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The effect of coimmobilizing heparin and fibronectin on titanium on hemocompatibility and endothelialization

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ABSTRACT

Currently available cardiovascular implants, such as heart valves and stents, exhibit suboptimal biocompatibility because of the incomplete endothelialization and sequential thrombosis formation especially after a long-term implantation. To improve the blood compatibility and endothelialization simultaneously and ensure the long-term effect of the cardiovascular implants, a technique of combining electrostatic interaction and coimmobilization was developed to form heparin and fibronectin (Hep/Fn) films on aminosilanized titanium (Ti) surfaces. The Hep/Fn coimmobilized films were stable after immersion in PBS for five days, probed by wettability studies and by the release kinetics of heparin and fibronectin. Blood compatibility tests showed that the coimmobilized Hep/Fn films displayed lower hemolysis rate, prolonged blood coagulation time, higher AT III binding density, less platelets activation and aggregation, and less fibrinogen conformational change compared with Ti surface. Endothelial cells (ECs) seeding and fibronectin bioactivity results showed more attached and proliferated ECs and exposed cell-binding sites on the Hep/Fn immobilized samples than that on Ti surfaces. Thus, the Hep/Fn coimmobilized films kept excellent bioactivity even after immersion in PBS for five days. Systemic evaluation suggests that the coimmobilization of Hep/Fn complex improves the blood compatibility and promotes the endothelialization simultaneously. We envisage that this method will provide a potential and effective selection for biomaterials surface modification of cardiovascular implants.

1. Introduction

The principal requirement for biomaterials is biocompatibility, and a truly biocompatibility biomaterial should perform its function without causing undue host response or resulting adverse clinical reaction [1,2]. As to the cardiovascular implants that contact directly with blood, such as endovascular stents, heart valves, hemodialysis membranes and pacemaker components [3], blood compatibility is the first consideration, especially in the initial implantation. The adsorption of plasma proteins and the adhesion of platelets may cause thrombus formation and implant failure as a reaction of host to the implanted biomaterials. Endothelialization is another consideration due to its excellent anticoagulant properties, particularly for the long-term implantation. It was reported that late thrombosis with adverse clinical outcomes occurred with delayed or absent stent endothelialisation [4].

A variety of techniques have been explored to improve the biocompatibility of the cardiovascular biomaterials, including physical and chemical methods (heating [5], Ti–O coating [6] and polymer coating [7]), and biomolecules immobilization (heparin [8], chitosan [9], tropoelastin [10] and RGD peptides [11]). Immobilizing biomolecules on biomaterials surface has attracted a lot of research in recent years, though the stability of these biomolecules in vivo exposure is still a common limitation to the clinical translation. There are many methods for immobilization of biomolecules onto a surface, e.g. physical adsorption, encapsulation, entrapment and covalent or ionic binding, etc. The advantages of covalent immobilization are that the immobilized biomolecules are not easy to be removed by rinsing and robust enough to withstand in vivo exposure. The immobilization of different biomolecules on biomaterial surfaces has been proven to enhance cell attachment and proliferation [12] or improve blood compatibility [9,13]. However, the previous studies have shown that when the antithrombotic effect is improved with the anticoagulant molecules, the growth of ECs will be inhibited [14], whereas when the growth of ECs is promoted with extracellular matrix molecules, the blood compatibility deteriorates. As of now, therefore, most studies just focus on one aspect of biocompatibility, i.e. blood compatibility or endothelialization. Few studies

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have been found to investigate the both aspects simultaneously [15,16]. Though the previous studies have shown that anticoagulation and endothelialization simultaneously are contradictory, to our best study, biomaterials coimmobilized with different biomolecules can possess the properties of each individual biomolecule [17]. Thus the biomaterials surface simultaneously immobilized with different biomolecules possessing anticoagulant and pro-endothelialization properties, respectively, is hypothesized to improve both aspects of anticoagulation and endothelialization, which may provide a potential application for long-term cardiovascular implants, though there seem to be only few studies in this direction [18].

In the present study, heparin and fibronectin were chosen as the constituents for this purpose, because heparin is an important anticoagulant in clinics to minimize thrombus formation on artificial surfaces [19]. It has an incontrovertible effect on inhibiting thrombus formation by catalytically increasing the affinity of antithrombin III (AT III) to thrombin [20]. While fibronectin is an extracellular matrix protein known to promote cell attachment and spreading, differentiation and phagocytosis. Covalent immobilization of heparin and chitosan on PLA was shown to reduce the platelets adhesion and increase the fibroblasts attachment [9]. While the effect of heparin immobilization on endothelial cell proliferation, that is inhibition or promotion, is not consistent [14]. Fibronectin immobilized on aminosilanized Ti surface was found to enhance the attachment of fibroblasts [21]. Fibronectin is also reported to participate in platelets adhesion and aggregation via the integrin αIIbβ3 (glycoprotein IIb/IIIa) receptors on platelet membrane [22]. However, heparin and fibronectin can form complexes by the heparin binding site on fibronectin chains under the physiological conditions or by electrostatic interaction under certain pH solution conditions, which may change their individual properties. An electrostatic interaction method is mainly used here to form Hep/Fn complexes, which might be more stable than that formed under the physiological conditions (electrostatic interaction together with spontaneous binding can enhance the stability of complexes). Electrostatic interaction is often used in layer-by-layer assembly (LBL). LBL of heparin and chitosan was shown to improve the biocompatibility of a coronary stent system [16] and provide antibacterial properties [23], but most biomolecules that used in self-assembly are polysaccharides or polyelectolytes, while few refer to extracellular matrix (ECM) proteins playing important role in cell attachment and proliferation. In our study, heparin carries negative charges even in the very low pH values, while the isoelectric point of fibronectin is 5.5, thus heparin and fibronectin could form complexes using electrostatic attraction by changing the charge of fibronectin via adjusting pH of solution. The Hep/Fn complex was then coimmobilized on silanized Ti surfaces after its formation. The aim of this work is to develop a ready-to-use biocompatibility surface on metallic or other inorganic substrates, which is anticipated to possess both anticoagulation and endothelialization properties simultaneously.

In the present work, we describe the construction and bioevaluation of the coimmobilized Hep/Fn films on Ti plates for blood-contacting implant applications. The modification of Ti substrate was characterized by Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). The stability and biofunctionality of Hep/Fn coimmobilized films before and after immersion in PBS for several days was evaluated. The related mechanism of blood compatibility (conformational change of fibrinogen and antithrombin III (AT III)) and endothelialization (exposure of RGD sequences on fibronectin) was also involved.

2. Materials and methods

2.1. Materials and reagents

Ti substrates (10 × 10 mm) were prepared using 99.5% pure Ti foils (Baoji, China). The foils were polished and ultrasonically cleaned twice successively with acetone, ethanol, and deionized water (dH_2O) for 5 min each and then were dried in an oven at 60 °C for 2 h before use. 0.067 M phosphate buffer saline (PBS, pH 7.3) was purchased from Hyclone Co. Ltd. and adjusted to pH 4 with HCl. 1 M H_2SO_4 in dH_2O was used as stop solution for immunochemistry assay. 3-aminopropyltriethoxysilane (APTE), Toluidine Blue O (TBO) and Acid Orange 7 (AO) were purchased from Sigma–Aldrich. Heparin (Hep, ≥160 u/mg) from porcine intestinal mucosa and fibronectin from human was purchased from Sigma–Aldrich. Mouse monoclonal antihuman fibronectin RGD antibody, mouse polyclonal antihuman fibronectin antibody, mouse monoclonal antihuman fibrinogen (FGN) γ chain antibody, mouse monoclonal antihuman antithrombin III (AT III) antibody, horseradish peroxidase (HRP) conjugated goat antimouse IgG antibody and TMB (3,3′,5,5′–tetramethylbenzidene) for the immunochemistry tests were purchased from BD Biosciences, San Jose, CA. Activated partial thromboplastin time (aPTT) and thrombin time (TT) kits for anticoagulation properties test were purchased from Sunbio, China. M199 culture medium and cell counting kit-8 (CCK-8) for ECs culture and proliferation test were purchased from BD Biosciences, San Jose, CA. The actin staining reagent kit (SABC-FITC) for platelet and ECs skeleton was purchased from Boshide, China. All the other reagents used in the experiments were of the highest analytical purity (>99.9%).

2.2. Coimmobilization of Hep/Fn complex on Ti substrate

Fig. 1 shows the fabrication process of Hep/Fn coimmobilized films on Ti substrate. The cleaned Ti plates were activated in 2.5 M NaOH solution at 80 °C for 24 h, and then thoroughly rinsed with dH_2O and blown dry. The NaOH-activated Ti substrate further on is referred to as TiOH. This activation process produced a very hydrophilic substrate, which was then silanized by immersing into a 2% v/v solution of APTE in 99.8% anhydrous ethanol for 10 h to generate an amino surface. The silanized TiOH surface by APTE was denoted TiOHA and was sonicated in ethanol in order to obtain the silane monolayer and to remove the physisorbed molecules. After that, the samples were kept in a 120 °C oven for 6 h to enhance the binding of APTES with the substrate [24]. 5 mg/ml heparin and 100 μg/ml fibronectin ($pI_{Fn} \approx 5.5$) were pre-diluted in pH 4 PBS and blended with the volume ratio of 1:1 at 37 °C for 1 h. And then TiOHA samples were immersed into Hep/Fn complex at 37 °C for 2 h and rinsed with PBS to remove the unattached heparin and fibronectin molecules. Thereafter the sample was denoted TiOHAHF.

2.3. FTIR

The infrared absorption spectra of the pristine Ti, TiOH, TiOHA and TiOHA coimmobilized with Hep/Fn was obtained from an FTIR spectrometer (NICOLET 5700, USA) in diffuse reflectance mode. For each spectrum obtained, a total of 64 scans were accumulated at 4 cm^{-1} resolution. Scanning was conducted in the range from 400 to 4500 cm^{-1}.

2.4. XPS

The elemental chemical surface composition was determined by XPS on an AXIS HSi spectrometer (Kratos Analytical Ltd, UK) with an AlKa X-ray source (1486.6 eV photons,150 W). The pressure in the chamber was below 2×10^{-9} Torr. The binding energy scale was referenced by setting Cls peak at 284.6 eV.

