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Synthetic ePTFE Grafts Coated with an Anti-CD133 Antibody-Functionalized Heparin/Collagen Multilayer with Rapid in vivo Endothelialization Properties

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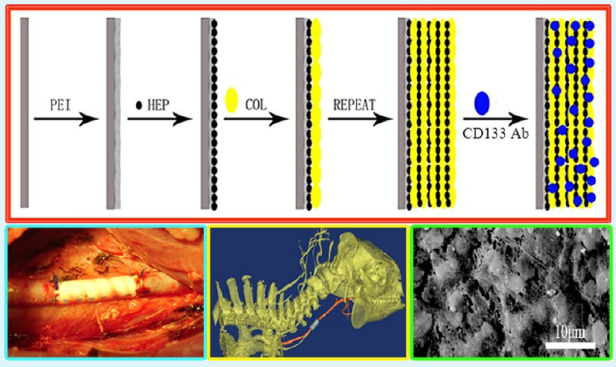
ABSTRACT: An anti-CD133 antibody multilayer functionalized by heparin/collagen on an expanded polytetrafluoroethylene (ePTFE) graft was developed to accelerate early endothelialization. The surface modification of ePTFE grafts demonstrated that the multilayer is stable in static incubation and shaking conditions and that the anti-CD133 antibodies were successfully cross-linked onto the surface. Blood compatibility tests revealed that the coimmobilized heparin/collagen films in the presence or absence of anti-CD133 antibodies prolonged the blood coagulation time and that there was less platelet activation and aggregation, whereas the hemolysis rate was comparable with the bare ePTFE grafts. Cellular proliferation was not inhibited, as the heparin/collagen synthetic vascular grafts coated with CD133 antibody showed little cytotoxicity. The endothelial cells adhered well to the modified ePTFE grafts during a cell adhesion assay. A porcine carotid artery transplantation model was used to evaluate the modified ePTFE grafts in vivo. The results of histopathological staining and scanning electron microscopy indicated that the anti-CD133 antibody was able to accelerate the attachment of vascular endothelial cells onto the ePTFE grafts, resulting in early rapid endothelialization. The success of the anti-CD133 antibody-functionalized heparin/collagen multilayer will provide an effective selection system for the surface modification of synthetic vascular grafts and improve their use in clinical applications.



KEYWORDS: *ePTFE grafts, surface modification, endothelialization, endothelial cells, platelet adhesion*

1. INTRODUCTION

Expanded polytetrafluoroethylene (ePTFE) is a favorable synthetic vascular material; large-diameter ePTFE grafts have been successfully used in clinical applications. Medium- to small-diameter ePTFE grafts, which are used for peripheral bypass conduits to the lower extremities, or as alternative arteriovenous (AV) grafts for hemodialysis, exhibit poor long-term patency because the adsorption of plasma proteins and the adhesion of platelets may induce early thrombus formation.[1,2] Endothelialization is a crucial factor for long-term implantation owing to its excellent anticoagulant properties. Rapid and complete coverage with a functional endothelial layer may effectively decrease thrombus formation, limit intimal hyperplasia, and prolong the implant's function.[1,3] Spontaneous in situ endothelialization of synthetic ePTFE vascular grafts is slow and difficult to achieve due to the graft's highly hydrophobic behavior toward endothelial cell adhesion, spreading, and growth.[4,5] Therefore, there is a need for the surface modification of ePTFE vascular grafts to avoid platelet aggregation along with better endothelialization. However, previous studies have shown that when the antithrombotic effect is improved with anticoagulant molecules, the growth of endothelial cells is inhibited, whereas when the growth of endothelial cells is promoted with extracellular matrix molecules, the blood compatibility deteriorates.[6] Thus, most studies just focus on one aspect of biocompatibility or endothelialization. In the present study, we intend to coimmobilize different biomolecules on biomaterials and improve both their antithrombotic properties and endothelialization. Heparin and collagen were selected as the constituents for this purpose, because heparin is the most popular clinically used anticoagulant for minimizing thrombus formation, whereas collagen is an extracellular matrix protein known to promote cell attachment, spreading, and differentiation.[7−9] CD133 is a cell surface antigen that is specifically expressed on endothelial progenitor cells. These cells are highly proliferative and play an important role in the regeneration and repair of damaged blood vessels.[10] Previous studies have shown that anti-CD34

Received: May 7, 2013
Accepted: July 16, 2013
Published: July 16, 2013

antibody-coated stents/vascular grafts promote rapid in situ endothelialization; however, long-term results show that these antirestenosis effects may not be as remarkable as expected.[8,11,12] CD133 is more specific than CD34 as a surface marker for endothelial progenitor cells. Therefore, the immobilization of an anti-CD133 antibody onto the heparin/collagen multilayer can achieve satisfactory antithrombotic and endothelialization results.

In this paper, we aimed to describe the construction and bioevaluation of an anti-CD133 antibody-functionalized heparin/collagen multilayer on ePTFE grafts. The construction of the heparin/collagen multilayer and immobilization of the anti-CD133 antibody have been described in detail. Blood compatibility and biocompatibility of the modified ePTFE vascular grafts were also tested. Finally, using a porcine carotid artery transplantation model, we demonstrated the early, rapid in situ endothelialization of the modified ePTFE synthetic vascular grafts.

2. MATERIALS AND METHODS

2.1. Materials. ePTFE films/grafts were commercially obtained from W.L. Gore & Associates, Inc. (6 mm in diameter; USA). Low-molecular-weight heparin (HEP; Aladdin Chemistry Co. Ltd.) and collagen (COL; Shanghai Yuanye Biology Co. Ltd.) were dissolved in acetic buffer solution (pH = 4.0) at a final concentration of 1 mg/mL. Polyethylenimine (PEI, mw = 70000; Sigma) solution was diluted in phosphate-buffered saline (PBS; pH = 7.4) at a final concentration of 3 mg/mL. The mouse monoclonal antihuman CD133 antibody was purchased from Miltenyi Biotec Inc. Germany. All other reagents used in the experiments were of the highest analytical purity.

2.2. Hep/Col Multilayer Construction and Anti-CD133 Antibody Immobilization. Synthetic ePTFE grafts coated with an anti-CD133 antibody-functionalized HEP/COL multilayer were prepared using a multistep process. The first step involved PEI adsorption for 1.5 h, which resulted in a stable positively charged graft surface. The substrates were then dipped alternately in HEP solution and COL solution to form a 5-bilayer HEP/COL polymer. Each step was followed with acetic buffer solution rinsing and nitrogen stream drying. Next, the HEP/COL multilayers were immersed in 0.25% glutaraldehyde in PBS for 2 h to promote cross-linking and to immobilize the anti-CD133 antibody.

