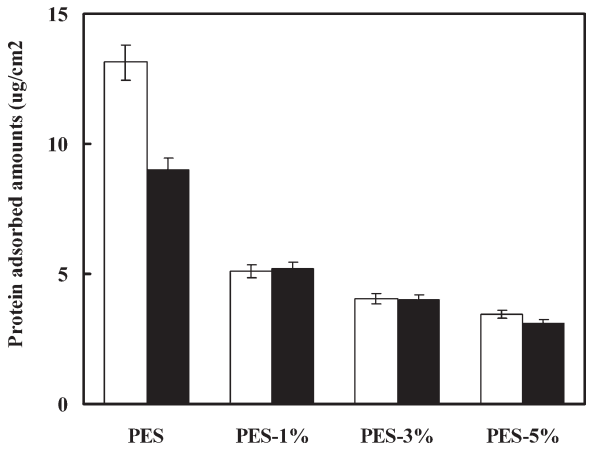

Figure 8. Protein adsorption on the PES membrane and copolymer-modified PES membranes: (□) BSA adsorption and (■) BFG adsorption ($n = 3$).

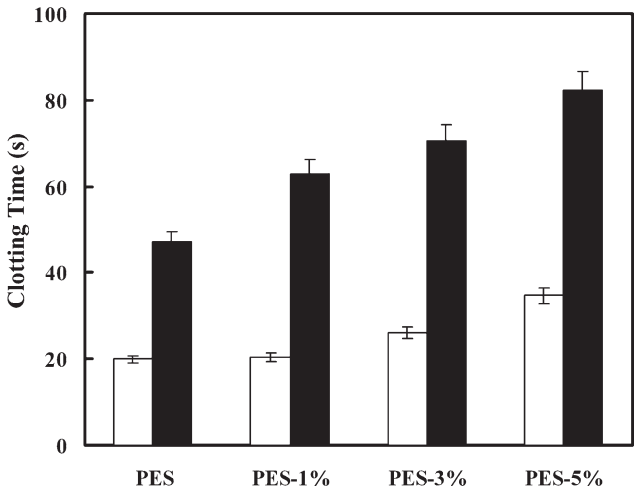

Figure 10. (□) TT and (■) APTT for the PES and modified membranes ($n = 3$).

compatibility of materials. It is generally recognized that the hydrophobic interaction between the material surface and protein plays a dominant role in the nonselective adsorption of protein. Materials possessing a hydrophilic surface commonly show a relatively low adsorption of proteins.[45]

A surface possessing a similar free energy with endangium always shows low protein adsorption. The surface free energy of a material can be decreased by the improvement of the hydrophilicity of the surface. The hydrogen bonds between the hydrophilic groups on the material surface and water lead to the formation of a water barrier layer that is firmly bonded to the surface, and this barrier layer can prevent protein adsorption.[43] The negatively charged surface prevents protein adsorption by electrostatic repulsion, as most plasma protein is electronegative. For instance, a surface with sulfonic acid and/or carboxyl group shows low protein adsorption.[46,47] For steric hindrance, it is commonly considered that surfaces with molecular brushes suppress protein adsorption by conformational changes of the molecular chain, the high density of the molecular chain, and long-chains; this results in a relatively low protein adsorption. For example, PEG brushes on a membrane surface could prevent protein adsorption.[48]

In addition, fibrinogen in blood plasma is particularly important for platelet adhesion because it can bind to the platelet glycoprotein IIb/IIIa receptor.[49] Thus, low protein adsorption is considered important in the improvement of blood compatibility.

