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Biomaterials 26 (2005) 1437–1444
Biomaterials
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Hemocompatibility and anaphylatoxin formation of protein-immobilizing polyacrylonitrile hemodialysis membrane

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Received 26 December 2003; accepted 23 April 2004

Available online 7 June 2004

Abstract

Plasma proteins were covalently immobilized onto polyacrylonitrile (PAN) membrane to evaluate the hemocompatibility and anaphylatoxin formation. This is used as a model to study the effect of protein-adsorption on the blood-contacting response of hemodializing membranes. The proteins used were either platelet-adhesion-promoting collagen (COL) or platelet-adhesion-inhibiting human serum albumin (HSA). The microstructure and characterization of the protein-immobilizing PAN membranes were evaluated by Coomassie dye assay, atomic force microscopy, X-ray photoelectron spectroscopy and water contact angle measurement. PAN–HSA membrane improved not only hemocompatibility including less platelet adhesion, longer blood coagulation times, and higher thrombin inactivity level, but also induced lower complement activation. On the other hand, PAN–COL membrane exhibited blood incompatibility, although induced less increase of C3, C4 antigens of serum. Overall results of this study demonstrated that the immobilization of HSA onto the surface of PAN membrane would be beneficial to improve the hemocompatibility and to reduce the anaphylatoxin formation during hemodialysis.

Keywords: Polyacrylonitrile; Hemocompatibility; Platelet adhesion; Anaphylatoxin formation; Leukopenia

1. Introduction

Antithrombogenic biomaterial has been of great interest as one of the ultimate objectives for developing artificial internal organs. Many studies have aimed to improve hemocompatibility of biomaterials by surface modification [1–7]. The most widely used blood anticoagulant, heparin, can catalytically increase the rate that antithrombin III (ATIII) inhibits thrombin and some other coagulation proteases [8–10]. Despite its widespread clinical use as an anticoagulant, heparin has a number of major disadvantages. Some patients suffered from the heparin-induced thrombocytopenia (HIT) following prolonged heparin exposure, thus precludes future heparin use in these individuals [11].

The surface characteristics can affect the protein adsorption levels. In our previous studies [12,13], we reported that heparinized PAN membrane can suppress the adsorption of human plasma proteins and adhesion of platelets, thereby effectively improve the hemocompatibility. Chenoweth et al. [14] reported that hemodialysis with reused dialyzers can reduce the adsorption of peripheral blood leukocyte by 20–30%. Correspondingly, C3a antigen formation within reused hemodialyzer was only 20% of that of a new hemodialyzer. Based on these studies, we believe that the proteins adsorption on the surface of hemodialyzer membranes could improve the hemocompatibility during the hemodialysis therapy.

In the literature, much effort has been made in curtailing the adsorption of plasma proteins onto the artificial surfaces to improve the hemocompatibility via the immobilization of heparin. We thought that by immobilizing certain plasma protein could also improve the hemocompatibility of hemodialyzing membranes.

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doi:10.1016/j.biomaterials.2004.04.039

To demonstrate this point, we would immobilize human serum albumin (HSA) and collagen (COL) on the surface of PAN membranes. The evaluations of the hemocompatibility were based on platelet adhesion, blood coagulating time and quantitatively evaluate the complement activating potential of modified PAN membranes. We hope the results of this preliminary study can not only contribute to interpret the relationship between protein adsorption and hemocompatibility as well as anaphylatoxin formation, but also broaden the application in blood-contacting devices.

2. Experimental

2.1. Materials

Polyacrylonitrile (PAN) was obtained from Aldrich, and *N*,*N*-dimethylformamide was purchased from Acros, USA. 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) was purchased from Sigma. Type I collagen (COL), human serum albumin (HSA, Mw 65,000) and human plasma fibrinogen (HPF, Mw 340,000) were purchased from Calbiochem. Heparin (5000 IU/ml, Mv: 15,000 Da, 179 IU/mg) was supplied by Leo Corp., USA. These chemicals were reagent grade and used without further purification. Anticoagulant citrate dextrose (ACD) human blood and plasma were provided from Blood Center in Taiwan.