2.5. AFM

The surface topography and roughness of the Ti substrates before and after immobilization of Hep/Fn complex were characterized using a Nanowizard II AFM (JPK Instruments, Berlin, Germany) in tapping mode. AFM was performed at room temperature in air at a rate of one line scan per second. Image analysis was performed using the CSPM Imager software.

2.6. Stability of the coimmobilized Hep/Fn films

The stability of the coimmobilized Hep/Fn films was determined as follows: the samples were immersed in PBS at 37 °C and shaken for 12 h, 1, 3 and 5 days in a sealed container, respectively, and the samples without PBS immersion were used as reference. Then all the PBS soaked samples and reference samples at each time point were taken out and dried, and the residual heparin and fibronectin on the samples were measured by contact angle measurement, heparin quantity (TBO) and fibronectin quantity (immunochemistry assay).

2.6.1. Contact angle measurement

Static (sessile drop) water contact angles were determined with a contact angle apparatus (JY-82, China). PBS soaked samples and reference samples were first dried and then fixed to a glass slide, a droplet of dH_2O was added to the surface to detect

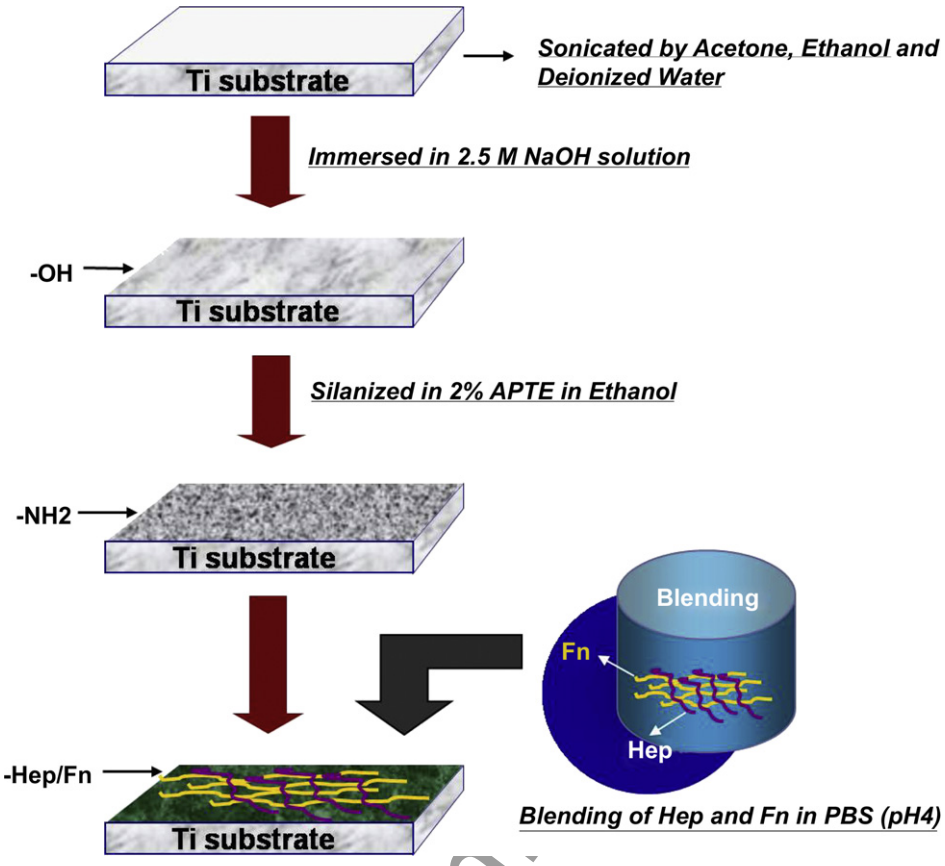

Fig. 1. Sketch map of coimmobilization of Hep/Fn complex on Ti substrate.

contact angle using a horizontal microscope. For each sample, the mean value of the contact angle was calculated from at least three individual measurements taken at different locations on the examined samples.

2.6.2. Heparin quantity and release- TBO assay

The amount of immobilized heparin on PBS soaked samples and reference samples was determined by a TBO assay. Briefly, the samples were incubated in 5 ml of a freshly prepared solution of 0.04 wt% TBO in aqueous 0.01 M HC1/0.2 wt% NaC1. Then the samples were gently shaken at 37 °C for 4 h and rinsed twice with dH_2O, and during this process Hep/TBO complex was formed on the samples surface. After that 5 ml of a 4/1 (v/v) mixture of ethanol and aqueous 0.1 M NaOH was added in, and the Hep/TBO complex was dissolved and released into the fluid phase. After complete dissolution of the complex, 200 μl supernatant was added to a 96-well plate and the absorbance was obtained using a microplate reader at 530 nm. The absorbance was used to calculate the amount of immobilized heparin via the calibration curve. Blanked Ti, TiOHA and Ti surface physisorbed Hep/Fn complex was used as comparison.

The calibration curve was prepared as following: To a heparin solution of known concentration, the same volume of a fresh prepared TBO solution was added. The mixture was also shaken at 37 °C for 4 h and Hep/TBO complex was spontaneously precipitated. Then the mixture was centrifuged at 3500 rpm for 10 min. Subsequently, the precipitate was rinsed with aqueous 0.01 M HC1/0.2 wt% NaC1 and dissolved in 5 ml of a 4/1 (v/v) mixture of ethanol and aqueous 0.1 M NaOH. The absorbance was obtained with a microplate reader at 530 nm.

2.6.3. Fibronectin quantity and release-immunochemistry test

The quantity of bound fibronectin on PBS soaked samples and reference samples was determined by immunochemistry. In brief, the samples were put into a 24-well culture plate and blocked with 1 wt% bovine serum albumin (BSA) in PBS at 37 °C for 30 min. Subsequently, 20 μl mouse monoclonal antihuman fibronectin antibody (diluted 1:250 in PBS) was added and incubated at 37 °C for 1 h, then the samples were thoroughly rinsed three times with PBS. Thereafter, 20 μl HRP conjugated goat antimouse IgG antibody (diluted 1:100 in PBS) was added and incubated at 37 °C for another 1 h. The samples were washed three times again with PBS. Further, 100 μl TMB solution was added onto the sample surfaces and reacted in dark for 10 min (blue color), and then 50 μl 1 M H_2SO_4 was used to stop the peroxidase catalyzed reaction (yellow color). Finally, 130 μl supernatant was transferred to a 96-well plate and the absorbance at 450 nm was measured on a microplate reader. The concentration of fibronectin was obtained compared with that of the calibration curve. Blanked Ti, TiOHA and Ti surface physisorbed Hep/Fn complex was used as comparison. All experiments were done in quadruple ($n = 4$).

2.7. Biofunctionality of the coimmobilized Hep/Fn films

The biofunctionality of the coimmobilized Hep/Fn films was determined in vitro by blood compatibility and endothelial cells compatibility, respectively. In the blood compatibility tests, stainless steel was used as positive controls, low-temperature isotropic carbon (LTIC) was used as negative controls.

2.7.1. Detection of AT III binding to heparin

Immobilized heparin on materials surface was reported to inactivate factor XIIa in the presence of AT III. Fresh anticoagulant (ACD) whole blood of a volunteer was centrifuged at 3000 rpm to obtain platelet-poor plasma (PPP). The samples without PBS immersion were put into 24-well culture plate and 100 μl PPP was added and incubated for 1 h at 37 °C. The binding of AT III to surface immobilized heparin was also detected using immunochemistry with the same procedure as in fibronectin adsorption test except for the difference of the first antibody. Here, the first antibody was mouse monoclonal antihuman AT III antibody (diluted 1:250 in PBS).

2.7.2. Hemolysis rate test

The samples without PBS immersion were soaked in diluted blood solution containing 2% fresh ACD human blood and 98% physiological salt solution, and incubated at 37 °C for 1 h. After centrifugation at 3000 rpm for 5 min, the absorbance at 450 nm of the solution was recorded as D_t. Under the same conditions, the solution containing 2% ACD blood and 98% physiological salt solution was used as a negative reference, the solution containing 2% ACD blood and 98% dH_2O was used as a positive reference. Their absorbencies were recorded as D_{nc} and D_{pc}, respectively. The hemolysis rate α of the samples was calculated via the following formula:

2.7.3. aPTT and TT

The anticoagulation property of Hep/Fn coimmobilized samples with PBS immersion and reference samples was determined by means of an aPTT and TT assay. aPTT was used to detect the intrinsic coagulation, i.e. the influence on the coagulation factors, and TT was used to detect the last step of coagulation, i.e. the thrombin-mediated fibrin formation. Therefore, aPTT and TT tests were commonly used to evaluate the in vitro anticoagulation properties of different biomaterials. For

aPTT test, fresh frozen human PPP was thawed at 37 °C, 500 μl PPP was added onto the samples and incubated at 37 °C for 15 min. Then 100 μl incubated PPP was transferred to a test tube and 100 μl aPTT reagent was added to the same test tube and incubated at 37 °C for 3 min. Subsequently, 100 μl of an aqueous 0.025 M $CaC1_2$ solution was added. The suspension was stirred by a magnetic stick and the coagulation time was determined at 37 °C using a coagulation instrument (Hospitex Diagnostics, Italy).

For TT test, 100 μl TT reagent was added into the tube with 100 μl incubated PPP and then incubated at 37 °C for 3 min. Subsequently, the suspension was stirred by a magnetic stick and the coagulation time was determined at 37 °C using the same coagulation instrument.

2.7.4. Platelet adhesion -Lactate dehydrogenase (LDH) test

LDH test was used to determine the amount of adherent platelets on samples without PBS immersion. Fresh ACD blood obtained from a healthy adult volunteer was centrifuged at 1500 rpm for 15 min and thus platelet-rich plasma (PRP) was obtained. Then the samples were put into a 24-well culture plate and 50 μl/well PRP was dropped onto the sample surface and incubated for 1 h at 37 °C. After thoroughly washing, the adherent platelets were lysed by 50 μl/well 1% triton solution (Triton X-100, Sigma) in PBS. The concentration of active LDH was determined as a quantitative description of adherent platelets by recording the change in absorbance at 340 nm. And the amount of adherent platelets was calculated according to the calibration curve.

