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Immobilization of Heparin on the Surface of Polypropylene Non-Woven Fabric for Improvement of the Hydrophilicity and Blood Compatibility

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Abstract

A polypropylene non-woven fabric (PPNWF) was exposed to oxygen plasma to produce peroxides on its surface. These peroxides were used to initiate graft polymerization of acrylic acid (AA) on the surface of PPNWF. Direct heparinization was accomplished *via* a reaction between heparin and PP-PAA (AA grafted PPNWF) which was activated by EDC (N-ethyl-N′-[3-(dimethylamino)propyl] carbodiimide). Indirect heparinized PPNWF was prepared by grafting poly(ethylene oxide) (PEO) on a PP-PAA surface to form PP-PAA-PEO, followed by reaction with heparin which was activated by EDC before use. The surface-modified PPNWFs were characterized by attenuated total reflection Fourier transform infrared (ATR–FT-IR) spectroscopy, electron spectroscopy for chemical analysis (ESCA) and contact angle goniometry. It was found that hydrophilicity was greatly improved, as indicated by the decrease of the water contact angle from 142 to 33°. *In vitro* blood compatibility evaluation of modified PPNWFs, including hemolysis rate, platelet adhesion, plasma protein adsorption and activated partial thromboplastin time (APTT) was investigated. The results suggested that both heparinized PPNWFs showed lower hemolysis rates and better platelet anti-adhesion than non-heparinized controls. Furthermore, PPNWF obtained *via* indirect immobilization of heparin showed better hydrophilicity and blood compatibility than direct heparinization of PPNWF.

Keywords

Polypropylene non-woven fabric, heparin, hydrophilicity, blood compatibility

1. Introduction

Polypropylene non-woven fabric (PPNWF), as one of the most popular materials, is used in many fields, such as biomedical separation and water purification, due

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to its random network of overlapped fibers, multiple connected pores, and thermal and chemical stability. However, when used as blood purification membrane, its surface tends to interact with plasma proteins and platelets, leading to thrombus formation, immune system response and other serious problems [1–6], because of its hydrophobicity and poor blood compatibility. Therefore, it was significant to improve hydrophilicity and blood compatibility of the material surface that might inhibit protein adsorption, platelet adhesion and thrombin formation while maintaining the mechanical and physical properties of underlying substrate [7–10].

Immobilization of biomolecules on polymer surfaces was proved to be an effective way to improve blood compatibility. Biomolecules, such as insulin [11], gelatin [12], collagen [13], chitosan [14, 15] and heparin [11, 16], have been widely immobilized on surfaces of different materials to improve blood compatibility.

Heparin, the most widely used blood anticoagulant, possesses a number of biological functions, such as anticoagulant activity, cell-growth stimulation and plasma clearing ability [17, 18]. The study of heparin immobilization *via* electrostatic interaction [19] has been replaced by covalent immobilization. This process has the advantages of being more permanent and providing enduring stability to the immobilized heparin [20]. Lin *et al.* reported that a hemodialyzer made of polyacrylonitrile membrane immobilized on a chitosan–heparin conjugate could significantly reduce the injection of heparin in clinical applications, and might even lead to heparin-free therapy [21]. Furthermore, such a hemodialyzer also exhibited antibacterial activity. Jiang *et al.* immobilized heparin on the surface of a polyethylene membrane *via* coupling with a polydopamine active layer [22]. They demonstrated that the hydrophilicity of the polyethylene membrane was remarkably improved after dopamine modification and heparin immobilization, and that the heparinized membrane showed very good hemocompatibility.

In this paper, attempts were made to immobilize heparin on the surface of PPNWF. Acrylic acid (AA) was firstly grafted on the PPNWF surface which was pretreated by oxygen plasma. Subsequently, heparin was directly immobilized on the PP-PAA surface and indirectly grafted on the surface of PP-PAA through the use of PEO as a spacer, respectively. The modified PPNWFs were characterized using attenuated total reflection Fourier transform infrared (ATR–FT-IR) spectroscopy, electron spectroscopy for chemical analysis (ESCA), and contact angle goniometry. *In vitro* blood compatibility evaluation, including hemolysis rate, platelet adhesion, plasma protein adsorption and activated partial thromboplastin time (APTT), was also investigated.

2. Materials and Methods

2.1. Materials

PPNWF (148 g/m^2) was obtained from Shanghai Shilong Hi-tech. Acrylic acid (AA), toluidine blue, N,N-dimethyl formamide (DMF), poly(ethylene oxide) (PEO), N-ethyl-N′-[3-(dimethylamino)propyl] carbodiimide (EDC), heparin so-

dium salt (⩾150 U/mg) and 4-dimethylamiopryidine (DMAP) were purchased from Sinopharm Chemical Reagent. AA was purified by vacuum distillation before use. The average molecular weight of PEO used in this study was 400. Human whole blood, total protein assay kit (Changchun Huili Biotech) and total protein standard substance (Changchun Huili Biotech) were kindly donated by Institute of Blood Transfusion, Chinese Academy of Medical Sciences. All other reagents were analytical grade and used without further purification.

