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ARTICLE

Preparation and characterization of a thrombin inhibitor grafted polyethersulfone blending membrane with improved antithrombotic property

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Systemic anticoagulation is indispensable for hemodialysis patients to prevent clotting, but patients at high risk of bleeding can't be given systemic anticoagulation. Hemodialyzer membrane modification maybe an effective program to solve this problem. In this study, we chose argatroban, a direct thrombin inhibitor, to modify polyethersulfone (PES) membrane. Firstly, we prepared argatroban (AG) grafted polyethersulfone (PES-AG) matrix by acetylating, oxidating and amidation reaction. Then, PES-AG was blended with PES at ratios of 2:1 and 1:1 respectively to prepare PES/PES-AG membrane by using phase inversion technique. FTIR and ^{1}H NMR spectroscopy were used to confirm that argatroban was grafted to PES successfully. Scanning electron microscope (SEM) was used to observe characteristic morphology of membranes. Activated partial thromboplastin time (APTT), prothrombin time (PT), Thrombin time (TT) and platelet function were detected to evaluate the antithrombotic property of modified membrane. The results indicate that the antithrombotic property of PES/PES-AG (1:1) membrane was superior to other three groups.

1. Introduction

Extracorporeal devices (such as hemodialysis, extracorporeal membrane oxygen (ECMO), percutaneous transluminal angioplasty) are required systemic anticoagulation to avoid thrombus formation. Heparin and low-molecular weight heparin are routinely administered during hemodialysis, but such systemic anticoagulation may aggravate hemorrhage complication. As hemodialysis membrane is the contact surface which induces thrombus, anticoagulant modification of hemodialysis membrane may decrease local clotting of hemodialyzer, and don't influence systemic coagulation status of patients.

As we all known, while biomaterials contacting with blood, a complex series of pathological responses would happen (i.e., coagulation initiation, platelet activation), and thrombosis occurs ultimately [1]. Owing to outstanding mechanical property as well as physicochemical properties [2], polyethersulfone (PES) hemodialyzers have been widely used in hemodialysis. However, it is not rare in clinic that PES dialyzers are blocked by thrombus in clinic. C. Zhao group [3, 4] prepared heparin-like PES membranes by introducing sulfonic acid and carboxylic groups to improve biocompatibility of PES. Over the past 40 years, many attempts have been made to prepare heparin-grafted extracorporeal devices to minimize systemic administration of heparin [5, 6]. HeprAN membrane, membrane of Evodial dialyzer, was originated from well-established AN69ST membrane technology where heparin was grafted to membrane [7, 8]. The latest HepZero study shows that heparin-coated dialyzer (Evodial, Gambro-Hospal, Meyzieu, France) is useful and safe to use for heparin-free hemodialysis in patients with hemorrhage tendency [9]. But heparin-coated dialyzer was only proved to be non-inferior to saline infusion; its treatment superiority is not demonstrated [10].

The average molecular weight of heparin is in the range of 12 to 15 kDa. Anticoagulation of heparin depends on antithrombin □ (AT□) [11]. In other word, heparin isn't suited to patients with AT□ deficiency. Heparin has high affinity for platelet factor 4 (PF4), which may induce heparin-induced thrombocytopenia (HIT) [12]. Argatroban (AG) is a synthetic

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direct thrombin inhibitor (526.65 Da) which has several advantages. Firstly, AG can bind to the catalytic site of thrombin selectly and directly [13-15]. The anti-thrombin action is fast and reversible [16]. Secondly, AG does not generate antibodies, so it doesn't induce HIT [17]. M.Beiderlinden et al.[18] considered AG as a feasible and effective anticoagulant for patients with suspected HIT undergoing ECMO. Thirdly, AG not only inhibits free thrombin but also blocks thrombin bonded with clots [19, 20]. Finally, argatroban is metabolized by liver totally, so patients with renal dysfunction needn't dosage adjustment [21].

Of course, argatroban also has disadvantages. Firstly, there is no known specific antidote for overdose of argatroban. But argatroban inhibits thrombin is reversible, so this disadvantage is not important. What's more, argatroban has been proved safely for clinical use by many researches[22-24]. Secondly, patients with hepatic diseases require dose adjustment. Thirdly, the cost of argatroban is higher than heparin. Even though, we still believe that argatroban is a good anticoagulant/antithrombin choice, especially for patients at high risk of bleeding in hemodialysis.

