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Original Article

Sodium alginate/heparin composites on PVC surfaces inhibit the thrombosis and platelet adhesion: applications in cardiac surgery

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Abstract: Thrombosis and hemocyte damage are the main problems of applied non-coated biomaterials to cardiac surgery that remain unsolved. The present study is aimed at the chemical modification of polyvinyl chloride (PVC) for applications in cardiac surgery and the biological property assessment of modified PVC. Sodium alginate (SA)/heparin (HEP) composites were covalently immobilized onto the surface of the PVC pipeline. The surface grafting density and protein adsorption were determined by ultraviolet spectrophotometry. The surface contact angles were evaluated by contact-angle measurement, whereas the surface characteristics were evaluated by Fourier-transform infrared spectroscopy. Blood coagulation time and platelet adhesion were measured using an automated blood coagulation analyzer and a hemocytometer, respectively. Surface morphologies of the thrombus and platelets were evaluated by scanning electron microscopy. The immobilization of SA/HEP reduced the contact angles of the coated surface. Protein adsorption was reduced by the immobilization of SA. The activated partial thrombin time and thrombin time of the coated PVC were significantly prolonged as compared with the non-coated PVC. Platelet adhesion and thrombus formation were all reduced by the immobilization of HEP. The results revealed that the SA/HEP coating can improve the antithrombogenicity of the PVC pipeline, as well as improve its biocompatibility and hemocompatibility, which are essential for cardiac pulmonary bypass surgery.

Keywords: Polyvinyl chloride (PVC), sodium alginate (SA), heparin (HEP), hemocompatibility, biocompatibility

Introduction

Cardiac pulmonary bypass (CPB) is an important clinical procedure in heart surgery and heart assistance. The central component of the CPB machine is a polyvinyl chloride (PVC) pipeline, which allows for blood transport. When the PVC surfaces are exposed to blood, the plasma proteins are adsorbed, which is followed by the activation of clotting factors and the adhesion of platelets [1-3], prior to the formation of non-soluble fibrin network [4]. Several host defense mechanisms are subsequently activated, the most evident being the hemostatic mechanism that causes thrombus formation, occlusion of medical devices, and embolization. Systemic anticoagulation, usually via the intravenous administration of heparin (HEP), counteracts the complications related to coagulation. Unfortunately, HEP may induce side effects such as hemorrhage, HEP-induced thrombocytopenia (HIT) [5], difficulty in breathing, closure of the throat, and swelling of the lips as well as less serious symptoms such as mild pain, increased body temperature, and hair loss. Meanwhile, the anticoagulant response to HEP is unpredictable because the HEP-antithrombin complex is unable to inhibit fibrin-bound thrombin [6].

Both HEP and sodium alginate (SA) are polysaccharides. HEP catalytically increases the formation rate of antithrombin III (AT III) as well as inhibits thrombin and other coagulating proteases, thereby proving its effectiveness in curtailing thrombosis when it is immobilized onto polymer surfaces [7, 8]. SA contains mannuronic (M) and glucuronic (G) groups [14], with prothrombic G-groups and anticoagulant M-groups [15]. SA has been used to develop skin substi-

