Article

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Electrospun Bilayer Composite Vascular Graft with an Inner Layer Modified by Polyethylene Glycol and Haparin to Regenerate the Blood Vessel

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In this study, we prepared a composite vascular graft with two layers. The inner layer, which was comprised of degradable Poly(lactic-co-glycolic acid) (PLGA)/Collagen (PC) nanofibers modified by mesoporous silica nanoparticles (MSN), was grafted with polyethylene glycol (PEG) and heparin to promote cell proliferation and to improve blood compatibility. The outer layer was comprised of polyurethane (PU) nanofibers in order to provide mechanical support. The growth and proliferation of human umbilical vein endothelial cells (HUVECs) in the inner layer was significant, and blood compatibility testing showed that the inner layer had good blood compatibility. The MSN-PEG-Heparin on the fiber surface was observed *in vitro* during the degradation of the inner layer. After 60 days, the weight of fiber membrane decreased by 92.4%. The inner layer did not cause an inflammatory reaction during the degradation process *in vivo* and there was uniform cellular growth on the PC/MSN-PEG-Heparin fiber membrane. Composite grafts implanted into the rabbit carotid artery were evaluated for 8 weeks by H&E and immunohistochemical staining, demonstrating that a monolayer of endothelium (CD31-labeled) and smooth muscle (αSMA-labeled) regenerated on the composite graft. Our results demonstrate that the composite graft, with a functional inner layer, has potential to be used for small-caliber blood vessels with long-term patency.

KEYWORDS: *PEG, Heparin, Electrospinning, Artificial Vascular Graft, Biocompatibility.*

INTRODUCTION

Cardiovascular disease is common and carries a high rate of mortality. The incidence of cardiac arrest caused by diseases, such as coronary heart disease, hemangioma, and peripheral vascular defects, is on the rise, accounting for about 30% of deaths worldwide.[1] The main treatment methods for cardiovascular disease fall into three categories: drug treatment, surgery, and percutaneous intervention.[2] Vascular graft implantation is the most effective interventional treatment for coronary heart disease. At present, commonly used vascular grafts in the clinic include autologous vascular, allogeneic vascular, and artificial vascular grafts. The availability of autologous or allogeneic vascular grafts is limited, so it is necessary to fabricate artificial vascular grafts. Large diameter (>6 mm) artificial vascular grafts have been successfully used in the clinic, but small diameter (<6 mm) vascular graft applications are not ideal.[3] Artificial small vascular grafts cannot quickly undergo endothelialization, cause intimal thickening, vascular stenosis, and thrombosis. Thus, the long-term patency rate of small caliber artificial vascular grafts is low. The preparation of small caliber vascular graft substitutes remains an urgent problem in the field of blood vessel transplantation.

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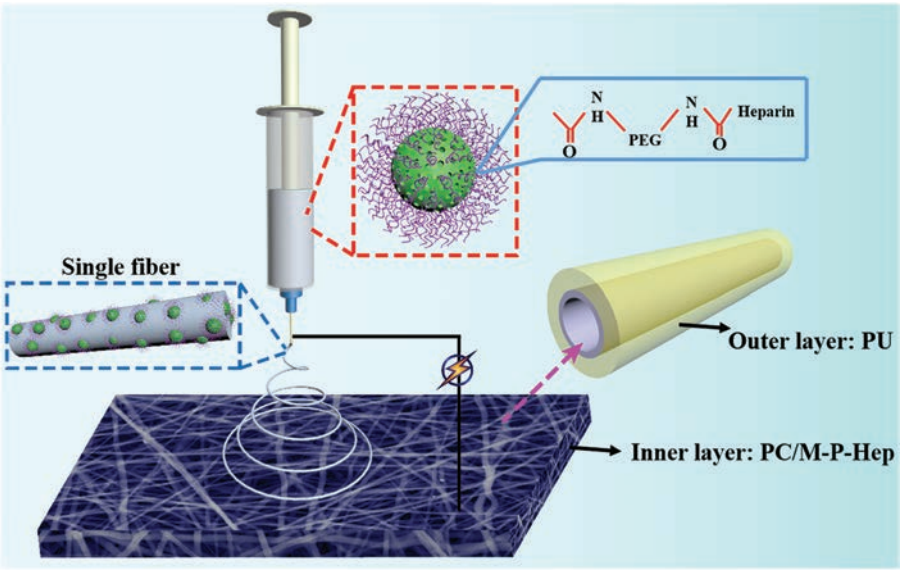