In recent years, argatroban has been considered as a wonderful choice to modify extracorporeal devices. For example, T. C. Major et al. [25] prepared AG/ hexane methylene/ nitric oxide releasing polymer-coated extracorporeal circulations to improve hemocompatibility of polymers. H. Chen et al.[26] modified surface of islets with liposomes carrying AG using an amphiphilic poly(ethylene glycol)-phospholipid conjugate derivative and DNA hybridization. S. Nishi et al.[27] reported that experimental aneurysms treated with AG-coated, polyurethane-covered stents were completely occluded without significant parent artery stenosis. C.Salvagnini et al. [28] synthesized graftable thrombin inhibitors for the preparation of blood-compatible polymer materials. Our team previous loaded AG onto a polyacrylonitrile dialyzer membrane by layer-by-layer method, and applied it in animal model of hemodialysis. The results demonstrated AG dialyzer is effective to use in heparin-free hemodialysis [29].

In this study, we introduced argatroban into PES by acetylating, oxidating and amidation reaction to prepare AG grafted polyethersulfone matrix (PES-AG). Then, PES-AG was blended with PES in different proportions to prepare PES/PES-AG membrane. The antithrombotic property of modified membranes were evaluated in terms of activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) and platelet function.

2. Materials and methods

2.1. Materials

PES (Ultrason E 6020P) was obtained from BASF Chemical Co., Germany. Acetyl chloride (C_2H_3ClO;98.5%), Aluminium trichloride anhydrous ($AlCl_3$;99%), N-Hydroxysuccinimide (NHS;98%)，1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC;98.5%) and triethylamine ($C_6H_{15}N$;99%) were purchased from Shanghai Aladdin Chemistry Co., Ltd. Potassium Permanganate ($KMnO_4$;98%) and Sodium hydroxide (NaOH;98%) were purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd. 1-Methyl-2-pyrrolidone (NMP;C_5H_9NO;99.5%,Super Dry) was used as a solvent and was a product of Beijing J&K Scientific Ltd. All of these chemical reagents were analytical reagent (AR) and were used without further purification unless otherwise described. Argatroban monohydrate (AG; $C_{23}H_{36}N_6O_5S \cdot H_2O$;99%;Fig. 1) was obtained from Shanghai Chemvon Biotechnology Co., Ltd. The FITC mouse anti-human CD41b (Platelet glycoprotein GP Ⅱb) and PE-Cy™ 5 mouse anti-human CD62P (P-selectin glycoprotein) were from BD Biosciences Pharmingen.

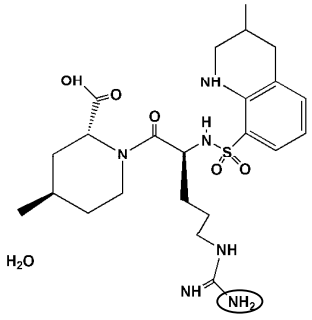

Fig. 1 Structure of argatroban ((2R,4R)-1-[(2S)-5-[(Aminominomethyl)amino]-1-oxo-2- [[(1,2,3,4-tetrahydro-3-methyl-8-quinolinyl)sulfony]amino]pentyl]-4-methyl-2-piperidinecarboxylic acid, monohydrate). The immobilization of argatroban was accomplished by linking the free primary amine (black circle) to carboxylic group of PES-COOH.

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2.2. Preparation and characterization of the PES/PES-AG membrane

2.2.1. Preparation of PES-COOH. Carboxylic polyethersulfone matrix (PES-COOH) was prepared by acetylating reaction and oxidating reaction in accordance with D.Wang et al.[30] reported work, but a subtle adjustment was made according to actual process of experiments.