2.2. Fabrication of modified PAN membrane

The membranes were prepared by the phase inversion technique to form a flat membrane of about 0.12 mm in thickness [12]. The hydrolysis of PAN membrane was carried out according to the chemical scheme [12,13] in Fig. 1. The PAN membranes were cut into pieces of $3 \times 3\,cm^2$, and hydrolyzed in 1 M NaOH at 40°C for 10 min to convert the CN groups into carboxyl groups. This was designated as PAN–AA. Afterwards, the protein and heparin immobilization was performed according to the chemical scheme also shown in Fig. 1. A piece ($2 \times 2\,cm^2$) of PAN–AA was immersed in 20 ml of buffered solution containing 0.01 M EDC in the pH range 4.0–4.5 with 0.02 M MES (4-morpholineethanesulfonic acid monohydrate, Aldrich) buffer. After gently shaking for 2 h at 4°C, the EDC-activated samples were washed with deionized water [15]. Subsequently, the membranes were immersed in 20 ml of solution containing 1 wt% HSA or 1 wt% COL dissolved in 1 wt% acetic acid at 4°C for at least 24 h. The resulting membranes were washed with PBS buffer 5 times and subsequently rinsed with DI water 5 times, which the modified membranes were designated as PAN–HSA and PAN–COL, respectively. The heparinized PAN (PAN–HEP) membrane was prepared by the procedure in our previous study [13]. The pure PAN membrane without carboxyl acid was followed the above procedure as blank control.

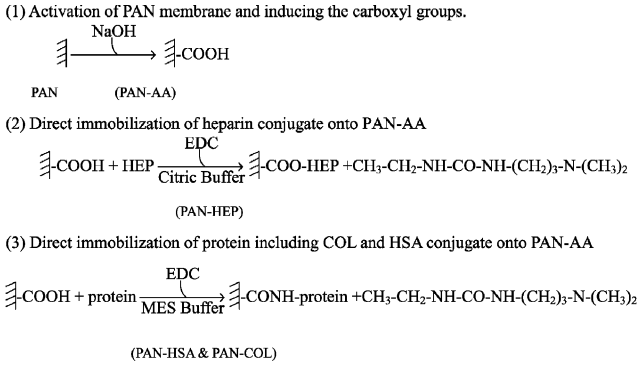

Fig. 1. Chemical schemes of heparin and protein-immobilized PAN membrane.

2.3. Surface density determination

The surface density of carboxyl groups and heparin were determined using Rhodamine 6G dye and toluidine blue O dye, respectively [15]. Coomassie brilliant blue G-250 (CBBG, Sigma) protein dye was used to verify the immobilization of proteins on the membrane surface [16]. Briefly, a piece of sample ($1 \times 1\,cm^2$) was immersed in 20 ml of 10 mg/dl CBBG solution for 30 min. Thereafter, the sample was extensively rinsed in deionized water and allowed to dry. A piece of PAN membrane was used as the control. The adsorbed dye molecules were desorbed with 20 ml of 10 mg/dl sodium dodecyl sulfate (SDS) solution for 5 h. The absorbance of the dye desorbed was measured at 468 nm using a spectrophotometer (UV-1201, Shimadzu, Kyoto, Japan), and the surface density of the functional groups was then calculated.

2.4. Characterization analysis

The functional groups on modified PAN membranes were analyzed using X-ray photoelectron spectroscopy (XPS) (ESCALAB 250, Thermo VG Scientific, West Sussex, UK) equipped with Mg Kα at 1253.6 eV at the anode. A survey scan of varying binding energy from 100 to 600 eV and N_{1s} (400–402 eV) were taken [13,16]. The images of protein-immobilized membrane surfaces were observed using an atomic force microscope (AFM) (MMAFM-2, Digital Instrument, Santa Barbara, CA).

2.5. Hydrophilicity test

The hydrophilicity of the surface was evaluated based on the water contact angle of the membrane measured with a contact angle goniometer (DSA 100, Krüss

GmbH, Hamburg, Germany) [5]. Each value was averaged from six measurements. Ultrafiltration measurements can be made using a simple experimental set-up. The pure water permeability (PWP) was calculated from following equations [13]:

$$\mathrm{PWP} = J/\Delta P,$$

where J is the permeate flux and ΔP is the applied pressure.

2.6. Fibrinogen adsorption

The adsorbing amount of human plasma fibrinogen was measured by means of bicinchoninic acid (BCA) protein assay, as described by our previous study [13].