The surface morphology of the HEP/COL multilayer was observed by scanning electron microscopy (SEM), and the whole HEP/COL multilayer fabrication process was also monitored by infrared spectroscopy. The immobilization and stability of the anti-CD133 antibody was measured by confocal laser scanning microscopy (Leica, Germany). In brief, the samples were immersed in PBS at 37 °C and shaken for 2, 6, 12, 24, or 48 h in a sealed container at 40 rpm. All the PBS soaked samples at each time point were then removed, dried, and observed under a confocal laser scanning microscope. Water contact angles were measured with a contact angle apparatus (JY-82; China) to reveal the hydrophilic changes of the modified ePTFE grafts.

2.3. In vitro Hemocompatibility and Biocompatibility Test. *2.3.1. Hemolysis Rate Test.* The samples were immersed into diluted blood solution containing 2% fresh anticoagulant blood and 98% physiological salt solution and then incubated at 37 °C for 1 h. Samples were then centrifuged at 3000 rpm for 5 min, and the absorbance of the solution recorded at 570 nm as A_t. Under the same conditions, the solution containing 2% fresh anticoagulant blood and 98% physiological salt solution was used as the negative control; the solution containing 2% fresh anticoagulant blood and 98% ddH_2O was used as the positive control. The absorbance values measured were recorded as A_{nc} and A_{pc}, respectively. The hemolysis rate α was calculated using the following formula:

$$\alpha = (A_t - A_{nc})/(A_{pc} - A_{nc}) \times 100\%$$

2.3.2. Plasma Recalcification Time (PRT). The PRT of the modified ePTFE biomaterial was measured, and the bare ePTFE biomaterial was used as the control. Blood was drawn from healthy human donors and was then centrifuged at 3000 rpm for 15 min to obtain platelet-poor plasma (PPP). The PPP and 0.025 mol/L $CaCl_2$ solution were warmed to 37 °C. PPP (0.1 mL), dropped onto the sample surfaces, and incubated for 1 min, after which $CaCl_2$ (0.1 mL) was added. The recalcified plasma was brought back to the surface by gentle stirring with a small stainless hook. The time taken for a silky fibrin to appear was recorded as the PRT.

2.3.3. Platelet Adhesion Test. Platelet adhesion of the modified and bare ePTFE biomaterials was qualitatively tested by SEM. Blood was drawn from healthy human donors and was then centrifuged at 1500 rpm for 15 min to obtain platelet-rich plasma (PRP). Modified and bare ePTFE films (0.5 cm × 0.5 cm) were sequentially added into the PRP solution and incubated at 37 °C with 5% CO_2 for 1 h. After incubation, all of these films were gently washed with PBS to remove nonspecifically adhered platelets. Finally, all samples were fixed, dehydrated, and coated with gold for SEM observation.

2.3.4. Cell Adhesion Test. Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical cords of newborns by using enzymatic digestion. Cells at passage 2−3 were used. The cell culture medium for the HUVECs was DMEM (Gibco, USA) supplemented with 20% FBS and 100 IU penicillin/100 mg/mL streptomycin. Cells were cultured in humidified air containing 5% CO_2 at 37 °C. At confluence, the cells were trypsinized for further passage or used for the tests.

Before the cell adhesion test, HUVECs were marked with CellTracker CM-DiI (Invitrogen). The ePTFE scaffolds were placed in a 96-well plate. Marked HUVECs were then added at 2×10^4 cells per well. The cells were allowed to attach for 4 h. At the indicated times, the cells were washed 3 times with PBS to remove unattached or weakly adherent cells. Subsequently, the cells were then fixed in 4% paraformaldehyde for 10 min at room temperature and washed with PBS, followed by DAPI (Invitrogen) staining for 5 min. The labeled cells were then visualized by fluorescence microscopy. Nine random fields for each experiment were analyzed with Image-Pro Plus image analysis software to calculate adherent cell numbers. The mean adhesion rate value was calculated based on three independent experiments.

2.3.5. Cell Cytotoxicity Test. The leaching solutions were prepared at 37 °C, 0.2 g/mL by mixing different ePTFE grafts with DMEM for 12, 24, and 48. Cell cytotoxicity was subsequently tested using the cell proliferation assay, CCK-8. The CCK-8 assay is based on the tetrazolium salt, WST-8, which produces a water-soluble compound, formazan, upon bioreduction by cellular dehydrogenases. The amount of formazan produced is directly proportional to the number of living cells. Briefly, HUVEC cells were seeded at 2×10^4 cells per well into 96-well plates and cultured in a 5% CO_2 incubator at 37 °C for 4 h. The medium in each well was then replaced with a mixture of fresh DMEM culture medium and different leaching solutions and cultured for 10 h. Finally, 10 μL of CCK-8 reagent (WST-8) was added. After 2 h of incubation with the cells, the WST-8 salt turned orange. The supernatant was then transferred to a new 96-well plate, and the OD_{450} value was measured with a Bioelisa Reader (Elx800; China). This experiment was carried out in triplicate with three independent replicates.

2.4. In vivo Animal Experiments. All animal experiments were approved by the Animal Care and Use Committee of Zhongshan Hospital, Fudan University (Shanghai, China). Healthy white male pigs (50 kg, provided by the Experimental Center of Zhongshan Hospital) were used for the experiments.

2.4.1. Synthetic ePTFE Graft Implantation. As described in our previous work, before each operation or termination, the pigs were fasted overnight. The following day, they were premedicated with intramuscular ketamine hydrochloride (10 mg/kg), diazepam (10 mg/kg), and atropine (0.5 mg). An ear-vein pathway was then established and used for the continuous administration of ketamine hydrochloride and diazepam (1:1) and an infusion of balanced fluids. The animals

