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Fabrication of thromboresistant multilayer thin film on plasma treated poly (vinyl chloride) surface

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Layer-by-layer deposited anticoagulant multilayer films were prepared on ammonia plasma treated poly (vinyl chloride) (PVC). Fourier transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR) and contact angle results revealed the presence of $-NH_2$ on the ammonia plasma treated PVC surfaces and the layer-by-layer self-assembly process. The stability of multilayer film was studied with the radio labeled method. The remainder bovine serum albumin (BSA) in cross-linked 5(heparin/BSA) multilayer films dipped in phosphate buffered saline (PBS, pH 7.4) was more than 90% in 40 days. The static platelet adhesion result indicated the anticoagulant multilayer films deposited on the plasma treated PVC reduced platelet adhesion drastically and no thrombus forming. The plasma recalcification time revealed that the multilayer modified surfaces greatly prolonged the plasma recalcification time. Such an easy processing and shape-independent method may have good potential for surface modification of cardiovascular devices.

1. Introduction

Poly (vinyl chloride) (PVC) is one of the most widely used polymers in biomedical devices such as blood bags, blood tubing, catheters, and inhalation masks, which have contributed significantly to the quality and effectiveness of the health care system [1, 2]. However, like other materials, PVC exposed to blood generally adsorbs a layer of proteins on their surfaces very rapidly. The composition of the protein layer varies with the surface properties of the materials and usually induces thrombosis and infection [3].

To minimize these interactions different methods for biomaterial surface modification have been attempted in order to obtain more biocompatible polymer materials. A lot of methods have been used to promote the thromboresistant surface of PVC, such as modification by coating or immobilization with heparin, immobilization of hirudin and coating with albumin [4–13].

In recently, the plasma modification technique was widely used in biomaterial surface modification [7, 8, 14–16]. The plasma surface modification is an effective and economical surface treatment technique and became a very active field. "Electrostatic self –assembly" (ESA) method, or layer-by-layer (LBL) method which is based on the alternating physisorption of oppositely charged polyelectrolytes, represents a new, alternative solution for biomaterial coating [17–19]. The buildup is easy and the procedure can be adapted to almost any type of surface, as long as surface charges are present. So, the plasma modification technique could provide charged surface for LBL method and the alternating physisorption of polyelectrolytes generate a dense polymer layer and functional group on such modified surface and eliminate the effect of the bulk material surface.

Herein we investigated the stepwise deposition of anticoagulant using heparin and BSA after ammonia plasma treatment with LBL method. Heparin is a highly sulfonated anionic polysaccharide and the polyampholyte albumin is a positively charged protein at pH 3.9 [18, 19]. So at this pH, heparin/BSA multiplayer films could be prepared. The multilayer films forming by LBL method that will serve as a buffer between the blood stream and the surface of an artificial organ and could completely screen the surface of materials but preserve the bulk properties [20, 21].

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2. Experiments

2.1. Substrate preparation

The matrix materials in our study for deposition multilayer thin film originated from a commercially available unplasticized polyvinyl chloride (UPPVC) (Hydro Polymers Ltd., UK). Heparin, sodium salt and Albumin bovine Fraction V were purchased from Shanghais Chemical Reagent Company of China.

PVC (10 w/w%) in tetrahydrofuran (THF) was cast on a glass culture dish, followed by drying the PVC specimen in a desiccator under vacuum with water suction pump for 10 h, then further drying at vacuum oven overnight to remove residual low molecular weight species (THF and H_2O).

2.2. Surface modification of PVC by ammonia plasma

Amino groups were introduced on cleaned PVC surfaces using ammonia plasma treatment. The detailed experimental technique of ammonia plasma generation and characterization of modified surfaces has been described in previous publications [22, 23]. In brief, a radio frequency (RF) plasma generator was coupled to a plasma reactor. Samples were placed in the plasma reactor downstream from the ammonia gas inlet. The plasma reactor was evacuated using two vacuum pumps. Plasma treatment of substrates was carried out at 40–50 Pa; this pressure was maintained by a constant in-flow of ammonia into the reactor. A pulsed RF power of 150 W was applied to the plasma reactor for 5 min. After returning the reactor to atmospheric pressure, the substrates with plasma-treated thin films were removed.