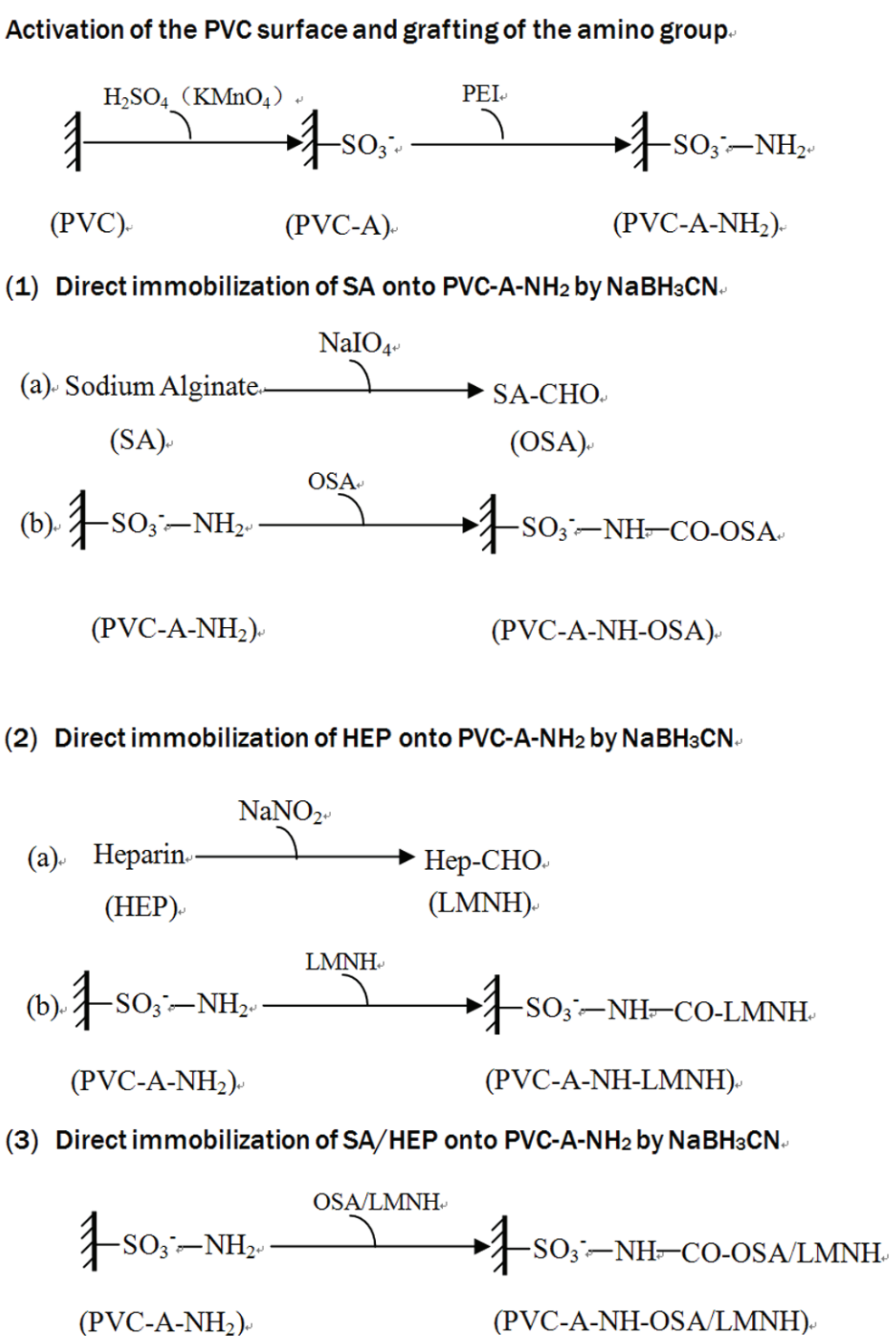

Figure 1. Chemical scheme of the coated PVC pipeline. Direct immobilization of (1) SA and (2) HEP; (3) Immobilization of composite SA/HEP.

tutes and wound dressing materials [9] as a matrix for the immobilization of enzymes and cells [10], as well as a vehicle for drug and gene delivery [11, 12]. The immobilization of nega-

tively charged sulfated polysaccharides allows surfaces to become more biocompatible because of the electrostatic repulsion of negatively charged components of blood. Furthermore, the increased wettability caused by the presence of negatively charged groups may diminish protein adsorption or make it more reversible [13]. Thus, sulfated polysaccharides should be investigated as attractive alternatives for HEP to decrease the thrombogenic activity and protein adsorption of artificial surfaces.

In this study, we modified a PVC pipeline to increase its biocompatibility and antithrombotic activity. The surface of the PVC pipeline was immobilized with a SA/HEP composite. The biocompatibility and anticoagulation activity of the SA/HEP-coated surface was compared with HEP-coated and SA-coated surfaces. The study revealed that chemical modification provides PVCs with important clinical value.

Materials and methods

Reagents

PVC pipelines (10 cm^2) were obtained from KE-Wei (Dong guan, China). SA, polyethyleneimine (PEI), and sodium periodate were procured from Sigma (St. Louis, USA). HEP (155 IU/mg) was supplied by Jin-xing Bio. (An-hui, China). Human serum albumin (HAS) was obtained from CSL Behring GmbH (Marburg, Germany), whereas human fibrinogen (HPF) was obtained from EMD Chemicals (San Diego, Germany). All other reagents were of analytical grade.