2.7.5. Platelet activation: P-selectin detection

The static platelets do not express P-selectin on their membrane while the activated platelets express this membrane protein. P-selectin expression was used to evaluate the activation of the adherent platelets on TiOHAHF samples without PBS immersion. The P-selectin expression was also determined by immunochemistry as depicted above. The samples were put into a 24-well culture plate and 100 μl PRP was added on and incubated at 37 °C for 1 h. And the only difference was that the first antibody was mouse monoclonal antihuman P-selectin antibody.

2.7.6. Platelet adhesion by immunofluorescence staining method

Platelet adhesion on Hep/Fn coimmobilized samples with PBS immersion and reference samples was also examined by immunofluorescence staining method. Briefly, the samples were immersed in 500 μl PRP and incubated at 37 °C for 1 h. Subsequently, the samples were rinsed thoroughly with PBS three times to remove non-adhering platelets and fixed with 2.5% glutaraldehyde for 2 h. Then, the samples were rinsed with PBS and blocked with 1% BSA at 37 °C for 30 min and rinsed with PBS for three times. Afterwards, rabbit antihuman actin antibody (1:100 diluted in PBS) was added and incubated at 37 °C for 30 min and rinsed with PBS for three times, then goat anti-rabbit IgG antibody (1:100 diluted in PBS) was added and incubated at 37 °C for 30 min and rinsed with PBS for three times again, Finally, FITC labeled antibody was introduced and incubated at 37 °C for 30 min and rinsed with PBS for five times. The stained samples were observed under an inverted fluorescence microscope (Leica, Germany).

2.7.7. Conformational change of fibrinogen- exposure of γ chain

The conformational change of fibrinogen, i.e. the exposure of γ chain, could bind to the GPIIb/IIIa receptor on platelet membrane and further cause the platelets aggregation. The exposure of fibrinogen γ chain on PBS soaked samples and reference samples was detected by immunochemistry with the same procedure as depicted above except for the difference of the first antibody. Here, PPP was used and the first antibody was mouse monoclonal antihuman fibrinogen γ chain antibody (diluted 1:250 in PBS).

2.7.8. ECs culture

ECs derived from human umbilical vein were isolated and cultured using the following method: The human umbilical cord was cannulated and washed thoroughly with PBS to remove the blood inside the lumen. And then 0.1% type II collagenase (Gibco BRL, USA) in medium 199 was introduced and incubated at 37 °C for 10 min. The detached cells were washed in serum-free medium and collected in complete M199 containing 20% fetal calf serum (FCS, Gibco BRL), 50 μg/ml EC growth factor (ECGF, Sigma), 100 μg/ml heparin, 20 mmol/L HEPES, 2 mmol/l L-glu, 100 U/ml penicillin and 100 μg/ml streptomycin. The suspended cells were then seeded in a single-used culture flask and incubated at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Replicated cultures were performed by trypsinization when cells were approaching confluence. Cells were fed with fresh prepared growth medium every 48 h. The 3rd generation of ECs was used to evaluate the proliferation behavior on samples.

2.7.9. ECs proliferation-CCK-8

All pristine Ti, TiOH, and TiOHA samples were sterilized in a steam autoclave at 120 °C for 2 h prior to ECs culture experiments. And Hep/Fn complex was filtered and immobilized onto the TiOHA samples prior to ECs culture experiments. Subsequently, all the sterilized samples were placed in a 24-well culture plate and 1 ml ECs suspension was added in. The concentration of ECs for seeding on the samples was 5×10^4 cells/ml.

ECs proliferation was investigated by CCK-8 kit after 1, 3 and 5 days of incubation, respectively. The medium was removed and the samples were washed twice with PBS. Subsequently, fresh medium (without phenol red) containing CCK-8 reagent was added to each sample and incubated at 37 °C for 3 h in standard culture conditions. Afterwards, 200 μl of the blue solutions were transferred to a 96-well plate. The absorbance was measured at 570 nm by a microplate reader. All proliferation experiments were performed in quadruple.

2.7.10. Actin staining of ECs- immunofluorescence staining

ECs were seeded on the samples with the concentration of 5×10^4 cells/ml and incubated at 37 °C for 1, 3 and 5 days, respectively. To study the extent of cell spreading and cytoskeleton formation of ECs on samples, immunofluorescence staining of actin using SABC-FITC kit was performed as described in the section of Platelet adhesion by immunofluorescence staining method.

2.7.11. Conformational change of fibronectin- exposure of RGD peptides

The conformational change of fibronectin, i.e. the exposure of RGD peptides ($Fn_{III9-10}$), could bind to the integrin receptor on ECs membrane and further promote their attachment. The exposure of RGD peptides was also detected by immunochemistry method as described in the section of fibronectin quantity and release except for the difference of the first antibody. Here, the first antibody was mouse monoclonal antihuman fibronectin RGD peptides antibody (diluted 1:250 in PBS).

2.8. Data analysis

The data were analyzed with the software SPSS 11.5 (Chicago, Illinois). Statistical evaluation of the data was performed using Student's paired t test. The probability (P) values $P < 0.05$ were considered to be statistically significant differences. The results were expressed as mean ± standard deviation (SD).

3. Results

3.1. FTIR and XPS

FTIR analysis was used to characterize the surfaces of pristine Ti and Hep/Fn immobilized Ti (Fig. 2A). It indicates the presence of specific functional groups on the grafted surface. Compared with the original Ti surface, TiOH surface showed a new peak in ~3400 cm^{-1}, which was approximate to the −OH group. The APTE grafted surface showed new weak peaks in ~2900 cm^{-1} corresponding to $-CH_2$ and $-CH_3$, and in ~1530 cm^{-1} corresponding to −NH, indicating APTE derived on the surface. A reduced −OH peak was observed compared with TiOH surface due to the reaction between the −OH groups on substrate and that on APTE. However, the peak of $-NH_2$ in 3200 cm^{-1} was not sensitive to FTIR and could hardly be found in the FTIR spectrum. The Hep/Fn immobilized Ti surface had shown substantial amount of hydroxyl and amide bond as indicated by −OH stretching vibrations (~3400–3200 cm^{-1}) and C═O stretching vibrations (~1650 cm^{-1}), respectively. This revealed the presence of Hep/Fn complex on Ti surfaces. Notablely, there were new peak in 2400 cm^{-1} for TiOH and TiOHAHF, while not for TiOHA, this peak is corresponding to the CO_2 from the air, and the differences were caused by the background remove. However, this peak could not affect the determination of other special groups. In addition, the peaks appeared in ~1600 cm^{-1} on TiOH and TiOHA samples were corresponding to the H_2O from the air.

XPS was also employed to determine the surface chemical compositions of original and the modified Ti in this study, The XPS widescan spectra of the samples and their corresponding surface elemental compositions are shown in Fig. 2B. Carbon is typically present from unavoidable hydrocarbon contamination, and it was used as an internal reference at 284.6 eV for calibrating peak positions. Compared with original Ti surface, new Ca2p peaks (~346.9 eV) appeared in the spectrum of TiOH and TiOHA samples, which may be attributed to the calcium contamination of NaOH during its preparation. In addition, Ti2p peaks (~460 eV) decreased and new N1s peaks (~400 eV) appeared corresponding to TiOHA sample, indicating the successful grafting of APTE. After heparin or fibronectin immobilization alone, N1s peaks increased and Ti2p peaks further decreased and C2p peaks disappeared, indicating the

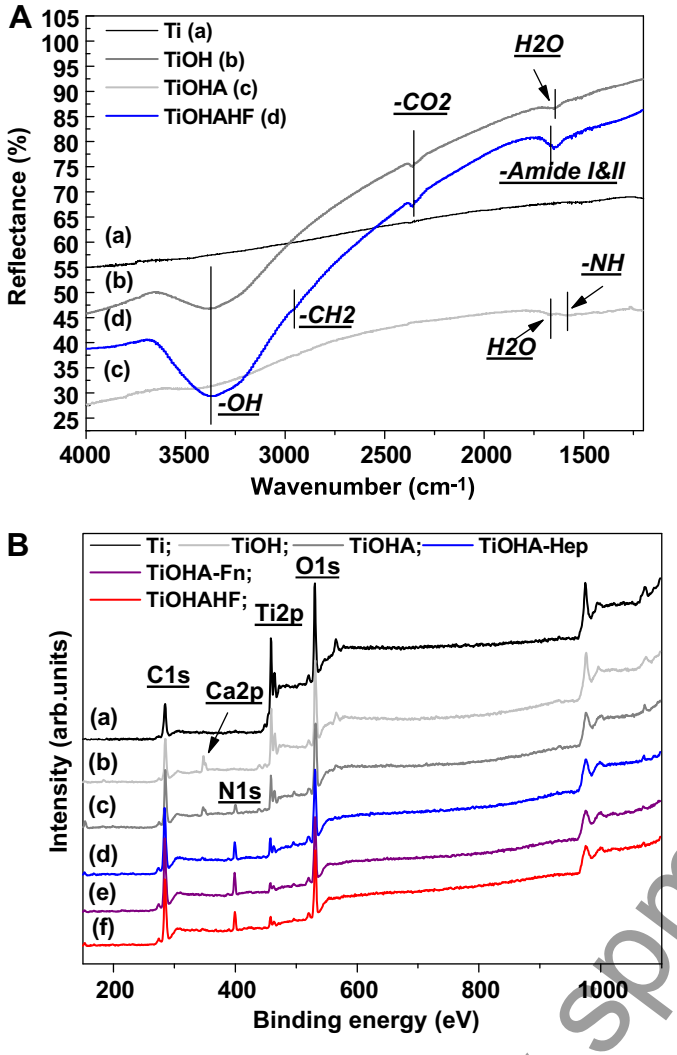

Fig. 2. Characterization of (A) FTIR spectra of: (a) pristine Ti, (b)TiOH, (c) TiOHA and (d) TiOHAHF; (B) XPS analysis of (a) pristine Ti, (b)TiOH, (c) TiOHA, (d) TiOHA-Hep, (e) TiOHA-Fn and (d) TiOHAHF.

immobilization of heparin or fibronectin. And after Hep/Fn complex immobilization, no significant difference appeared in XPS spectra compared with the sample immobilized with heparin or fibronectin alone. It is indicated that the Hep/Fn complex had been immobilized onto the Ti OHA surface. This result was consistent with that of the FTIR spectra. In addition, it has been proven that a protonated N element is formed on TiOHA surface by the high-resolution spectra of N1s (data are not shown here). Moreover, there are many reactive groups (carboxyl, sulfonic, etc) carrying negative charges in Hep/Fn complex, whereas the amino groups in the silanized surface carry positive charges. Thus we speculate that Hep/Fn complex is immobilized in the silanized surface by electrostatic interaction. However, it is difficult to distinguish the two biomolecules due to the overlapping of carboxyl stretching vibrations (1715–1650 cm^{-1}) on heparin and amide groups (1650 cm^{-1}) on fibronectin in FTIR spectra and the overlapping of N1s elements for both biomolecules. Other experiments should be performed to identify each.