2.7. Blood coagulation time

Anticoagulant citrate dextrose (ACD) human blood (30 ml) was provided from the Blood Center in Taiwan. To obtain platelet-rich plasma (PRP), the ACD blood was centrifuged at 100g for 20 min at 4°C to separate the blood corpuscles. Subsequently, portion of the PRP was further centrifuged at 2000g for 20 min at 4°C to obtain the platelet-poor plasma (PPP). A sample membrane ($1 \times 1\,\mathrm{cm}^2$) was put into 0.5 ml PPP and was incubated at 37°C for 1 h. The coagulation times, including activated partial thrombin time (APTT), prothrombin time (PT) and fibrinogen time (FT) were determined using an automated blood coagulation analyzer (CA-50, Sysmex Corp., Kobe, Japan). The control was measured against the PPP without polymer sample [13].

2.8. Thrombin inactivation assay [17]

An aliquote of 0.2 ml PPP with 0.05 ml Tris buffer containing the test sample was incubated at 37°C for 15 min. Then 0.05 ml of Tris buffer containing thrombin (12.5 U/ml) was added to the solution and the clotting time was measured using the blood coagulation analyzer. The control was 0.05 ml of Tris buffer without a test sample.

2.9. Whole blood coagulation time (WBCT) and thrombus formation

The WBCT was measured according to the modified Okamoto method [18]. In brief, blood from healthy volunteers was transferred to two glass tubes at a volume of 1 ml each. One tube contained a sample piece of $1 \times 1\,\mathrm{cm}^2$ in size, and the other tube without sample was used as the control. Both tubes were incubated in water bath at 37°C. The blood coagulation was observed by inclining the tube at 10 s intervals. The blood coagulation time was taken when the blood flow was not observed even if the tube was inclined at a 90° angle.

Thrombus formation was tested following our previous study [19]. Briefly, the samples were rinsed with doubly distilled water and placed into the wells of 24-well culture plates in contact with 1.5 ml of human whole blood without anticoagulant and were incubated at 37°C for 120 min, and then the samples were washed 5 times with PBS (pH 7.4). The samples were then dehydrated with graded ethanol and dried by the critical-point procedure with CO_2. The degree of thrombosis (DT) of the membrane is defined as follows:

$$\mathrm{DT} = \frac{W_t - W_{\mathrm{dry}}}{W_{\mathrm{dry}}} \times 100\%,$$

where W_{dry} and W_t are the weight of the dry membrane and the weight of the membrane at time t, respectively.

2.10. Evaluation of platelet adhesion

The determination of platelet adhesion was in accordance with our previous study [13]. Briefly, rinsed samples ($1 \times 1\,\mathrm{cm}^2$) were placed into the wells of 24-well culture plates in contact with 1.5 ml of human PRP and were incubated at 37°C for 2 h. Then 6 ml of PBS was added to the PRP and stood for 1 min to stop further platelets adhesion. Adhering platelets were fixed with 2% (w/v) glutaraldehyde solution in PBS for 1 h. Samples were dehydrated with graded ethanol and dried by the critical-point procedure with carbon dioxide. The number of platelets adhered to the samples was determined by the lactate dehydrogenase (LDH) method [20], and the kit was purchased from Caro, Germany. A calibration curve was obtained between the LDH activity and the concentration of platelets counted with a hemocytometer (K-1000, Sysmex Corp., Kobe, Japan). The adhered platelets on the membranes were then calculated.

2.11. Blood-cell count [14]

A $1 \times 1\,\mathrm{cm}^2$ sample was washed three times with double-distilled water and placed into a well of 24-well culture plate in contact with 2 ml of ACD whole blood. Then, the plate was incubated at 37°C for 30–120 min. The numbers of white blood cells (WBC) in the whole blood were counted with a hemocytometer.

2.12. Complement activation [21]

Human blood was obtained from healthy adult volunteers without the addition of anticoagulant. After incubating at 37°C for 1 h, the clotted blood was centrifuged at 2000 g for 20 min to separate the serum. Specimens of $2 \times 2\,\mathrm{cm}^2$ were immersed in 2 ml serum and incubated at 37°C for 1 h. The concentrations of

complement component C3, C4 in the serum were determined with enzyme-linked immunosorbant assay (ELISA, Quidel, San Diego, CA).

2.13. Statistical analysis

A statistical analysis was performed using Student's *t*-test for 2 groups.