Surface modification

The PVC pipelines were cut into 10 cm^2 pieces and acidized with concentrated sulfuric acid (with 2 g/l potassium permanganate) to form new carboxyl groups on the PVC surface. The acidized PVC samples were rinsed with double-distilled water three times to remove the unreacted sulfuric acid. The carboxyl-bearing PVCs were immersed in PEI with multi-amino groups. The process of direct HEP coating is described in the chemical scheme in **Figure 1-(2)**. The process of direct SA coating is described in the chemical scheme in **Figure 1-(1)**. Using a different method, the SA/HEP-immobilized PVC surfaces were prepared according to the scheme in **Figure 1-(3)**. Amino-bearing PVC samples were immersed in SA/HEP solution (pH 3.5) containing NaBH3CN at 40 °C for 3 h. NaBH3CN was used to couple the polysaccharides with the amino-bearing PVC surfaces.

Determination of surface grafting density

The surface density of the SA-bearing aldehyde groups (OSA) and the HEP-bearing aldehyde groups (LMNH) were measured using the phenol sulfuric acid procedure and the toluidine blue O method, respectively. The dye concentration was determined using an ultraviolet spectrometer (UV-2800; Hitachi, Japan).

Modified PVC characterization

The functional groups on modified PVC pipelines were analyzed using Fourier-transform infrared spectroscopy (FTIR; NICOLET 6700; Thermo, USA). The SA and HEP standards were determined using the potassium bromide tablet mode (detector: DTGs KBr; beam splitter: KBr; wavelength range: 650 nm-4000 nm).

Protein adsorption measurements

The protein adsorption was measured for HAS and HPF using the bicinchoninic acid assay, as described by Ishihara et al. [16].

Blood coagulation time

Human whole blood from a healthy volunteer was collected and centrifuged at 800×g for 10 min at 4 °C to separate the blood corpuscles. The resulting platelet-rich plasma (PRP) was used for the platelet adhesion experiment. Subsequently, half PRP was centrifuged at 3000×g for 10 min at 4 °C to obtain the platelet-poor plasma (PPP) for the test of human plasma protein adsorption. A sample PVC pipeline (10 cm^2) was incubated in 0.5 ml of PPP at 37 °C for 1 h. The activated partial thrombin time (APTT), thrombin time (TT), prothrombin time (PT), and fibrinogen time (FT) of PPP were then determined using an automated blood coagulation analyzer (STA-R Evolution®; Diagnostica Stago, France).

Evaluation of platelet adhesion

The concentration of human PRP before adhesion (PLT1) was determined with a hemocytometer (ADVIA2120; Siemens, Germany). The PVC pipeline samples (0.5 cm × 0.5 cm; 10 cm^2) were washed three times with double-distilled

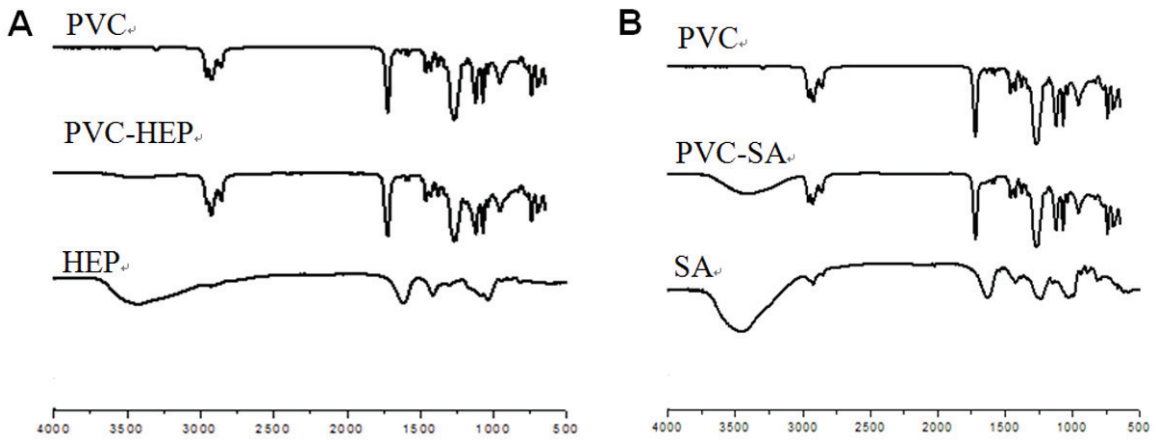

Figure 2. Qualitative analysis of immobilized HEP and immobilized SA. FTIR was used to detect the characteristic peak of uncoated PVC, HEP-coated, and SA-coated PVC, as well as the KBr tablets of HEP and SA. A. A strong and wide O-H adsorption peak appeared at 3352 cm-1 in the FITC of HEP and HEP-coated PVC, whereas the non-uncoated surface had no characteristic peaks. B. A characteristic absorption peak appeared at 3450 cm-1 in the FITC of SA and SA-coated surface.