3.2. AFM

Commonly, activation by NaOH could cause a rough Ti surface, and the grafting of short chain alkyl, i.e. APTE, further enlarged the surface roughness. The morphology change of the surface observed by AFM is shown in Fig. 3. The surface of the pristine Ti was flat with an average roughness of 3.2 ± 0.2 nm, and became rougher with a roughness of 23 ± 0.6 nm after NaOH activation. Then much rougher (36.9 ± 0.4 nm) surface was obtained after grafting APTE, indicating the presence of APTE on the TiOH surface. However, the surface roughness of the Hep/Fn immobilized surface reduced to 31.2 ± 0.4 nm although it seemed the same. The variation could be ascribed to the clustering of heparin and fibronectin on silanized Ti surface or the crosslinking caused by the binding of Hep/Fn complex to APTE. For implants in contact with blood such as artificial heart valve and cardiovascular stents, the surfaces should be smoother and the roughness at the level of protein adsorption (<50 nm), otherwise platelets may adhere and thrombogenesis may occur. In this study, surface roughness of TiOHAHF is small enough for hemocompatibility improvement.

3.3. Contact angle

The water contact angles were measured as a function of process of Ti substrate (Fig. 4). Compared with the pristine Ti, the water contact angles dramatically decreased to about 0° after activation by NaOH (data are not shown in plot here), while increased to 26.5 ± 3.6° after silanization by APTE, indicating that the TiOHA surfaces were more hydrophobic than TiOH surfaces. For a solid material, surface chemical composition and roughness are two of the key factors to determine the surface wettability. NaOH activation and immobilization of biomolecules could introduce a lot of hydrophilic groups, such as −OH and −COOH, and thus improve the surface hydrophilicity. Otherwise, surface roughness could also influence the surface hydrophilicity, as reported by Shibuichi et al. [25], the material hydrophilic or hydrophobic character is enhanced when the roughness increases for a same kind of surface, whether the initial contact angle value is lower or higher than 90°. Heparin and fibronectin are both hydrophilic biomolecules, together with roughed surface, the contact angle of the Hep/Fn coimmobilized sample decreased to about 17.3 ± 3.5°, indicating a very hydrophilic surface and the presence of heparin and fibronectin. After immersion in PBS for five days, the Hep/Fn immobilized samples still kept much more hydrophilic than the samples of TiOHA and Ti, it could also be seen that the contact angle became smaller with the increased immersion time in PBS, but not significantly different. The reason might be ascribed to the formation of hydrogen bonding of water and hydrophilic groups on heparin and fibronectin. However, the results suggest the formation of a relatively stable Hep/Fn films on Ti surfaces.

3.4. Quantity and release of heparin and fibronectin

The quantity and release of heparin and fibronectin on different samples are shown in Fig. 5. From Fig. 5a, it could be seen clearly that TiOHAHF displayed much larger amounts of heparin than Ti, TiOHA and the physisorbed Ti before PBS immersion (0d). The immobilized heparin was more stable than the physisorbed. It was obvious that the coimmobilized biomolecules were not easy to be removed by rinsing and robust enough to withstand in vivo exposure. The amount of heparin on the sample of TiOHAHF kept at a relatively high level before the 3rd day, while it decreased to a low level at the 3rd day. The heparin density at the 5th day was about 2.6 μg/cm^2, which was significantly higher than that achieved in previous reports describing covalent grafting of heparin [26,27]. Heparin with the density of 2.6 μg/cm^2 on PU surface was found to significantly inhibit platelets adhesion [28], thus in our study, after PBS immersion for five days, the residual heparin was still enough to exhibit the anticoagulant properties. However, the amount of physisorbed heparin on Ti surface dramatically decreased to an

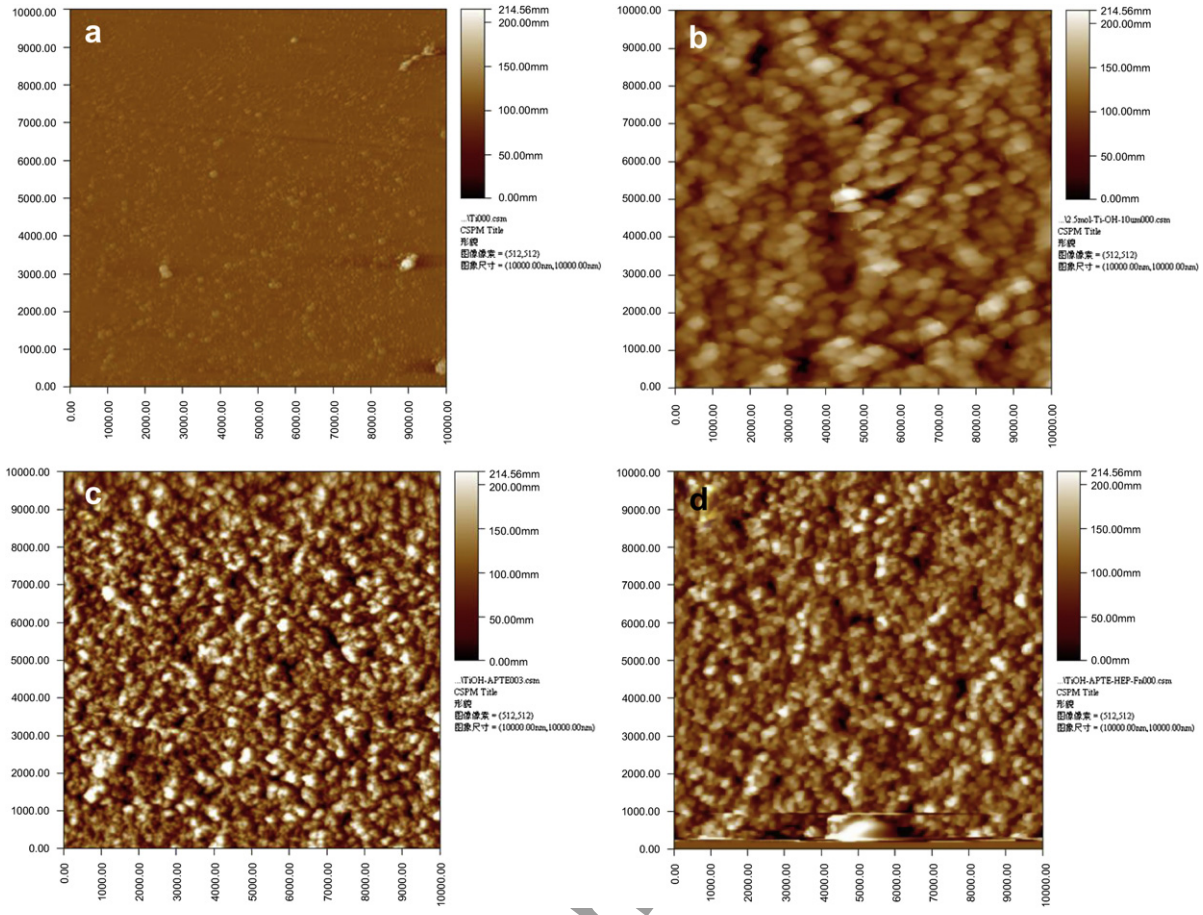

Fig. 3. AFM images of the morphology of: (a) pristine Ti, (b) TiOH, (c) TiOHA, and (d) TiOHAHF.

extremely low level even at the 1st day, which was equivalent to that on TiOHA surface without heparin adsorption, and further decreased to a level equivalent to Ti BLK (blank sample), indicating a through loss of heparin on physisorbed Ti surface. Note that there was a burst release of heparin after one day immersion in PBS, the reason may be that the heparin molecule was smaller than fibronectin, and the interaction of heparin and surface amino groups was destroyed after one day shaking, and heparin molecules released to PBS solution. A burst release of heparin from fibers was reported to prevent smooth muscle cells (SMC) proliferation [29], though the effect on SMC in our study was still not clear, however, the phenomenon was worthy of further study. Though some heparin released from the films, water contact angle still kept at a low level, suggesting a relatively stable Hep/Fn complex on surface.

Fig. 5b depicts the quantity and release of fibronectin on different samples. It could be seen clearly that the quantity of immobilized fibronectin was much larger than that physisorbed after PBS immersion for several days. Unlike heparin release, a sustained release for fibronectin was observed on the sample of TiOHAHF over 5 days, and no significant difference was observed for fibronectin release at each time point, while an obvious release of fibronectin occurred after one day immersion in PBS on physisorbed Ti. Fibronectin on TiOHAHF kept a high amount during the 5 day immersion period, while dramatically decreased to an extremely low level at the 3rd day on physisorbed Ti, which was almost equivalent with the TiOHA surface without fibronectin adsorption (blank sample), indicating a through loss of fibronectin on physisorbed Ti surface. Fibronectin molecule with long chain structure was much larger than heparin molecule, thus there were more binding sites for fibronectin and the substrate. Therefore, fibronectin would not release to PBS even one or more binding sites were destroyed during immersion periods. The results of quantity

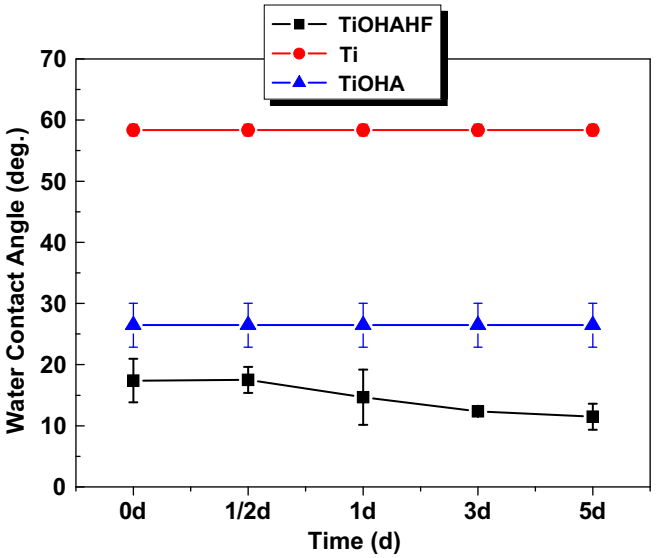

Fig. 4. Water contact angle as a function of immersion of Hep/Fn immobilized samples in PBS for different time. Four drops were analyzed with contact angles measured on the samples of TiOHAHF. Ti and TiOHA were used as control (mean ± SD, $N = 4$).