2.2. Graft Polymerization of AA on the Surface of PPNWF

The PPNWF was washed with acetone and dried, and then PPNWF was put into low temperature plasma (HD-1B, Jiangsu). Prior to treatment, a base pressure of approx. 4 Pa was achieved by use of a rotary vane vacuum pump. Then, oxygen gas (99.99%) was introduced into the chamber, the gas pressure was maintained at 15 Pa, and the working power (30 W) and treatment time (3 min) were fixed. After treatment, it was immersed in solution made up of AA and de-ionized water. The solution was bubbling with nitrogen gas for 20 min in order to displace oxygen. The glass tube was put in a water-bath at 80°C for 4 h. The scheme of graft polymerization of AA on PPNWF surface is illustrated in Fig. 1. Then, the formed PP-PAA was taken out and washed by a lot of de-ionized water to remove physically absorbed AA and PAA, and then was dried to a constant weight at 40°C in an air-dry oven. The grafting density (GD, mg/cm^2) was calculated by the following equation:

$$\mathrm{GD} = \frac{W_1 - W_0}{A_0}, \tag{1}$$

where W_0 and W_1 are the weight of the pristine PPNWF and PP-PAA, respectively, and A_0 represents the area of the pristine PPNWF.

2.3. Immobilization of Heparin

The direct heparination [23, 24] and indirect heparination [25, 26] are described in the chemical scheme in Fig. 1.

Direct immobilization: PP-PAA (10 cm ×10 cm) was immersed in 500 ml citric acid buffer solution (pH 4.8) containing EDC (10 mg/ml). The reaction was allowed to proceed at room temperature for 24 h to activate the carboxylic acid. The activated PP-PAA was slightly washed by de-ionized water and then was put in citric acid buffer solution (pH 4.8) containing heparin (10 mg/ml). The reaction was carried out at 20°C for 24 h. The formed PP-PAA-Hep was washed with excess de-ionized water in an ultrasonic cleaner to remove residual un-reacted heparin and by-product.

Indirect immobilization: PP-PAA was immersed in PEG containing concentrated sulfuric acid (1%, v/v) at 100°C for 24 h under nitrogen protection [27], followed by washing with excess de-ionized water in an ultrasonic cleaner (10 min) 3 times and dried at room temperature.

The formed PP-PAA-PEO was immersed in DMF solution including heparin (10 mg/ml) which had been activated by a sufficient amount of EDC at 4°C for

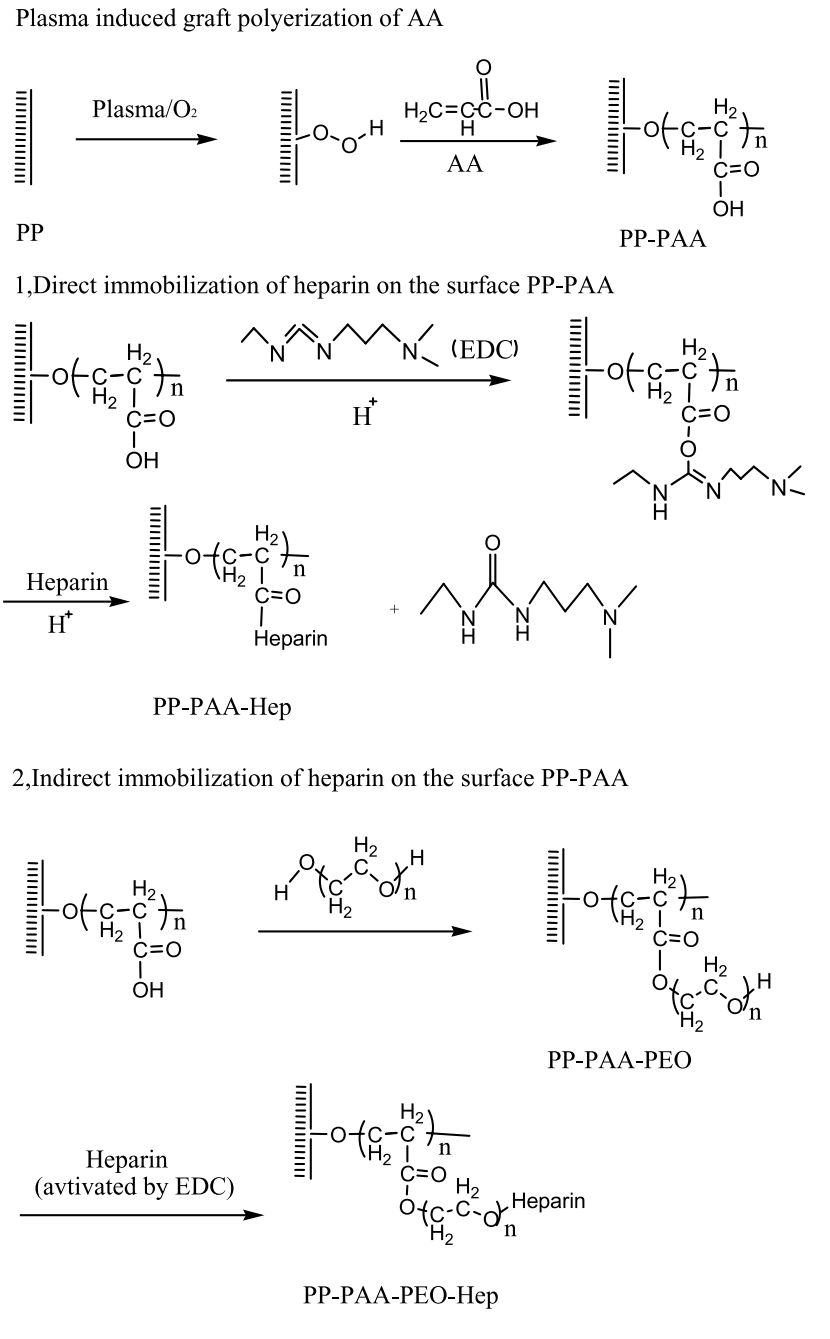


Figure 1. Scheme of the synthesis of HEP-immobilized PPNWF by (1) direct immobilization of heparin and (2) indirect immobilization of heparin.