Firstly, 10 grams of polyethersulfone had been dried in a vacuum oven at 110 ℃ for 1 h, and then the PES was dissolved in 40 ml NMP super dry solvent. The concentration of PES is 20 wt.%. Then, a solution of $AlCl_3$ (5.5 g, used as catalyzer), C_2H_3ClO (8 ml, used as acetylating reagent) and NMP (100 ml, used as solvent) was added to the PES solution slowly under stirring at 90 ℃ for about 2 hours. The whole process was under N_2 atmosphere to inhibit the side reaction. While the reaction cooled down to room temperature, the white solids (PES-$COCH_3$) was precipitated, filtrated and washed with double-distilled water until the pH value of the filtrate was the same as double-distilled water. After the above reactions, the obtained PES-$COCH_3$ was dried in a vacuum oven for 24 hours for the oxidating reaction.

Secondly, PES-$COCH_3$ (10 g) was dissolved in NMP (40 ml) with the concentration of 20 wt.%. The solution of $KMnO_4$ (1.2g), NaOH (3.7 g), double distilled water (9 g) and NMP (40 ml) was added dropwise into PES-$COCH_3$ solution under stirring at 80 ℃ for 4 hours. The black solution was centrifugated at 5000 rpm for 30 min to separate solids from liquid. Then, the supernatant liquid was dialyzed against acid solution (PH=1), and the white material (PES-COOH) was obtained. At last, the PES-COOH was dried in a vacuum oven for 24 h.

2.2.2. Preparation of PES-AG. Argatroban was introduced into PES matrix via the formation of amide linkage (-CO-NH-) between the free primary amine group of argatroban and the carboxylic group of PES-COOH.

PES–COOH (16 g) was dissolved in NMP with the concentration of 20 wt.%. Then, AG (10 g), EDC (6.4 g), NHS (2.5 g) and $C_6H_{15}N$ (5 ml) were added to PES–COOH solution at room temperature (RT) with stirring for 4 h. Lastly, the product (PES-AG) was washed with double-distilled water for several times, and dried in a vacuum oven for 24 h. The reaction pathway is shown in Fig. 2.

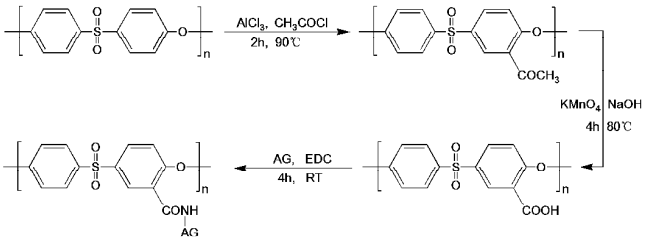

Fig. 2 Chemical synthesis of AG grafted polyethersulfone (PES-AG).

2.2.3. Preparation of PES/PES-AG blending membrane. As D.Wang et al.[30] previous published, the hydrophilicity of PES/PES-COOH membrane was much better than PES membrane, and the best blending ratio was 2:1. So, PES/PES-COOH (2:1) membrane was chose as control group. Because pure PES-AG could not prepare flat sheet membrane successfully, PES-AG was blended with PES. The blending ratios were 2:1 and 1:1 respectively. Liquid-liquid phase inversion technique was used to prepare blending membranes. The total concentration of polymers was kept at 10 wt.%. The prepared membrane with pure PES (10 wt.%) was named A; the membrane with PES/PES-COOH ratio of 6.7/3.3 (wt.%) was termed B; the membrane with PES/PES-AG ratios of 6.7/3.3 and 5/5 (wt.%) were termed C and D. As shown in table 1.

Table 1 Compositions of the casting solutions for preparing the membranes.

Membrane No.	PES (wt.%)	PES-COOH (wt.%)	PES-AG (wt.%)	NMP (wt.%)
A	10	0	0	90
B	6.7	3.3	0	90
C	6.7	0	3.3	90
D	5	0	5	90

2.2.4. Characterization of PES, PES-$COCH_3$, PES-COOH and PES-AG. The FTIR spectra of PES and modified membranes were recorded with potassium bromide (KBr) discs using a Nicolet

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6700 Fourier transform infrared spectrometer (Thermo Fisher Scientific Inc., USA).

The ^{1}H NMR (500 MHz) spectra of PES, PES-$COCH_3$, PES-COOH and PES-AG were recorded on an Avance III-500MHz spectrometer (Bruker Co., Switzerland). PES was dissolved in deuterated chloroform solution ($CDCl_3$). PES-$COCH_3$, PES-COOH and PES-AG were dissolved in deuterated dimethyl sulfoxide (DMSO-d6) to prepare samples.