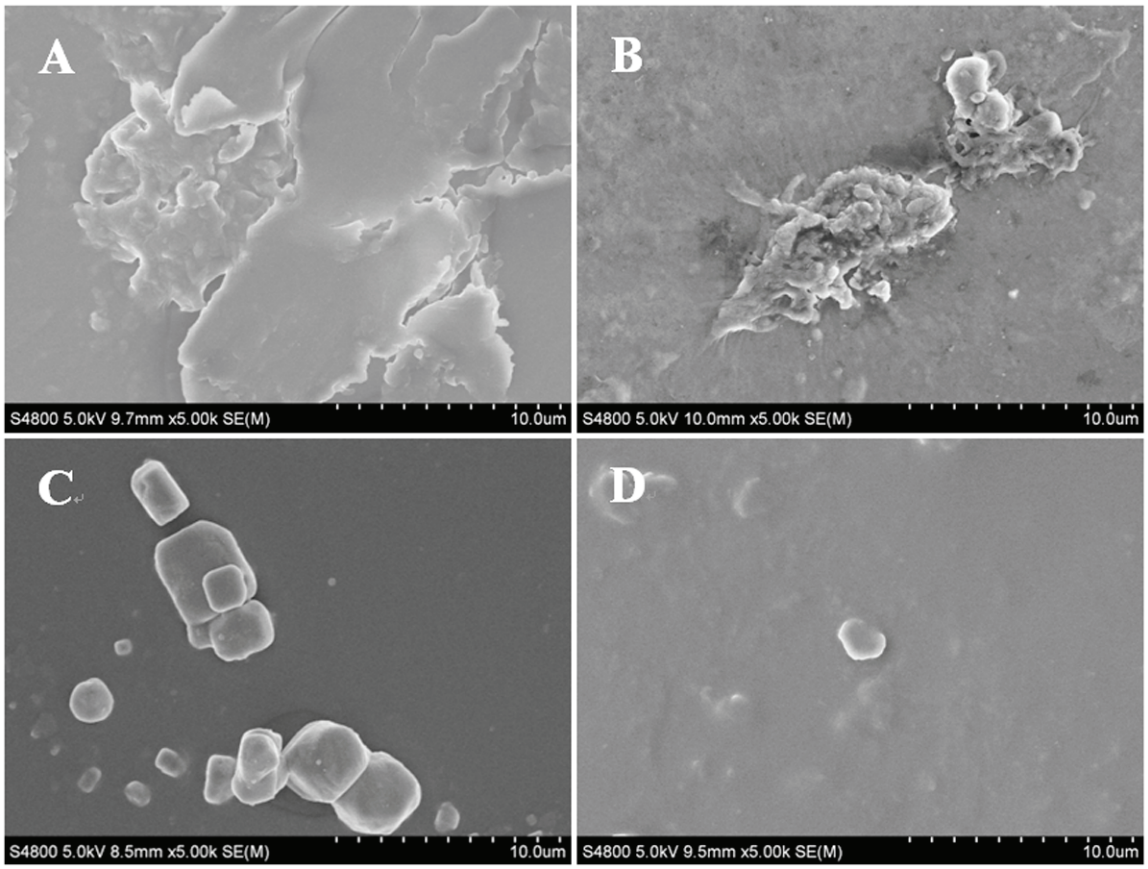

Figure 3. Platelet adhesion on different PVC surfaces: A. uncoated, (B) SA-coated, (C) HEP-coated, and (D) SA/HEP-coated. SEM (×5000) was used to detect the morphology of the PVC surface after platelet adhesion. Numerous platelets adhered to the uncoated surface (Figure A). Slightly less platelets adhered to the SA-coated and HEP-coated surfaces (Figure B and C, respectively). Much fewer platelets adhered to the SA/HEP-coated surface. SA and HEP improved the hemocompatibility of the PVC surface. SA/HEP coating can significantly inhibit platelet adhesion.