24 h and the reaction was allowed to proceed at 20°C for 24 h using of DMAP (2 wt%) as catalyst. The formed PP-PAA-PEO-Hep was rinsed with a large amount

of de-ionized water in an ultrasonic cleaner (10 min) 3 times, and dried at room temperature. The absence of heparin in the washing water was confirmed using toluidine blue.

The immobilized density of PEO (mg/cm^2) was calculated by the following equation [27]:

$$\mathrm{GD} = \frac{W_2 - W_1}{A_0}, \tag{2}$$

where W_1 and W_2 are the mass of the PP-PAA and PP-PAA-PEO, respectively.

The density of immobilized heparin was determined by the toluidine blue (TB) colorimetric method as described in the literature [28–30]. Each value was the average of 3 independent measurements.

2.4. Surface Characterization

ATR–FT-IR spectra of modified PPNWFs were obtained using a FT-IR spectrometer (Avatar 360, Nicolet Instruments).

The surface composition of modified PPNWFs was determined *via* X-ray photoelectron spectroscopy (AXIS Ultra^{DLD}, Kratos) with an Al/K anode mono-X-ray source. All the samples were completely vacuum dried prior to use. The releasing angle of the photoelectron for each atom was fixed at 90°. Surface spectra were collected over a range of 0–1200 eV and high-resolution spectra of the C_{1s}, N_{1s}, O_{1s} and S_{2p} region were collected. The atomic concentrations of the elements were calculated by their corresponding peak areas.

Hydrophilicity of the samples was characterized on the basis of water contact angle measurement [27] using a THETA Optical Tensiometer (KSV Instrument) equipped with video capture. A total of 5 μl distilled water was dropped on the air-side surface of the samples at room temperature. At least 5 contact angles were averaged to get a value.

2.5. Hemolysis Assay

Hemolysis assay [31] was performed to evaluate blood compatibility of pristine PPNWF, PP-PAA, PP-PAA-Hep, PP-PAA-PEO and PP-PAA-PEO-Hep. Tested nonwoven fabric (0.5 g) was cut into pieces before transferred into polystyrene tube. Normal saline (0.9%, 10 ml) was poured into each of the tubes and kept at 37°C in a shaking water bath. Then the red blood cells were washed three times with 0.9% normal saline. After 4 h in incubation, 400 μl of washed red blood cell (2 ml red blood cell was diluted with 10 ml normal saline) was added into each of the tubes. All of the tubes were further incubated at 37°C for 60 min. Similarly, positive and negative controls were produced in separate tubes by adding 400 μl of the diluted blood to 10 ml distilled water and normal saline, respectively. The fluid was then transferred into a fresh polystyrene tube and centrifuged at 2500 rpm for 5 min. The absorbance of supernatants was measured at 545 nm by UV-Vis spectrophotometry (model 7200, UNICO Instruments).

The hemolysis rate was calculated according to the following formula:

$$\mathrm{HR} = \left(\frac{A_\mathrm{s} - A_\mathrm{nc}}{A_\mathrm{pc} - A_\mathrm{nc}} \right) \times 100\%, \tag{3}$$

where A_s, A_pc and A_nc are the absorbance of sample supernatant, positive control and negative control, respectively.

2.6. Platelet Adhesion

Whole blood was collected from healthy volunteers in a 30 ml disposable syringe containing 3 ml of an aqueous solution of 4.0 wt% sodium citrate. The citrated whole blood was immediately centrifuged for 15 min at 2000 rpm to obtain citrated platelet-rich plasma (PRP). PPNWF (1 cm × 1 cm) was immersed in the PRP for 60 min at 37°C. The tested sample was rinsed gently with PBS, after which the adhered platelets were fixed with 2.5 wt% glutaraldehyde in PBS for 30 min. Finally, these samples were washed with PBS, and dehydrated with a series of ethanol/water mixtures of increasing ethanol concentration (30, 40, 50, 60, 70, 80, 90 and 100% ethanol, 30 min in each mixture) [32, 33].

The membrane surface was coated with gold and observed by scanning electron microscopy (SEM) at 3000× magnification.

2.7. Plasma Protein Adsorption

Modified nonwoven fabric (0.1 g) was cut into pieces before transfer into polystyrene tubes. Fresh plasma (2 ml) was put into each tube and kept at 37°C in a shaking water bath. After 3 h incubation, 50 µl plasma was taken out of each tube and put into fresh polystyrene tubes. Then 2 ml total protein assay reagent was put into each tube and the tubes were shaken well. The absorbance of fluid from each tube at 545 nm was measured by UV-Vis spectrophotometry. The concentrations of total protein in the plasma samples were calculated according to the following formula:

$$C_\mathrm{b} \text{ (or } C_\mathrm{a}) = \left(\frac{A_\mathrm{b} \text{ (or } A_\mathrm{a})}{A_\mathrm{ss}} \right) \times C_\mathrm{ss}, \tag{4}$$

where C_b and C_a are the concentrations of total protein in the plasma samples before and after adsorption, respectively, A_b and A_a are the absorbance of plasma samples before and after adsorption, respectively, and C_ss (70 g/l) and A_ss (0.500 ± 0.001) are the concentration and absorbance of total protein standard substance, respectively.

