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Polymer 46 (2005) 11060–11065
polymer
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Acrylonitrile-based copolymer membranes containing reactive groups: Surface modification by the immobilization of biomacromolecules

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Received 2 June 2005; received in revised form 8 September 2005; accepted 13 September 2005
Available online 29 September 2005

Abstract

Asymmetric membranes fabricated from poly(acrylonitrile-*co*-maleic acid) (PANCMA) were immobilized with heparin and/or insulin to improve their surface properties. These biomacromolecule-immobilized PANCMA membranes were prepared by the amination of the membrane surface with ethylenediamine, followed by the reaction of the amino groups with heparin and/or insulin in the presence of 1-ethyl-3-(3-dimethyl amidopropyl) carbodiimide. The surface-modified membranes were analyzed by X-ray photoelectron spectroscopy to confirm the immobilization of the biomacromolecules. Morphological changes on the membrane surface and in the cross section were characterized by scanning electron microscopy. The surface hydrophilicity and hemocompatibility of the studied membranes were evaluated on the basis of water contact angle, platelets adhesion and cell attachment measurements. It was found that, after the immobilization of the biomacromolecules, the water contact angle and the amount of adhered platelets and macrophages on the membrane decreased significantly when compared with the nascent ones, indicating the improvement of surface hydrophilicity. Furthermore, the heparin immobilized membrane showed the best hemocompatibility among the corresponding membranes studied.

Keywords: Acrylonitrile copolymer membrane; Surface modification; Biomacromolecule immobilization

1. Introduction

Polyacrylonitrile (PAN) which possesses excellent properties, such as good thermal and mechanical stability, has been successfully applied as membrane materials in the fields of water treatment [1,2], pervaporation [3,4], gas separation [5,6], biochemical product purification [7], enzyme immobilization [8,9], and biomedical applications [10–12]. Moreover, various comonomers had been copolymerized with acrylonitrile to improve the physical/chemical properties and to extent the applications of PAN-based membranes [13–17]. In particular, PAN-based hollow fiber membranes such as AN69 (Produced by HOSPAL, fabricated from acrylonitrile/methallyl sulfonate copolymer) have already been used as dialyzers that enable low to middle molecule protein removals and high-flux dialysis therapy [18,19]. However, the hemocompatibility for this type of membrane is still insufficient and thus the continuous addition of anticoagulant (such as heparin) is required during hemodialysis. Therefore, in recent years, there has been much attention in developing PAN-based membranes with improved hemocompatibility and/or antifouling property [12,20–24]. For example, it was described by Yang et al. [12] that hemodialysis membrane with excellent hemocompatibility and antibacterial activity can be prepared through the covalent immobilization of chitosan/heparin complex onto the PAN membrane surface. It was also reported by Lavaud et al. [23] that the hemocompatibility of AN69 membrane can be further improved when they coated polycationic heparin onto the membrane surface.

The main objective of our series study is to enhance the surface performance of PAN-based membranes through surface modification and/or copolymerization. In our previous work, sugar moiety [10], *N*-vinyl-2-pyrrolidone (NVP) [24], or phospholipid analogues [25] was incorporated into polyacrylonitrile by copolymerization processes to improve the surface hemocompatibility of PAN-based membranes. Poly(ethylene glycol)s (PEG) with various molecular weights were immobilized on the poly(acrylonitrile-*co*-maleic acid) (PANCMA) membranes also for the same purpose [22]. In this case, step for the partial hydrolysis of nitrile groups (–C≡N) on the PAN membrane surface to generate carboxyl groups (–COOH) as described by

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doi:10.1016/j.polymer.2005.09.042

