Letter to the Editor

Inhibiting Smooth Muscle Cell Proliferation via Immobilization of Heparin/Fibronectin Complexes on Titanium Surfaces*

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The aim of this study was to investigate the inhibitory effect of heparin/fibronectin (Hep/Fn) complexes on neointimal hyperplasia following endovascular intervention. Hep/Fn complexes were immobilized onto titanium (Ti) surfaces, with subsequent X-ray photoelectron spectroscopy (XPS), Toluidine Blue O (TBO) and immunohistochemistry methods were used to characterize surface properties. Smooth muscle cell (SMC) cultures were used to evaluate the effect of Hep/Fn complexes on SMC proliferation. Results showed that Hep/Fn complexes successfully immobilized onto Ti surfaces and resulted in an inhibition of SMC proliferation. This study suggests that Hep/Fn surface-immobilized biomaterials develop as a new generation of biomaterials to prevent neointimal hyperplasia, particularly for use in cardiovascular implants.

Biocompatibility is of paramount importance for cardiovascular implants such as the endovascular stents and heart valves that directly come into contact with blood. Neointimal hyperplasia caused by smooth muscle cell (SMC) migration and proliferation is still a major reason for restenosis and atherosclerosis after endovascular intervention[1]. In the present study, fibronectin and heparin were chosen as the constituents for this purpose because fibronectin is an extracellular matrix protein known to promote cell attachment, spreading, differentiation, and phagocytosis, whereas heparin has been shown to effectively inhibit the growth of SMC by preventing the activation of mitogen-activated protein kinase[2]. Heparin and fibronectin form complexes via electrostatic attraction when adjusting a solution's pH. In our previous study, we showed that the immobilization of a Hep/Fn complex on a Ti surface shows both anticoagulant and endothelialization properties[3]; therefore, the main purpose of the present work was to measure the suppressive effect of the Hep/Fn complex on SMC proliferation. In this study, we describe the construction and bioevaluation of the immobilized Hep/Fn complexes on Ti plates for its potential use in blood-contacting implant applications. The modification of the Ti substrate was quantitatively characterized by XPS, quantitative test of heparin and fibronectin. Cell culture was grown and used to evaluate the effect of Hep/Fn Ti films on SMC attachment and proliferation, and the potential mechanisms for SMC inhibition are speculated and discussed.

Ti substrates (1 cm × 1 cm) were prepared using pure Ti plates (99.8%, Shanxi, China). Heparin (Hep), fibronectin, TBO, and 3-aminopropyltriethoxysilane (APTE) were purchased from Sigma-Aldrich. The antibodies, Dulbecco's Modified Eagle Medium (DMEM), and cell counting kit-8 (CCK-8) were all purchased from BD Biosciences (San Jose, CA). The actin staining reagent kit (SABC-FITC) was purchased from Boshide (China). All other reagents were of the highest analytical purity (>99.9%).

The fabrication process of Hep/Fn immobilization on Ti substrate is mentioned in our previous study[3]. Briefly, the Ti substrate was first activated by NaOH (2.5 mol/L) and silanized by APTE (2%); the silanized Ti was subsequently immersed into the blending solution (pH=4) of heparin (5 mg/mL) and fibronectin (100 μg/mL). Finally, the samples were taken out and air dried. The samples were named as follows: The NaOH-activated Ti substrate is referred to as TiOH, the silanized TiOH surface by APTE is denoted as TiOHA, and the TiOHA sample immobilized with the Hep/Fn mixture is denoted as HF-Ti. The elemental chemical binding properties of the surfaces were determined by high-resolution scanning XPS (Perkin Elmer 16PC).

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The amount of surface immobilized heparin and exposed RGD sequences on HF-Ti surfaces was determined by a TBO assay and immunochemistry, as described in our previous study[3]. Next, SMCs were explanted from human umbilical artery sources and cultured in DMEM supplemented with 10% FCS, 0.68 mmol/L l-glutamine (0.68 mmol/L, Sigma Chemical Co., St. Louis, MO), 100 U/mL penicillin, and 100 μg/mL streptomycin, and the medium was changed every 2-3 d. The third passage of SMC culture was used to evaluate the viability and proliferation with a CCK-8 assay kit after 1, 3, and 5 d of incubation. Finally, immunofluorescence staining of SMC actin, using an SABC-FITC kit, was performed according to the instructions of the kit, and the samples were photographed using an inverted fluorescence microscope (Leica, Germany).