2.3. Multilayer film preparation

The films were formed by the successive adsorption of heparin and BSA from aqueous solution at pH 3.9 on the ammonia plasma treated PVC sheets. Firstly plasma treated PVC sheets were protonized with hydrochloride aqueous solution pH 2, secondly dipped into 2 mg/ml heparin aqueous solution for 15 min, followed by rinsed with water (pH 3.9) and then dipped in 2 mg/ml BSA aqueous solution for another 15 min rinsed with water (pH 3.9). Repeating this procedure, until desired layer numbers. All the dipping conditions and rinsing are at pH 3.9.

The multilayer films for stability and *in vitro* static blood-compatibility tests were cross-linked with glutaraldehyde solution: The desired multilayer films were dipped in 1% glutaraldehyde in PBS (pH 7.4) for 30 min, and then thoroughly washed with de-ionized water.

Finally the multilayer film was dried with a stream of nitrogen, and further removed the residue of water at vacuum oven for overnight at room temperature.

2.4. Attenuated total reflectance (ATR) infrared spectra

Attenuated total reflectance (ATR) infrared spectra were obtained with a Bruker Vector 22 spectrometer using a germanium ATR element supported on an ATR accessory. The sample was placed against the ATR element, and the spectra were collected in the range 1000–4000 cm^{-1} with a resolution of 1 cm^{-1}.

2.5. Contact angle measurements

The sessile method of contact angle measurement was carried out on a KRUSS DSA 10-MK2 goniometer on sheet-samples. The tested PVC sheet samples were dried at vacuum oven overnight before measurement. A water drop was made on the tip of a syringe and placed on a sample by moving the substrate vertically until contact was made between the water drop and the sample.

2.6. Stability of multilayer film

The stability of mutilayer coating was investigated by ^{125}I labeled technique. BSA was labeled using the iodo-gen method as described in references [24, 25]. Purification of the labeled protein was performed using Sephadex G-25 M columns (PD-10, Amersham Pharmacia biotech). The yield of iodination reaction was 93.8% for BSA determined by precipitating the ^{125}I-labelled protein with 20% trichloro acetic acid (TCA, Merck). ^{125}I-labelled protein was added to unlabelled protein solution in order to obtain a final activity of $\sim 10^7$ cpm/mg. The ESA mutilayer of heparin/BSA was prepared as above, except that ^{125}I-labelled albumin was used. The amount of adsorbed BSA (Γ, $\mu g/cm^2$) was calculated by dividing the surface radioactivity (cpm) by the specific radioactivity of BSA solution (K, cpm/μg) and the surface area (A, cm^2) as follows.

$$\Gamma = \frac{CPM}{2KA}$$

The PVC sheets coated by mutilayer of heparin/^{125}I-BSA cross-linked with glutaraldehyde were kept in PBS solution. The PBS solution was detracted to test the radioactivity every 5 days. The amount of protein fall from mutilayer coating was therefore monitored.

At least five experiments were conducted for each set of conditions. Standard deviation based on all measurements considered being at steady-state range from 10% of the mean at low surface concentration to 5% at high surface concentration.

2.7. *In vitro* test of static blood-compatibility

2.7.1. In vitro static platelet adhesion test

At the room temperature, 20 μl of fresh platelet rich plasma (PRT) was dropped onto the sample's surface. It was kept in touch with the surface for 30 min, then the treated samples were gentle rinsed in PBS and immobilized in 1% solution of glutaraldehyde for 15 min, leached with tri-distilled water several times, the samples were dehydrated with gradient solution of alcohol/water. The blood-contacting surfaces were finally analyzed by scanning electron micrograph (SEM, stereo Scan 600). The number of

adherent platelets was estimated from the SEM images. Eight different fields were randomly counted for each sample.

2.7.2. Plasma recalcification time (PRT)

PVC (2 w/w%) in tetrahydrofuran (THF) was vertically dropped into the glass tube (10 × 75 mm), the drying, the multilayer film preparation and the mutlilayer film cross-linked process as the same to the process of preparation PVC sheet, multilayer films and mutlilayer film cross-linked on the flat sheets as described above.