3. Results and discussion

3.1. Surface modification of PAN membranes

As listed in Table 1, the surface density of the carboxyl groups on PAN membrane attained 69.4 nmol/cm^2 after 10 min hydrolysis with 1 M NaOH at 40°C. The chemical scheme for immobilizing heparin and protein on the surface of PAN membrane was shown in Fig. 1. The carboxyl groups of PAN–AA were reacted with the hydroxyl group of heparin and the amino groups of proteins. The surface density of heparin on the PAN membrane was 1.03 μg/cm^2, determined by toluidine blue O dye assay. The immobilizing densities of proteins determined by Coomassie assay were 13.2 μg/cm^2 for HSA and 6.5 μg/cm^2 for COL, respectively. This result was basically consistent with Kang et al. study [16]. They treated poly(methyl methacrylate) (PMMA) films with oxygen plasma and followed by grafting with acrylic acid, then coupled with proteins (13.75 μg/cm^2 for albumin and 7.25 μg/cm^2 for collagen). Table 1 also shows that the immobilizing amount of HSA is greater than that of COL ($p = 3.9 \times 10^{-5}$). This was probably due to the smaller molecular weight for HSA and higher steric hindrance for COL [22].

3.2. Surface characterization

Fig. 2(a) shows the XPS spectra of PAN, PAN–AA and PAN–HEP from binding energy 100 to 600 eV. Both spectra of PAN–AA and PAN–HEP have the peak

Table 1
Characterization of modified PAN membranes (mean ± std)

Membrane type	Surface density	Water contact angle (θ)	PWP (cm/h-MPa)
PAN	—	41.6 ± 0.6	11.4 ± 0.4
PAN–AA	69.4 ± 2.8[a]	40.2 ± 1.3	12.6 ± 0.6
PAN–HEP	1.03 ± 0.9[b]	35.8 ± 1.9	16.3 ± 1.3
PAN–HSA	13.2 ± 1.2[c]	36.2 ± 0.8	13.4 ± 0.9
PAN–COL	6.5 ± 0.07[c]	38.9 ± 1.3	12.1 ± 0.7

[a] Carboxyl groups (nmol/cm^2), determined with Rhodamine 6G dye assay ($n = 6$).
[b] (μg/cm^2), determined with TB assay ($n = 6$).
[c] (μg/cm^2), determined with CBBG assay ($n = 6$).