SPSS 13.0 (Stanford, USA) was used to analyze the data by one-way ANOVA analysis. Probability (*P*) value <0.05 was considered significant in the study.

A peak-fitted XPS spectrum for the C1s core level of TiOHA is shown in Figure 1A. The spectrum satisfactorily fit a combination of four distinct peaks: the peak at 282.3 eV corresponded to C-Si groups, the peak at 284.8 eV to C-C and C-H moieties, the peak at 285.9 eV to C-NH_2 functional groups, and the peak at 287.9 eV to C-O groups, with relative intensities obtained from the fit. After heparin immobilization on TiOHA (Figure 1B), the peak at 285.9 eV shifted to 286.1 corresponding to C-N, C-O, and C-OH groups, while a new peak resulted at 288.5 eV for the -COOR or -COOH groups from heparin. After fibronectin immobilization on TiOHA (Figure 1C), the peak at 286 eV corresponded to C-NHx moieties, and the peak at 287.9 corresponded to C=O groups from fibronectin. For HF-Ti (Figure 1D), the peak at 286 eV corresponded to C-N, C-OH, and CH_2-CNO groups from heparin and fibronectin. Because both heparin and fibronectin contain C-N functional groups, there was a possibility of overlapping peaks in the C1s region, thereby complicating the XPS analysis. Nevertheless, our XPS analysis revealed a successful immobilization of Hep/Fn complexes on TiOHA surfaces.

Table 1 shows that the HF-Ti surface displayed significantly larger (*P*<0.05) amounts of heparin and fibronectin than TiOHA and bare Ti surfaces. The amount of heparin was approximately 3.8 μg/cm^2, which was significantly higher than that achieved in previous reports describing the covalent grafting of heparin[4], but was slightly lower than that reported by Byun et al.[5]. The amount of fibronectin was also

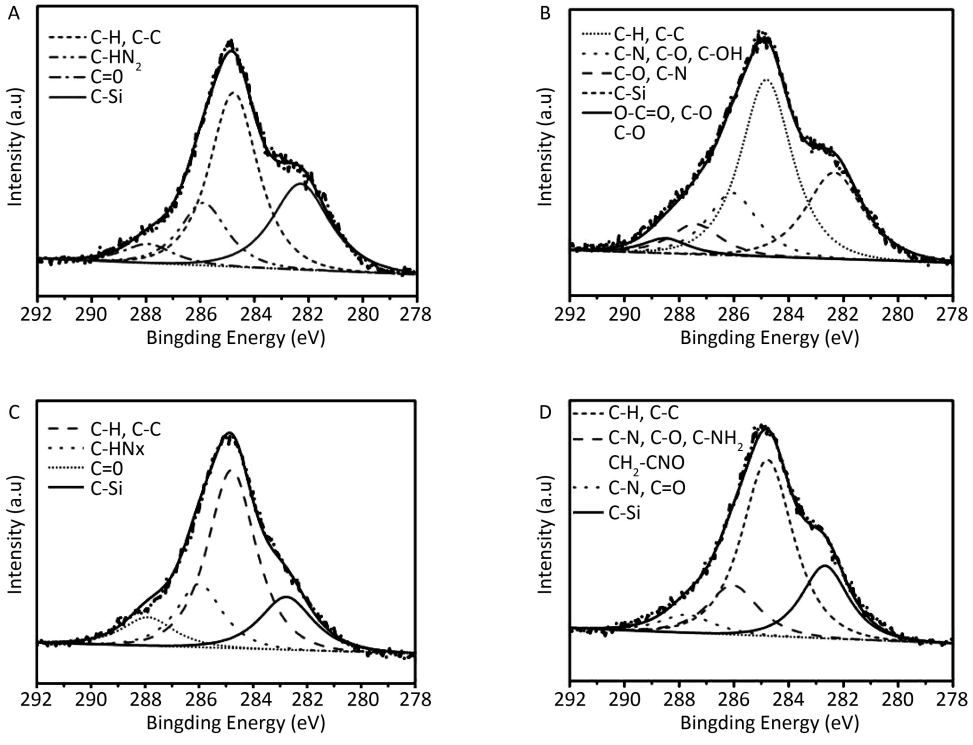

Figure 1. High-resolution XPS analysis of C1s spectra of surface coatings in the presence of the surface immobilized biomolecules. (A) TiOHA; (B) TiOHA-Hep; (C) TiOHA-Fn; (D) HF-Ti.