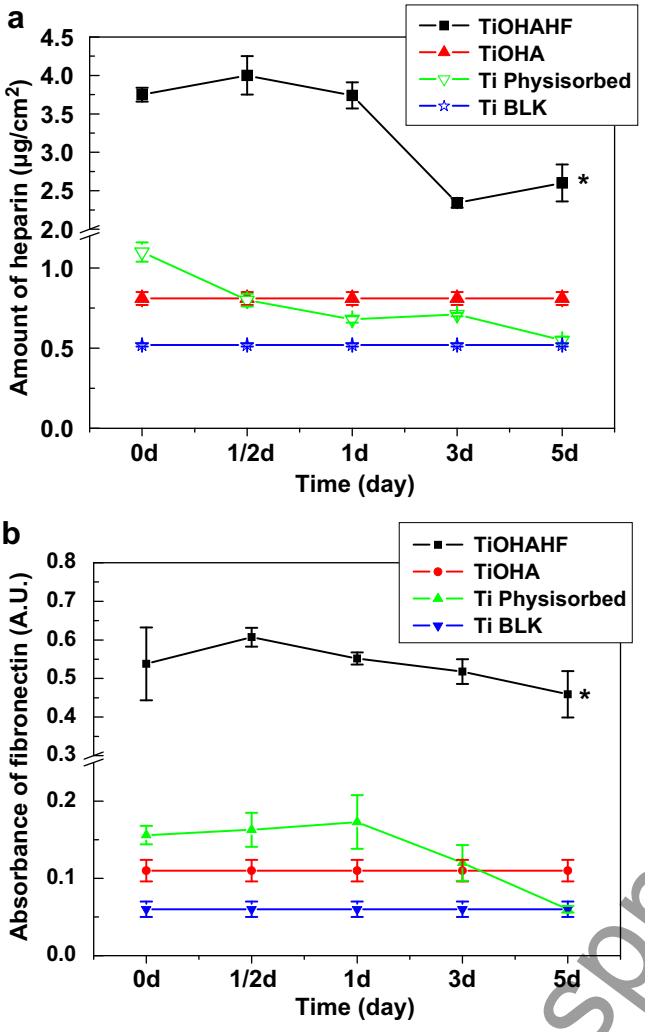

Fig. 5. Quantity and release of heparin (a) and fibronectin (b) as a function of immersion time of Hep/Fn immobilized samples in PBS for different time. The physisorbed Hep/Fn on Ti surface was used as comparison and Ti, TiOHA surface without Hep/Fn adsorption was used as blank (*$p < 0.05$, mean ± SD, $N = 4$).

and release of heparin and fibronectin demonstrated that the fibronectin molecules in the coimmobilized Hep/Fn films on Ti surface were stable and resistent to the hydrolytic degradation.

3.5. Blood compatibility

3.5.1. Results of AT III binding and hemolysis rate

Generally, heparin performs its anticoagulation properties by binding to AT III and thus accelerated the inactivation of thrombin. For verifying the anticoagulant activities of the immobilized heparin on the sample of TiOHAHF, the density of AT III binding to the immobilized heparin was measured and shown in Fig. 6a. It could be seen that TiOHAHF displayed a significantly higher density of AT III than the control samples and TiOHA ($p < 0.05$). The result here directly demonstrated that the immobilized heparin on TiOHAHF possessed a high bioactivity to bind to AT III and therefore a good anticoagulant property. Moreover, from the result we found that AT III adsorbed on LTIC was low, since LTIC was known for its excellent blood compatibility, we postulated that another mechanism dominated that.

Hemolysis rate is an important factor for characterization of the blood compatibility. The lower the hemolysis rate, the better the blood compatibility. Fig. 6b shows the hemolysis rate of the pristine Ti and modified Ti surfaces. It could be seen that the hemolysis rate of the Hep/Fn immobilized samples was significantly lower than that of the control samples, TiOH and TiOHA ($p < 0.05$), the hemolysis rate of Ti was about 1.5%, while less than 0.4% for the Hep/Fn immobilized samples. As a novel material, the Hep/Fn film is used for the surface modification of the blood-contacting implants, and its hemolysis is far below the accepted threshold value of 5% [30], implying a good hemocompatibility.

3.5.2. aPTT and TT

aPTT and TT tests were performed to evaluate the anticoagulant activities of the samples (Fig. 7). The pristine Ti, TiOH, TiOHA and plasma (PPP) were used as reference samples, SS and LTIC were used as positive and negative controls, respectively. It was found that the aPTT values (Fig. 7a) of the TiOHAHF samples (without PBS immersion and PBS immersion for 1 d) were significantly prolonged compared with the plasma and control samples ($p < 0.05$). After immersion in PBS for 3 and 5 days, the aPTT values of TiOHAHF were reduced to 52.7 ± 2 s and 50 ± 6.5 s, respectively, which was equivalent to that of the PPP, however, these values were still much longer than that of the pristine Ti ($p < 0.05$). The TT values for all the samples are shown in Fig. 7b, all Hep/Fn immobilized samples with or without PBS immersion displayed significant longer TT values than PPP and the controls. Moreover, the TT values

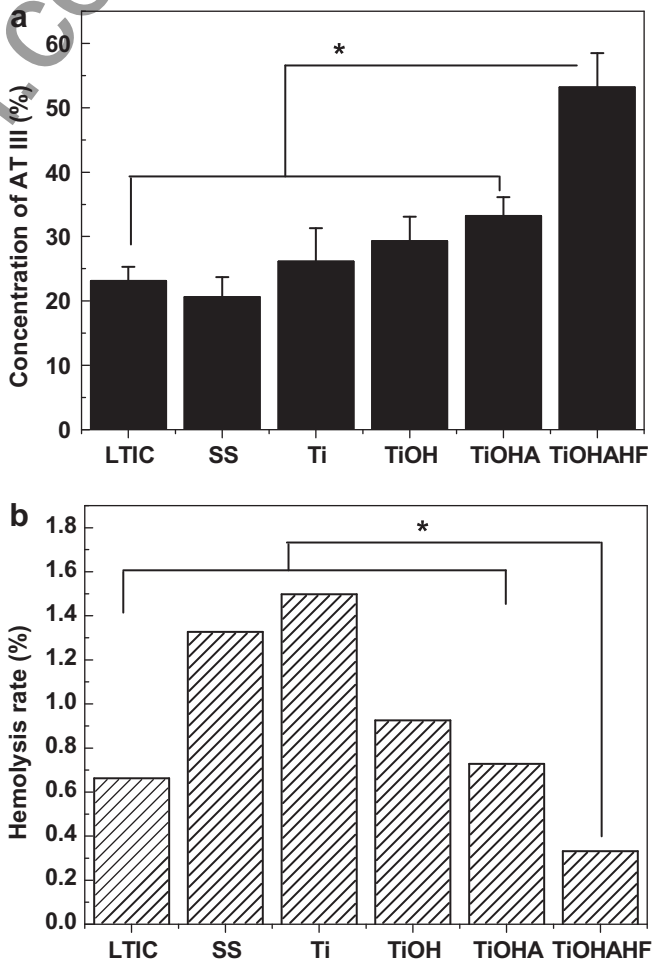

Fig. 6. Results of AT III binding (a) and hemolysis rate (b) of the pristine Ti, TiOH, TiOHA and Hep/Fn immobilized samples, LTIC was used as negative control and SS was used as positive control (*$p < 0.05$, mean ± SD, $N = 4$).

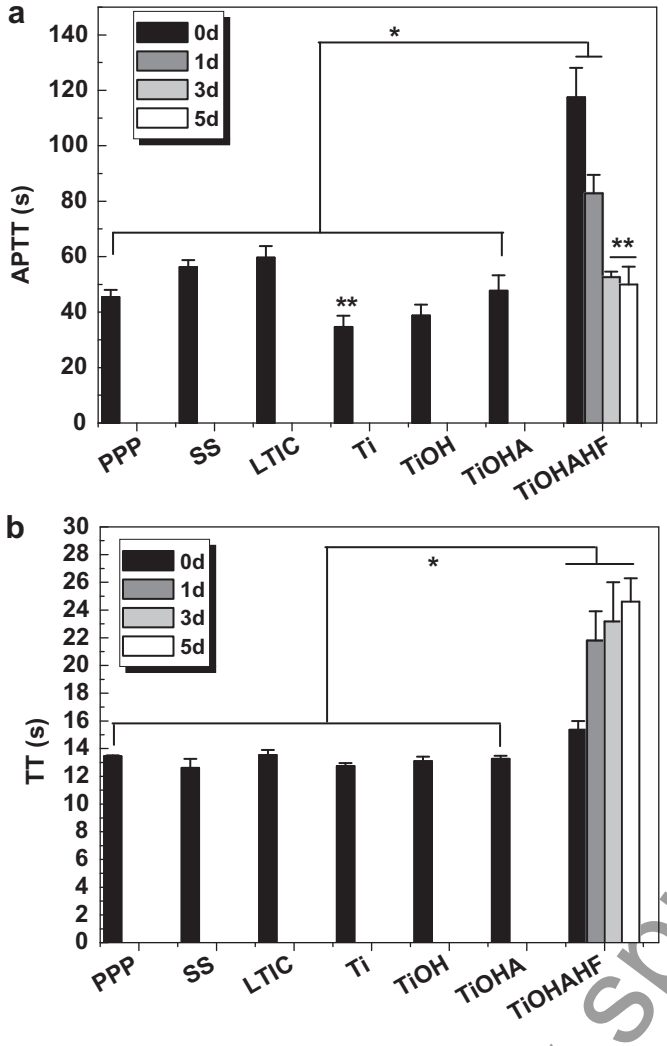

Fig. 7. Anticoagulation properties of Hep/Fn immobilized surfaces with or without PBS immersion. The pristine Ti, TiOH, TiOHA and and plasma were used as references, LTIC and SS were used as negative and positive controls, respectively. (a) APTT; (b) TT. (*$p < 0.05$, **$p < 0.05$, mean ± SD, $N = 4$).

of Hep/Fn immobilized samples with PBS immersion for 1, 3 and 5 d were further prolonged and larger than 20 s compared with the samples without PBS immersion. The detailed mechanism was still unclear, possibly, before immersion in PBS (0 d), the anticoagulation properties of heparin were affected due to the large amount of heparin and fibronectin on the surface, and the bioactive sites were shield. While after immersion in PBS for certain time, more bioactive sites of heparin on the surface were exposed with the release of heparin or fibronectin. In addition, aPTT and TT reflected different coagulation pathway, the inconsistent results for the two assays need further study. However, the results of aPTT and TT suggested that the Hep/Fn coimmobilized samples possessed good anticoagulation properties, which were sustained well after immersion in PBS for several days.