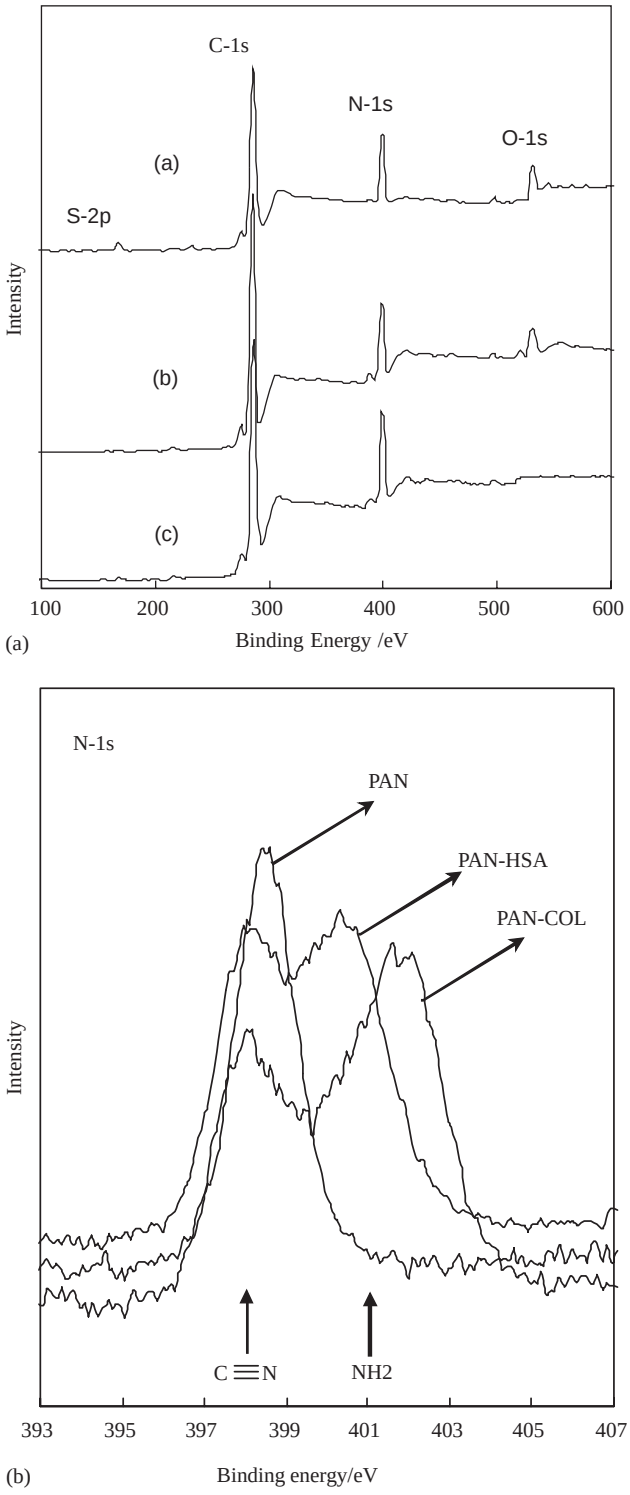

Fig. 2. XPS survey scan spectra of PAN membrane. (a) relative intensity vs binding energy (100 to 600 eV) a: PAN–COL; b: PAN–AA; c: PAN; (b) for N_{1s} scan spectra (binding energy 400 eV).

of O_{1s} (binding energy 532 eV) while the spectrum of PAN has only the N_{1s} peak. The additional S_{2p} peak (binding energy 167 eV) was observed in the spectrum of PAN–HEP. Fig. 2b shows the N_{1s} (binding energy,

400 eV) scan spectra of PAN, PAN–HSA, and PAN–COL. The NH_2 peak (401–402 eV) can be observed for PAN–HSA and PAN–COL, while only CN peak (398 eV) can be found for PAN. The results indicate that heparin and protein were effectively grafted to the PAN membrane.

Table 1 showed that the hydrophilicity was improved after the surface modification, as indicated by the decrease of water contact angle. In addition, Table 1 also shows that the PWP from ultrafiltration results are improved due to the surface modification. The PWP was 16.3 cm/h-MPa for PAN–HEP membrane and 13.4 cm/h-MPa for PAN–HSA. These values were respectively about 1.43 and 1.17 times of the untreated PAN membrane. In the literature, Laurent et al. [2] reported that the Cuprophan hollow fiber coupled with bovine serum albumin (BSA) showed a slight increase in the ultrafiltration coefficient.

Fig. 3 shows the AFM topographic images of PAN, PAN–HSA and PAN–COL. The surfaces of PAN–HSA and PAN–COL had wrinkles and were much rougher than that of PAN. The roughness (R_q) determined by AFM showed 11.9, 18.1 and 45.9 nm for PAN, PAN–HSA and PAN–COL, respectively. This kind of rougher surface would be beneficial to prevent the adhesion of platelet [23].

3.3. Platelet adhesion and fibrinogen adsorption

Platelet adhesion and fibrinogen adsorption are the two main factors in evaluating the hemocompatibility of artificial surfaces [7,19]. Fig. 4 shows the effect of surface modification on the adhesion of platelets. The maximum number of adhering platelets (6689 cells/mm^2) appeared on PAN–COL membrane after 2 h incubation, whereas the minimum (1623 cells/mm^2) appeared on PAN–HEP. The PAN–HSA showed the medium adhesion of platelets. The platelet adhesion can be reduced by electrostatic repulsion between platelets and negatively charged heparin, as reported in our previous study [13,19]. In this work, PAN–HSA behaved similarly to PAN–HEP in reducing the platelet adhesion. This is because HSA is known as a platelet adhesion inhibiting protein, whereas COL is a platelet adhesion promoting protein [24]. When blood contacts a foreign material, such as a hemodialyzer, catheter or angioaccess, plasma proteins would always be adsorbed onto the material surfaces, and provoke the adhesion of platelets, white blood cells and some red blood cells onto the plasma protein layer and lead to the formation of fibrin [25,26].