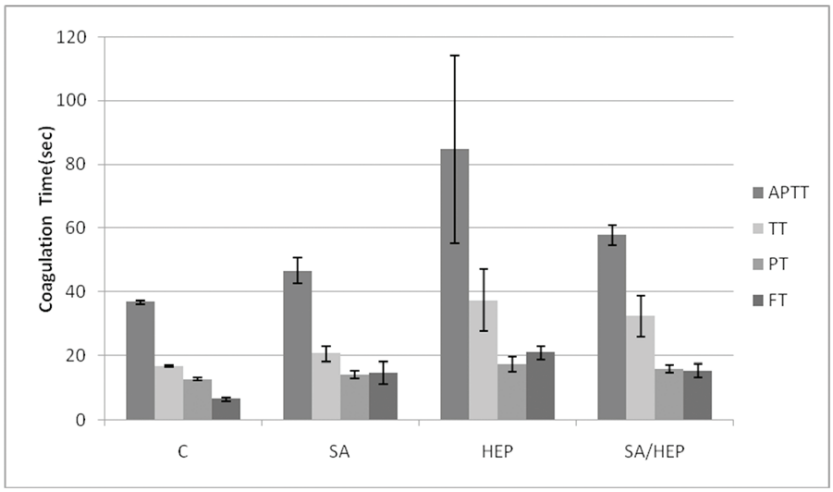

Figure 4. Comparison of anticoagulation time of different surfaces: (C) uncoated, (SA) SA-coated, (HEP) HEP-coated, and (SA/HEP) coated with the SA/HEP composite. The coagulation time of APTT, TT, PT, and FT (n = 6) were evaluated by an automated blood coagulation analyzer. APTT and TT of HEP-coated surface were significantly longer than those of non-coated surface. The SA-coated and SA/HEP-coated surface significantly prolonged the coagulation time of APTT and TT. Both HEP and SA demonstrated their anticoagulant properties by prolonging APTT and TT.

water and placed into 24-well culture plates incubated with human PRP (750 μl/well) in 5% CO_2 at 37 °C for 60 min. After which, the concentration of human PRP after adhesion (PLT2) was obtained.

Adhesion quantity = PLT1 - PLT2

Adhesion rate = (PLT1 - PLT2) / PLT1

The PVC pipeline samples were washed three times after incubation with PRP, and then fixed with 3% (w/v) GA solution in PBS for 2 h. The fixed samples were dehydrated with graded ethanol (50%, 60%, 70%, 80%, 90%, v/v; absolute alcohol) and dried using the critical-point procedure with CO_2. The platelet adhesion morphology was observed using SEM after metal spraying.

Thrombus formation

The PVC pipeline samples were washed thrice with double-distilled water and placed into 24-well culture plates with 1.5 ml human whole blood per well. After which, the samples were incubated in 5% CO_2 at 37 °C for 60 min, and then washed three times with PBS. The formed thrombus was fixed with 3% (w/v) GA solution in PBS for 1 h. The samples were dehydrated with graded ethanol and dried by the critical-point procedure with CO_2. The degree of thrombosis (DT) of the PVC pipeline at a given time was defined as follows:

DT = (W2 - W1) / W1,

where W1 and W2 are the weights of the dry PVC and blood coagulation samples, respectively.

Statistical methods

Statistical analysis was performed using SPSS (version 17.0). All quantitative data were expressed as the mean ± standard deviation. Comparisons among groups were performed using ANOVA (analysis of variance), whereas pairwise comparisons used the Student-Newman-Kuels (SNK) q test. Differences with $p < 0.05$ were considered statistically significant.

Results

Surface characterization

SA/HEP-coated PVC surface is provided with a characteristic peak. A strong and wide O–H adsorption peak appeared at 3352 cm-1 in the fluorescein isothiocyanate (FITC) labeling of the HEP tablet and HEP-coated surface (**Figure 2A**). Characteristic absorption peaks appeared at 3450 cm-1 in FITC labeling of the SA tablet and SA-coated surface (**Figure 2B**).

Surface grafting density

The SA and HEP grafting densities were measured by dye staining of phenol-sulfuric acid and toluidine blue O, respectively. Significantly more HEP was bound to the HEP-coated and SA/HEP-coated surface than to the uncoated surface. However, no significant differences were observed in the HEP density of the HEP-coated and SA/HEP-coated surfaces, with values of 3.12 ± 0.20 and 2.86 ± 0.27 μg/cm^2 HEP, respectively. Significantly more SA was bound to the SA-coated and SA/HEP-coated surfaces than to the uncoated surface. As

Table 1. Grafting density on different surfaces (mean ± SD)

Surface type	*n*	Sodium alginate[1]		Heparin[2]	
		μg	μg/cm^2	μg	μg/cm^2
C	6	2.22 ± 0.15	0.45 ± 0.03	1.01 ± 0.60	0.50 ± 0.30
SA	6	52.76 ± 0.62*	10.55 ± 0.12*	_	_
HEP	6	_	_	6.24 ± 0.41*	3.12 ± 0.20*
SA/HEP	6	38.18 ± 0.58*	7.64 ± 0.12*	5.72 ± 0.54*	2.86 ± 0.27*

F_1 = 10213.17, F_2 = 110.19, *p < 0.05.