All of flat sheet membranes were snapped in liquid nitrogen and then coated with a thin layer of gold. Cross section and surface morphology of membranes was observed by scanning electron using a JSM-5900LV scanning microscope (JEOL Inc., Japan).

2.2.5. APTT, PT, TT. Blood from healthy female volunteers (27 years old) was collected by vacuum anticoagulant tubes (Shandong Weigao Group Medical Polymer Co., China) which contained 3.2 % sodium citrate. Then, the collected blood was centrifuged at 2730×g for 15 min to obtain the platelet poor plasma (PPP). At the same time, four kinds of membranes (1cm×1cm) were immersed in phosphate buffer saline (PBS, PH=7.4) for 30 min. Then, PBS was removed and 1 ml of fresh PPP was dropped. All the samples were vibrated for 15 min to make membranes contact with plasma fully. At last, samples were measured by Destiny Max automated blood coagulation analyzer (Trinity Biotech Plc., Ireland). All the tests were completed within 2 hours. Every sample was measured three times. The results was averaged to get reliable value, and then analyzed by statistical method.

2.2.6. Platelet adhesion and activation. According to above APTT, PT and TT results, the anticoagulant property of PES/PES-AG (1:1) membrane was superior than the other three groups. So, we regarded PES membrane as control group, and used immunofluorescence technique to observe platelets that were activated by PES membrane or PES/PES-AG (1:1) membrane. Whole blood samples were collected as above method. And then, the platelet rich plasma (PRP) was prepared by centrifuging at 110×g for 15 min. At the same time, two kinds of membranes (0.5cm × 0.5cm) were washed with PBS three times. Then membranes incubated in fresh PRP for 1 h, 20μl anti-CD41b and 20μl anti-CD62P were added to the samples and incubated for 30 min. Stained samples were fixed in 4% paraformaldehyde, and washed with PBS 3 times. Then the samples were put onto slides, and fluorescence quenching agent was dropped before covered by cover glasses. Finally, fluorescent microscope was used to visualize fluorescently labeled platelets adherent on the membrane surface.

2.2.7. Statistical analysis. Data (APTT, PT and TT) were expressed as mean ± SEM (standard error of the mean) and analyzed with a one-way ANOVA, followed by Fisher's least significant difference (LSD) method for pair-wise multiple comparisons. All statistical analyses were carried out using a SPSS17.0 software. Statistical significance was set at $P< 0.05$.

3. Results and discussion

3.1. FTIR spectroscopy and ^{1}H NMR analysis

FTIR and ^{1}H NMR spectra (500 MHz) were used to verify the existence of -$COCH_3$, -COOH and -CO-NH- in modified PES matrix. FTIR spectra of the samples were shown in Fig. 3. After the acetylating reaction, a peak at 1678 cm^{-1} was observed and indicated the presence of the carbonyl group which belonged to PES-$COCH_3$. After the oxidation reaction, the peak at 3377 cm^{-1} was attributed to the presence of the carboxylic group for PES-COOH. In addition, the peak at 1654 cm^{-1} was observed, which indicated the presence of the -C=O in PES-AG. These featured peaks weren't detected in the pure PES membrane spectrum.

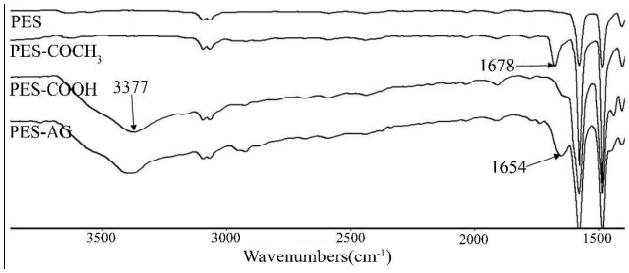

Fig. 3 FTIR spectra of PES and modified PES membranes.