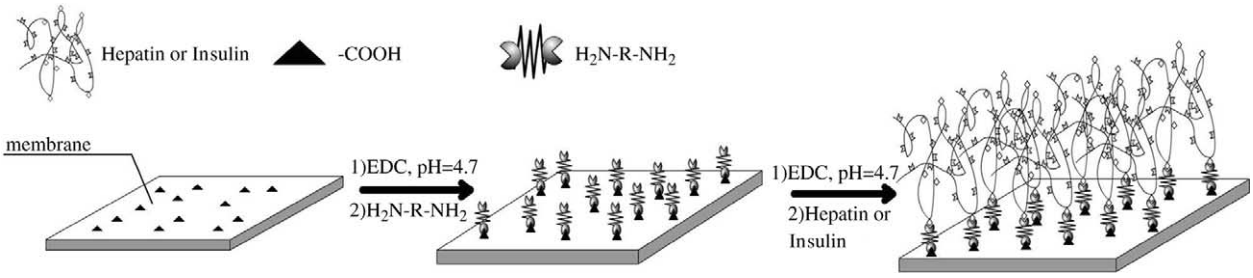

Fig. 1. Schematic diagram showing the immobilization of biomacromolecules on the PANCAM membrane.

Yang et al. [12] can be eliminated. Another advantage for our protocol maybe that –COOH on the membrane surface can be simply controlled by the monomer ratio in the process of copolymer synthesis. Through a coupling reaction, these carboxyl groups on the PANCMA membrane surface can be further used for the chemical immobilization of biomacromolecules to overcome an unavoidable disadvantage for the coating protocol that the coated biomacromolecules will elute into the blood fluid from the membrane during hemodialysis (especially when the molecular weight of the coating is not enough high). In this work, therefore, we try to promote the surface hydrophilicity and hemocompatibility of these membranes through the immobilization of biomacromolecule such as heparin or insulin. Both heparin and insulin have been frequently immobilized on biomaterials to enhance the biocompatibility or cytocompatibility for tissue engineering. For example, Kim et al. [26] demonstrated that platelet adhesion on the poly(ethylene terephthalate) film can be reduced by heparin or insulin immobilization. Heparin, a well-known anticoagulant used as an additive in hemodialysis, was also immobilized on the membranes such as polyacrylonitrile [12,27] and poly(vinylidene fluoride) [28] to improve their surface hemocompatibility. In our case, we also envisaged that, after the immobilization of the biomacromolecules, the studied membranes should possess a dual-layer structure with a biomimetic surface layer, which could create a specific microenvironment for the enzymes and thus benefit the enzyme activity [29].

2. Experimental

2.1. Materials

All chemicals used in this work were analytical grade. Heparin in sodium salt, insulin and 1-ethyl-3-(3-dimethyl amidopropyl) carbodiimide (EDC) were purchased from Sigma Chemical Company. Three PANCMA copolymers, designated as PANCMA04, PANCMA07 and PANCMA11 in the following text, were synthesized in our laboratory with a water-phase precipitation copolymerization process [22]. The numbers 04, 07 and 11 in the designation indicate that the mole fraction of maleic acid in the copolymer is 3.69, 7.48 and 11.45 mol%, respectively. The molecular weight (M_n) of these copolymers is 15.8×10^4 g/mol for PANCMA04, 15.1×10^4 g/mol for PANCMA07, and 14.5×10^4 g/mol for PANCMA11. PANCMA asymmetric membranes were prepared by dissolving the copolymer powder in dimethyl sulfoxide (DMSO). After the air bubbles in the solution disappeared through heat preservation at 100 °C for 24 h, the casting solution (PANCMA/H_2O/DMSO = 20/6/74) was cast onto a clean glass plate using a casting knife with 150 μm gate opening. The nascent membrane was placed in air (50 ± 1 °C, 45–50% relative humidity) for 10 min, and then immersed in 25 ± 1 °C ultrafiltrated water for 24 h. Before surface modification, the membranes were washed with a water–ethanol–hexane sequence to clean impurity, and then dried in a vacuum oven at room temperature.

2.2. Amination of the membrane surface

Amination of the PANCMA membrane with ethylenediamine was carried out through activating the carboxyl groups on the membrane surface by a coupling reagent EDC [30], which (100 mg) was dissolved in a sodium citrate buffer solution (100 mL, pH 4.7) to produce a 0.1 wt% EDC aqueous solution. PANCMA membrane (10×10 cm^2) was immersed in the EDC solution at 4 °C for 2 h to activate the carboxyl groups. The membrane was then gently washed twice with deionized water and incubated in an aqueous solution containing an excessive amount of ethylenediamine at 4 °C for 24 h to produce a aminated PANCMA membrane as represented in Fig. 1.