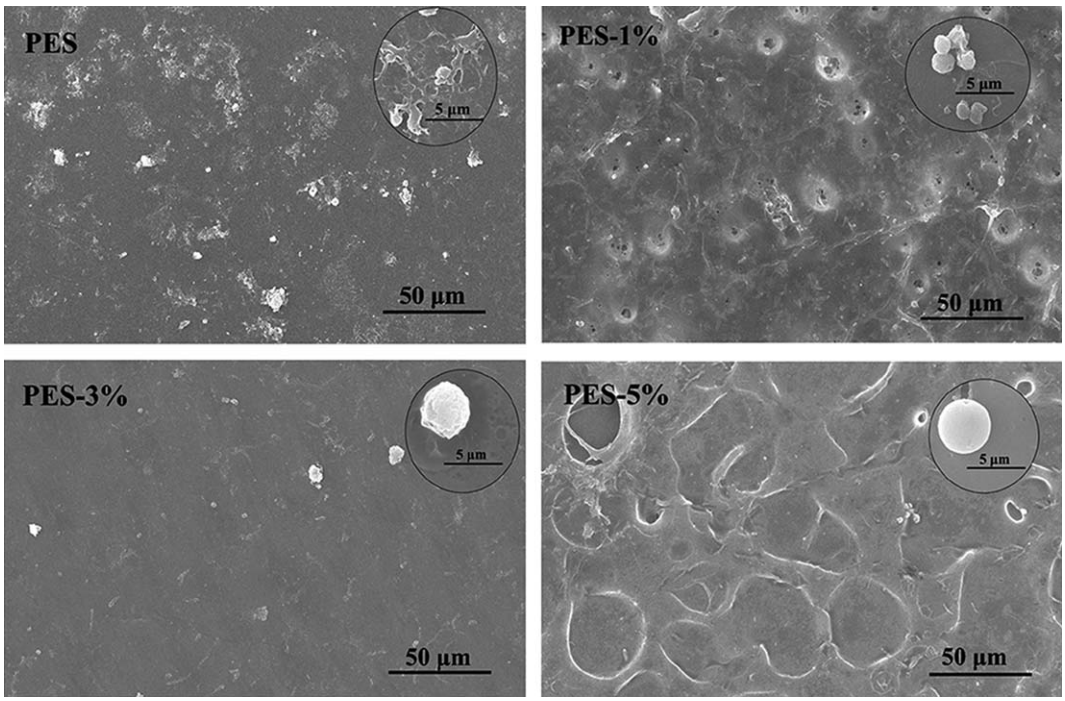

Figure 9. SEM images of the platelets adhering to the PES and modified PES membranes.

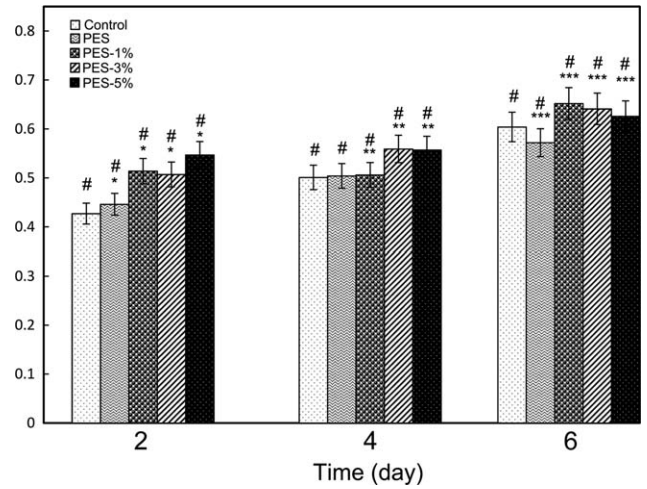

Figure 11. MTT tetrazolium assay. The Formazan absorbance is expressed as a function of time from hepatocytes seeded onto the pristine and modified PES membranes and the controls. The values are expressed as means plus or minus the standard deviation ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.05$, and $^{***}p < 0.05$ compared with the values for the control sample at 2, 4, and 6 days, respectively. $^{\#}p < 0.05$ between different days for the same sample.

In this study, the surfaces of the membranes were studied in relation to the adsorption of BSA/BFG *in vitro*, as shown in Figure 8. It was found that all of the modified membranes had lower BSA and BFG adsorption amounts than the PES membrane, and the values decreased with increasing copolymer content in the membranes. This was caused by the improved hydrophilicity of the modified membrane surfaces and the electrostatic repulsion of the negatively charged surfaces. The improved protein-resistant properties might have improved the blood compatibility of the modified PES membranes.

Platelet Adhesions. The adhesion of platelets to blood-contacting medical devices is a key problem in thrombus formation on material surfaces. Platelets can bind to the adsorbed proteins, and this leads to platelet activation. The activated platelets can promote thrombin formation and platelet aggregation.[50]

In this study, platelet adhesion on the membrane surface was investigated. Figure 9 shows the SEM images of platelets adhering to the PES and modified PES membranes. By comparing the figures at low magnification, we observed that numerous platelets aggregated and accumulated on the PES membrane surface. For the modified membranes, few platelets were observed. At high magnification, platelets adhering to the PES membrane had a flattened and irregular shape. On the contrary, the platelets adhering to the modified membranes had a nearly rounded morphology with no pseudopodium formation. These results demonstrate that the platelets adhering to the modified membranes were not activated. We concluded that both the spreading and pseudopodium formation in the modified membranes were distinctly suppressed.