^{1}H NMR spectra of the samples were shown in Fig. 4. The peak at δ=2.39 was the chemical shift of -$COCH_3$, which was grafted onto the PES matrix by acetylating reaction. The peak at δ=10.69 was the chemical shift of -COOH, which was grafted onto PES by oxidating reaction. When added 10 μl deuteroxide

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(D_2O) to PES-COOH sample, the peak of -COOH was disappeared, which further confirmed that carboxylic group was successfully introduced to PES material. After amidation reaction, the peak at δ=3.39 was the chemical shift of amide linkages (-CO-NH-) between AG and carboxyl groups of PES-COOH. The result showed that PES-AG was successfully synthesized. For the PES, the peaks at δ=7.00 and δ=7.83 were the chemical shifts of the phenyl on the PES main chain.

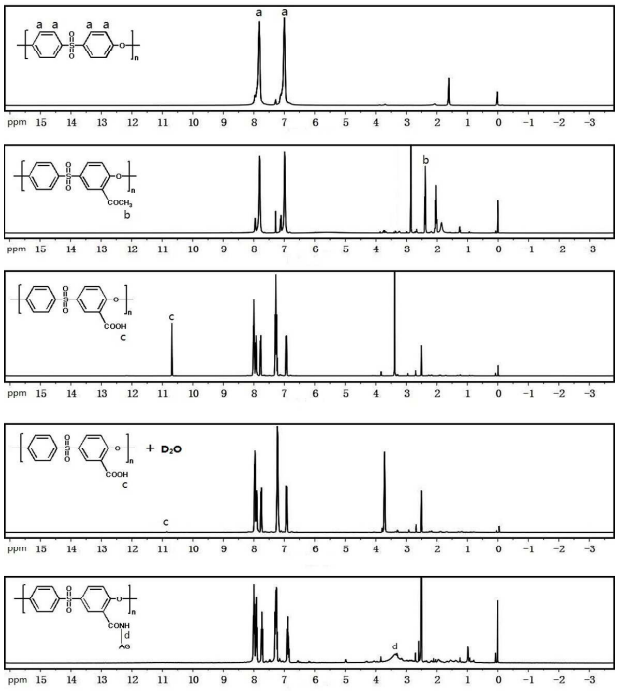

Fig. 4 ^{1}H NMR spectra of PES and modified PES membranes.

3.2. Cross-section and surface structures of membranes

The cross-sectional and surface SEM micrographs of PES without additive and with PES/PES-COOH or PES/PES-AG of different molecular weights are shown in Fig. 5 and Fig. 6 respectively. As Fig. 5 shown, all the membranes were asymmetric membrane consisting of a dense top-layer, a finger-like structure and porous sub-layer at the bottom. The pore diameter of finger-like structure expanded to the bottom, large numbers of micropores on the surface of the macrovoids were observed, which maybe due to the interaction between PES and PES-COOH or PES-AG in the casting dope. The above results were similar with previous research [30].

SEM images for the membranes surfaces are shown in Fig.6. We can observe that the surface was changed from smooth to rough with the addition of PES-COOH and PES-AG. There were some particles on the modified membrane surfaces, which need further investigations.