The fresh human plasma, from which the Ca^{2+} was removed, and the solution of $CaCl_2$, 0.025 mol/l, were warmed up to 37 °C.

1 ml warmed plasma was taken into the tested tube incubated for 1 min, then 1 ml warmed $CaCl_2$ solution was added into the tube, at the same time, we began to stir the recalcified plasma with a small stainless steel hook and started to time the process until the silky fibrin appeared. The time was recorded as PRT.

3. Results and discussion

Water contact angle analysis was used to characterize the surface hydrophilicity of control and ammonia plasma treated PVC. The contact angle decreasing from 91.6 ± 1.3 to 47.6 ± 1.3 after ammonia plasma treatment indicated the hydrophilic group was introduced onto the PVC surface. FTIR-ATR analysis indicated the presence of specific functional groups on the plasma treated surface. The control PVC surface showed no peaks in the amine region (1700–1500 cm^{-1}) and –NH stretching vibrations (3500–3200 cm^{-1}), but broad shoulders appeared in this two regions of the plasma treated PVC as show in Fig. 1. The peaks at 1700–1500 cm^{-1} indicated the presence of primary ammine and the 3500–3200 cm^{-1} indicated the presence of –NH. The plasma treated PVC sheets were dipping in hydrochloric acid aqueous solution (pH 2) for 5 min, and then the $–NH_2$ on the PVC surfaces were protonized, followed by alternative adsorption of heparin and albumin from aqueous solution of pH 3.9 as was shown in Scheme 1. Each cycle consists of heparin or albumin adsorption and rinsing with water of pH 3.9.

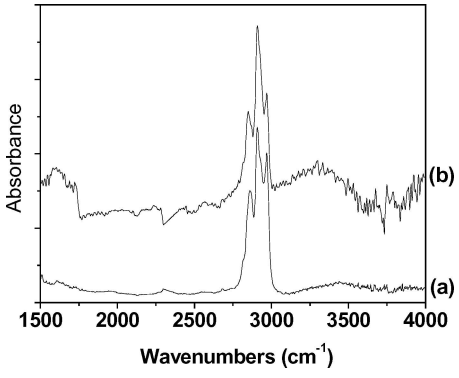
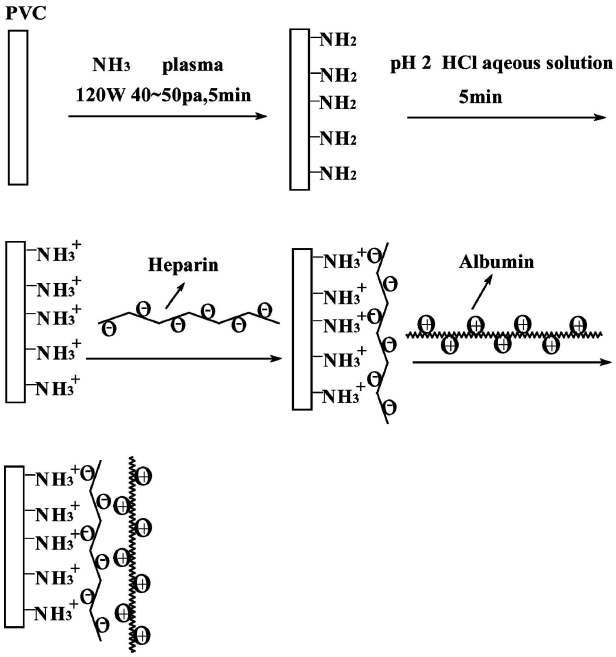


Figure 1 FTIR-ATR spectra of control PVC (a) and ammonia plasma treated PVC (b).



Scheme 1 The ammonia plasma treated PVC and the subsequent layer-by-layer deposition of heparin and BSA process.

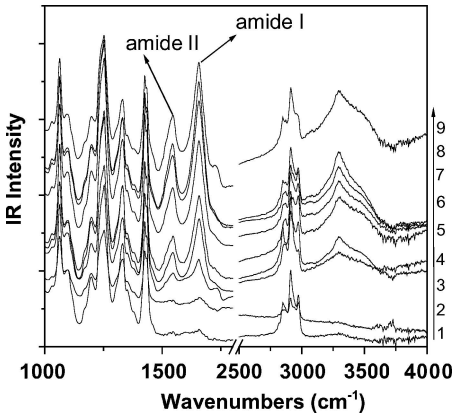


Figure 2 ATR-FTIR spectra of heparin/BSA multilayer film as a function of the number of bilayer depositions.