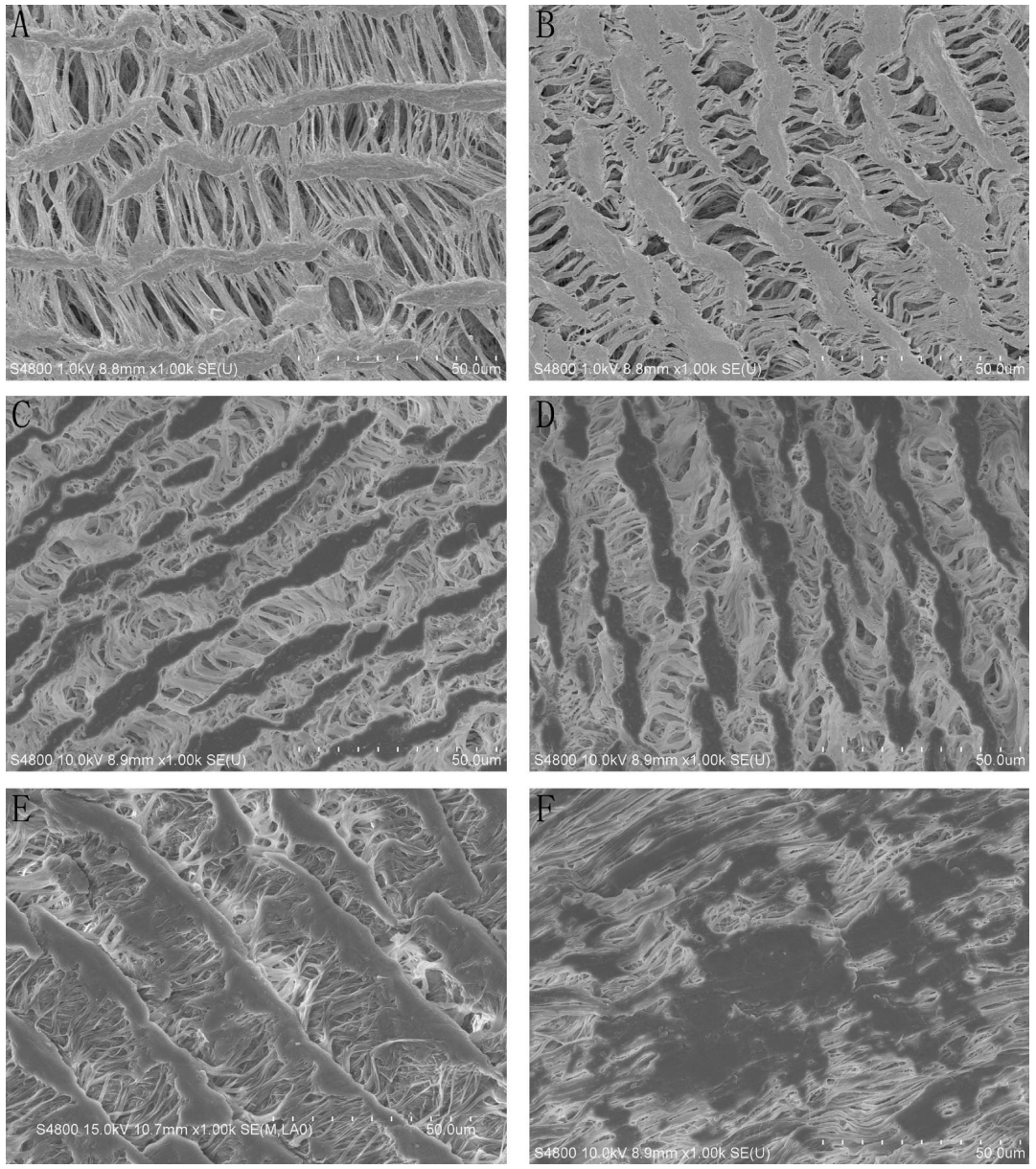


Figure 1. Scanning electron micrograph of bare ePTFE grafts and $(HEP/COL)_n$ multilayer modified ePTFE grafts. The term n represents the number of HEP/COL bilayers: (A) $n = 0$; (B) $n = 1$; (C) $n = 2$; (D) $n = 3$; (E) $n = 4$; (F) $n = 5$; original magnification: 1000×.

were then intubated and ventilated (50% O_2) with the assistance of breathing machines.

The neck skin was prepared and draped in a sterile fashion. Graft implantations were performed by experienced cardiovascular surgeons (X.S. and S.L.). Heparin (125 U/kg, IV) was administered to maintain an activated clotting time of greater than 250 s before transplantation of the synthetic grafts. The common carotid artery was dissected freely and clamped at the proximal and distal ends. After cutting the common carotid artery, papaverin (5 mg/mL) was applied locally to prevent vascular spasm. An end-to-end anastomosis was then carried out using a continuous suture of 5-0 polypropylene (Ethicon), and the circulation was restored after careful deairing. All ePTFE grafts (3 bare ePTFE grafts, 3 HEP/COL-modified ePTFE grafts, and 3 HEP/COL-CD133 antibody-modified ePTFE grafts) were 6 mm in diameter and 25 mm in length. All animals were administrated with aspirin enteric-coated tablets (100 mg, bid), warfarin sodium (2.5 mg, bid), and subcutaneous low-molecular-weight heparin calcium (5000 IU/d) on postoperative days.

2.4.2. Tissue Preparation and Histological Analysis. After 7 days of follow-up, the pigs were anesthetized as described previously. Heparin (12 500 U) was administered before the operation. The implanted graft was removed and cut longitudinally to observe the presence of thrombus and anastomotic stoma. The graft was then cut into several sections, some were immersed in 4% formalin for histological analysis and the others were fixed in 2% glutaraldehyde for SEM.

After fixation with 4% formalin for 24 h, the grafts were cut into 5-mm blocks and embedded in paraffin. HE staining was performed to analyze intimal coverage. Immunohistochemistry of von Willebrand Factor (vWF) was carried out to analyze the intimal components.

2.4.3. Scanning Electron Microscopy. The integrity of the cellular coverage of the modified and bare ePTFE grafts was observed by SEM. The extracted ePTFE grafts were fixed in 2% glutaraldehyde and then dehydrated by increasing concentrations of ethanol (80%−100%). Subsequently, the samples were dried, coated with a thin layer of gold, and analyzed with SEM.

2.5. Statistical Analysis. The results are presented as mean ± standard deviation (SD). Comparisons were made using a *t*-test, Pearson X^2 test, or nonparametric equivalent Mann−Whitney test.

The significance level was set at $p < 0.05$. Statistical analysis was performed using SPSS 14.0 software (SPSS Inc., Chicago).

3. RESULTS

3.1. Characterization of the HEP/COL Multilayer Fabrication and CD133 Antibody Immobilization. *3.1.1. Characterization of the HEP/COL Multilayer Fabrication.* The buildup of the $(HEP/COL)_n$ multilayer coating was monitored by SEM (Figure 1) and infrared spectroscopy (Figure 2). Here, "*n*" represents the number of HEP/COL

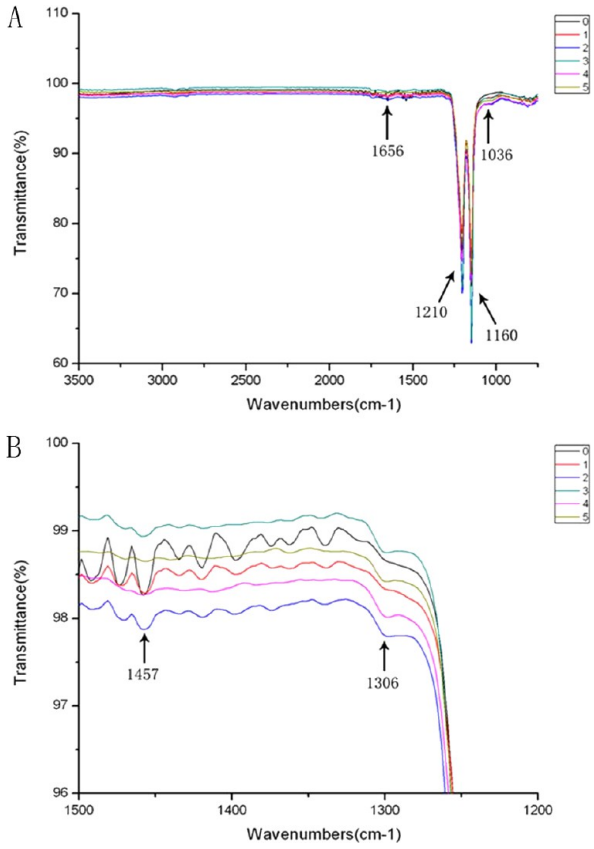


Figure 2. Distinctive peaks from the infrared spectroscopy: (A) sulfonic group ($-OSO_3$, 1036 cm^{-1}) and hydroxy group (−OH, 1656 cm^{-1}) of heparin; (B) collagen (1457 and 1306 cm^{-1}). The term *n* represents the number of HEP/COL bilayers.