Adsorption percentage (AP) of total protein was calculated according to the following equation:

$$\mathrm{AP} = \left(\frac{C_\mathrm{b} - C_\mathrm{a}}{C_\mathrm{b}} \right) \times 100\%, \tag{5}$$

where C_b and C_a are the same as in equation (4).

2.8. Activated Partial Thromboplastin Time (APTT)

The tested nonwoven fabrics (1 cm × 1 cm) were incubated with 1 ml plasma in a plastic tube for 15 min at 37°C. Then the tested samples were taken out immediately and the remaining plasma was kept at 4°C. Actin-activated cephaloplastin reagent (50 µl, Baxter Diagnostics) was added to the remaining plasma (50 µl), followed by the addition of a 0.025 M $CaCl_2$ solution (50 µl) after a 5 min incubation. All of these actions were automatically carried out by an automated blood coagulation analyzer (ACL Elite, Beckman Coulter). The clotting time of the treated plasma was exported from the analyzer. The experiment was repeated three times and a mean value was calculated.

3. Results and Discussion

3.1. Characterization of Surface-Modified PPNWFs

The composition of the tested samples is shown in Table 1.

The ATR–FT-IR spectra of pristine and modified PPNWFs are shown in Fig. 2. Compared with pristine PPNWF, PP-PAA showed a new peak appearing at 1714 cm^{-1}, which was based on the carbonyl (C=O) of the carboxylic acid groups in grafted AA. In comparison with PP-PAA, both PP-PAA-Hep and PP-PAA-PEO-Hep showed a new peak at around 1035 cm^{-1} [31] due to the symmetric stretching of S=O in the sulfonic group ($–SO_3^-$) of heparin and another peak at about 1329 cm^{-1} due to asymmetric stretching vibration of the $–SO_3^-$ group. As shown in spectrum d, there was a new peak at about 1224 cm^{-1} which resulted from asymmetric vibration of the C–O of PEO.

As shown in Fig. 3a, pristine PPNWF had a peak corresponding to C_{1s}, while PP-PAA-Hep and PP-PAA-PEO-Hep had peaks corresponding to C_{1s} (binding energy 284.8 eV) and O_{1s} (binding energy 531.9 eV) and N_{1s} (binding energy 400 eV). In addition, PP-PAA-Hep and PP-PAA-PEO-Hep had a weak peak at around 168 eV, as seen in Fig. 3b and c, which was assigned to the $–SO_3^-$ of immobilized heparin [21]. The chemical composition of the modified PPNWFs, calculated from the XPS spectra, is shown in Table 2. The oxygen content (30.62%) of the PP-PAA resulted

Table 1.
Amount of carboxyl group, PEO and heparin immobilized on the surface of PPNWFs

Substrate	Carboxyl group (mg/cm^2)	PEO (mg/cm^2)	Heparin ($µg/cm^2$)
PP-PAA	1.16	—	—
PP-PAA-Hep	—	—	3.53
PP-PAA-PEO	—	0.08	—
PP-PAA-PEO-Hep	—	—	1.84

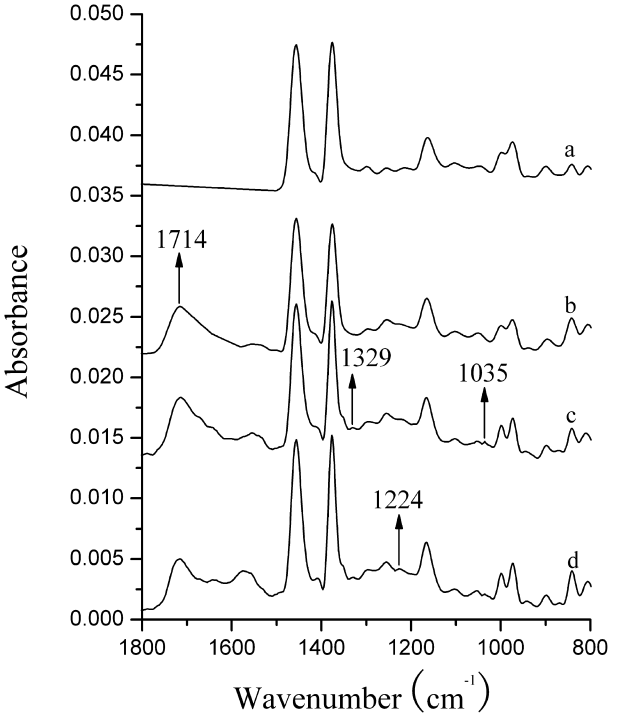


Figure 2. ATR–FT-IR spectra of (a) pristine PPNWF, (b) PP-PAA, (c) PP-PAA-Hep and (d) PP–PAA-PEO-Hep.

from successful graft polymerization of AA. The oxygen content of the PP-PAA-PEO-Hep was slightly higher than that of PP-PAA-Hep because of grafted PEO.

Figure 4 shows the C_{1s} core level scan spectra of (a) PP-PAA, (b) PP-PAA-Hep and (c) PP-PAA-PEO-Hep surfaces as typical examples. The percentage contribution of the ESCA C_{1s} components to the surface-modified PPNWFs, calculated from the C_{1s} core level spectra (see Fig. 4), is shown in Table 3. After immobilization of heparin, the peak at 288.9 eV (COO) of PP-PAA-Hep decreased significantly, whereas the peak at 287.8 eV (CONH) increased significantly. The peak at 285.9 eV (C–O) of PP-PAA-PEO increased significantly due to the immobilized PEO. For PP-PAA-PEO-Hep, the peak at 285.9 eV (C–O) increased in comparison with PP-PAA-PEO due to the immobilization of heparin. In short, the above results clearly demonstrated that PAA, PEO and heparin were successfully grafted on PPNWF surface.