2.3. Immobilization of heparin

A sample of 100 mg non-fractionated heparin (sodium salt) was dissolved in a sodium citrate buffer solution (80 mL, pH 4.7), mixed with EDC (40 mg), and kept at 4 °C for 5 h to activate the carboxyl groups. Then, the amino-bound PANCMA membrane (10×10 cm^2) was immersed in a sodium citrate buffer solution (80 mL, pH 4.7) containing heparin previously activated with EDC, and kept at 4 °C for 24 h to immobilize heparin onto the membrane surface (designated as PANCMA-heparin, Fig. 1). After the amidation reaction, the membrane was washed with phosphate buffered saline (PBS, pH 7.2) and a 0.1% Triton X-100 aqueous solution and then subsequently rinsed with deionized water in an ultrasonic cleaner for 5 min to remove the physically adsorbed heparin.

2.4. Immobilization of insulin

A sample of 16 mg insulin (from bovine pancreas, Sigma Chemical Co.) was dissolved in a 0.1 wt% EDC aqueous solution (80 mL, pH 4.7) and incubated at 4 °C for 5 h to

activate the carboxyl groups. Then, the surface-aminated PANCMA membrane ($10\times10\ cm^2$) was immersed in this solution to immobilize insulin on the membrane surface as mentioned above. The resulted membrane was designated as PANCMA-insulin, see Fig. 1.

2.5. Surface characterization

To study the chemical changes between the nascent and surface-modified PANCMA membranes and to confirm the immobilization of the biomacromolecule onto the membrane surface, X-ray photoelectron spectroscopy (XPS, Perkin–Elmer Instruments, USA) equipped with Al K_α at 1486.6 eV and 300 W power at the anode was used. The energy scale of the spectrometer was calibrated using the lowest binding energy component present in the superficial layer. A survey scan spectrum was taken and the surface elemental composition relative to C_{1S} was calculated from the peak area with a correction for atomic sensitivity [31].

The morphological changes between the nascent and biomacromolecule-immobilized membrane surfaces were examined by scanning electron microscopy (SEM, Cambridge S-260, UK). The sponge-like structure in the cross section of the membranes was observed with a Sirion FEG-SEM (FEI, USA).

The hydrophilicity of the membrane surface was characterized on the basis of water contact angle measurement using a contact angle goniometer (OCA20, Dataphysics, Germany) equipped with video capture. A total of 5 μL ultrafiltrated water was dropped on the air-side surface of the membrane at 25 °C and the contact angle was measured after 10 s. At least 10 contact angles were averaged to get a reliable value.

2.6. Adhesion of blood platelets

A sample of 20 mL human fresh blood was taken by venipuncture of a sole healthy donor. The blood was mixed with trisodium citrate (one part to nine parts of blood) and centrifuged at 250*g* for 10 min to obtain platelet-rich plasma (PRP). The studied membranes ($1\times1\ cm^2$) were placed in a tissue culture plate, 20 μL fresh PRP was dropped on the membrane center and then incubated at 37 °C for 30 min. The membrane was rinsed gently with a phosphate buffered saline (PBS, pH 7.2), after which the adhered platelets were fixed with 2.5 wt% glutaraldehyde in PBS for 30 min. Finally, this sample was washed with PBS, and dehydrated with a series of ethanol/water mixtures of increasing ethanol concentration (30, 40, 50, 60, 70, 80, 90, 100% ethanol, 30 min in each mixture) [32]. At least five SEM photographs with magnification of 1000 were taken randomly from each sample surface after gold sputtering, and the amount of platelets adsorbed on unit membrane surface was counted. Mean value for each membrane with standard deviation is reported.