bilayers; the bare ePTFE material represents a kind of polyporous netty material (Figure 1A). The surface morphologies of the $(HEP/COL)_{1-5}$ multilayer change with the multilayer growth indicate the successful deposition of the polyelectrolyte multilayer onto the substrates (Figure 1B−F). When the fifth HEP/COL bilayer was completed, there was an obvious membranous electrolyte film on the surface of the ePTFE graft (Figure 1F).

The transmittance of ePTFE grafts changed with every new HEP/COL bilayer, indicating the adsorption of the HEP or the COL molecules onto the material surface (Figure 2). Additionally, distinctive peaks of the heparin sulfonic group ($-OSO_3$) symmetric vibrational band centered at approximately 1036 cm^{-1} and the hydroxy group (OH) centered at approximately 1656 cm^{-1} were observed (Figure 2A). The peaks at 1457 and 1306 cm^{-1} indicate that the collagen retains its triple-helical structure (Figure 2B). All these results indicate that collagen and heparin exist in the coating films.

3.1.2. Anti-CD133 Antibody Immobilization and Stability Investigation. Anti-CD133 antibody immobilization and stability were investigated using PE and APC-marked CD133 antibodies with or without PBS immersion; the results are shown in Figure 3. Compared with the control ePTFE graft (Figure 3A), red (PE) fluorescence was observed by fluorescence microscope indicating that the anti-CD133 antibodies were successfully coated onto the surface of the HEP/COL multilayer (Figure 3B). The stability is of greater importance when the immobilized anti-CD133 antibody functionalized HEP/COL multilayer films are used for synthetic vascular graft coating. After shaking with PBS for 2, 6, 12, 24, and 48 h, the modified ePTFE grafts continued to be observed with bright red fluorescence (Figure 3C−G), which indicates a reliable stability of the anti-CD133 antibody immobilization.

3.1.3. Contact Angle Test. The water contact angles were measured as a function of the process of the ePTFE substrate modification (Figure 4A). Heparin and collagen are both hydrophilic biomolecules, and immobilization of these two biomolecules could introduce several hydrophilic groups and thus improve the ePTFE surface hydrophilicity. A contact angle test showed that the hydrophilicity increased as the contact angle decreased from 127.2° to 106.9° before and after the ePTFE surface was modified with heparin, collagen, and CD133 antibodies (Figure 4B). These results also suggest the formation of HEP/COL films on the ePTFE surfaces.

3.2. Hemocompatibility and Biocompatibility Assessment. *3.2.1. Hemolysis Rate Test.* The hemolysis rate is an important factor for the characterization of blood compatibility. The lower the hemolysis rate, the better the blood compatibility. The hemolysis rates of the HEP/COL-CD133 antibody-modified ePTFE, HEP/COL-modified ePTFE, and ePTFE were 1.56%, 0.71%, and 2.40%, respectively ($p > 0.05$; Table 1). It could be seen that the hemolysis rates of both modified ePTFEs were lower than the pristine ePTFE and far below the accepted threshold value of 5% (GB/T 16886 and ISO 10993), implying a good hemocompatibility.

3.2.2. Plasma Recalcification Time (PRT). Hemocompatibility of the HEP/COL-CD133 antibody multilayer, HEP/COL multilayer, and ePTFE were also assessed using PRT. The plasma clotting time of ePTFE is approximately 510 ± 137.5 s. The plasma clotting time is significantly prolonged after HEP/COL or HEP/COL-CD133 antibody multilayer modification. No clotting was detected at 20 min. The excellent hemocompatibility of the HEP/COL multilayer before and after anti-CD133 antibody functionalization may be attributed to the anticoagulant heparin.

3.2.3. Platelet Adhesion Test. The results of the platelet adhesion on different samples are shown in Figure 5. It can be observed that after incubation in PRP for 1 h, a marked platelet adhesion was observed on the ePTFE surface. The HEP/COL-modified ePTFE, in the presence or absence of the CD133 antibody, showed less platelet adhesion compared with ePTFE (HEP/COL-CD133, HEP/COL vs ePTFE: 3.0 ± 2.8, 2.5 ± 2.4 vs 28.5 ± 4.8; $p < 0.05$).

3.2.4. Cell adhEsion Test. Because excellent cell compatibility of a synthetic blood vessel is the first step of endothelialization, we checked the adhesion of HUVECs onto the HEP/COL in the presence or absence of the CD133 antibody-modified ePTFE grafts, and we compared it to the pristine ePTFE graft. HUVECs were able to attach onto all grafts after 4 h of incubation (Figure 6A−C). The number of