Figure 1. **The process of fabricating the PC/MSN-PEG-Hep fiber.**

It is essential to promote early endothelialization and reduce anti-acute thrombosis of artificial vessels to maintain patency and to prevent thrombosis.[4] Polyurethane (PU) has excellent elasticity and can provide a similar mechanical environment to that of natural vascular tissue.[5,6] In addition, PU has excellent blood compatibility and histocompatibility.[7] However, the PU degradation cycle is slow, and cells cannot migrate into the inner graft *over* a short period of time. Poly(lactic-co-glycolic acid) (PLGA) is a functional and biodegradable organic polymer[8] that has good biocompatibility, innocuity, and film formation. PLGA is widely used in pharmaceutical products, medical engineering materials, and modern industrial fields.[9,10] Collagen is widely used in biological materials research,[11] and combining collagen with polymer materials such as PLGA can improve the biocompatibility of polymers.[12] Polyethylene glycol (PEG) has a biological decontamination effect and is widely used for the surface modification of artificial medical materials.[13] Additionally, it can inhibit protein adhesion.[14] As a result, PEG is considered to be the most valuable polymer to modify blood contact materials, and it has been reported that PEG-modified surfaces have an anti-platelet adhesion propterty.[15–17] Heparin inhibits blood coagulation and can promote the growth of HUVECs.[19] Many previous studies have reported that a heparin coating on a polymer material surface directly.[20] The anticoagulant effect of heparin coatings is effective because the heparin is in direct contact with the blood,[21] but the number of surface materials that can be combined with heparin is limited and the amount of heparin that can be loaded onto these materials is low. Additionally, heparin coatings cannot produce a long-term anticoagulant effect due to the fact that these coatings are easily dissolved into the blood circulation.[22,23] Preventing this heparin loss remains a great challenge. Mesoporous silica nanoparticles (MSN) have excellent biocompatibility, and the surface is easy to modify.[24–26] MSNs are a good medium for grafting. Electrospinning is an effective way to prepare scaffolds for tissue engineering applications, because electrospinning nanofiber scaffolds can simulate the morphology and structure of natural extracellular matrix and provide a good microenvironment for cell adhesion and proliferation.[27]

The objective of the current study was to prepare a composite scaffold that can overcome the problems of graft stenosis and thrombosis. First, we chose a fast degradation material, PLGA mixed with collagen (PC), to form the inner layer. We hypothesized that the degradation of the inner layer would match that which is required for the endodermal regeneration process. Then, PEG and heparin were grafted onto MSNs to modify the PC layer in order to achieve adequate heparin loading and retention. If the graft were to degrade too rapidly, it would not provide enough mechanical support at the later stages of revascularization. Therefore, we selected PU as the outer layer, which can provide long-term mechanical support because PU is not biodegradable. The process of fabricating the PC/MSN-PEG-Hep fiber can see Figure 1.

MATERIALS AND METHODS

Materials

PLGA and PU were supplied by Jinan Daigang Biotechnology Co., Ltd. (Jinan, China). Mesoporous silica nanoparticles (MSN) was synthesized by the Donghua University Institute of Biology. Shandong International Biotechnology Park provided Collagen Type I (molecular weight 3×10^5 Da). The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), hydroxysulfosuccinimide (NHS), and

2-(N-morpholino) ethanesulfonic acid (MES) were purchased from Sigma-Aldrich. The 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was acquired from Shanghai Darui Fine Chemical Co., Ltd. (Shanghai, China). Absolute ethyl alcohol (Changshu Hongsheng Fine Chemical Co., Ltd.) was used. HUVECs were purchased from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, China). Counting Kit-8 (CCK-8), RPMI Medium Modified (RPMI-1640), fetal bovine serum (FBS), glutaraldehyde, and phosphate buffered saline (PBS) were acquired from Shanghai Limin Industrial Co., Ltd. (Shanghai, China). All of the cell staining reagents were provided by Gibco Life Technologies Co., (USA) unless specified.

Preparation of MSN-PEG-Heparin Nanoparticles

For the preparation of MSN-PEG, MSN was ultrasonically dispersed in a 0.01 N NaOH solution, stirred 10 minutes, and then centrifuged for 20 minutes. MSNs were washed 3 times with water. Then, MSNs were separately dispersed in a mixed solution of 0.05 M MES and 0.5 M NaCl. After one hour of stirring, 0.12 M EDC, 2 mg/mL PEG, and 0.06 M NHS were added. The solution was stirred at room temperature for 16 hours. Finally, MSN-PEG was obtained after centrifugation and was washed 3 times with water. The prepared MSN-PEG was dispersed in a 15% heparin solution and stirred for 24 hours. MSN-PEG-Hep was obtained after centrifugation.[28, 29]