3.5.3. Platelet adhesion and activation

The LDH and P-selectin tests were performed to evaluate the amount and activation of the adherent platelets on Hep/Fn immobilized sample before PBS immersion. SS and LTIC were used as positive and negative controls, Ti, TiOH and TiOHA were used as reference. The results of platelets adhesion and activation (P-selectin expression) on different samples were shown in Fig. 8. It could be seen that after incubation in PRP for 1 h, a remarkable platelet adhesion was observed on SS, Ti and TiOHA surfaces, which also showed the highest platelets activation compared with LTIC and TiOHAHF sample ($p < 0.05$). LTIC showed the least platelets adhesion and activation compared with all the other samples. The number of the adherent platelets on different samples decreased in the order SS > TiOHA > Ti > TiOH > TiOHAHF > LTIC, and the P-selectin expression on different samples decreased in the order SS=Ti=TiOHA > TiOH > TiOHAHF > LTIC. P-selectin was only present on activated platelets membrane, and thus could be used as a marker of platelet activation on biomaterials. Therefore, P-selectin assay gave a direct indication regarding the activation of the platelets as the order SS=Ti=TiOHA > TiOH > TiOHAHF > LTIC. The results of platelets adhesion and activation suggested that the Hep/Fn immobilized surface possessed a better blood compatibility than SS and Ti.

3.5.4. Fluorescence staining of adherent platelets

A fluorescence staining method was used to identify the adherent platelets on Hep/Fn immobilized samples with or without PBS immersion and the results are shown in Fig. 9, the stained platelets displayed green fluorescence. It could be seen that the control samples, i.e. SS, Ti and TiOHA showed a significantly larger amount of platelets adhesion than the TiOHAHF samples with or without PBS immersion, and more platelets aggregated on SS, Ti and TiOHA samples than that on Hep/Fn immobilized samples. Moreover, the amount of the adherent platelets on TiOHAHF samples increased with the immersion periods, and platelet aggregation appeared after PBS immersion for five days. The Hep/Fn immobilized sample without PBS immersion (0 d) showed an equivalent platelets adhesion with LTIC, which had the least amount of platelets adhesion. The fluorescence staining of platelets indicated that the Hep/Fn immobilized surface possessed better blood compatibility even after immersion in PBS for several days, which could reduce the likelihood of thrombus formation upon biomaterial surface contacting with blood. This result was consistent with the results of aPTT and TT test.

3.5.5. Conformational change of fibrinogen- exposure of γ chain

The conformational change of fibrinogen, i.e. exposure of γ chain (HHLGGAKQAGDV at γ 400–411) [31], plays a critical role in platelet activation and aggregation. Resting platelets can interact with immobilized but not with soluble fibrinogen. Fibrinogen is

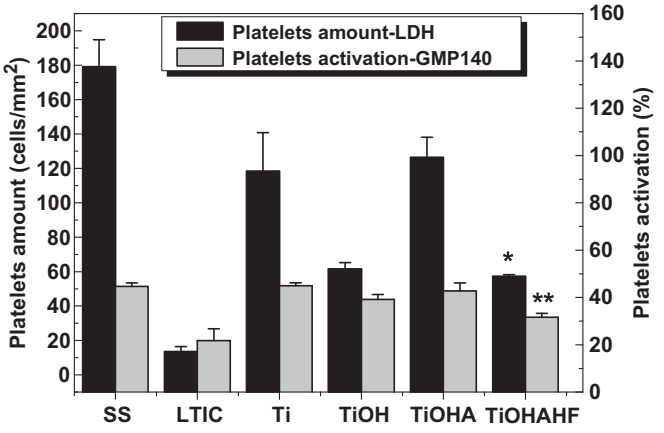

Fig. 8. Amount and activation of adherent platelets on various sample surfaces measured by LDH test and P-selectin test after incubation with PRP for 1 h. The samples included the pristine Ti, TiOH, TiOHA and TiOHAHF, LTIC and SS were used as negative and positive controls. (*$p < 0.05$; **$p < 0.05$. mean ± SD, $N = 4$)

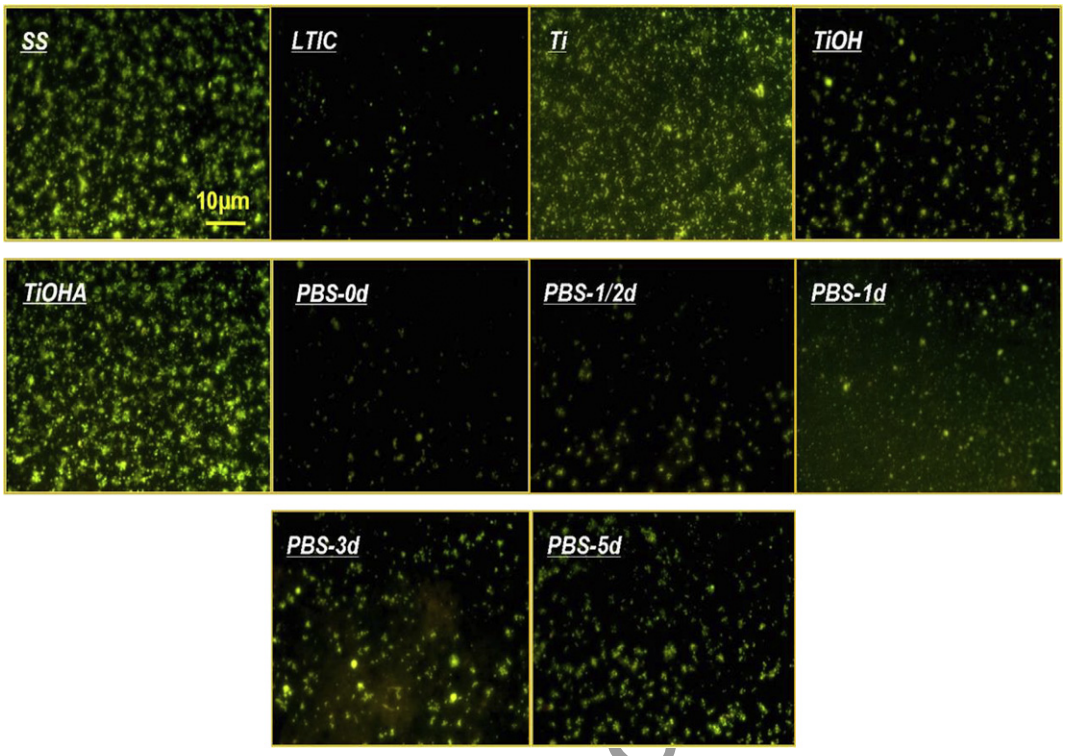

Fig. 9. Platelet adhesion fluorescence microscope images of pristine Ti, TiOH, TiOHA, SS, LTIC and Hep/Fn immobilized samples before and after PBS immersion for various periods.

folded in plasma and little γ chain exposed, but unfolded or conformational changed when adsorbed on the implants and γ chain exposed. The exposure of the protected γ chain sequences allows fibrinogen to bind to the GPIIb/IIIa integrin receptor on platelet membrane and further cause the adhesion and aggregation of platelets. Therefore, fibrinogen conformational changes measured by the immunochemistry method could reveal the thrombosis tendency. The conformational changes of fibrinogen determined from the control samples and the Hep/Fn immobilized samples with or without PBS immersion are shown in Fig. 10. All Hep/Fn immobilized samples before and after PBS immersion showed significantly lower absorbance compared with the controls, TiOH and TiOHA samples ($p < 0.05$). The results indicated that PBS immersion for several days did not worsen the thrombosis tendency of the Hep/Fn films on Ti surface. Fibrinogens adsorbed on Ti and SS surfaces underwent larger conformational changes than those on TiOHAHF samples and LTIC, suggesting a good blood compatibility of Hep/Fn immobilized samples. The results of fibrinogen conformation change may be used to explain the results of APTT, TT and platelets adhesion test.