In Table 2, the surface with higher number of equilibrium platelet adhesion has higher adsorbing amount of HPF. The maximum adsorption (588 μg/cm^2) of HPF appeared on PAN–COL, whereas the minimum (208 μg/cm^2) appeared on PAN–HEP. The PAN–COL surface caused more platelet adhesion

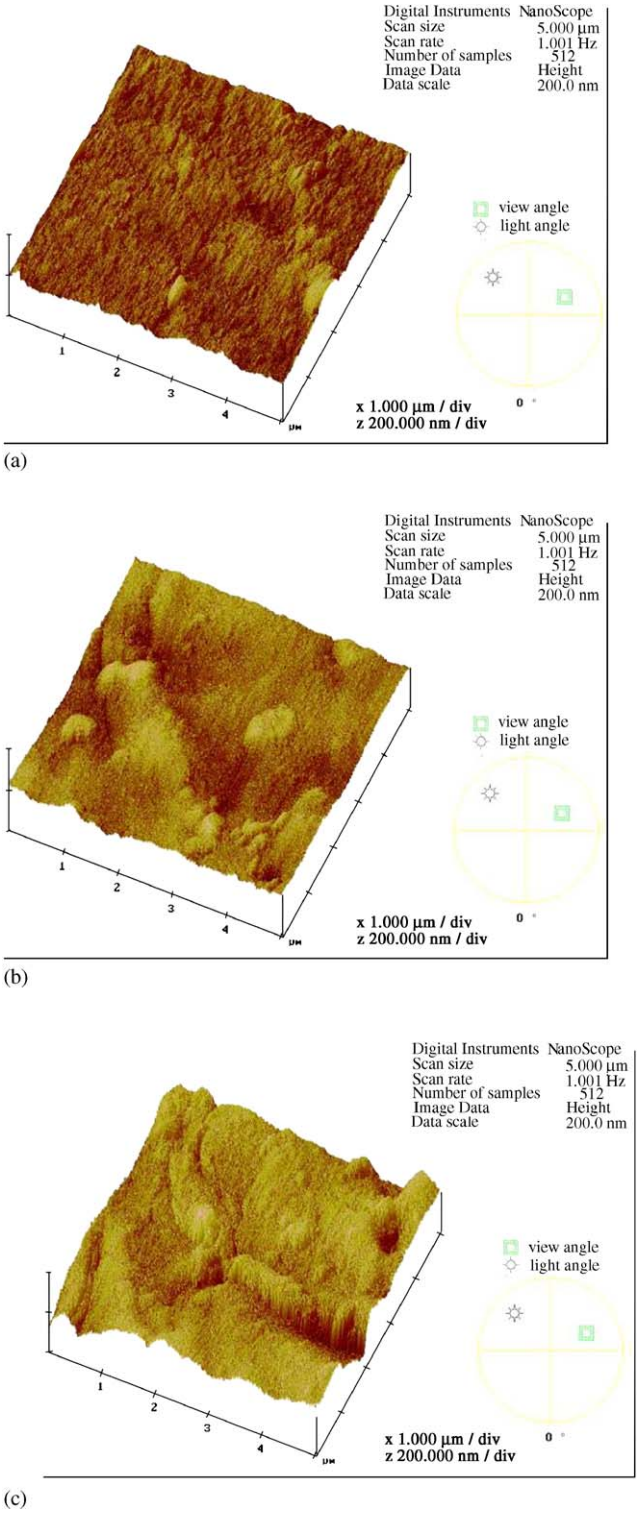

Fig. 3. AFM images of the PAN membrane (a) PAN, (b) PAN–HSA, (c) PAN–COL.

($p = 4.6 \times 10^{-5}$) compared to PAN. This is because that COL interacts with some proteins such as fibronectin and fibrinogen as well as cells such as platelets and fibroblasts [27]. Wissink et al. [28] pointed

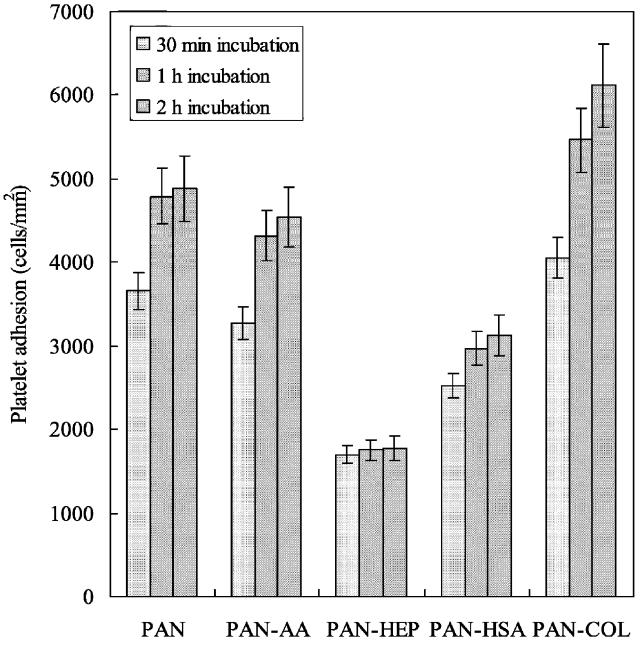

Fig. 4. Comparison of platelet adhesion of membranes after 30 min, 1 and 2 h incubation ($n = 3$).