Table 4 shows the water contact angles of the samples. As seen in Table 4, the water contact angle (WCA) of the pristine PPNWF was about 142°. The WCA reduced from 142 to 70° because of graft polymerization of AA on the surface of PPNWF, and the WCA was further decreased from 68 to 33° with the immobilization of heparin and PEO-Heparin. These results indicated that surfaces of modified PPNWFs became more wettable after surface modification.

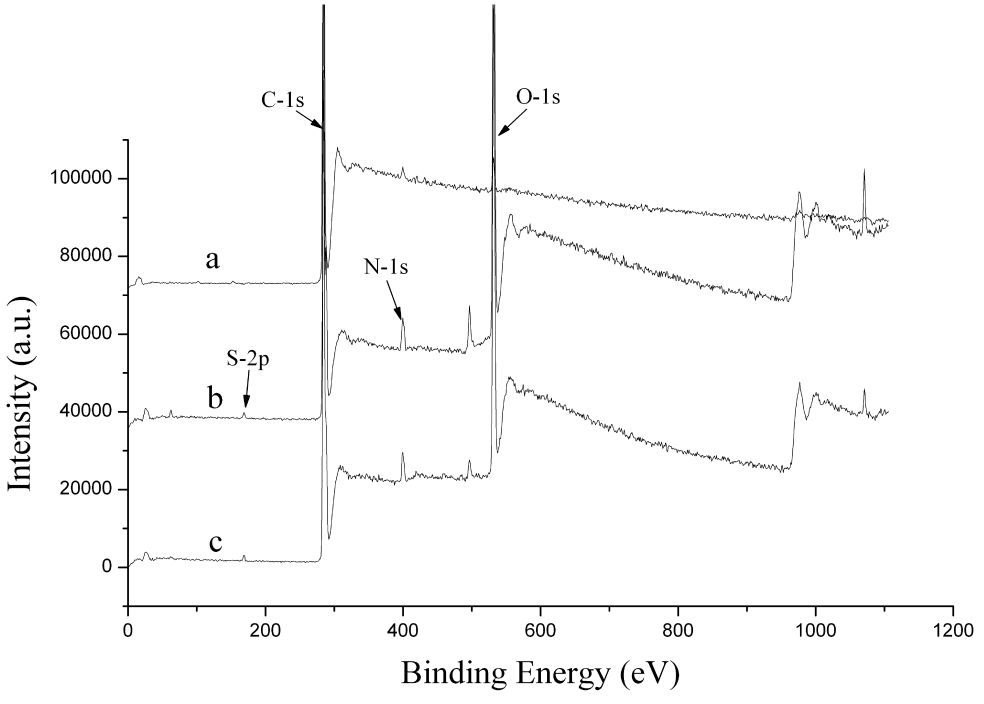


Figure 3. XPS spectra of (a) pristine PPNWF, (b) PP-PAA-Hep and (c) PP-PAA-PEO-Hep.

Table 2.
Chemical composition of surface-modified PPNWFs (in at%) from XPS

Substrate	C	O	N	S
Pristine PPNWF	100	—	—	
PP-PAA	69.38	30.62	—	—
PP-PAA-Hep	67.98	30.45	1.33	0.24
PP-PAA-PEO	67.83	32.17	—	—
PP-PAA-PEO-Hep	67.68	31.44	0.74	0.14

3.2. Hemolysis Assay

The hemolysis rate (HR) was adopted to evaluate the erythrocyte compatibility of biomaterials. The HR of pristine PPNWF, PP-PAA, PP-PAA-Hep, PP-PAA-PEO and PP-PAA-PEO-Hep is given in Table 5. The results indicated that PP-PAA-Hep and PP-PAA-PEO-Hep had good erythrocyte compatibility, while PP-PAA could cause severe hemolysis. The high HR of PP-PAA could be attributed to the acidity of PAA. Erythrocytes were very sensitive to environmental pH. Murthy *et al.* found that acidic poly(alkylacrylic acid) caused severe hemolysis [34]. Cao *et al.* also proved that increasing grafting density of AA could cause an increase of the HR