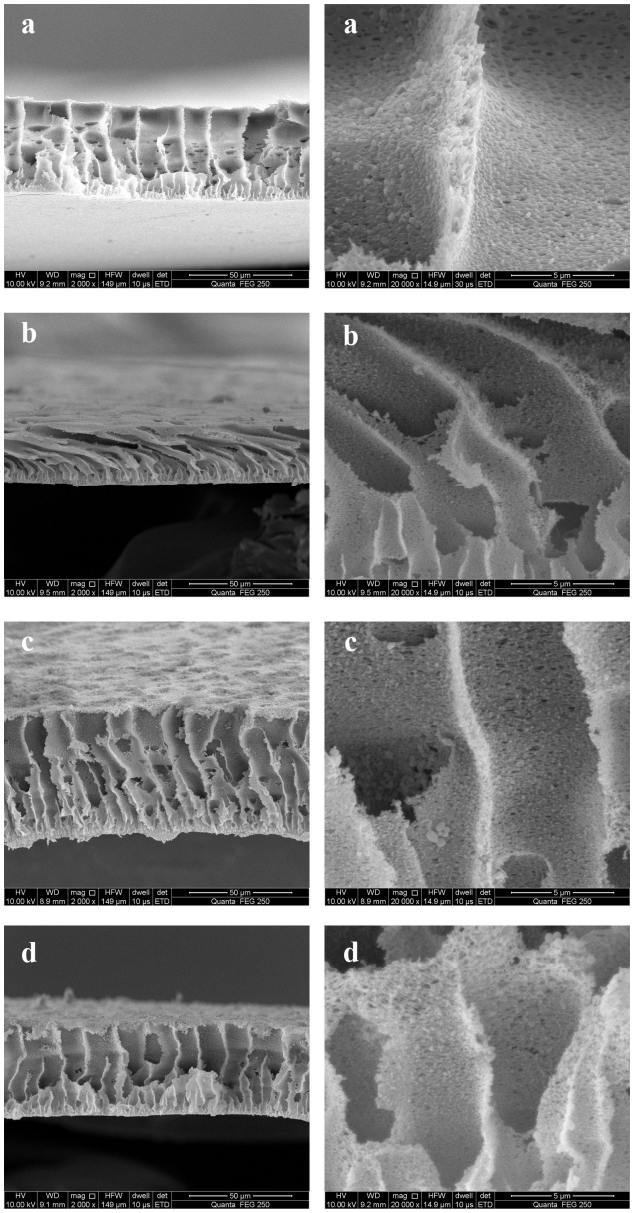

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Fig. 5 Cross-sectional SEM micrographs of PES and modified membranes, a: PES membrane, b: PES/PES-COOH (2:1) membrane, c: PES/PES-AG (2:1) membrane, d: PES/PES-AG (1:1) membrane.

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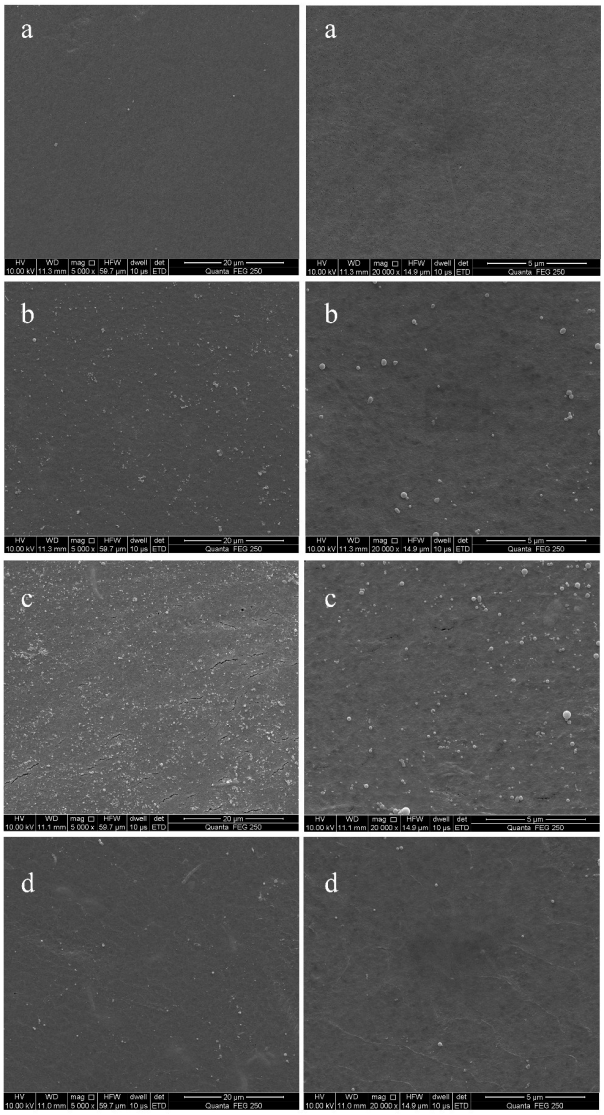

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Fig. 6 SEM images of the top-views PES and modified membranes, a: PES membrane, b: PES/PES-COOH (2:1) membrane, c: PES/PES-AG (2:1) membrane, d: PES/PES-AG (1:1) membrane.