Preparation of Nanofibers and Composite Graft

For the preparation of the PC/MSN-PEG-Hep fiber (PC/M-P-Hep), MSN-PEG-Hep were separately dispersed in HFIP (3 mg/mL), then processed by ultrasonic vibration for 30 minutes to ensure uniform dispersion. Then, PLGA and collagen (w/w, 6:4) were dissolved in the dispersion at a concentration of 10%. The applied voltage and solution flow of fabricated PC/M-P-Hep fiber were 10 KV and 1.0 ml/h respectively. The fiber of PC, PC/MSN-PEG (PC/M-P) were then prepared. The PC/M-P-Hep inner layer of the composite graft was fabricated over the course of an hour using a rotating stainless-steel rod as the collector. Then, the outer layer was generated using a 10% PU solution, a voltage of 12 kV, and a flow rate of 1.0 mL/h, and electrospinning for 4 hours. The temperature was 25 °C and the humidity was 50% during all electrospinning process. All of the samples were cross-linked in vapor generated by the evaporation of 10 mL of 25% glutaraldehyde (GA) aqueous solution at room temperature for 20 minutes. Lastly, all of the samples were dried in a vacuum chamber for 7 days at room temperature and were stored at low temperature for ultimate use.

Scaffold Characterization

The morphology of nanofiber membranes and the structure of the composite scaffold was observed by scanning electron microscopy (SEM) (PhenomSL, China). The structure of MSNs and single fibers was investigated by transmission electron microscopy (TEM) (JEOL, JEM-2100, Japan). At the same time, the uniformity of MSN-PEG-Hep on the fiber surface was analyzed by energy dispersive spectrometry (SEM-EDS) (FEI, Quanta250, Czech Republic). The contact angle of each sample was measured three times using a contact angle instrument (OCA40, Dataphysics, Germany).

Biodegradability

Biodegradability was evaluated *in vitro* over the course of 2 months. Every PC/MSN-PEG-Hep fiber membrane sample was cut into 0.2 g chips. Three samples were prepared for parallel examination at each time point. All of the samples were placed into a centrifuge tube with 15 mL of PBS (pH = 7.2 ± 0.1), and centrifuge tubes were placed in a water bath at 37 °C. 2.0 mg sodium azide per 10 mL PBS was added to inhibit mold growth.[30] The PBS was replenished each week. The weight of the samples was measured after 15, 30, 45 and 60 days. Samples to be tested were washed completely with water and dried to constant weight in a vacuum chamber. After weighing the dried samples, the morphology of samples was observed by SEM. The weight loss rate can be calculated by follow formula:

$$\text{weight loss } (\%) = \frac{W_0 - W_1}{W_0} \times 100\%$$

where W_0 is the initial weight of samples and W_1 is the dry weight of samples after degradation.[31]

In Vitro Cell Culture

Before seeding cells, all fiber membranes were sterilized with alcohol vapor for 48 hours, and then washed three times with PBS. Then, all of the samples were placed into 24-well plates individually and secured by stainless rings. Finally, HUVECs were cultured into 24-well plates that contained RPMI-1640 with 10% fetal bovine serum and 1% antibiotic-antimycotic then placed in a 37 °C incubator containing 5% CO_2. The media was replenished every two days. The density of HUVECs was 2.0×10^4 cells/well, and the cell proliferation was evaluated on after 1, 4, and 7 days. The cytocompatibility of fibers was assessed using CCK-8. The cells and matrices were incubated with 400 μl CCK-8 Solution containing 10% CCK-8 and 90% RPMI-1640 for 2 h. Then, 100 μl of CCK-8 solution from each sample was transferred into separate wells of a 96-well plate to test the OD value at 450 nm using a microplate reader (MK3, Thermo, USA). The mean and standard deviation from triplicate wells for each sample are reported. Meanwhile, the viability and morphology of cells were observed by fluorescence microscopy using calcein staining.

Blood Compatibility

Measurement of Plasma Recalcification Profiles

Platelet-poor plasma (PPP) was prepared from whole blood (whole blood was centrifuged at 5000 r/min for 15 minutes, and the supernatant was taken).[32] Fiber membranes of PC, PC/M-P, PC/M-P-Hep were placed in a 24-well plate covering the entire bottom surface of the dish, and 500 μl of PPP was added to each well. The positive control consisted of culture-treated plastic exposed to PPP with $CaCl_2$ and the negative control was culture-treated plastic exposed to PPP without $CaCl_2$. After incubating for 1 hour at 37 °C, 100 μl of PPP was transferred from each well plate to a 96-well plate, repeated 5 times, and followed by rapidly adding 100 μl of 0.025 M $CaCl_2$ into each well (except the negative control wells). The 96-well plate was placed in a fully automated microplate reader and absorbance at 405 nm was measured every 30 seconds for 45 minutes.