The ATR-FT-IR spectra of multilayer films deposition of different heparin/BSA bilayer are shown in Fig. 2. The spectrum exhibited the absorption bands at 1546 cm^{-1}, 1656 cm^{-1} which correspond the amide II and amide I respectively. This indicated the albumin was deposited onto the ammonia plasma treated PVC surface. In addition the –NH stretching vibrations (3500–3300 cm^{-1}) also the evident on the multilayer film deposited PVC sheets. Absorption peak intensities had showed an increase with the increase of the bilayer number in multilayer films. The ratios of amide I/–CH_2– (1425 cm^{-1}) and amide II/–CH_2– (1425 cm^{-1}) change with different bilayer numbers are showed in Fig. 3. As seen from the Fig. 3, the ratios almost linearly increase with the bilayer number; this indicated the albumin increasing with alternating adsorption heparin and BSA cycle.

The assembly of heparin and BSA multilayer film was characterized via contact angle. Fig. 4 shows the static contact angle with pure water measured in atmospheric air at constant temperature (20 °C) and constant

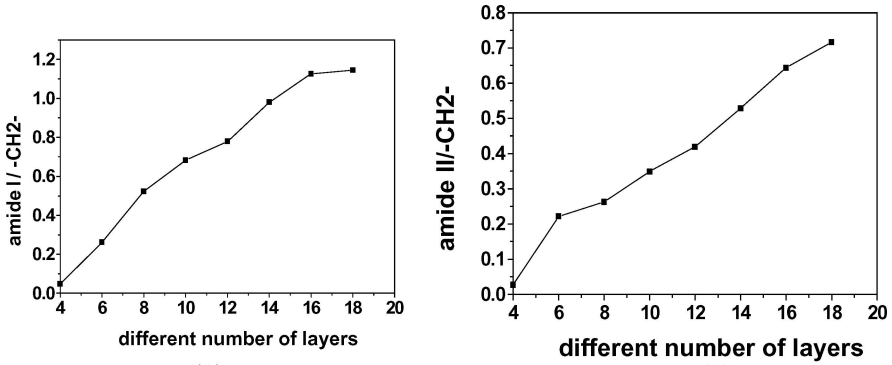


Figure 3 Ratios of ATR-FTIR data: (A) (amide I)/(methylene groups, -CH_2-) and (B) (amide II)/(methylene groups, -CH_2-). The samples are deposited different heparin/BSA bilayer on ammonia plasma treated PVC, respectively.

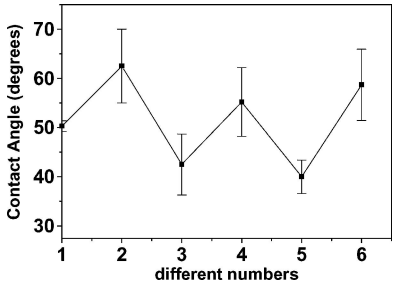


Figure 4 Contact angle measured from films containing a different number of adsorbed layers of heparin/BSA. Even numbers represent films with heparin as the outmost layer and odd numbers represent films with BSA as the outmost layer.

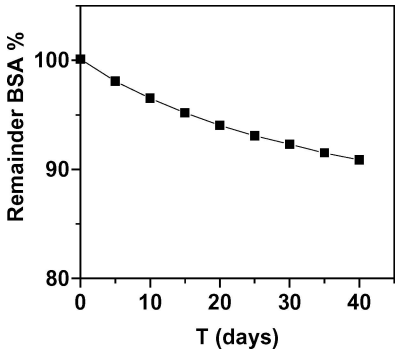


Figure 5 The remainder BSA in 5(heparin/BSA) multilayer films dipped in PBS (pH 7.4) for 40 days.

humidity at each assembly step. The angle was alternately changed, also confirming stepwise heparin and BSA assembly. At the first layer of heparin step, the angle was 50.3 ± 1.1, when the heparin/BSA bilayer film formed on the plasma treated PVC the contact angle was determined to be 62.5 ± 7.5. Following the heparin and BSA adsorption cycles the contact angle of the film fluctuates periodically. The obvious alternate change of contact angle data verified the LBL buildup of the film by alternate deposition of heparin and BSA.