2.7. Macrophage adhesion

The murine macrophage suspension was prepared with the method reported previously [10]. The suspension was isolated from freshly killed mice using chloroform. The skin was sprayed with alcohol and the abdomen opened. A sample of 10 mL Roswell Park Memorial Institute (RPMI) 1640 containing 10% foetal bovine serum (FBS), 100 g/mL penicillin, and 100 μm/mL streptomycin was injected into the peritoneal cavity, and then the abdomen was gently massaged by fingers for 5 min. The peritoneum was carefully punctured, and then the washings were removed by a sterile pipette and placed in a sterile container to be centrifuged at 1000 rpm for 10 min to collect the macrophages. The macrophages obtained were grown in RPMI 1640 to obtain the macrophage suspension in which the cell concentration was 1×10^6 cells/mL.

The membrane ($1\times1\ cm^2$) was cleaned sequentially in an ultrasonic bath of ethanol solution for 10 min and rinsed in PBS. Then the sample was immersed in physiological saline (pH 7.4) to recondition for several hours. The cell suspension was inoculated on the surface of the membrane to assess the cell attachment. The incubation period was 48 h for the cell attachment test in a humidified atmosphere of 5% CO_2 in air at 37 °C. Then the supernatant was removed, and the membrane was washed cautiously five times using PBS prior to fixation. The adherent cell density on the films was quantified on the basis of measurements obtained visually from at least five randomly selected fields ($0.24\times0.36\ mm^2$) using an Olympus TE300 phase contrast optical microscope. Average value for each membrane with standard deviation is reported.

3. Results and discussion

3.1. Fabrication and characterization of the membranes

Fig. 2 shows the typical XPS spectra of PANCMA07 (a), PANCMA07-heparin (b), and PANCMA07-insulin (c) membrane surfaces. It can be seen that the nascent PANCMA07 membrane showed three peaks corresponding to C_{1S}

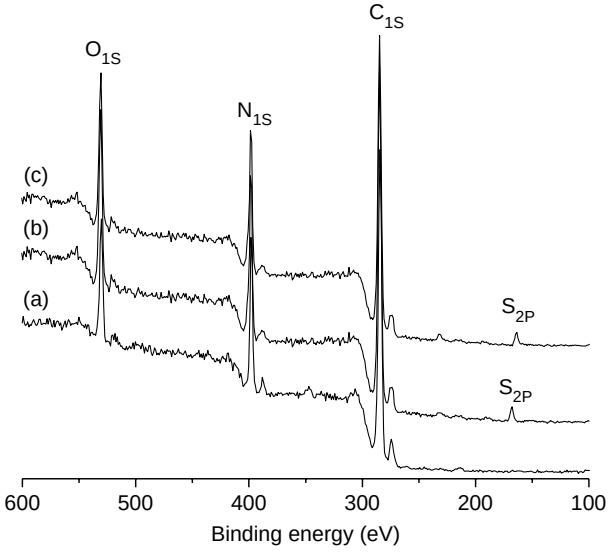

Fig. 2. XPS survey scan spectra of (a) the nascent, (b) the heparin-immobilized and (c) insulin-immobilized PANCMA07 membranes.

Table 1
Chemical composition of the nascent and heparin or insulin-immobilized PANCMA membranes calculated from XPS scan spectra

Membrane	Atom percent (mol%)			
	C	O	S	N
PANCMA07	76.05	10.35	0.00	13.60
PANCMA07-heparin	71.58	17.42	0.80	10.20
PANCMA07-insulin	68.50	14.82	1.20	15.48

(binding energy, 285 eV), N_{1S} (binding energy, 400 eV) and O_{1S} (binding energy, 532 eV), while the PANCMA07-heparin and the PANCMA04-insulin membranes had one additional peak attributed to S_{2P} (binding energy, around 165 eV). The chemical composition of the surface-modified PANCMA07 membranes calculated from the XPS spectra is shown in Table 1. The oxygen content (10.35 mol%) of the PANCMA07 membrane surface was increased by the heparin immobilization (17.42 mol%) and also by the insulin immobilization (14.82 mol%). In addition, the sulfur content of the PANCMA07-heparin and PANCMA07-insulin membranes increased obviously from 0 to 0.80 mol% and 1.20 mol%, respectively.