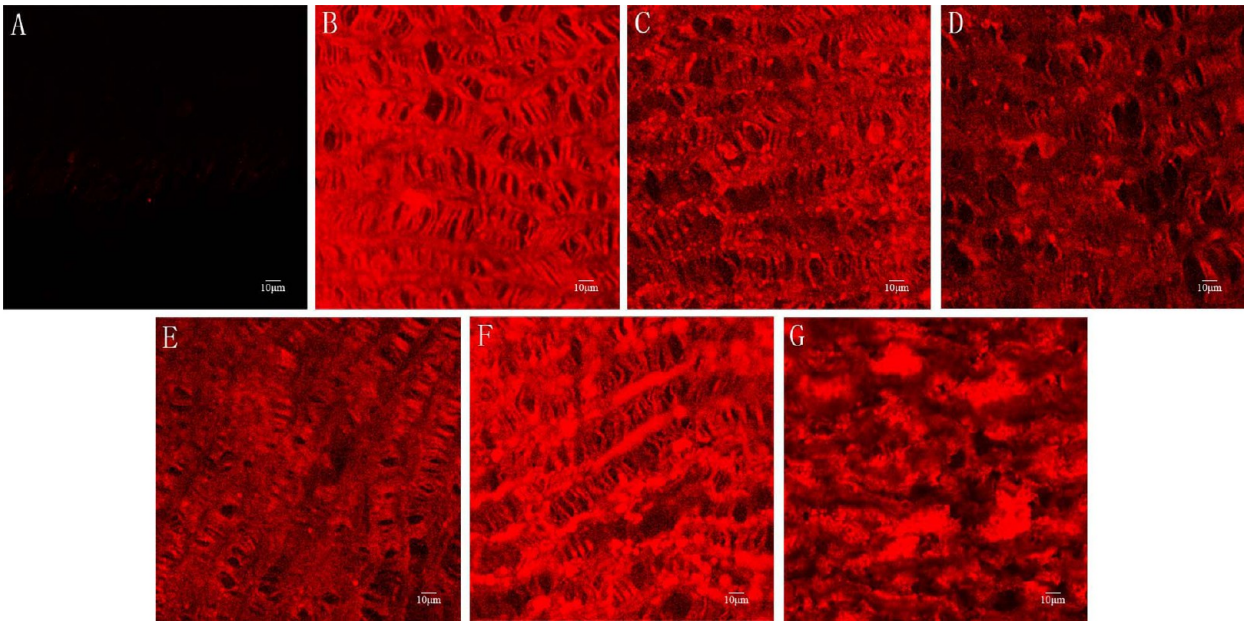


Figure 3. Confocal laser scanning microscopy images of $(HEP/COL)_5$ multilayer modified ePTFE grafts without (A) and with anti-CD133-PE antibody (B) and washed with PBS for 2, 6, 12, 24, and 48 h (C−G) (original magnification: 63×).

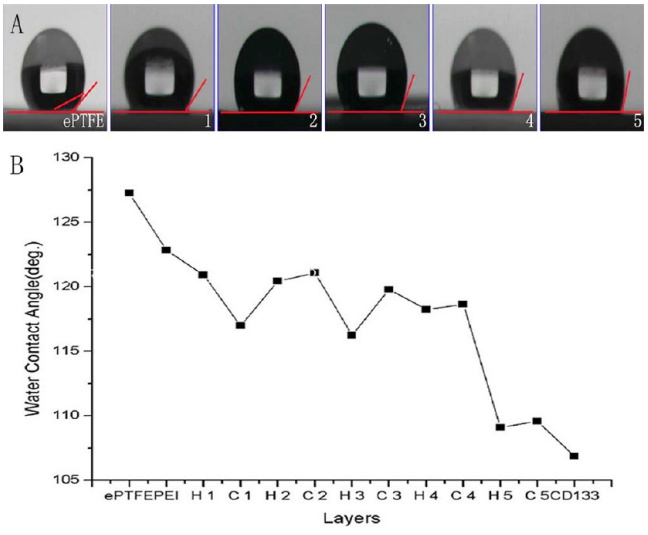


Figure 4. Water contact angle measurements of the bare ePTFE graft and $(HEP/COL)_{1-5}$-modified ePTFE grafts (A) and the trend of the decreasing water contact angle shown by dots-line graph (B).

Table 1. Hemolysis Rate Test

group	OD value	hemolysis rate (%)
HEP/COL-CD133 ePTFE	0.063 ± 0.004	1.56
HEP/COL ePTFE	0.061 ± 0.006	0.71
bare ePTFE	0.065 ± 0.009	2.40
positive control	0.295 ± 0.004	
negative control	0.060 ± 0.004	

adhered HUVECs in the HEP/COL in the presence or absence of the CD133 antibody-modified ePTFE groups were higher than those in the ePTFE control group (HEP/COL-CD133, HEP/COL vs ePTFE; $p < 0.05$; Figure 6D).

3.2.5. Cell Cytotoxicity Test. We used a CCK-8 assay to evaluate the possible toxicities of HEP/COL with or without CD133 antibody-modified ePTFE vascular grafts, by monitoring the endothelial cell survival in biomaterial-conditioned medium. Cytotoxicity was calculated from average optical density values at 450 nm. There was no significant difference in cell proliferation among the three groups when the HUVECs were treated with different leaching solutions (12, 24, and 48 h) as described above ($p > 0.05$; Figure 7). Therefore, the modified ePTFE vascular prosthesis developed in our study showed little cytotoxicity.

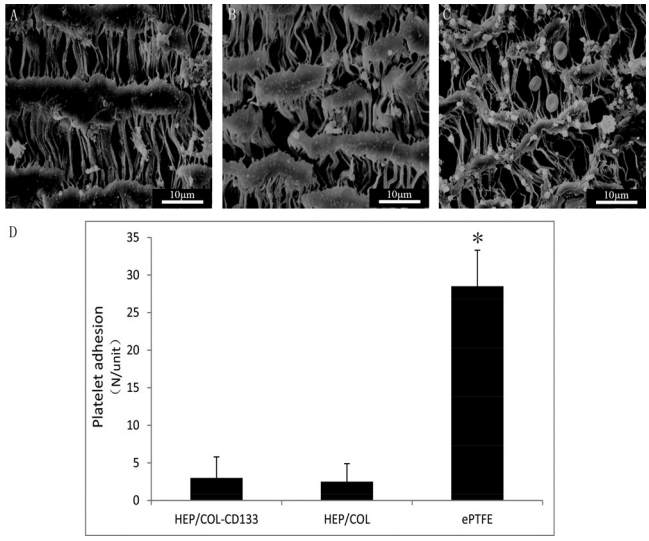


Figure 5. Platelet adhesion test for the anti-CD133 antibody-functionalized $(HEP/COL)_5$ multilayer (A), $(HEP/COL)_5$ multilayer (B), and bare ePTFE grafts (C). Significant differences were observed between the $(HEP/COL)_5$ modification with or without CD133 antibody ePTFE grafts and bare ePTFE grafts (HEP/COL-CD133, HEP/COL vs ePTFE: 3.0 ± 2.8, 2.5 ± 2.4 vs 28.5 ± 4.8; $p < 0.05$, D) (original magnification 1000×).

3.3. Porcine Carotid Artery Transplantation Model Evaluation. *3.3.1. Postoperative Patency Examination.* Seven days after graft implantation, all pigs were examined with Doppler ultrasonography or CT angiography before

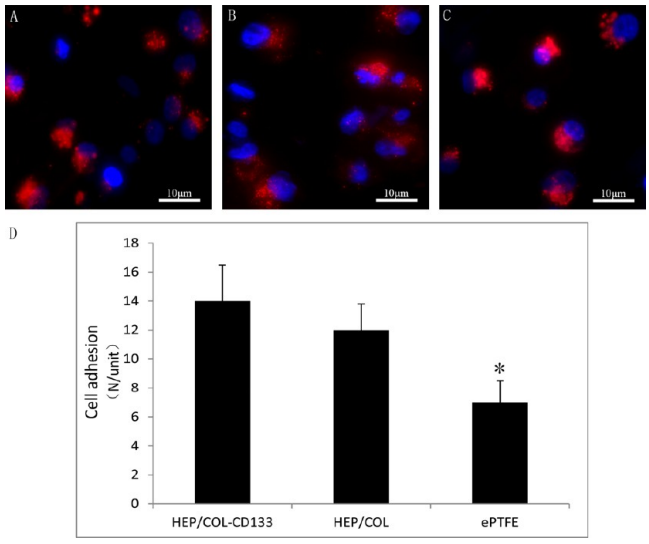


Figure 6. Cell adhesion test for ECV304 with $(HEP/COL)_5$ with or without CD133 antibody-modified or bare ePTFE grafts (A–C). Significant differences were observed between the $(HEP/COL)_5$ modified with or without CD133 antibody ePTFE grafts and the bare ePTFE grafts (HEP/COL-CD133, HEP/COL vs ePTFE; $p < 0.05$, D) (original magnification: 400×).