Anticoagulant Activity. TT is an overall assessment of the effect of thrombin during the coagulation stage. The transformation of fibrinogen into fibrin occurs in the presence of thrombin. Whether in the intrinsic pathway or in the extrinsic pathway, the formation of thrombus cannot be separated from fibrin; this a consequence of thrombin activity.[51] Therefore, a TT test can reveal the influence of blood-contacting materials on thrombin activity. The APTT test is a widely used method to evaluate coagulation abnormalities in the intrinsic pathway.[52] Through the detection of functional deficiencies in factors II, V, and X and fibrinogen, a prolonged APTT would be observed if blood-contacting materials reacted to or combine with the coagulation factors.

To further study the blood compatibility of the modified membranes, TT and APTT were measured, as shown in Figure 10. It was found that TT and APTT of the modified membranes increased compared with those of the PES membrane, and with increasing copolymer amounts, TT and APTT increased and were over twice those of the PES membrane when 5% copolymer was blended. We concluded that the prolonged TT and APTT of the modified membranes were caused by low BFG adsorption, suppressed platelet adhesion, and activation.

Cytocompatibility

Considering the hydrophilicity of the VP and AA and the bioinertness of MMA, the material was supposed to be biocompatible. We used an MTT assay to determine the cell toxicity of the material, and the cell morphology was observed by SEM.

Cell Toxicity. The data of the MTT assay are shown in Figure 11. The formazan absorbance indicated that the hepatocytes seeded onto the control sample (the polystyrene cell culture plate), and different membranes were able to convert MTT into a blue formazan product. We observed that the viability of cells on each membrane was positively related to the culture time ($p < 0.05$). Moreover, for 2, 4, and 6 days of cell culturing, the viability of the cells on the modified PES membranes was higher than that on the pristine PES membrane at different levels ($p < 0.05$). The modified membranes had a better viability; this indicated that the introduction of the poly(VP-*co*-MMA-*co*-AA) copolymer might have had a positive influence on the cytocompatibility of the PES membranes and could prevent inflammatory risks. Therefore, we concluded that the copolymer-modified membranes have the potential to be used as bioartificial liver supports.

Cell Morphology. In most cases, cells would alter their morphology to stabilize the cell–material interface after contacting biomaterials. The entire process of cell adhesion and spreading consists of cell attachment, filopodial growth, cytoplasmic webbing, flattening of the cell mass, and ruffling of the peripheral cytoplasm, which progress in a sequential fashion.

Figure 12 shows the morphology of the hepatocytes cultured for 6 days on the pristine PES and modified PES membranes. As shown in the figure, the hepatocytes presented a spreading morphology and adhered onto the membranes by pseudopodia. Furthermore, with increasing copolymer content, the number of adherent hepatocytes increased. The results indicate that the modified membranes could promote cell attachment and growth.