3.3 APTT, PT and TT

The hemodialysis membrane can activate the coagulation cascade through intrinsic and extrinsic pathways [31]. The intrinsic pathway starts with activation of factor Ⅻ, and the extrinsic pathway is initiated by tissue factor (TF). APTT, PT and TT were used to evaluate PES membranes and modified membranes effects on activity of intrinsic coagulation pathway, extrinsic coagulation pathway and fibrinolytic system respectively. Thrombin plays a central role in thrombosis. Consequently, blocking the activity of thrombin or preventing its generation is one of the most current antithrombotic treatment strategies [32, 33].

As is shown in Fig. 7, Comparing to the blank group (pure plasma), PT and TT levels of PES membranes were shortened (Fig.7B and Fig.7C), but APTTs of PES membrane had no different to blank group (Fig.7A). The results implied that PES membrane might activate coagulation cascade mainly through activation of extrinsic pathway. Activation of mononuclear leukocytes and platelets within the extra-corporeal circuit leads to release of surface membrane blebs that act as a source of TF [34-36].

Comparing to the PES membrane, APTT, PT and TT levels of PES/PES-COOH (2:1) membrane was prolonged ($p<0.05$), which showed that PES/PES-COOH (2:1) membrane had better anticoagulant property than PES membrane by grafting carboxylic groups. Polymer incorporated with carboxylic groups could gain increased hydrophilic property, depressed platelet adhesion. This result accorded with other researches[37-39]

Compared to the PES/PES-COOH (2:1) membrane, the clotting times of PES/PES-AG membrane (except APTT levels of PES/PES-AG (2:1) membrane) were slightly prolonged ($p<0.05$), which indicated that PES/PES-AG membrane had better antithrombotic property than PES/PES-COOH (2:1) membrane by introducing AG. It is owing to two functional structural groups (4-methyl-2-piperidine carboxylic acid group and the methyltetrahydroquinoline sulfonyl group) of AG binding to the active site of thrombin [40]. Previous work had showed that immobilizing AG at the free primary amine to polymers did not affect the antithrombotic property of AG [41, 42]. So, PES-AG still has antithrombotic property. However, the prolonged clotting times of PES/PES-AG membrane did not get our expectation yet. It might because the AG grafting was low, which needed to be improved in the future.

Compared to the PES/PES-AG (2:1) membrane, APTT, PT and TT levels of PES/PES-AG (1:1) membranes were prolonged ($p<0.05$). It declared that increased blending weight ratio of

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PES-AG could improve the antithrombotic property of PES/PES-AG membrane. And PES/PES-AG membrane might have a dose dependent relationship for this antithrombotic property.

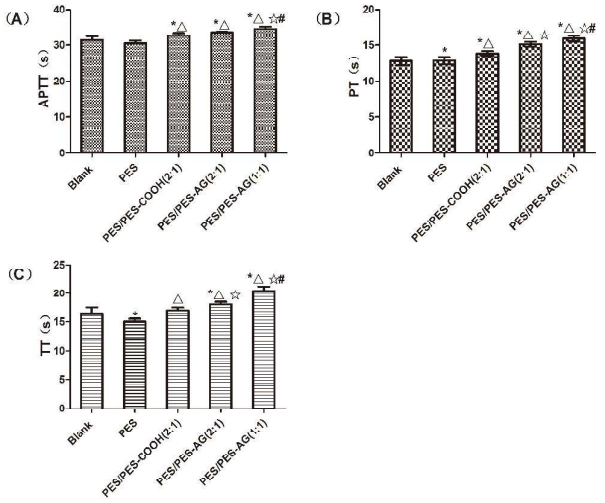

Fig. 7 Comparision APTT, PT and TT of 4 kinds of membranes (for n=6 experiments with each membranes). * = $p<0.05$, blank group (blood plasma) vs. 4 kinds of membranes; △ $p<0.05$, PES membrane vs. PES/PES-COOH (2:1) membrane, PES/PES-AG (2:1) membrane and PES/PES-AG (1:1) membrane; ☆ $p<0.05$, PES/PES-COOH (2:1) membrane vs. PES/PES-AG (2:1) membrane and PES/PES-AG (1:1) membrane; #=$p<0.05$, PES/PES-AG(2:1) membrane vs. PES/PES-AG (1:1) membrane. The data are mean ± SEM.