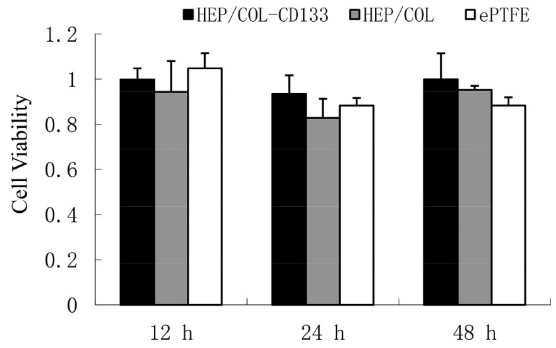


Figure 7. Cell toxicity test of ECV304 treated with different leaching solutions (12, 24, and 48 h; $p > 0.05$).

termination to verify whether a thrombus had formed or not. All implanted ePTFE grafts, including the HEP/COL-CE133 antibody-modified, HEP/COL-modified, and bare ePTFE grafts, maintained patency, and their morphologies were excellent as shown by Doppler ultrasonography (Figure 8A and B) and CT angiography (Figure 8C and D).

3.3.2. Gross Examination and Histological Analysis. Gross visualization of the explanted grafts revealed that the luminal surface of all patent HEP/COL-CD133 antibody-modified ePTFE grafts exhibited a glistening luminal surface free of any identifiable thrombus (Figure 9A). The luminal lining was homogeneous across the entire surface. The patent control grafts of HEP/COL modified and the bare ePTFE grafts exhibited some areas of red thrombus on the luminal surface and gross evidence of luminal cellularization was not obvious as in the former cases (Figure 9B and C).

The HEP/COL in the presence or absence of CD133 antibody modification and bare ePTFE grafts were sampled for HE staining (Figure 9D–F) and immunohistochemistry analysis of vWF (Figure 9G–I), respectively. Figure 9D and G illustrate the luminal formation of both a neointima and neomedia on the patent HEP/COL-CD133-modified grafts, and cellular infiltration and extracellular matrix deposition in the interstices of the ePTFE grafts. There is no evidence of formation of intima on the bare ePTFE grafts (Figure 9F and I). The intima of HEP/COL-modified ePTFE grafts were incomplete (Figure 9E and H).

3.3.3. Scanning Electron Microscopy (SEM). Samples of the patent implanted grafts were prepared for SEM evaluation. The patent HEP/COL-CD133 antibody-modified grafts exhibited a luminal lining of cells with characteristics typical of the endothelium (Figure 10A). There was a lack of thrombus deposition consistent with the antithrombogenic nature of endothelium. The patent HEP/COL-modified grafts exhibited incomplete endothelialization and the deposition of platelets, fibrin, and red blood cells (Figure 10B). The patent control bare ePTFE grafts show no evidence of spontaneous endothelialization of the luminal surface (Figure 10C).

4. DISCUSSION

The results of the present study show that coimmobilization of HEP/COL biomolecules, formed with a layer-by-layer self-assembly technique, can simultaneously improve the blood compatibility, biocompatibility, and endothelialization of synthetic ePTFE vascular grafts, which has the potential to promote its clinical application for bypassing surgeries.

As is already known, biocompatibility is the most important factor for biomaterials implanted into the human body. Thus far, many methods have been developed for the surface modification of biomaterials. These methods include physical adsorption, chemical modification, encapsulation, entrapment, and covalent or ionic binding.[13−15] Simple physical adsorption or encapsulation has the drawback of poor stability of biomolecules when the biomaterials are implanted long-term in the human body. Additionally, the absorbed molecules show poor resistance to fluidic shear stress.[16] Covalent or ionic immobilization of biomolecules has the advantage that the immobilized molecules are not easily removed by rinsing.[6,17] However, the molecular conformation is usually influenced and decreases or loses their original bioactivity. The layer-by-layer self-assembly technique is a novel method, which is based on the alternate adsorption of oppositely charged biomolecules, and it has been developed and widely used in many fields.[18−21] With such a method, biomaterials coimmobilized with different biomolecules can possess the properties that each biomolecule has.[8,22] In our study, heparin and collagen were selected as the constituents to complete the whole process of surface modification on biomaterials. Heparin is a commonly used anticoagulant and performs anticoagulant properties by binding to AT III and indirectly impacting the intrinsic coagulation pathway.[23] Collagen is the main component of the extracelluar matrix, based on which endothelial cells could adhere, proliferate, and differentiate.[24] The HEP/COL multilayer presents synergic properties of thromboresistance and cytocompatibility.

Successful immobilization of heparin/collagen on ePTFE grafts was monitored by SEM, infrared spectroscopy, and confocal laser scanning microscopy, although qualitative information was provided. Meng et al. and Lin et al. once applied quartz crystal measurements to monitor the layer-by-layer buildup of two different biomacromolecule processes through frequency shift vs time curves and validated it as a practical method.[7,8] The ePTFE itself is a kind of polyporous material. Therefore, the surface modification of such materials is not like metal materials. SEM results show that there was an