Fig. 3 shows the C_{1S} core level scan spectra of the PANCMA07 (a), PANCMA07-heparin (b), and

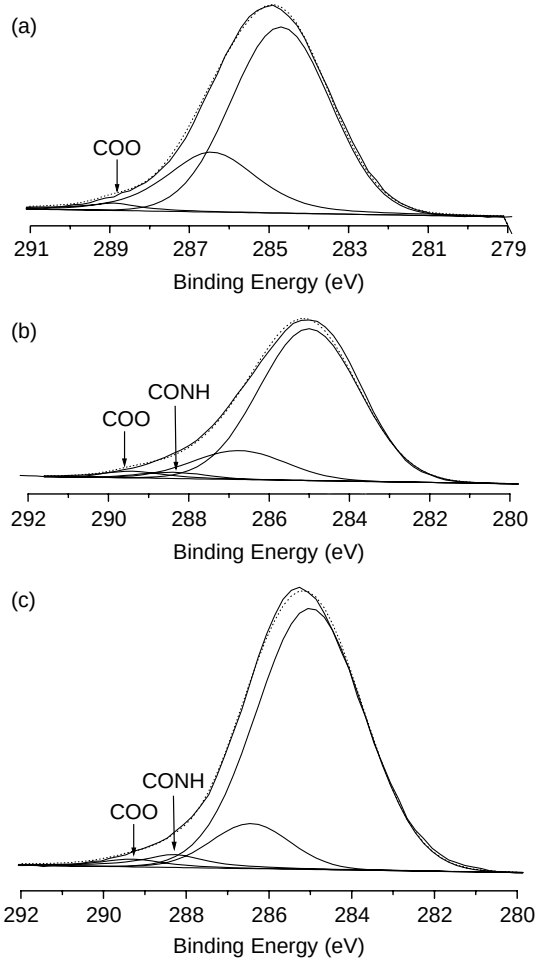

Fig. 3. XPS C_{1S} core level spectra of (a) the nascent, (b) heparin-immobilized and (c) insulin-immobilized PANCMA07 membranes.

Table 2
Percentage contribution of XPS C_{1S} components for heparin or insulin-immobilized PANCMA membranes

Membrane	Contribution of C_{1S} components			
	C–C (284.7 eV)	C≡N (286.4 eV)	CONH (288.1 eV)	COO (289.1 eV)
PANCMA07	72.25	25.13	0	2.62
PANCMA07-heparin	80.59	14.55	2.66	2.18
PANCMA07-insulin	81.3	13.22	3.48	1.95

PANCMA07-insulin (c) membrane surfaces. It seems that the spectrum of PANCAM07 membrane could be resolved into three peaks corresponding to C–C (binding energy, 284.7 eV), C≡N (binding energy, 286.4 eV) and COO (binding energy, 289.1 eV), while the spectra of PANCMA07-heparin and PANCMA07-insulin membranes showed one new peak attributed to CONH (binding energy, 288.1 eV). The percentage contribution of the C_{1S} components on the surface-modified PANCMA07 membranes, calculated from the C_{1S} core level spectra (Fig. 3), is shown in Table 2. It is clearly that, after the immobilization of the biomacromolecule, the peak area at 288.1 eV (CONH) increased, while the peak area at 289.1 eV (COO) decreased due to the surface covering by the immobilized biomacromolecule. This result suggested that heparin or insulin could be successfully immobilized onto the PANCMA membranes.

Morphological changes for the nascent and the biomacromolecule-immobilized PANCMA membranes were examined with SEM. Typical pictures are shown in Fig. 4. It can be seen that, because the reaction temperatures of the modification processes were far below from the T_g of PAN (105–115 °C), despite a slight decrease of pore size in the sponge bulk of the membranes, no significant morphological changes were observed on the PANCMA membrane surface and in the bulk.

Water contact angle (WCA) has been commonly used to determine the relative hydrophilicity or hydrophobicity of the membrane surface. For membranes with comparable structures, relatively low WCA value normally means high hydrophilicity. Static water contact angles for the studied membranes are summarized in Table 3. Results indicated that the PANCMA04 membrane had a maximum WCA value (48.2°), and the values of all PANCMA and corresponding modified membranes decreased with the content of maleic acid in the PANCMA copolymer increasing from 3.69 to 11.45 mol%. Furthermore, for the same PANCMA membrane, the WCA value on the heparin-modified membrane was lower than those of the nascent and the insulin-modified PANCMA membranes. Results of WCA demonstrated that the surface of PANCMA membrane became more hydrophilic after immobilizing the chosen biomacromolecules such as heparin or insulin onto the membrane surface.