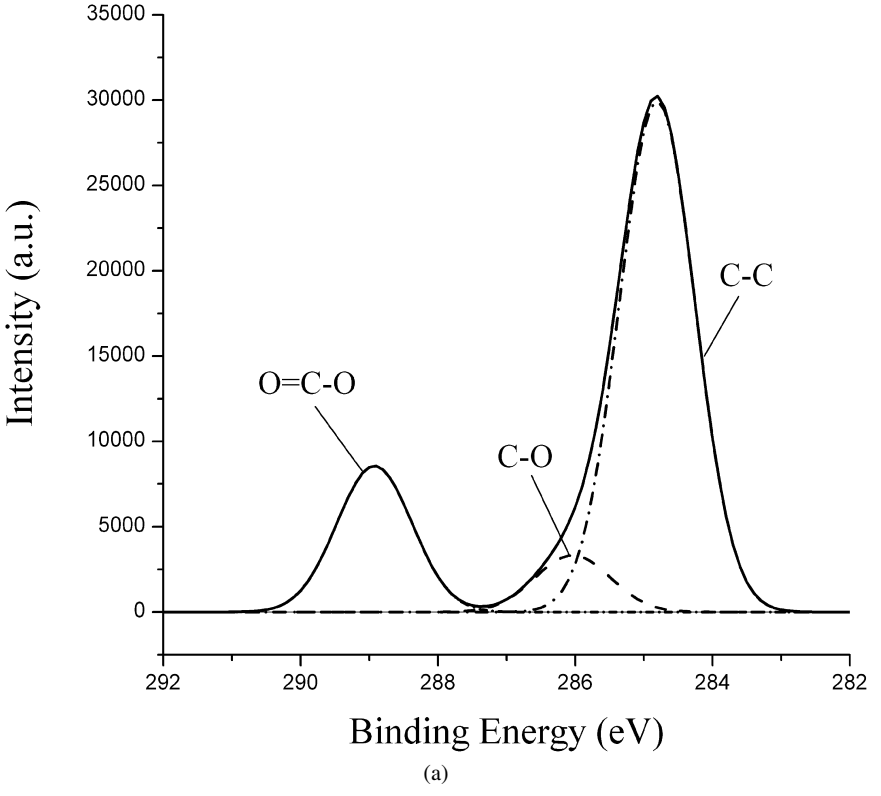
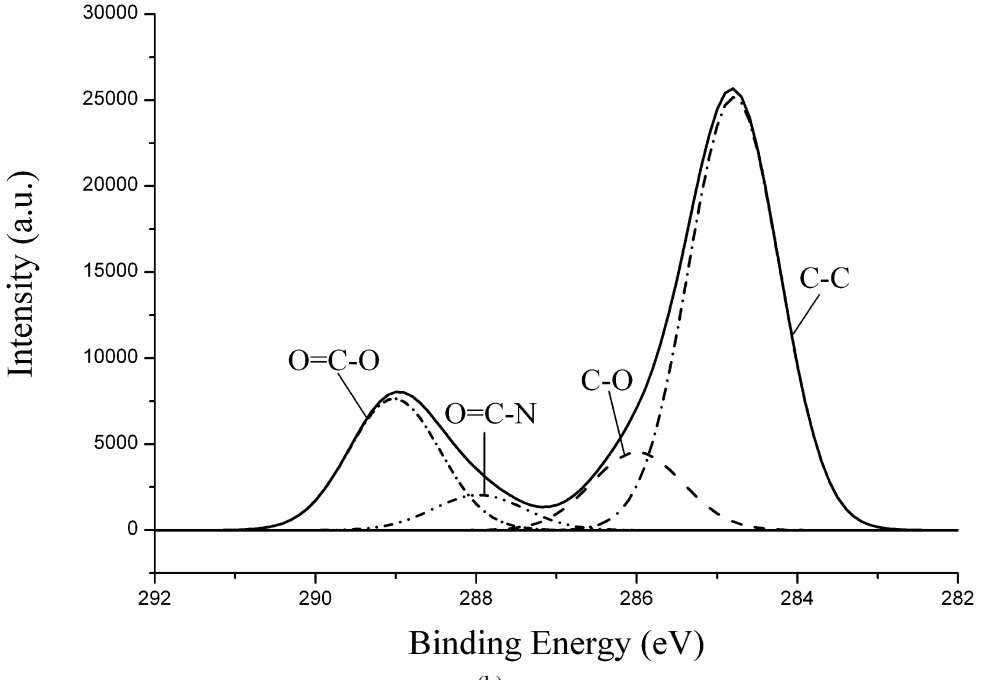


Figure 4. ESCA C_{1s} core level scan spectra of (a) PP-PAA, (b) PP-PAA-Hep and (c) PP-PAA-PEO-Hep.

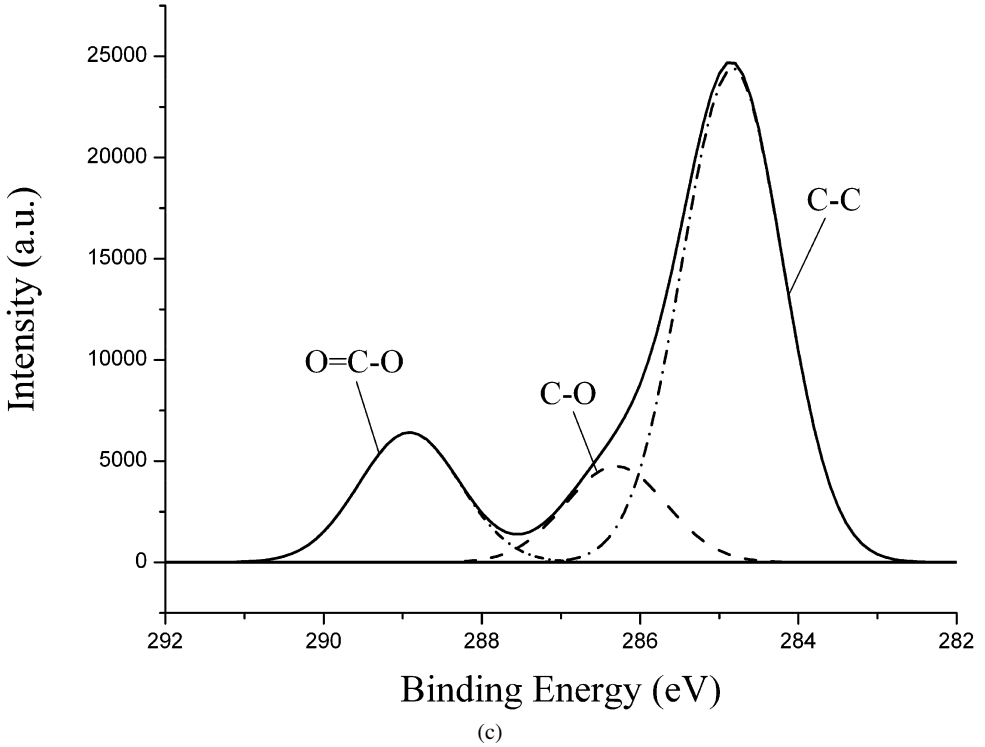
[31]. It was demonstrated that immobilization of heparin on the surface of PPNWF could improve its blood compatibility.