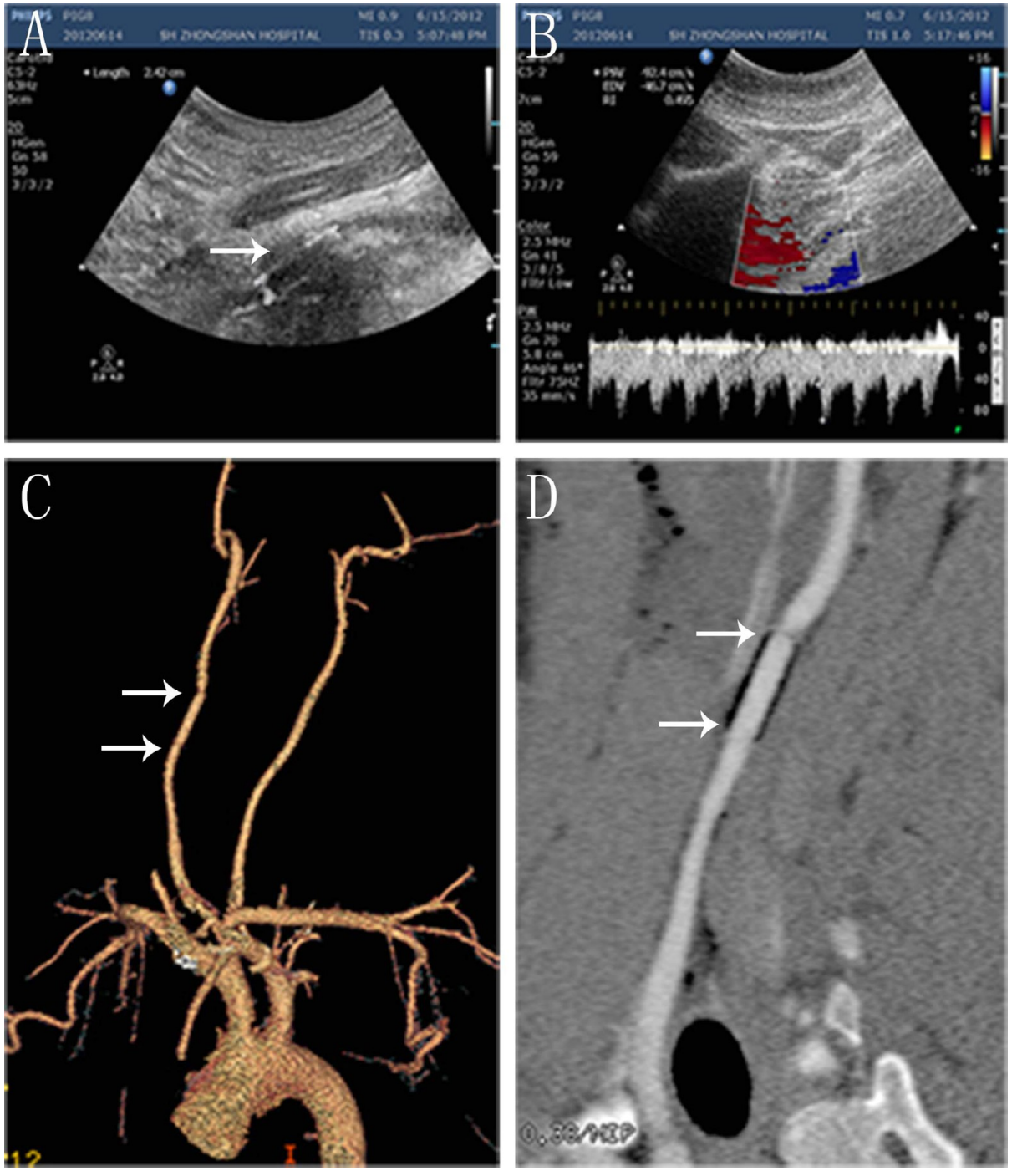


Figure 8. Ultrasonography (A and B) and CT angiography (C and D) examination of implanted ePTFE graft. Red and blue colors in part B indicate the blood flow signals of the implanted synthetic grafts. White arrows indicate the location of the implanted synthetic grafts.

obvious membranous film on the bare ePTFE graft after repeated immersion in the heparin and collagen solutions. The existence of heparin and collagen was also indirectly verified by the appearance of distinctive peaks corresponding to the heparin sulfonic and hydroxy groups and to collagen, as revealed by infrared spectroscopy. The ePTFE is a highly hydrophobic material and is not good for endothelial cells adhesion and spreading.[5] The hydrophilicity of this material was greatly improved after surface modification as the water contact angle decreased from 127.2° to 106.9°. Confocal laser scanning microscopy showed that anti-CD133-PE antibodies were successfully immobilized onto the membranous film of the ePTFE graft because a bright red fluorescence signal was detected. As the stability of the immobilized anti-CD133 antibody is an important property for the function maintenance of the modified ePTFE graft, a stability test was conducted. A bright red fluorescence signal could still be detected after the modified ePTFE material was immersed in PBS solution and put on a shaker (40 rpm) for more than 48 h.

Blood compatibility is a very important factor for modified biomaterials, and consequently, various in vitro evaluations were performed in the present study. Our results show that the hemolysis rate of both the modified and bare ePTFE graft were less than 5%, according to the medical biomaterial evaluation