3.6. Endothelialization

3.6.1. CCK-8 for EC proliferation

ECs from human umbilical vein were seeded on samples without PBS immersion. The effect of Hep/Fn immobilized films on endothelial cells attachment and proliferation was investigated by a CCK-8 viability assay after incubation for 1, 3 and 5 days, respectively. Fig. 11 shows the amount and proliferation of ECs on the surface of pristine Ti, TiOHA and the surface immobilized with Hep/Fn. The absorbance was proportional to the amount of the cells, the more the cells on the surface, the larger the absorbance. It could be seen that after culture for 1 and 5 days respectively, ECs on TiOHAHF surfaces exhibited significantly increased attachment and proliferation compared with those on control Ti ($p < 0.05$), while displayed less attachment and proliferation than the pristine Ti after culture for 3 days. The amount of ECs on the pristine Ti increased from the 1st day to the 3rd day, while reduced from the 3rd day to the 5th day, and ECs on the pristine Ti exhibited the largest proliferation at the 3rd day. In contrast, the amount of ECs on TiOHAHF decreased from the 1st day to the 3rd day firstly and then increased from the 3rd day to the 5th day. The reason may be ascribed to the burst release of heparin after the 1st day incubation, which inhibited the proliferation of ECs. Otherwise, it was reasonable to see that TiOHA showed the largest ECs attachment and proliferation during the whole culture periods due to the amino groups exposure, which were beneficial to ECs growth,

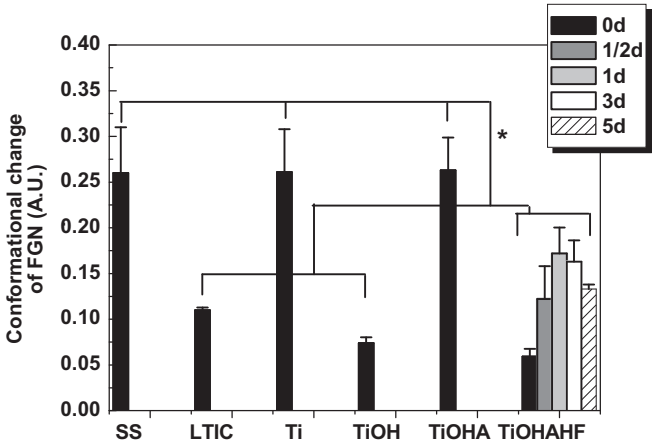

Fig. 10. Conformational change of fibrinogen on the Hep/Fn immobilized samples with PBS or Un-PBS immersed. SS and LTIC were used as positive and negative controls. (*$p < 0.05$. mean ± SD, $N = 4$).

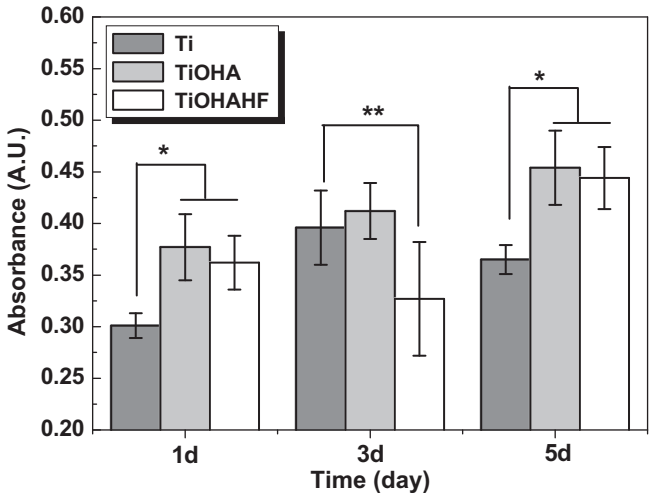

Fig. 11. Endothelial cells attachment and proliferation on pristine Ti, TiOHA and Hep/Fn immobilized samples before PBS immersion using CCK-8 assay. (*$p < 0.05$, **$p > 0.05$. mean ± SD, $N = 4$).

however, TiOHAHF displayed the similar ECs amount to TiOHA at the 5th day. The results demonstrated that immobilization of Hep/Fn complex could promote ECs attachment and proliferation.

3.6.2. Immunofluorescence staining of actin for EC skeleton and morphology

The morphology and skeleton arrangement of ECs on Hep/Fn immobilized surfaces were detected by the immunofluorescence staining for actin expression. Fig. 12 depicts the cytoskeleton of ECs after incubation for 1, 3 and 5 days, respectively. The results showed that ECs seeded on all samples exhibited elliptic, rounded or polygonal morphology. Consistent with the result of CCK-8, TiOHAHF surfaces exhibited more ECs attachment and proliferation than the pristine Ti at the 1st and 5th day, and ECs cultured on TiOHAHF surfaces displayed less proliferation than those cultured on pristine Ti at the 3rd day. TiOHA showed more ECs attachment and proliferation than the pristine Ti during the whole culture periods, and displayed the similar ECs coverage to TiOHAHF at the 5th day. The morphology of ECs on pristine Ti, TiOHA and TiOHAHF was similar with each other. Interestingly, both the TiOHA and TiOHAHF samples showed almost confluent coverage of ECs with elliptic and cobblestone morphology after 5 days culture, no ECs confluence was observed on pristine Ti surface. These results showed that the distribution and proliferation of ECs on the sample of TiOHAHF was enhanced, the Hep/Fn immobilized surface performed better endothelialization than Ti.

3.6.3. Exposure of fibronectin RGD peptides

To penetrate the possible mechanism of ECs attachment and proliferation on pristine Ti, TiOHA and TiOHAHF samples, the exposure of RGD peptides of fibronectin on Hep/Fn immobilized samples before and after PBS immersion was determined using immunochemistry based on the recognization of antibody and antigen. This approach was previously used to study substrate-induced changes in adsorbed protein. The exposure of RGD peptides on fibronectin played an important role on ECs adhesion. RGD peptides could bind to the $\alpha 5\beta 1$ integrin receptor on ECs membrane and promote the adhesion and spreading of ECs. The exposure of RGD peptides on all the samples is shown in Fig. 13. The result showed that all the Hep/Fn immobilized surfaces exhibited much more available cell-binding sites than the pristine Ti and TiOHA surface, even though there was a decreased exposure of the RGD peptides after PBS immersion. It could be easily explained by the reduced quantity of fibronectin with the increased immersion periods. The result here provided evidence that the cell-binding sites were available even after immersed in PBS for several days, therefore, a better ECs attachment and proliferation was anticipated corresponding well to the results of CCK-8 and cytoskeleton staining.

4. Discussion

It has been anticipated that a functional stent would inhibit thrombus formation while accelerating endothelial regeneration. Until now, many methods have been applied to modify the biomaterials surface for improving the blood compatibility or endothelialization. These methods include physical or chemical modification, or direct biomolecules immobilization on biomaterials surface [6–9]. Among these the immobilization of biomolecules recently aiming at different targets has attracted a lot of researches [10,32]. A variety of methods has been used to immobilize biomolecules on biomaterials surface. The preliminary method is physical adsorption of biomolecules onto biomaterials surface [33], and the interaction between the surface and the biomolecules mainly depend on electrostatic interaction, hydrogen bond, hydrophobic force and Van der Waals force, etc, but the stability of the biomolecules is still a bottleneck especially for the long-term implanted devices. LBL self-assembly is another method, based on alternate adsorption of oppositely charged components, and has been developed and widely investigated for many purposes during the past 15 years [34,35], and a comprehensive review related to protein LBL assemblies has been published by Lvov et al. [36]. Nevertheless, LBL self-assembly technique is rather time-consuming and troublesome, which may limit its clinical application. In contrast, covalent or ionic immobilization of biomolecules has the advantages that the method is easy to be realized and the immobilized biomolecules are not easy to be removed by rinsing and robust enough to withstand in vivo exposure [12,37], though the bioactivity of the biomolecules may be influenced, which, however, could be improved by change the immobilization conditions. To our best knowledge, biomaterials coimmobilized with different biomolecules can possess the properties that each biomolecule has [17]. The immobilization of different biomolecules on biomaterial surfaces has been proven to improve blood compatibility [9] or enhance cell attachment and proliferation [12].

In our study, heparin and fibronectin were first blended and then coimmobilized to Ti surface, because heparin is known to have excellent anticoagulation properties, while fibronectin can promote the attachment and spreading of endothelial cells. Heparin binding to fibronectin may prevent platelet adhesion and blood coagulation in the period after implantation when the implants are not yet completely covered by endothelial cells. Ti is used as model substrates because of its good biocompatibility. Silane-based strategies for organic moieties have already been applied to implant metals such as Co–Cr–Mo [38] and titanium oxide [39]. APTE is selected as the spacer molecules because it possesses two functional groups, the silane group is used for attachment to Ti surface while the amino group is used to couple biological molecules [40]. The silanized layer is proven to be stable in air for periods of months and in cell culture for periods of several days [41,42]. In addition, it has been shown previously that binding of fibronectin to an aminosilanized surface does not alter its biological activity [21]. The Hep/Fn immobilized surfaces displayed anticoagulation and endothelialization properties as demonstrated by the evaluation experiments of blood compatibility and ECs culture. Moreover, this immobilized Hep/Fn films are stable after

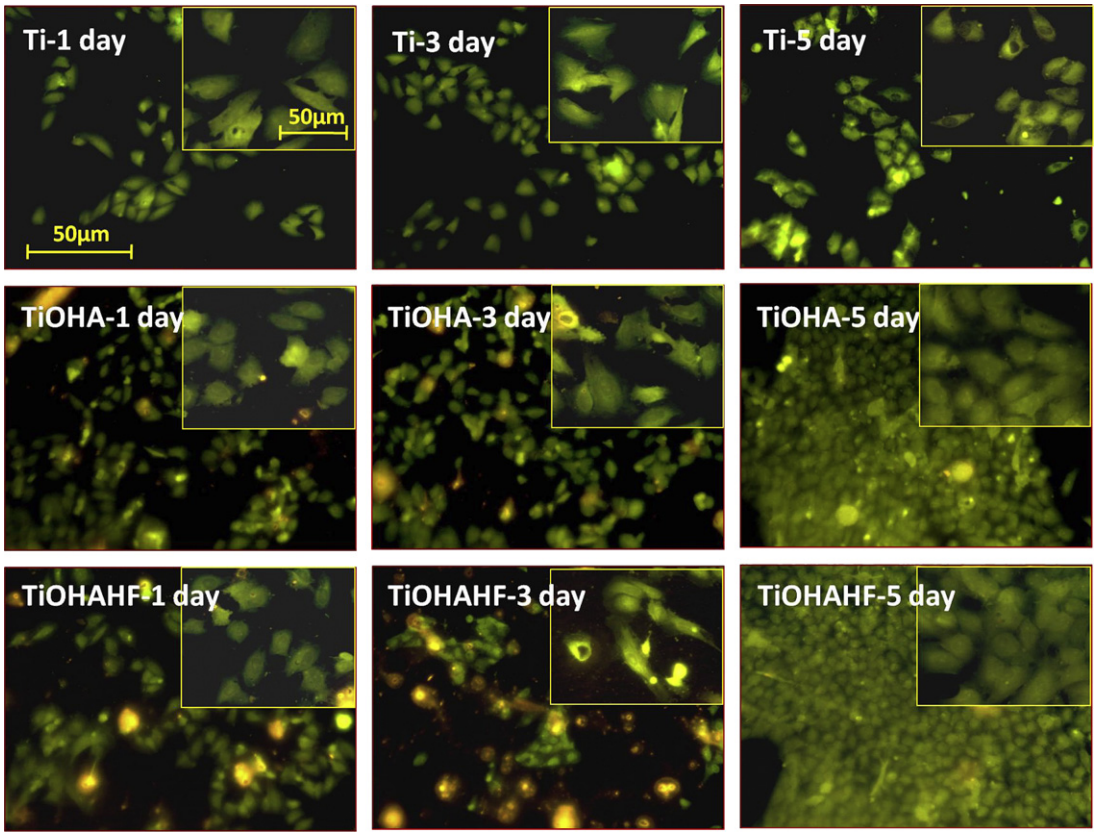

Fig. 12. Immunofluorescence staining micrographs of endothelial cells actin after cultured on pristine Ti, TiOHA and Hep/Fn immobilized surface for 1, 3 and 5 day, respectively.