Table 2. Plasma protein adsorption onto the PVC surfaces (mean ± SD)

PVC pipeline type	*n*	HAS adsorption[1] (μg/cm^2)	HPF adsorption[2] (μg/cm^2)
C	6	15.11 ± 0.64	23.66 ± 0.43
SA	6	3.49 ± 0.86*	9.24 ± 1.71*
HEP	6	13.88 ± 1.52	24.06 ± 0.42
SA/HEP	6	5.33 ± 0.94*	8.02 ± 2.93*

F_1 = 87.29, F_2 = 85.38, *p < 0.05.

Table 3. Platelet adhesion onto different PVC surfaces (mean ± SD)

Surface type	*n*	Adhesion[1]	Adhesion rate (%)[2]
C	6	49.67 ± 11.33	18.31 ± 4.18
SA	6	12.83 ± 2.83	4.73 ± 1.04*
HEP	6	1.17 ± 1.22*	0.43 ± 0.45*
SA/HEP	6	4.33 ± 2.44*	1.60 ± 0.90*

F_1 = 45.94, F_2 = 45.90, *p < 0.05.

Table 4. Thrombin formation of different surfaces (mean ± SD)

Group type	*n*	Adhesion weight (mg)[1]	DT (%)[2]
C	5	67.00 ± 3.2	7.08 ± 0.34
SA	5	48.94 ± 1.27*	5.17 ± 0.13*
HEP	5	45.32 ± 0.45*	4.79 ± 0.05*
SA/HEP	5	46.86 ± 1.63*	4.95 ± 0.17*

F_1 = 67.20, F_2 = 39.80, *p < 0.05.

shown in **Table 1**, slightly more SA was bound to the SA-coated surface than to the SA/HEP-coated surface.

Protein adsorption

Protein adsorption was measured by ultraviolet spectrometry from solutions containing various concentrations of purified HAS and HPF. The amount of protein adsorbed by the uncoated, SA-coated, HEP-coated, and SA/HEP-coated surfaces was depicted in **Table 2**, as a function of their concentration in the free solution. The two proteins that were adsorbed by the SA-coated surface were lower (HAS 3.49 ± 0.86, HPF 9.24 ± 1.71) than those adsorbed by the non-coated surface (HAS 15.11 ± 0.64, HPF 23.66 ± 0.43) with a significant difference. Slightly more proteins were adsorbed by the SA/HEP-coated surface than by the SA-coated surface. The HEP-coated surface adsorbed higher amounts of HAS and HPF than the SA-coated surface.

Platelet adhesion

The equilibrium platelet adhesion and activation on the surface was related to the protein adhesion. The maximum number of adhering platelets on the PVC surfaces after the 2 h incubation appeared on the uncoated surface (**Figure 3A**). The platelet adhesion on the HEP-coated and SA-coated surfaces slightly changed after the 2 h incubation, as shown in **Table 3** and **Figure 3**.

Coagulation time

The anticoagulant effects of HEP and SA can be observed by comparing the APTT, TT, PT, and FT of the uncoated surface with those of the HEP-coated, SA-coated, and SA/HEP-coated surfaces. Results showed the different clotting times of different surface types. The clotting times of the uncoated surface were nearly the same as those of the human plasma, whereas those of