3.4 Platelet adhesion and activation

According to above results, PES/PES-AG (1:1) membrane had the best antithrombotic property in four kinds of membranes. Platelets activated by PES membrane or PES/PES-AG (1:1) membrane were tested by immunofluorescence technique. CD62P (P-selectin) is known as platelet activation-dependent granule membrane protein, which reflected platelet activation. CD41b/CD61 is a transmembrane glycoprotein that can combine with CD61 to form the CD41/CD61 (GP□b/□a). This complex is expressed on platelets and involved in platelet activation, aggregation and adhesion. In this study, CD62P and CD41b were stained with PE-Cy ™ 5 mouse anti-human CD62P and FITC mouse anti-human CD41b respectively.

As is shown in Fig. 8, the green and red fluorescents of PES/PES-AG (1:1) membrane were much less than PES membrane, which indicated PES/PES-AG (1:1) membrane could obviously inhibit platelet adhesion and activation. Previous work showed that argatroban was able to prevent thrombin-induced platelet adherence to thrombin-treated endothelial cells [43]. Several researches indicated that argatroban could attenuate P-selectin expression in platelets when exposed to activating stimuli or extracorporeal circulations [44-46].

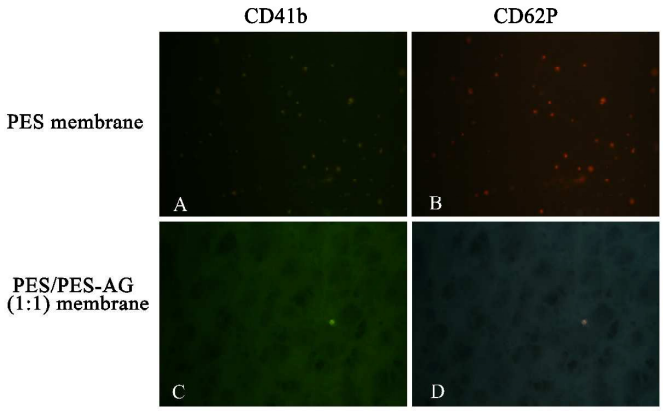

Fig. 8 PES membranes and PES/PES-AG (1:1) membranes were exposed to human plasma for 30min at 37□. Platelet CD41b and CD62P adhered on PES membrane or PES/PES-AG (1:1) membrane surface were detected by immunofluorescence technique.

4. Conclusions

The innovative part of this study was that argatroban grafted polyethersulfone matrix (PES-AG) was prepared by acetylating, oxidating and amidation reaction. FTIR and ^{1}H NMR spectroscopy were confirmed that AG was grafted to PES successfully. Then, liquid-liquid phase inversion technique was used to prepare PES/PES-AG blending membrane. The characteristic morphology of PES and modified PES membranes all had asymmetric structure which was observed by SEM. APTT; PT and TT results indicated that the antithrombotic property of PES/PES-AG membrane was superior to other groups, and needed to be improved further. In addition, the antithrombotic property of PES/PES-AG (1:1) membrane was better than PES/PES-AG (2:1) membrane. The results of platelets immunofluorescence showed PES/PES-AG

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(1:1) membrane could obviously inhibit activation and adhesion of platelets. In short, PES/PES-AG (1:1) membrane might be a promising material in extracorporeal circulation devices for antithrombosis, especially suitable for the patients who had hemorrhagic tendency. Further studies are required to explore the properties of PES/PES-AG (1:1) membrane comprehensively, and improved the hemocompatibiliy and stability of PES/PES-AG (1:1) membrane further.

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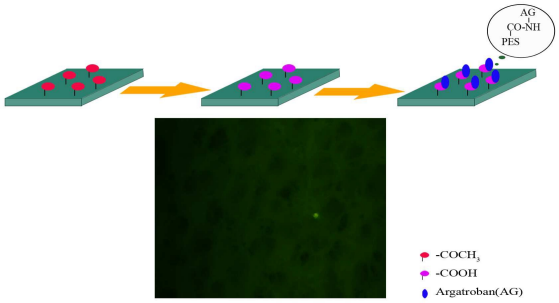

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