immersion in PBS for five days proven by heparin and fibronectin release. The successful immobilization of Hep/Fn on Ti surfaces was monitored by FTIR, XPS and AFM, though what they provided was qualitative information. The existence of heparin and fibronectin were indirectly verified by the appearance of amide I peaks at about 1650 cm^{-1} by FTIR, the appearance of N1s peaks and disappearance of Ca2p peaks in XPS spectra, and the surface roughness changes by AFM. It was difficult to distinguish the two biomolecules because of the overlapped FTIR spectrum and the random distribution of the two biomolecules on the silanized surface. However, the quantitative information of the immobilized heparin and fibronectin was obtained from the quantity and release assays of heparin and fibronectin, which could verify the immobilization of heparin and fibronectin, respectively.

Stability of the immobilized biomolecules is an important aspect for the function maintaince of the implants. The exposure of the substrate with the loss of biomolecules after contacting with blood may cause coagulation and thrombus formation. A stable Hep/Fn coimmobilized film was obtained by detecting the contact angle shift of the Hep/Fn immobilized surface, and the remained quantity of heparin and fibronectin after contacting with PBS for several days at 37 °C. Generally speaking, drug release from a polymer-based matrix takes place with an initial burst release [43]. The silanized surface with Hep/Fn complex could be considered as a polymer-based matrix here. As shown in Fig. 5a, there was also a burst release of heparin from the coimmobilized Hep/Fn films after one day immersion in PBS, and the amount of the remained heparin at the 5th day was almost at the same level as that at the 3rd day, no further release was observed. Heparin was low molecular weight molecule with short half-life in the body, the binding of heparin to the silanized surface might be destroyed, but the detailed reason needs further study. A burst release of heparin from heparin/PCL fibers was also found by Luong-Van E et al. [44]. An immediate release of heparin from the fibers was also considered to effectively inhibit the proliferation of smooth muscle cells [45]. However, no burst release of fibronectin was observed over a period of 5 days. Fibronectin as an ECM protein has been known as a cell adhesion molecule with duplexes structure connected by disulfide at the carboxyl terminal. Therefore, fibronectin molecule can bond to the silanized surface through many anchorages and thus not readily to

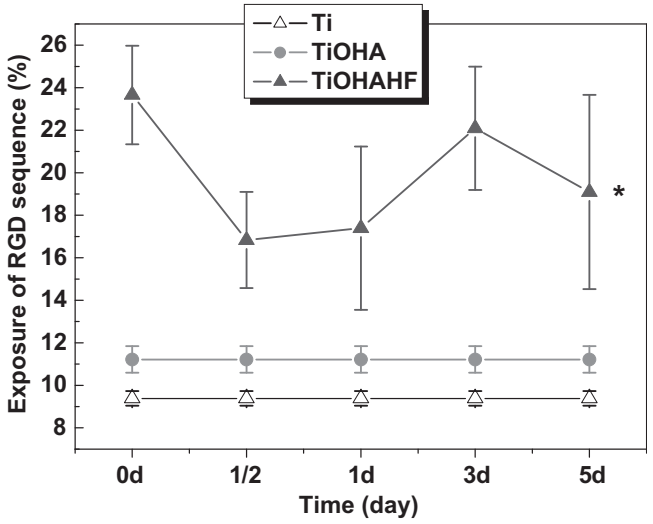

Fig. 13. Exposure of RGD peptides on pristine Ti, TiOHA and Hep/Fn immobilized samples before and after PBS immersed by immunochemistry method. (*$p < 0.05$, mean ± SD, $N = 4$).

be removed from the surface. As of now, no reports directly involving fibronectin release from the biomaterials surface have been found, though some investigations referring to fibronectin displacement and desorption by other serum proteins were reported [46]. We may speculate from the release difference of the two biomolecules that combination of heparin and fibronectin by electrostatic interaction was disturbed after immersion, and the stability of heparin and fibronectin depended on several different elements, including the concentration, the molecular weight and structure, and the integration degree with the substrate, etc. Though there was release of heparin and fibronectin from the Hep/Fn coimmobilized films, it was still reasonable to believe that the Hep/Fn complex was stable enough for performing its biofunctionality in terms of the residual amount and the studies by others [28].

Various in vitro evaluations of blood compatibility and endothelialization of the Hep/Fn coimmobilized samples before and after PBS immersion were performed. The result of hemolysis rate suggested that the amount of the immobilized heparin was not sufficient to cause hemolysis and bleeding complications, which provided the likelihood as a precondition to be used in clinical. The Hep/Fn immobilized samples showed excellent blood compatibility before PBS immersion, such as more AT III binding, less platelets adhesion and activation, and prolonged clotting time, etc. The interaction between heparin and AT III was a direct reason for the excellent blood compatibility of the Hep/Fn immobilized samples. As was proved by the result of AT III in Fig. 6a, the Hep/Fn immobilized surface showed a significant larger AT III density than the other samples. Heparin in solution mainly performs its anticoagulant properties by binding to AT III and indirectly impacting the intrinsic coagulation pathway (aPTT), however, anticoagulant activity of the immobilized heparin depends on different reaction conditions. Both AT III and fibronectin could bind to heparin via their anion binding sites [47], and most AT III binding to heparin could be displaced by fibronectin with an increasing bulk concentration to more than 100 μg/ml [48], thus the bulk concentration of fibronectin may influence the binding of AT III to heparin. In our study, the actual bulk concentration of fibronectin was 50 μg/ml (V_{Hep}:V_{Fn} = 1:1) and not enough to inhibit the binding of AT III to heparin. In addition, the bulk concentration of fibronectin was also proven not to influence the binding of AT III to heparin by an assistant experiment (data were not shown here).

Notice that, after immersion in PBS for several days, aPTT was reduced and more platelets adhered, while TT remained prolonged further. It is easy to understand the former, because heparin released with the immersion time. Nevertheless, it is unclear about the prolonged TT. Possibly, the interaction mechanisms underlying of aPTT and TT are different, aPTT detects the intrinsic coagulation, i.e., an influence on coagulation factors and high molecular weight kininogen, TT detects the last step of coagulation: i.e., the thrombin-mediated fibrin formation. And fibronectin-exposure might affect TT, however, it needs further investigation. From the results of aPTT, TT and immunofluorescence staining of the adherent platelets, we put forward tentatively that though a burst release is observed after one day immersion, the bioactivity of heparin can still keep well after immersion in PBS for several days, which will ensure the application properties. The increased platelets adhesion may be ascribed to the more conformational change of fibrinogen with the immersion time, indicating that the existence of heparin may also influence the adsorption and conformation of fibrinogen. For the evaluation of the effect of the Hep/Fn immobilized films on endothelialization, ECs were cultured on Ti and the Hep/Fn immobilized samples for 1, 3 and 5 days, and the amount and morphology of ECs were evaluated by CCK-8 assay and immunofluorescence staining. Interestingly, significantly reduced ECs on Hep/Fn immobilized surface was observed compared with that on pristine Ti at the 3rd day as shown in Figs. 11 and 12. It was obvious that there was a burst release of heparin after Hep/Fn immobilized samples were immersed in PBS for one day. The released heparin in cell medium may inhibit the proliferation of ECs, which was also reported by Nojiri et al. [14]. Though the exact influence of heparin on ECs prolifereation, inhibiting [14] or promoting [26], was not consistent. Additionally, during the incubation period, an almost confluent coverage of ECs on the Hep/Fn immobilized surface and the silanized surface was observed at the 5th day. The amino groups in silanized surface could promote cell attachment and growth, while the introduction of heparin affected ECs growth. However, as shown in the result of heparin release, the possible inhibition effect of heparin on ECs also decreased with the increased immersion time. Further study of the exposure of RGD peptides on fibronectin after PBS immersion further verified the bioactivity of the immobilized fibronectin. As is known, restenosis caused by SMC proliferation is a serious problem after implantation of biomedical devices. In theory, a sustained released of anti-restenotic drugs for at least three weeks is required to prevent SMC migration and proliferation [49]. Heparin was also proven to have the ability to inhibit SMC proliferation [50]. In our study, a confluent coverage layer of ECs formed at the 5th day culture during which time heparin still kept anticoagulation properties. Incontrovertibly, endothelialization of the implants surface will be the most ideal situation for their long-term implantation. Therefore, the coimmobilization of Hep/Fn provides us a potential and promising method to develop the cardiovascular implants with both anticoagulation and endothelialization properties. Further investigation will focus on the bioevaluation of the Hep/Fn immobilized films under flow conditions in vitro and in vivo experiments.

5. Conclusion

The feasibility, stability and biocompatibility of combining electrostatic interaction and coimmobilization to fabricate Hep/Fn films on cardiovascular biomaterials surface are assessed in the present work. The Hep/Fn coimmobilized films are proven stable and bioactive after immersed in PBS for several days. The bioevaluation results consistently demonstrate that the coimmobilization of Hep/Fn complex simultaneously improves the blood compatibility and endothelialization of biomaterials, which will show potentially great significance for the implantation of biomedical devices in human body. This coimmobilizing method can be extended to other biomolecules to immobilize on different biomaterials or tissue engineering graft. In our opinion, the present surface biomodification technology may offer a potential application of implanted biomaterial devices that are directly in contact with blood and tissue.

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