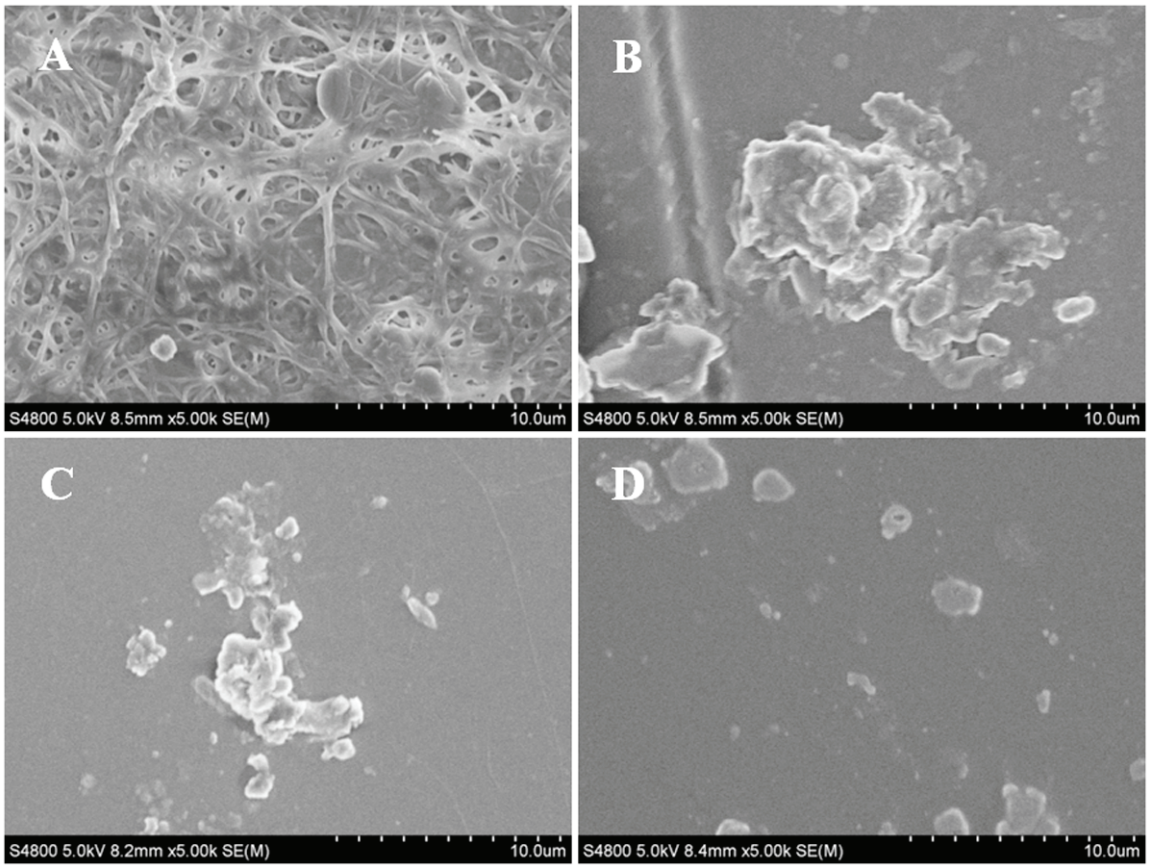

Figure 5. Thrombin formation of different surfaces: A. uncoated, (B) SA-coated, (C) HEP-coated, and (D) SA/HEP-coated. SEM (×5000) was used to detect the morphology of PVC surfaces after 2 hour incubation in fresh whole blood cells. The thrombin complex appeared on uncoated PVC (Figure A). Slightly less thrombin appeared on the SA-coated surface (Figure B). Much less thrombin appeared on the HEP-coated and SA/HEP-coated surfaces (Figure C and D, respectively). SA and HEP are both antithrombogenic, with the same trends as those of the platelet adhesion test.

the SA/HEP-coated surfaces were longer than those of the uncoated surfaces. The clotting times of HEP-coated surface were 50, 21, 5, 14 s (for APTT, TT, PT and FT, respectively), which were longer than those of the uncoated surface. The SA/HEP-coated surface prolonged the APTT, TT, PT and TT, such that the APTT and TT of the HEP-coated surface were almost twice that of the uncoated surface, as shown in **Figure 4**.

Thrombin formation

Thrombin formation is undesirable during CPB. Antithrombogenic materials have been of great interest in the development of cardiac surgery. The effect of surface modification on thrombin formation is shown in **Table 4** and **Figure 5**. The HEP-coated surface can reduce thrombin formation by about two-thirds as compared with the uncoated surface. The SA-coated surface can likewise significantly decrease thrombus formation. The SA/HEP-coated surface has slightly less thrombin formation than the SA-coated surface. Furthermore, thrombus formation increased with incubation time, which agreed with the results of Kang et al. [3].

Discussion

Thrombosis and hemocyte damage are the main problems of uncoated biomaterials that are applied to cardiac surgery. HEP, as an anticoagulant, has been used to prevent thrombosis during CPB. However, the systemic intravenous administration of HEP can cause serious side effects, including hemorrhage and HIT. Biomedical materials are broadly used in extracorporeal circulation, hemodialysis, and hemofiltration. Biocompatibility and hemocompatibil-