3.2. Surface biocompatibility of the membranes

When a foreign material contacts with blood, proteins are adsorbed instantaneously on the material surfaces and deformed, then platelets are adsorbed, activated, and

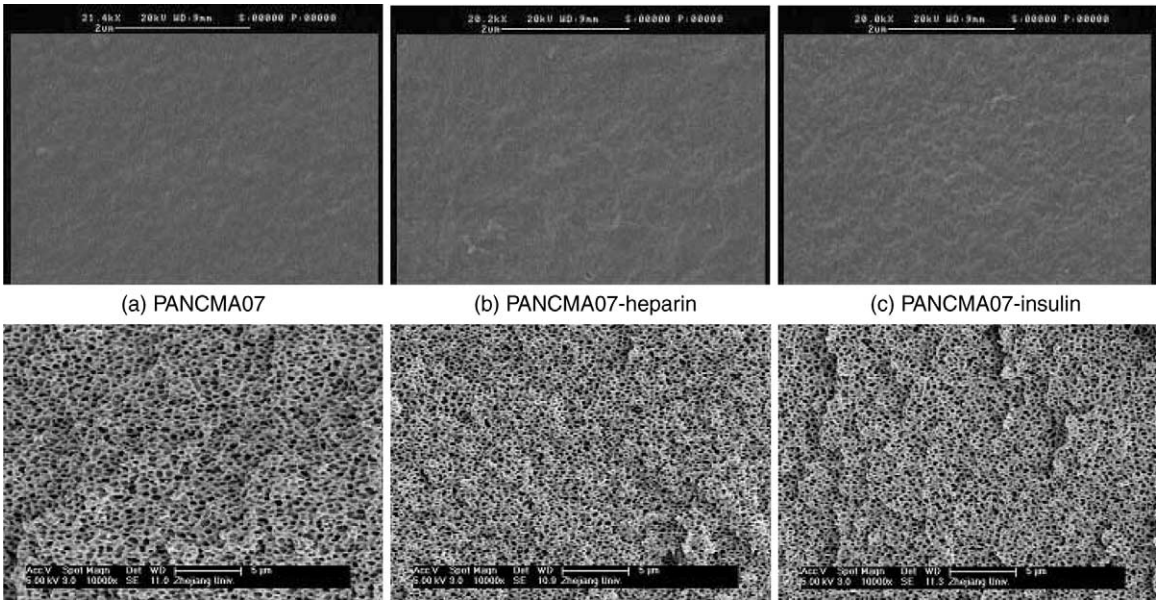

Fig. 4. SEM images of (a) the nascent, (b) the heparin-immobilized and (c) insulin-immobilized PANCMA07 membranes. Up, surface; Down, cross section.

aggregated so that platelets play a major role in the thrombus formation. Therefore, a study on platelets adhesion to evaluate the hemocompatibility of the membrane surface is important for a separation membrane applied in biomedical fields. Fig. 5 shows the platelet number adhered on the nascent and the modified PANCMA membrane surfaces from platelets-rich plasma (PRP). It was found that many platelets adhered on the nascent PANCMA membrane surfaces, while all biomacromolecule-immobilized membranes showed a sharp decline of platelets adhesion. Moreover, with the content of maleic acid in the PANCMA increased, platelets adhered on both the nascent and the biomacromolecule-immobilized membranes decreased significantly. The platelets adhered on the PANCMA-heparin membrane was less than the corresponding PANCMA-insulin membrane. Therefore, macrophage adhesion on the PANCMA-heparin membranes was further studied to evaluate the surface hemocompatibility. It is well known that macrophage is a kind of immune cell and performs various functions such as migration, phagocytosis, secretion, antigen presentation, and survival through precisely modulated adhesion, in living bodies. However, the molecular mechanism in macrophage adhesion is complex, dynamic, and not yet fully understood. Generally speaking, the fewer amount of macrophage adhered onto the material surface indicates the better hemocompatibility for the material, which is to say that the immunological reaction or immunological rejection will decrease after the material is planted into the living body. Results for macrophages adhesion on the nascent and heparin-modified membrane surfaces are shown in Fig. 6. It clearly demonstrated that with the immobilization of biomacromolecule heparin, the number of macrophages adhered on the modified membrane decreased remarkably. Moreover, with the content of maleic acid increased, the number of macrophages adhered on all PANCMA and PANCMA-heparin membranes decreased gradually, which indicated that the increase of maleic acid content was beneficial for increasing the immobilization density of heparin on the membrane surface and induced the reduction of macrophage adhesion. All these results confirmed that the hemocompatibility of the PANCMA membranes was improved highly by immobilizing biomacromolecules on the membrane surface.