Table 2
Platelet adhesion and HPF adsorption (mean ± std)

Membrane type	Platelet adhesion numbers (cells/mm^2)[a]	HPF adsorption (μg/cm^2)[b]
PAN	4810 ± 38	560 ± 17
PAN–AA	3811 ± 28	521 ± 15
PAN–HEP	1623 ± 16	208 ± 11
PAN–HSA	2584 ± 26	237 ± 14
PAN–COL	6689 ± 57	588 ± 29

[a] Incubated for 2 h and determined by LDH assay ($n = 3$).
[b] Incubated for 2 h and determined by BCA assay ($n = 3$).

out that COL is a highly thrombogenic material, and induces platelet adhesion and aggregation as well as activation of intrinsic blood coagulation. Marconi et al. [29] reported that albumin-coated surfaces exhibited remarkable inhibition of platelet adhesion and aggregation phenomena occurring usually on a blood-contacting foreign material. This agrees with our results.

3.4. In vitro evaluation of hemocompatibility

The effect of surface modification on the blood thrombus formation and whole blood clotting time (WBCT) can be seen in Table 3. Comparing to the original PAN membrane, PAN–HEP and PAN–HSA membranes can, respectively, reduce the thrombus formation by about 61% and 47%, while PAN–COL membrane can increase the thrombus formation by 25%. The WBCT (253 s) of PAN–COL was even shorter than that of the untreated PAN (320 s). All the other modified PAN membranes extended the WBCT.

Table 3
Blood coagulation characterization (mean ± std, $n = 3$)

Membrane type	Degree of thrombosis (%)	WBCT (s)	Thrombin inhibition ratio (%)
PAN	17.4 ± 2.8	320 ± 21	1.9
PAN–AA	16.4 ± 2.7	345 ± 13	6.1
PAN–HEP	6.7 ± 0.9	851 ± 25	24.1
PAN–HSA	9.2 ± 1.2	692 ± 31	14.8
PAN–COL	21.8 ± 1.3	253 ± 13	—

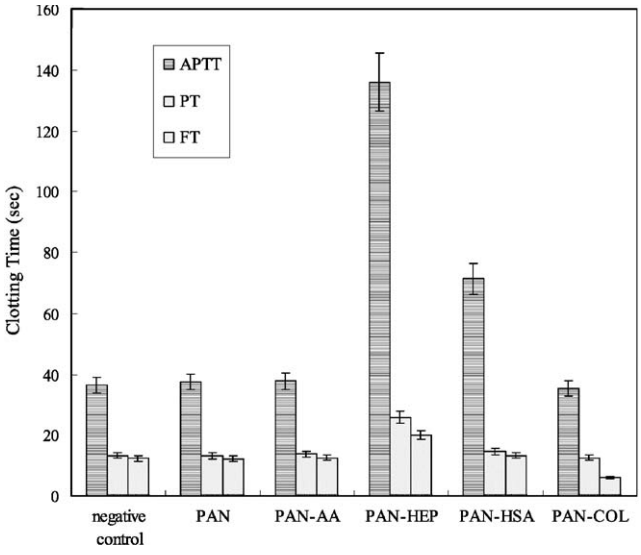

Fig. 5. Comparison of blood coagulation time (APTT, PT and FT) of unmodified and modified PAN membranes ($n = 3$).

Fig. 5 shows that the clotting times of PAN, PAN–AA, and PAN–COL membranes are nearly the same as the human plasma (the negative control), whereas these clotting times of PAN–HSA membrane is much longer, though shorter than those of PAN–HEP. The results show that APTT, PT, and FT of PAN–HEP are 3.5, 2.0, and 1.6 times longer than those of PAN, respectively. Heparin has been proved to be an effective agent in curtailing thrombosis and is effective when immobilized onto polymer surfaces [8]. Heparin binds and catalyzes the interaction of plasma proteins involving in the intrinsic and extrinsic clotting cascade. Table 3 lists the results of thrombin inactivation level for modified PAN membrane. The data showed that thrombin inactivation was up to 24.1% for PAN–HEP and 14.8% for PAN–HSA, as compared with PPP control. These results indicate that HSA-immobilization can still improve the hemocompatibility of PAN membrane.