3.3. Blood Platelet Adhesion

Platelets play an important role in the thrombogenicity of biomaterials in blood contacting application. Resistance of platelet adhesion and activation could be considered as one of the most important parameters of the blood compatibility of materials [35]. Thus, this paper also employed this test to evaluate the blood compatibility of surface-modified PPNWFs. Figure 5 shows platelet adhesion on surface of pristine PPNWF, PP-PAA-Hep and PP-PAA-PEO-Hep. Large amounts of platelets aggregated and adhered on the surface of pristine PPNWF (Fig. 5a). In contrast, the platelets adhering on the surface of PP-PAA-Hep and PP-PAA-PEO-Hep (Fig. 5b and c) were greatly decreased as compared with the virgin PPNWF. This meant that heparinized surface of PPNWF including direct and indirect immobilization possessed the excellent property to resist adhesion of platelets while the neat PPNWF showed strong adhesion of platelets [36]. It was considered that the improved blood compatibility could be attributed to the $-SO_3^-$ of



(b)



(c)

Figure 4. (Continued.)

Table 3.
Contribution of ESCA C_{1s} components (in %) to surface-modified PPNWFs

Substrate	C–C (284.8 eV)	C–O (285.9 eV)	C=O (287.8 eV)	COO (288.9 eV)
PP-PAA	68.03	6.48	—	25.49
PP-PAA-Hep	63.95	11.43	5.19	19.43
PP-PAA-PEO	67.63	14.46	—	17.91
PP-PAA-PEO-Hep	65.63	17.43	—	16.94

Table 4.
Static water contact angles of surface-modified PPNWFs ($n = 5$)

Substrate	WCA (°)
Pristine PPNWF	142 ± 1
PP-PAA	70 ± 2
PP-PAA-Hep	68 ± 3
PP-PAA-PEO	59 ± 3
PP-PAA-PEO-Hep	33 ± 5

Table 5.
Hemolysis rate of surface-modified PPNWFs ($n = 4$)

Substrate	Hemolysis rate (%)
Pristine PPNWF	0.25 ± 0.02
PP-PAA	36.27 ± 0.17
PP- PAA-PEO	18.08 ± 0.35
PP- PAA-Hep	1.78 ± 0.18
PP-PAA-PEO-Hep	0 ± 0.01

immobilized heparin through negative-charge repulsion with the negative-charged platelets and surface hydrophilicity to resist adhesion of platelets. These results agreed with a previous report on the surface modification with bioactive macromolecules [37].

3.4. Plasma Protein Adsorption

It is well known that the behavior of protein adsorption onto substrates depends significantly on the surface characteristics, such as hydrophilicity, roughness, charge and chemistry. Winterton *et al.* studied the reduced adsorption of plasma protein, such as fibrinogen and albumin, onto heparin-immobilized surfaces and the results showed that both albumin and fibrinogen had no binding affinity to heparin at physi-