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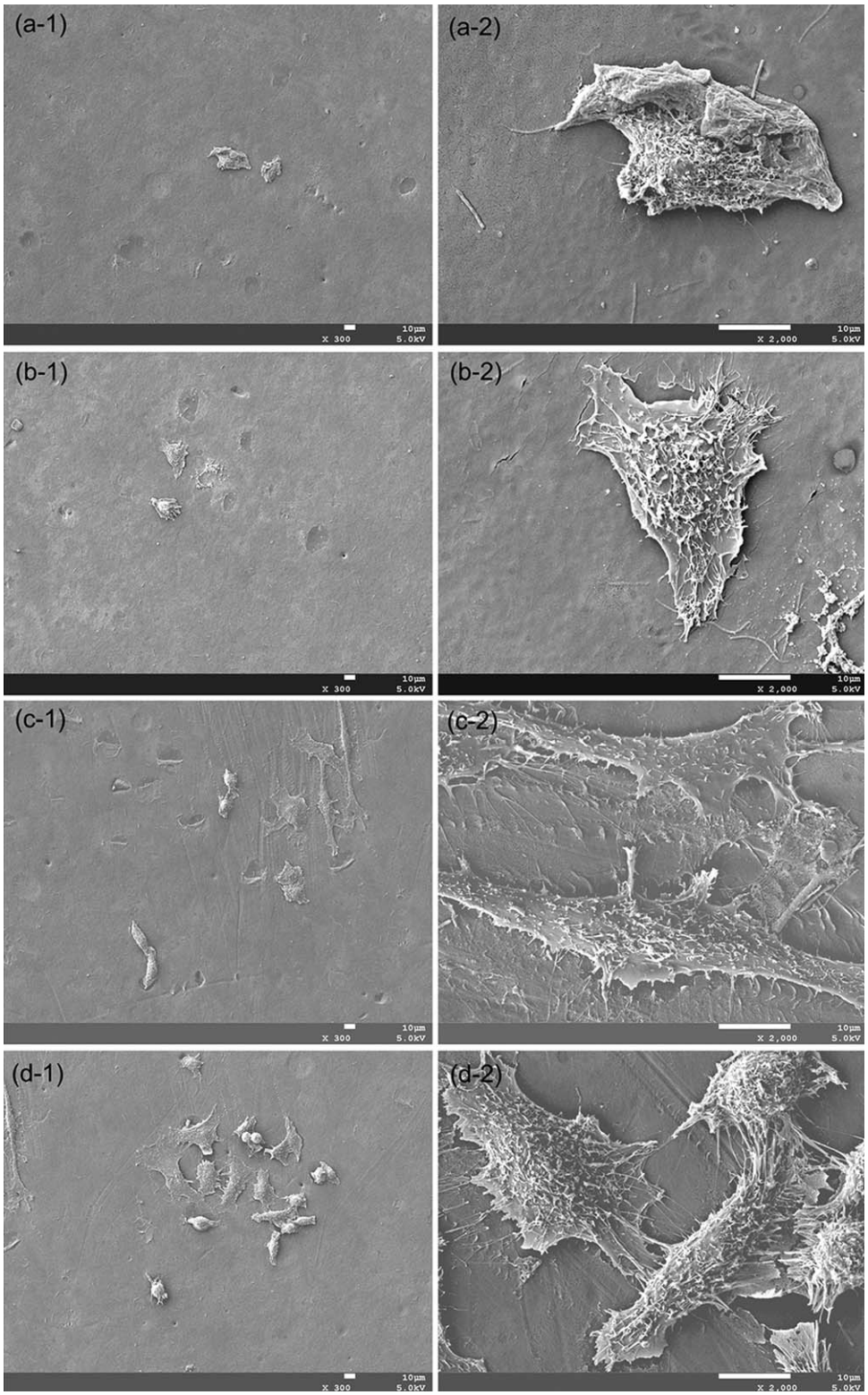

Figure 12. SEM images of LO2 human liver cells cultured on the PES and PES modified membranes after 4 days: (a) pristine membrane and (b) 1, (c) 3, and (d) 5% copolymer-modified PES membranes, respectively, at (1) 300× and (2) 2000× magnification.

Antifouling Properties of the Membranes

UF experiments were carried out to study the antifouling properties of the modified membranes. Figure 13 shows the time-dependent flux during three recycles of BSA–PBS solution UF operation. At the beginning, PBS was passed for 1 h to get a stable flux. As shown in the figure, the modified membranes

had a much larger flux than the PES membrane before the BSA solutions were used. This was caused by the PVP chains because the PVP could be regarded as a pore-forming agent and resulted in an increase in the permeability.[41,53,54] The flux decreased sharply when the solution changed from PBS to BSA solution; this was caused by the deposition and adsorption of protein molecules on the membrane surfaces and in the membrane pores. When the adsorption amount reached a stable value, a relatively steady flux was obtained. Furthermore, with increasing copolymer proportion, the protein solution flux for the modified membranes increased. This was attributed to the increased hydrophilicity and larger pore size, as shown in Figure 5.