significantly higher than that on TiOHA and Ti surfaces but was less than that in the literature[6]. This may be due to the different amount and orientation of surface amino groups, the competition of heparin with fibronectin in the Hep/Fn complex, and the complex competing to bind to the amine-coated surface. Additionally, the exposure of RGD sequences on fibronectin was significantly higher than the control Ti and TiOHA (P<0.05), also indicating the existence of fibronectin. These results further demonstrate that heparin and fibronectin were successfully immobilized on the surface.

Figure 2A shows the proliferation of SMC after incubation for 1, 3, and 5 d, respectively. SMC on pristine Ti and TiOHA surfaces exhibited significantly increased proliferation compared with that on all Hep/Fn immobilized surfaces (P<0.05), while SMC on Hep/Fn immobilized surfaces displayed less proliferation than the pristine Ti after culture for 3 days and showed no proliferation at day 5. The amount of SMC on pristine Ti surface increased linearly from day 1 to day 5. In contrast, the amount of SMC on HF-Ti increased from day 1 to day 3 and decreased from day 3 to day 5. SMC on HF-Ti exhibited the largest proliferation at day 3. This may be due to a burst release of heparin after the first day of incubation[7]. Heparin was previously shown to inhibit SMC proliferation[8]; however, the study noted that there was also a heparin release into the medium, and the inhibitory effect of residual heparin on SMC growth. Therefore, SMC proliferation was observed. Notably, the proliferation was temporal and did not get rid of the inhibitory effect of heparin after further incubation in this study. Our results demonstrated that immobilization of Hep/Fn complexes could significantly inhibit SMC proliferation.

Figure 2B depicts the cytoskeleton of SMC. The Hep/Fn immobilized surface exhibited less SMC attachment and proliferation than the pristine Ti on the 1st, 3rd, and 5th day. SMC cultured on Hep/Fn immobilized surfaces displayed more proliferation on the 3rd day than those on the 1st and 5th day, which were both consistent with CCK-8. SMCs with hypertrophic rectangular shapes showed an almost confluent coverage on pristine Ti surfaces after 5 d culture, while spindle-shaped SMCs without confluence were observed on HF-Ti surfaces. SMC dedifferentiation from a contractile to a synthetic state was considered to be an obligatory precursor in the development of proliferative intimal lesions, e.g., from spindle-shape to a more hypertrophic, rectangular appearance. It could be seen that SMC

Table 1. Amounts of Heparin and Fibronectin and the Conformational Change of Fibronectin on Various Samples

Sample	Heparin (μg/cm^2)	Fibronectin (OD)	RGD Exposure (%)
Ti	0.52±0.01	0.06±0.01	9.39±0.15
TiOHA	0.81±0.04	0.11±0.01	11.32±1.16
HF-Ti	3.75±0.09*	0.54±0.09*	23.66±2.32*

Note. *P<0.05 compared with Ti and TiOHA samples, mean±SD, n=4.