As albumin is polyampholyte (*p*I 4.9), when heparin/BSA multilayer films assembled at pH 3.9 contact with high pH environment, the charge in albumin perhaps will reverse and lead to the multilayer film unstable, so we used glutaraldehyde cross-link heparin/BSA multilayer films. The stability of mutlilayer films was studied with I^{125} radiolabeled BSA. As was seen from the Fig. 5 the remainder of BSA in cross-linked 5(heparin/BSA) multilayer films was gradually decreasing, but the remainder of BSA in the multilayer films was about 90% in PBS for 40 days. This indicated the multilayer films were stable and only very small amount of BSA was desorbed in 40 days.

The platelet reactivity was evaluated here on the basis of the number of platelet and the thrombus. Fig. 6 shows the typical scanning electron micrographs of the adherent platelets on control, plasma treated and heparin/BSA multilayer modified PVC. The adhesion platelets were aggregated on control and plasma treated PVC, while the aggregation of the platelets was suppressed on heparin/BSA multilayer modified PVC sheets.

The control PVC sheet adsorbed a high number of platelets (Fig. 7). The number of platelets adhering to plasma treated PVC sheet was slightly increased. Whereas the number of platelets adhering to the heparin and BSA deposited multilayer films on plasma treated PVC was decreased drastically and the number of adhered platelets is negligible on the 5(heparin/BSA) multilayer films. The LBL deposited mutilayer films demonstrated suppressed platelet aggregation and less platelet adhesion when compared with the control and plasma treated PVC.

The plasma recalcification time test (Fig. 8) indicated that the clotting time of the ammonia plasma treated PVC was prolonged slightly, whereas no clots form was found at the 4(heparin/BSA) + heparin multilayer films in 20 min whether cross-linked with glutaraldehyde or not. The plasma recalcification time on glutaraldehyde cross-linking 5(heparin/BSA) multilayer film is prolonged to 402 ± 18 seconds and no clot forming on the uncross-linked 5(heparin/BSA) multilayer films. The better anti-clot of uncross-linked 5(heparin/BSA) multilayers may due to the heparin release from multilayer coating. However, when the heparin was the outmost layer, the stable cross-linked multilayer has the same anti-clot property as well. The platelet adherent and the plasma recalcification time tests indicated that the LBL deposited multilayer films on plasma treated PVC improved the blood-compatibility of PVC.