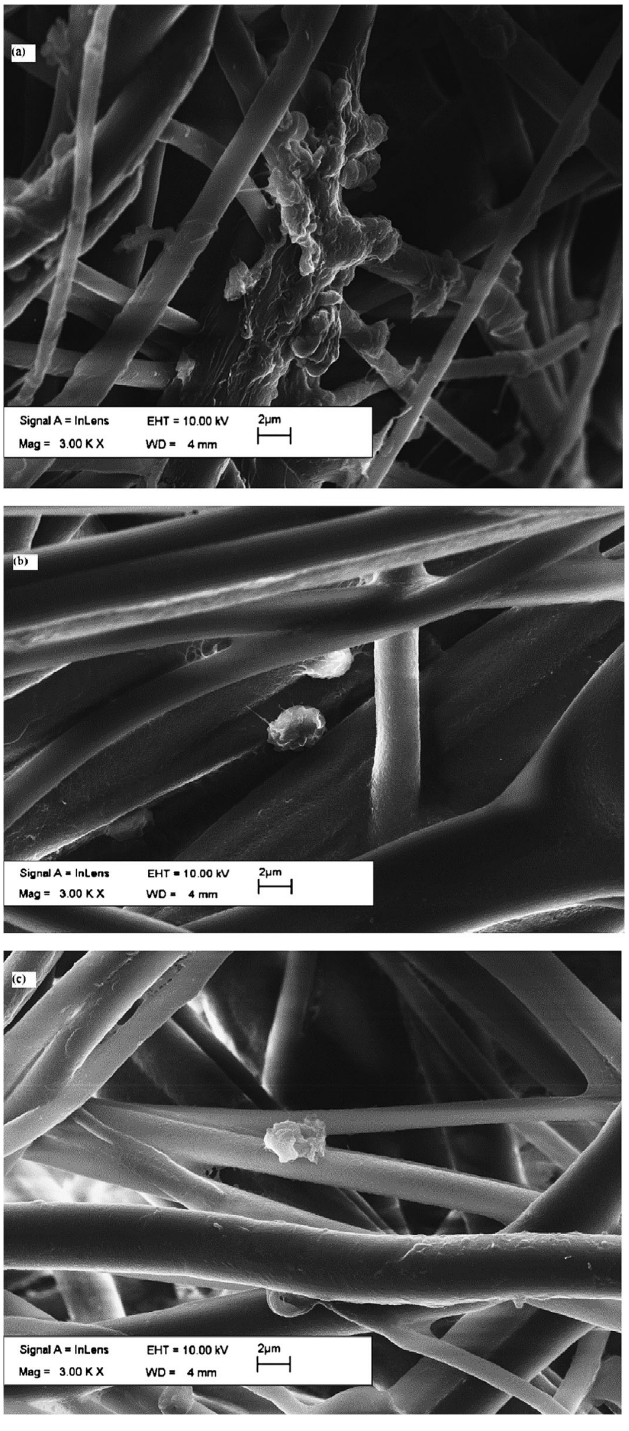


Figure 5. Platelet adhesion on the surface of (a) pristine PPNWF, (b) PP-PAA-Hep and (c) PP-PAA-PEO-Hep.

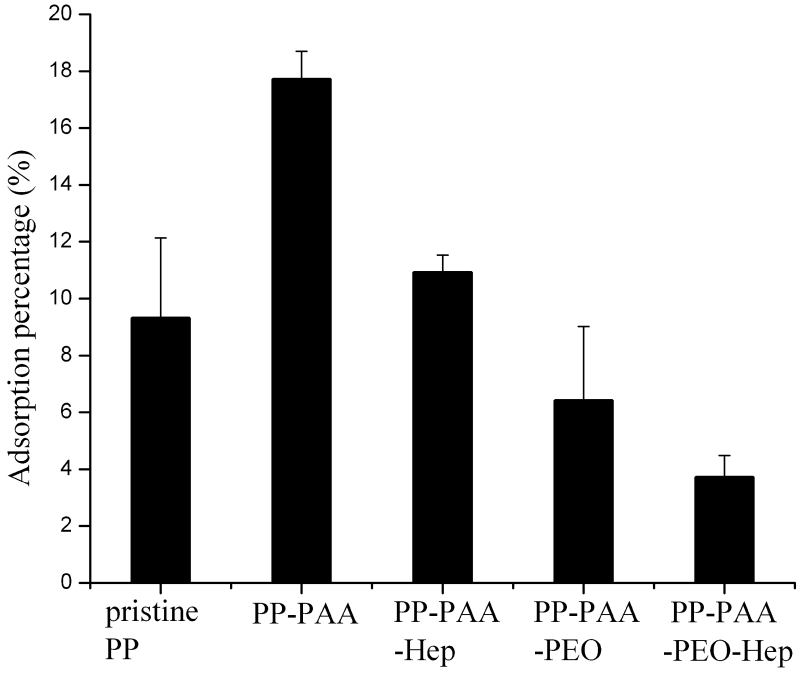


Figure 6. Adsorption percentage of total protein on surface-modified PPNWFs after 3 h incubation ($n = 4$, mean ± SD, $P < 0.01$ from value on plasma protein adsorption within each group).

ological pH [38]. In this paper, as shown in Fig. 6, adsorption percentage of plasma protein decreased from 17.7 (PP-PAA) to 3.7% (PP-PAA-PEO-Hep). The results indicated that indirect heparinized PPNWF (PP-PAA-PEO-Hep) could better reduce plasma protein adsorption than direct heparinized PPNWF (PP-PAA-Hep). This might result from that grafted PEO could improve the bioactivity of heparin. Besides, good hydrophilicity of modified PPNWF could also effectively inhibit adsorption of plasma protein.

3.5. APTT

This APTT experiment was used as a method to evaluate the blood compatibility. Table 6 shows the APTT of plasma treated with pristine PPNWF, PP-PAA, PP-PAA-Hep, PP-PAA-PEO and PP-PAA-PEO-Hep. The better the blood compatibility of the modified PPNWF, the less the APTT of plasma treated with the modified PPNWF increased compared to untreated plasma [31]. As shown in Table 6, we could see that the APTT of PP-PAA and PP-PAA-Hep was remarkably prolonged in comparison with negative control. However, it was remarkably shortened by the immobilization of PEO and PEO-heparin, suggesting that PP-PAA-PEO-Hep had good blood compatibility as compared with PP-PAA-Hep.

Table 6.
Blood compatibility evaluated by APTT

Substrate	APTT (s)
Pristine PPNWF	28 ± 1
PP-PAA	46 ± 4
PP-PAA-Hep	40 ± 3
PP-PAA-PEO	28 ± 1
PP-PAA-PEO-Hep	29 ± 1
PPP negative control	30 ± 1

Data are presented as mean $\pm$ SD of three different experiments.

4. Conclusions

PPNWF was successfully modified by graft polymerization of AA and immobilization of heparin. Water contact angle measurements illustrated that hydrophilicity of the modified PPNWFs was greatly improved by surface modification. Both heparinized PPNWFs showed better hemolysis rates, platelet adhesion than non-heparinized controls. Furthermore, the adsorption percentage of plasma protein and APTT of PP-PAA-PEO-Hep decreased in comparison with PP-PAA-Hep. This may result from that the grafted PEO as a spacer could enhance the bioactivity of heparin. This work demonstrated that the PPNWF modified *via* indirect immobilization of heparin possessed good hydrophilicity and blood compatibility, and can potentially be applied in plasma purification and separation.

Acknowledgements

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