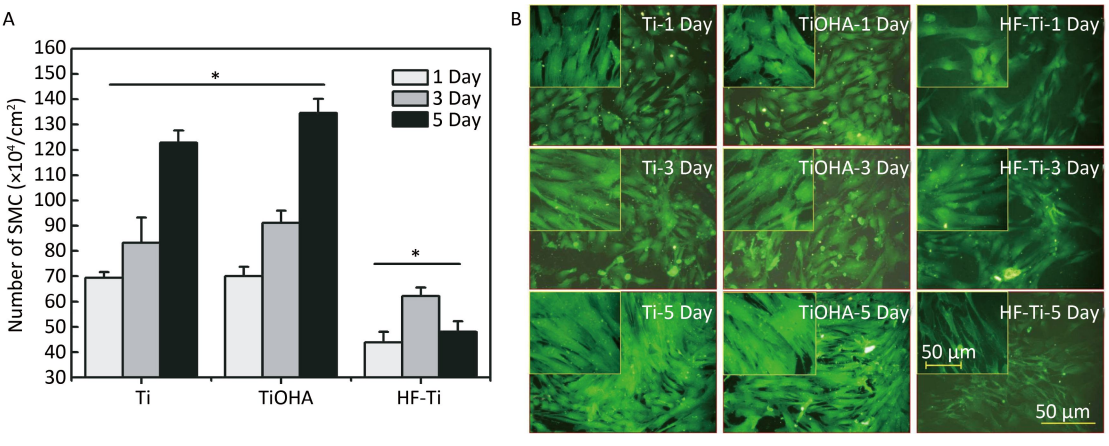

Figure 2. Attachment and proliferation (A) and immunofluorescence staining (B) of SMC cultures on pristine Ti, TiOHA, and HF-Ti for 1, 3, and 5 d, respectively. (*P<0.05, mean±SD, n=4).

displayed a more rectangular shape on Ti surfaces than that on HF-Ti surfaces, and SMC exhibited more contractile shapes on HF-Ti surfaces on the 5th day than that of the 1st and 3rd days. These results indicate that the distribution and proliferation of SMC on HF-Ti surfaces were effectively inhibited, and the Hep/Fn immobilized sample promoted better inhibitory properties than pristine Ti.

Decreasing platelet activation and suppressing vascular intimal hyperplasia has been simultaneously achieved by Scott et al.[1]. However, as is known, endothelialization is the most ideal situation for long-term cardiovascular implants, while intimal hyperplasia and restenosis caused by SMC proliferation are serious problems that lead to the failure of biomedical devices; thus, it is necessary to inhibit SMC proliferation on the surface of implants after implantation. In our study, heparin has been used to inhibit SMC proliferation, and fibronectin was used to target endothelial cell attachment and growth. Interestingly, fibronectin has a cell-binding site for both EC and SMC; therefore, fibronectin could potentially promote both EC and SMC proliferation simultaneously. However, in our study, heparin and fibronectin formed a complex first, and this formation of the Hep/Fn complex changed the individual properties and conformation of each biomolecule. Heparin-binding fragments of fibronectin (HBFF), an amino terminal 29 kDa segment and a carboxyl terminal 40 kDa segment, have been shown to be apparently specific, potent inhibitors of bovine aortic EC growth[9], though the effect of heparin on EC growth is still debated. It should be mentioned that in this study, the formation of the Hep/Fn complex inhibits this potential EC growth effect because the potent inhibitors (HBFF) of EC on fibronectin are blocked by the bound heparin, while the cell-binding sites (RGD, 120 kDa) on fibronectin are not affected. Thus, a weak inhibitory effect on EC growth is observed, and a confluent coverage layer of EC formed on Hep/Fn immobilized surfaces at the 5th day of culture.[3]

Lundmark et al.[10] have reported that in the presence of heparin, rat aortic SMC adhesion to fibronectin was inhibited in a dose-dependent manner with a maximal inhibition at 1 μg/mL of heparin, a concentration much higher than that used in our study. Moreover in that study, heparin did not affect the amount of surface-bound fibronectin. Thus, mixing fibronectin and heparin prior to coating was necessary for the anti-adhesive effect of heparin. This was consistent with our preparation of Hep/Fn complexes, where we saw that the binding of heparin can cause conformational changes in soluble fibronectin, allowing us to use it as an inhibitor of SMC binding when it is immobilized. In addition, we demonstrate here that inhibition of SMC adhesion to fibronectin by heparin is not dependent on the main heparin-binding domains of fibronectin (HBFF) because heparin also prevented SMC adhesion to the 105 and 120 kDa cell-binding fragments (RGD) of fibronectin. Therefore, it is feasible to accelerate endothelialization while simultaneously inhibiting SMC proliferation via immobilization of Hep/Fn complexes.

In summary, Hep/Fn complexes can effectively inhibit the proliferation of SMC; thus, the immobilization of Hep/Fn complexes provides a method that may be used to prevent neointimal hyperplasia to Ti. This promising initial study provides a lead for the development of a new generation of cardiovascular biomaterials and tissue engineering devices. Ongoing work includes conducting studies that will elucidate the molecular mechanism of Hep/Fn complexes and their effect on biocompatibility *in vivo.*

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