ity are the main problems during the application of synthetic biomedical materials [17], which may trigger the host defense mechanism when exposed to blood, thereby causing a cascade of thrombosis and thromboembolism. Coating techniques [18] can improve the biocompatibility and hemocompatibility of biomedical materials via the modification of the materials' surface. The anticoagulant properties of biomedical materials can be obtained by suppressing or preventing four pathways, including coagulation factor activation, platelet adhesion and aggregation, erythrocyte adhesion, and complement system activation. Several principles are used to design the coating materials, including protein adhesion resistance, decreased platelet adhesion and aggregation, inhibition of intrinsic coagulation factor activation, and inhibition of thrombosis. HEP, as an acid mucopolysaccharide, exerts its anticoagulant property by accelerating or increasing the activity of coagulation inhibiting factors, especially AT-III [19, 20]. The clinical application of HEP is mainly via intravenous injection, which is coupled with side effects [21] such as hemorrhage and thrombocytopenia. Moreover, intravenous HEP cannot facilitate continuous anticoagulation. A previous study indicated that HEP coating has poor biocompatibility and could not decrease protein adhesion [22]. SA, as a polyanionic macromolecule, is natural and non-toxic, with high biocompatibility and anticoagulant effects [20, 23, 24]. A composite coating with HEP and SA may improve the biocompatibility and hemocompatibility of biomedical materials.

The results in this study revealed that SA and HEP were constantly immobilized on the PVC surface by surface modification. The partial degradation of HP [25, 26] and SA [27, 28] with nitrous acid and sodium periodate gives rise to the fraction of molecules with highly reactive aldehyde terminal groups. The end-point bond is established between the aldehyde terminal groups and primary amino functions by reductive amination [29-31]. The surface density and stability of functional molecules are important because the enzyme inhibiting capacity may be associated with the surface density. The quantitative experiment showed that the coating density of SA or HEP has no significant influence on the single coating and composite coating. The surface contact angles are significantly decreased in the SA-coated and SA/HEP-coated surfaces as compared with the HEP-coated surface, thereby revealing that the SA coating can improve the hydrophilicity of the PVC surface. The nature of the adsorbed protein is believed to determine all adverse events that impair the use of biomaterials in medical devices: thrombus formation, platelet activation, initiation of coagulation [32-34], and activation of the complement system, which, in turn, causes leukocyte adhesion and activation [35]. The SA-coated surface can significantly decrease the level of protein adhesion. The SA/HEP-coated surface has slightly less protein adhesion than the HEP-coated surface. The protein adhesion study showed that the application of SA can improve the biocompatibility of the single HEP-coated surface and decrease the adverse effects.

Hemocompatibility is an important property of modified PVC. The partial degradation and covalent binding of HEP and SA could inevitably lead to the complete loss of antithrombin-binding. The ideal concept of SA and HEP binding includes an inherently stable covalent end-point bond of the molecules, thereby leaving the other parts of the molecule intact to react with the blood constituents [36]. The blood coagulation cascade includes intrinsic, extrinsic, and common pathways. APTT and PT are used to examine the intrinsic and common pathways, whereas FT is used to measure the time for transferring fibrinogen into fibrin [37, 38]. Results showed that the HEP coating can significantly prolong the coagulation time of APTT and TT by inhibiting the intrinsic coagulation pathway. The SA coating produced a slight anticoagulation effect by prolonging the APTT. The anticoagulation effect of SA/HEP is inferior to that of the single HEP coating, but the APTT and TT of SA/HEP are likewise significantly prolonged. The results revealed that composite coating demonstrated its anticoagulant property by inhibiting the intrinsic coagulation pathway. Furthermore, the fragmentation of SA and HEP did not destroy their functional molecules, and the binding procedure did not shield the anticoagulant molecules. The SA/HEP composite coating can decrease platelet adhesion and thrombosis, with the same trend as that of the coagulation time.

The immobilization of SA/HEP on PVC surfaces to incorporate endothelium-like antithrombogenic properties appears to be based on sound

rationale. The ideal density and stability of the SA/HEP coating may mimic the function of natural endothelial cells and express continuous anticoagulant properties.

Conclusion

The immobilized SA/HEP composite can improve the hemocompatibility of the PVC surface. The SA coating combined with the HEP coating could increase the hemocompatibility, and enhance the biocompatibility of the PVC surfaces. Thus, the process for immobilizing SA/HEP is applicable for the PVC pipeline in CPB. The results suggested that PVC pipelines immobilized with SA/HEP can significantly reduce the administration of HEP in cardiac surgery, and may even lead to HEP-free therapy. The SA/HEP coatings represent the beginning of PVCs with improved hemocompatibility and biocompatibility in the presence of human blood or tissues. Intelligent CPB materials with near-complete physiological surfaces will be available for surgical use in the near future.

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