Table 3
Water contact angle of the nascent and heparinor insulin-immobilized PANCMA membranes

Membrane	Contact angle (°)
PANCMA04	48.2±0.2
PANCMA07	42.4±0.3
PANCMA11	37.1±0.2
PANCMA04-heparin	33.1±0.3
PANCMA07-heparin	27.5±0.1
PANCMA11-heparin	24.3±0.1
PANCMA04-insulin	34.4±0.2
PANCMA07-insulin	30.5±0.3
PANCMA11-insulin	27.1±0.1

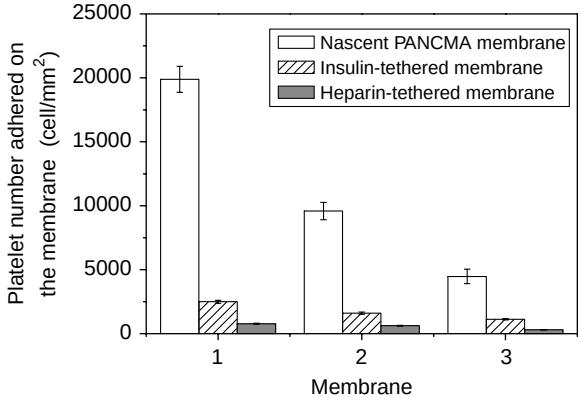

Fig. 5. Effect of the immobilization of heparin and or insulin on the adhesion of plasma platelet. (1) PANCMA04; (2) PANCMA07; (3) PANCMA11.

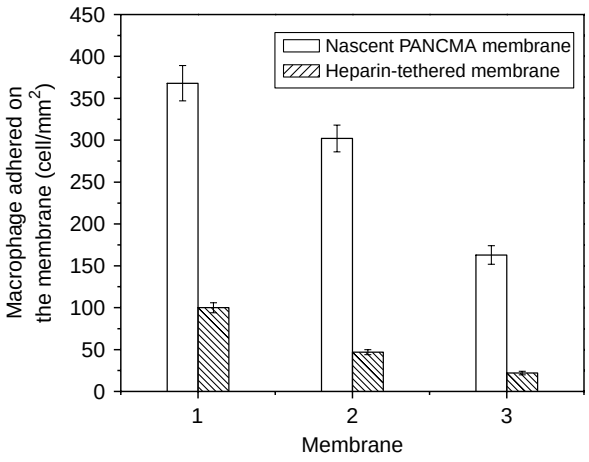

Fig. 6. Effect of the heparin immobilization on the adhesion of macrophage. (1) PANCMA04; (2) PANCMA07; (3) PANCMA11.

4. Conclusion

Two kind of biomacromolecules, heparin and insulin, were immobilized onto the PANCMA membranes via amidation reaction. XPS measurements confirmed the chemical changes on the membrane surface. Water contact angle measurement demonstrated that the hydrophilicity of the membranes was improved. The amount of platelets and macrophages adhering on the modified membranes decreased significantly when compared with those on the nascent PANCMA one. These results proved that the hydrophilicity and hemocompatibility of the PANCMA membranes could be effectively improved by adjusting the content of maleic acid in the PANCMA copolymer and immobilizing the biomacromolecules on the membrane surface.

Acknowledgement

Financial support from the National Nature Science Foundation of China (Grant no. 50273032) is gratefully acknowledged.

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