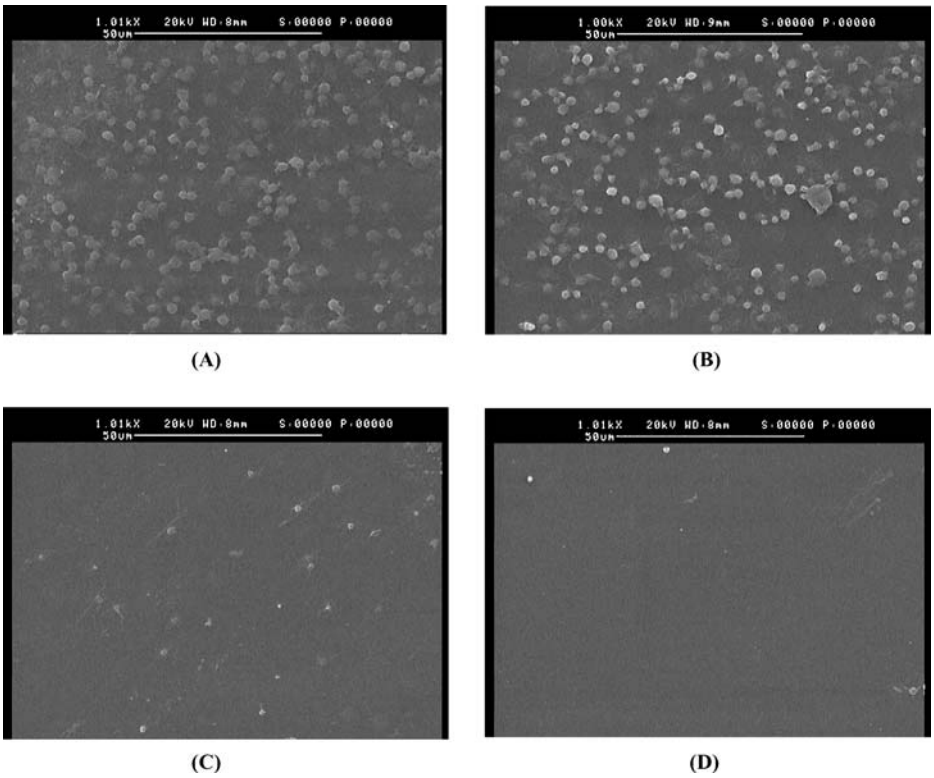


Figure 6 Scanning electron micrographs of different materials surface following PRP perfusions: control PVC (A), ammonia plasma treated PVC (B), 4(heparin/BSA) + heparin deposited multilayer film (C) and 5(heparin/BSA) deposited multilayer film (D).

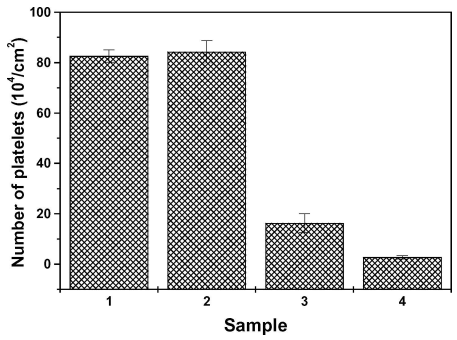


Figure 7 The adhesion number of platelet on control PVC (1), ammonia plasma treated PVC (2), 4(heparin/BSA) + heparin deposited multilayer film (3) and 5(heparin/BSA) deposited multilayer film (4). The error bars represent standard deviation ($n = 8$).

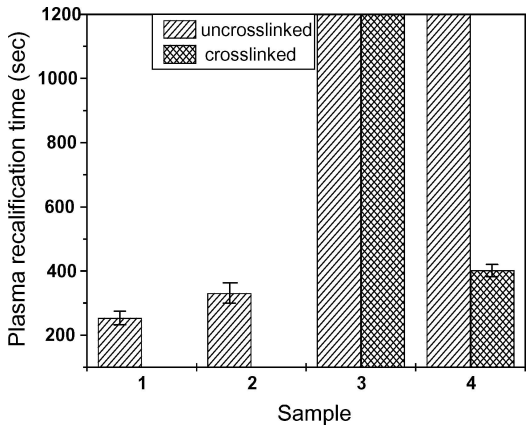


Figure 8 Data of plasma recalcification time (PRT). The samples are, respectively, control PVC 1, ammonia plasma treated PVC (2), 4(heparin/BSA) + heparin deposited multilayer film (3) and 5(heparin/BSA) deposited multilayer film (4). The error bars represent standard deviation ($n = 5$).

4. Conclusions

Electrostatic self-assembly of heparin/BSA are explored to construct thromboresistant multilayer film onto ammonia plasma treated biomedical PVC. The ATR-FTIR spectra revealed that BSA was increasingly deposited onto the plasma treated PVC with the number of bilayer heparin and BSA. The cycle change of the contact angle data indicated the layer-by-layer buildup of the film by alternative deposition of the heparin and BSA. The radio labeled test show the cross-linked mutilayer films are stable in PBS for 40 days. The result of the *in vitro* test of static hemo-compatibility indicated that heparin and BSA multilayer deposited on the plasma treated PVC reduced the platelet adhesion drastically, suppressed the platelets aggregation and greatly prolonged the plasma recalification time.

All above results demonstrated that electrostatic self-assembly of albumin/heparin can construct non-thrombogenic coating on plasma treated PVC. This "Electrostatic self-assembly" (ESA) method, which is based on the alternating physisorption of oppositely charged polyelectrolyte, represents a new, alternative solution for biomedical PVC surface modification. The buildup is easy and valid whatever the shape of the solid. These distinct advantages over the traditional methods of surface modification may be important for surface treating PVC intravascular device, such as tube and catheter etc.

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