3.5. Serum complement studies

Complement activation has been shown to occur during hemodialysis [14]. The extent of serum

Table 4
Blood-contacting characterization (mean ± std)

Membrane type	Relative WBC count[a] (%)	C3[b] (ng/ml)	C4[b] (ng/ml)
Control	100	120 ± 31	34 ± 7
PAN	84.5 ± 5.6	326 ± 41	46 ± 8
PAN–AA	85.6 ± 4.4	250 ± 31	45 ± 8
PAN–HEP	95.3 ± 5.6	214 ± 35	42 ± 10
PAN–HSA	92.7 ± 4.6	184 ± 23	38 ± 6
PAN–COL	78.3 ± 5.9	154 ± 26	37 ± 5

[a] Whole blood added with citric anticoagulant after 30 min incubation ($n = 3$).
[b] Incubated for 1 h in serum without anticoagulant ($n = 3$).

complement activation was used to assess the in vitro hemocompatibility of hemodialysis membranes.

As shown in Table 4, the serum C3 concentration (214 ng/ml) in contact with PAN–HSA membrane was lower than that of original PAN (326 ng/ml) and higher than that of PAN–COL (154 ng/ml). Consequently, the WBC count decreased to 84.5% for the original PAN, however, PAN–HSA merely decreased to 92.7%. On the other hand, the PAN–COL membrane decreased the relative WBC to 78.3% with slight increase in the complement activation of C3 and C4 compared to those of original PAN or PAN–HSA. These results demonstrated that the leukopenia is not necessarily accompanying with anaphylatoxin formation. Both HEP and HSA immobilization can reduce the leukopenia and C3a formation of the PAN, whereas COL immobilization can increase the leukopenia although its C3a level was lower than those of PAN–HEP and PAN–HSA.

Up till now, the relationship between complement activation and plasma protein adsorption for reused hemodialyzers is not fully clarified. The reused hemodialyzers usually suppress the leukopenia, anaphylatoxin formation and avoid inducing the first use syndrome [14]. The plasma proteins will always more or less adsorb onto the surface of hemodialyzer, even after extensive washing. Furthermore, formalin-based sterilization agents may facilitate the coating of membranes with significant quantities of plasma proteins via Shiff base reaction. The concentration of platelet-adhesion-inhibiting proteins in human plasma, such as albumin, is much higher than that of platelet-adhesion-promoting proteins, such as fibrinogen. In addition, the platelet-adhesion-promoting proteins are not presented at the membrane surface due to the Vroman effect [30]. Therefore, albumin should be the main protein residual on reused hemodialyzer membranes. In summary, these phenomena suggest that the adsorption or immobilization of albumin cannot only mask the complement-activating sites but also reduce thrombosis.

4. Conclusions

The surface of PAN membrane was hydrolyzed and then immobilized with proteins. The immobilization of platelet-adhesion-inhibiting HSA resulted in a rougher surface morphology of PAN membrane, reduced the platelet adhesion, fibrinogen adsorption, prolonged the blood coagulation times, as well as reduced the leukopenia and anaphylatoxin formation. On the other hand, the platelet-adhesion-promoting collagen immobilizing PAN membrane exhibited the opposite effect when contacting with blood, though inducing the least complement activation. This demonstrates that not all of plasma proteins are capable of improving the hemocompatibility. The results can also explain why reused hemodialyzers are beneficial to hemodialysis therapy. The reused hemodialyzers usually suppress the leukopenia, anaphylatoxin formation and avoid inducing the first use syndrome. These phenomena most likely are due to the fact that the complement-activating sites may be masked or modified by albumin. Therefore this study demonstrated that the immobilization of HSA onto the surface of PAN membrane was useful and practicable in improving the hemocompatibility and diminishing the anaphylatoxin formation during hemodialysis treatment.

Acknowledgements

The authors would like to express their gratitude to the National Science Council of ROC for the financial support to this project (NSC 91-2216-E-011-022).

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