After 1 h of BSA solution UF, the membrane was washed with flowing PBS for 20 min, and then, the flux was measured, and the R_{FF} was calculated, as shown in Table IV. For the PES membrane, R_{FF} was 41.14%, and for the modified membranes, it ranged from 69.87 to 105.01%. This could be explained as following: the VP chains passivated the membrane surface and reduced the protein adsorption and thus increased R_{FF}.[41,53,54]

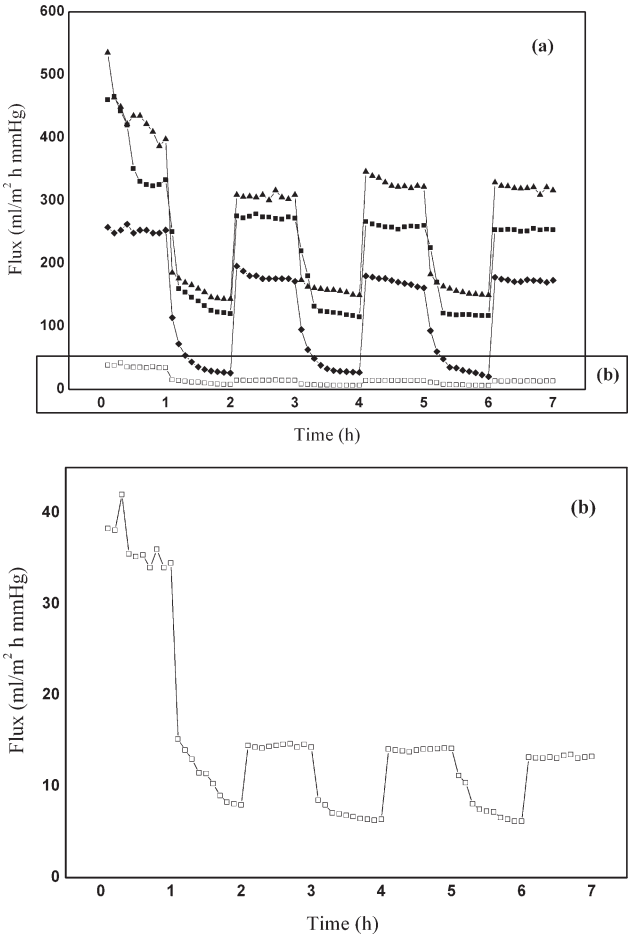

Figure 13. Time-dependent fluxes of the membranes during a process of three recycles at room temperature: (□) PES membrane and the modified PES membranes [(◆) 1, (■) 3, and (▲) for 5% blended membranes]. Conditions: PBS buffer for times of 0–1, 2–3, 4–5, and 6–7 h, and BSA solution for times of 1–2, 3–4, and 5–6 h. Duplicate experiments showed similar results.

Table IV. R_0 and R_{FF} Values of Different Membranes Through the UF Experiment

Sample	PES	PES-1%	PES-3%	PES-5%
R_0 (%)	100	89.35	89.87	90.16
R_{FF} (%)[a]	41.14	69.87	83.58	105.01

[a] R_{FF} averaged from three recycles data.

Meanwhile, AA provided ions to affect the UF behavior;[55] the ion dissociation during the washing procedure caused the restriction of the AA-modified surface and thus increased the removal of BSA from the surface.[56]

The BSA rejection ratios (R_0's) were also calculated, as shown in Table IV. For the modified membranes, R_0 ranged from 89.35 to 90.16%; this might have been caused by the large pore diameter and different membrane compositions.

To further study R_{FF}, the UF experiment was carried out three times. The R_{FF} values of the modified membranes ranged from 68.25 to 103.92% after three cycles of BSA solution UF, whereas the value of PES was only 37.86%. These results demonstrate that the copolymer-modified membranes presented outstanding and stable protein antifouling properties.

CONCLUSIONS

The purpose of this study was to develop an easy way to improve the blood compatibility and antifouling properties of PES membranes. This was successfully accomplished by the blending of a copolymer of poly(VP-*co*-MMA-*co*-AA), which was synthesized via a one-pot method via RAFT polymerization to control the molecular weight and MWD. After the modification, the water contact angle decreased due to the VP and AA chains; this indicated that the hydrophilicity of membranes were improved. The blood compatibility of the modified membranes was also investigated. Compared with the pristine PES membrane, the modified ones possessed a low hemolysis ratio, lower protein adsorption amount, depressed platelet adhesion, increased coagulation time, and good cytocompatibility. The results of biological tests indicated that the modified PES membranes were biocompatible. The copolymer-modified membranes also showed remarkable protein antifouling properties. These easily obtained PES membranes could be used in the biomedical field, such as in blood purification and medical devices.

ACKNOWLEDGMENTS

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