dx.doi.org/10.1021/am401706w | ACS Appl. Mater. Interfaces 2013, 5, 7360−7369

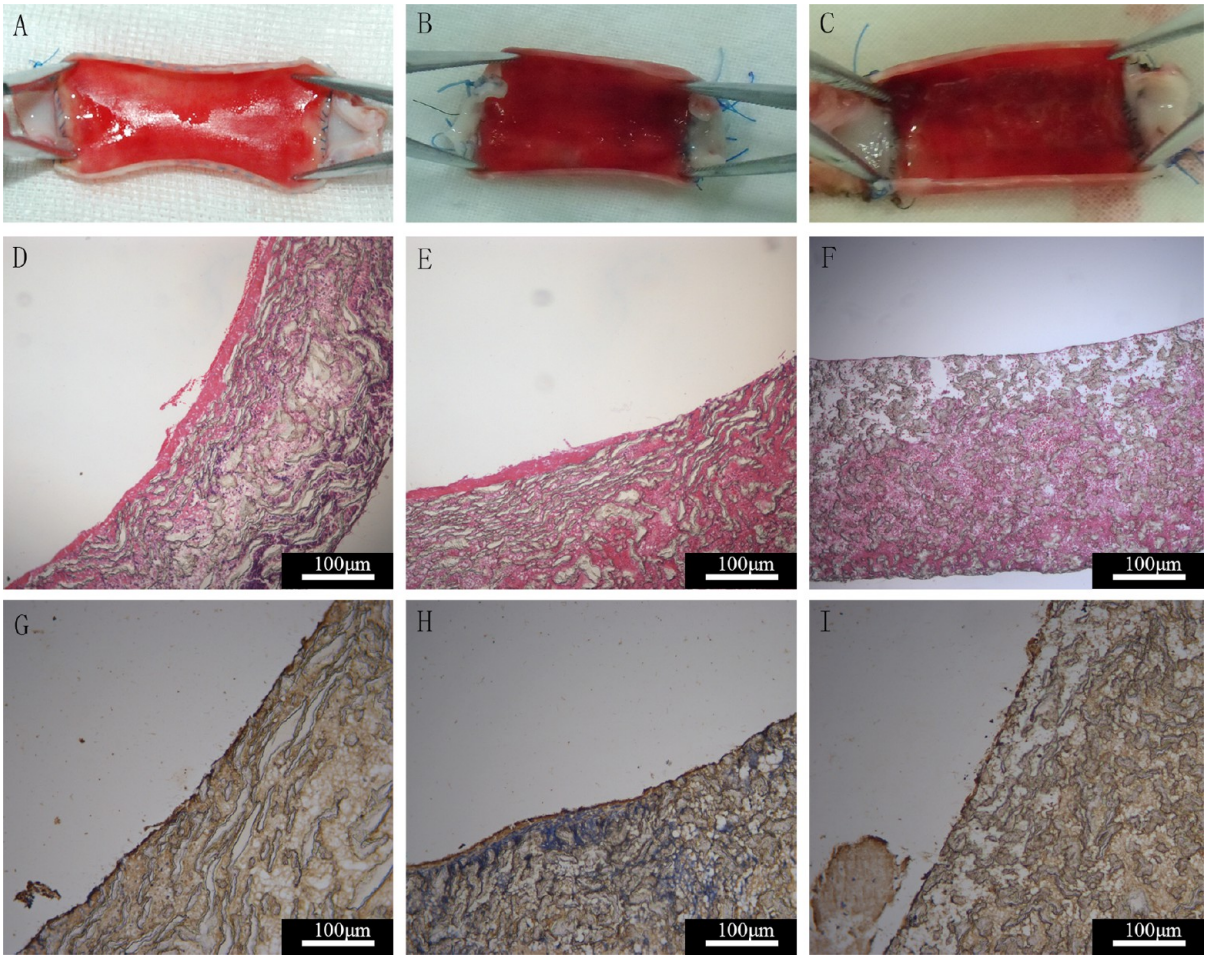


Figure 9. Gross observation (A–C), HE staining (D–F), and IHC of vWF (G–I) of the implanted anti-CD133 antibody functionalized $(HEP/COL)_5$ multilayer, $(HEP/COL)_5$ multilayer, and the bare ePTFE grafts (D–I, original magnification: 200×).

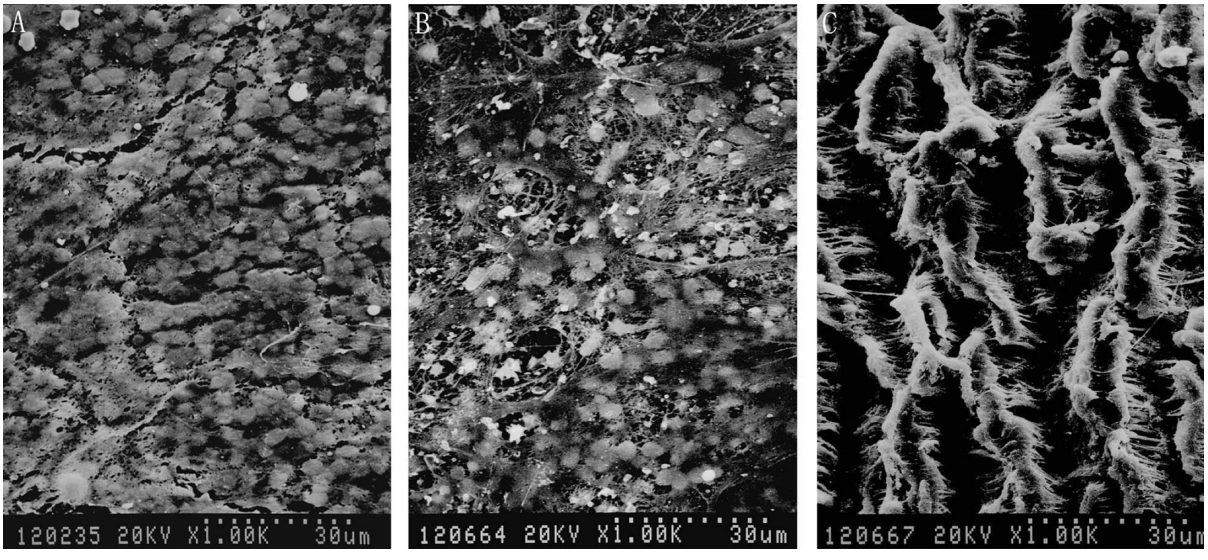


Figure 10. Endothelialization of anti-CD133 antibody-functionalized $(HEP/COL)_5$ multilayer (A), $(HEP/COL)_5$ multilayer (B), and bare ePTFE grafts (C) observed by scanning electron microscopy (original magnification: 1000×).

standard of GB/T16886 and ISO 10993. These results suggest that the amount of immobilized heparin or collagen was not sufficient to cause hemolysis and bleeding complications, which provides the possibility of its preconditional use in clinical settings. Furthermore, it should be noted that the plasma recalcification time was elevated and less platelets adhered, whereas the bare ePTFE biomaterial was immobilized with the anti-CD133 antibody functionalized HEP/COL multilayer. As

previously discussed, heparin is a diffusive polyelectrolyte and the diffusion and interpenetration of the polyelectrolyte in the multilayer results in the observed anticoagulant properties. Additionally, cell adhesion and cytotoxicity tests show the HEP/COL-CD133 antibody-modified ePTFE grafts provide an ideal surface environment for endothelial cell adhesion and that immobilized biomolecules have less cytotoxicity.

For evaluating the effect of the anti-CD133 antibody-functionalized HEP/COL multilayer on endothelialization, a porcine carotid artery transplantation model was used. A previous study had affirmed that anti-CD34 antibody-coated grafts could result in rapid endothelialization, while intimal hyperplasia markedly increased.[16] Several factors may have contributed to the adverse effects observed for the anti-CD34 antibody-coated grafts. First, previous studies reported that CD34+ cells can differentiate into various cell types, including vascular smooth muscle cells, endothelial cells, and macrophages.[25,26] Second, CD34+ cells have the capacity to release potent pro-angiogenic growth factors such as vascular endothelial growth factor and hepatocyte growth factor, which may promote the proliferative and migration of smooth muscle cells.[27] Third, local hemodynamic disturbances in implanted synthetic grafts may impede the ability of captured CD34+ cells to differentiate into endothelial cells.[28] CD133 is a conservative cell surface antigen, with a more specific expression pattern than the CD34 antigen on endothelial progenitor cells. These cells have high proliferative potential.[29] Therefore, we selected the CD133 antibody immobilized onto ePTFE vascular grafts to present endothelial progenitor cells selectively and to achieve rapid in situ endothelialization. Our preliminary results show that this was a practical method, but long-term patency will still require further study for confirmation.

Study Limitations. There are some inevitable limitations to the present study. First, the synthetic ePTFE vascular grafts used in our study were 6 mm in diameter. We are not certain as to the effect of our modifications on synthetic grafts less than 6 mm in diameter. Second, our anti-CD133 antibody was antihuman due to the unavailability of anti-CD133 monoclonal antibodies for porcine endothelial progenitor cells. However, some researchers have recognized that antihuman-CD133 antibody-coated ePTFE grafts have been shown to exhibit cross-reactivity in porcine graft explants. Finally, the samples of the in vivo experiments in this study are relatively small.

5. CONCLUSION

Synthetic ePTFE grafts coated with an anti-CD133 antibody-functionalized HEP/COL multilayer may achieve early rapid endothelialization but with limited platelet adhesion. Such a method may provide an effective selection system for the surface modification of medium- to small-diameter vascular grafts to improve their use in clinical settings.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (grant 81000105). We extend appreciation to Dr. Jianguo Jia, Dr. Bingyu Li, Prof. Keqiang Wang, and Prof. Xiangdong Yang for technical support and expertise.

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