Platelet Adhesion

To prepare platelet-rich plasma (PRP), whole blood was centrifuged at 1500 r/min for 15 min to obtain a supernatant. The fiber membranes of PC, PC/M-P, and PC/M-P-Hep were placed in a 24-well plate covering the entire bottom surface of the dish, and each sample was incubated with 500 μl of PRP at 37 °C for 2 h. Samples were gently washed with PBS to remove non-adherent cells. Then, samples were submerged in paraformaldehyde for at least 2 hours. Afterward, an ethanol concentration gradient (30%, 50%, 70%, 80%, 90%, and 100%) was used to dehydrate the samples. When the samples were completely dehydrated, they were sputter-coated with a 7-nm gold layer and assessed via scanning electron microscopy (SEM). (SEM 3400N, Electron Probe Instrumentation Center, Northwestern University).[33]

In Vivo Biocompatibility

Healthy male SD rats weighing 50–60 g were used. PC/M-P-Hep and PU fiber membranes were cut into 5-mm segments and sterilized with ethylene oxide. Then, the membranes were embedded subcutaneously. Finally, fiber membranes were removed after 7, 14, and 28 days and stained with hematoxylin and eosin (H&E) for evaluation.

Graft Evaluation *In Vivo*

Healthy New Zealand White Rabbits were used for carotid artery transplantation. Composite grafts and the control grafts (PU only) were cut into 15-mm segments (2 mm in inner diameter) and sterilized. Then, the grafts were sutured to the proximal and distal ends of the carotid artery. Slices of explanted grafts were stained with hematoxylin and eosin (H&E) for evaluation after 8 weeks of implantation. The regeneration of endothelium and smooth muscle was detected by immunohistochemistry using CD-31 and αSMA to label endothelia and smooth muscle, respectively. Nuclei were stained with DAPI.

Statistical Analysis

One-way ANOVA was used to evaluate the statistical significance of our results. A p value <0.05 was considered to be statistically significant. Results that meet this threshold are indicated with an asterisk.

RESULTS AND DISCUSSION

Tests About MSN-PEG-Hep

After preparing MSN-PEG and MSN-PEG-Hep, we performed a series of characterizations. MSN and MSN-PEG were analyzed by FT-IR and two apparent absorption peaks appeared at 2980 nm and 2890 nm (Fig. 2(A)), indicating that PEG was successfully grafted onto MSN. Following thermogravimetric analysis, we determined that the weight loss rate of MSN, MSN-PEG and MSN-PEG-Hep was significantly different (Fig. 2(B)). The weight of MSN, MSN-PEG, and MSN-PEG-Hep decreased rapidly when the temperature was over 200 °C. As the temperature increased, the PEG and heparin grafted onto MSN began to decompose, and the weight of MSN-PEG and MSN-PEG-Heparin decreased faster than that of MSN alone. The total weight loss of MSN-PEG was greater than that of MSN because PEG was grafted to MSN, and likewise when heparin was grafted to MSN-PEG, the weight loss rate of MSN-PEG-Hep was higher than that of MSN-PEG alone. MSN-PEG-Hep and MSN-PEG fiber membranes were soaked in toluidine blue solution, which reacts with heparin and stains it blue. MSN-PEG-Hep fiber membranes were stained blue in both the dry and wet states (Fig. 2(C)). However, the MSN-PEG fiber membranes

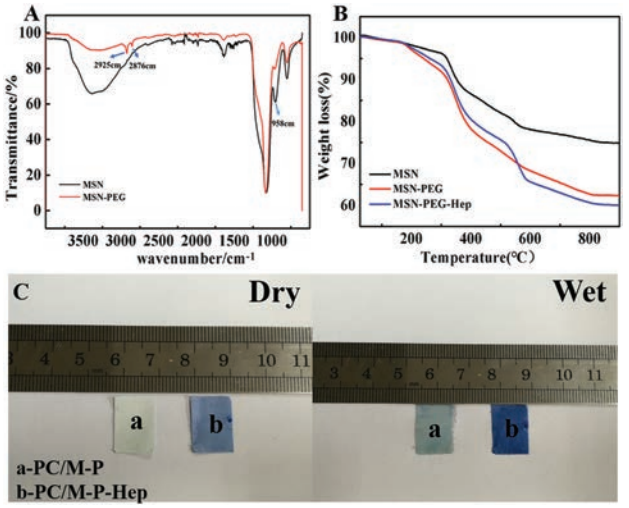

Figure 2. Analysis of MSN, MSN-PEG and MSN-PEG-Hep. (A) MSN and MSN-PEG were analyzed by FT-IR, (B) thermogravimetric analysis of MSN, MSN-PEG and MSN-PEG-Hep, (C) PC/M-P-Hep and PC/M-P fiber membranes stained by toluidine blue.

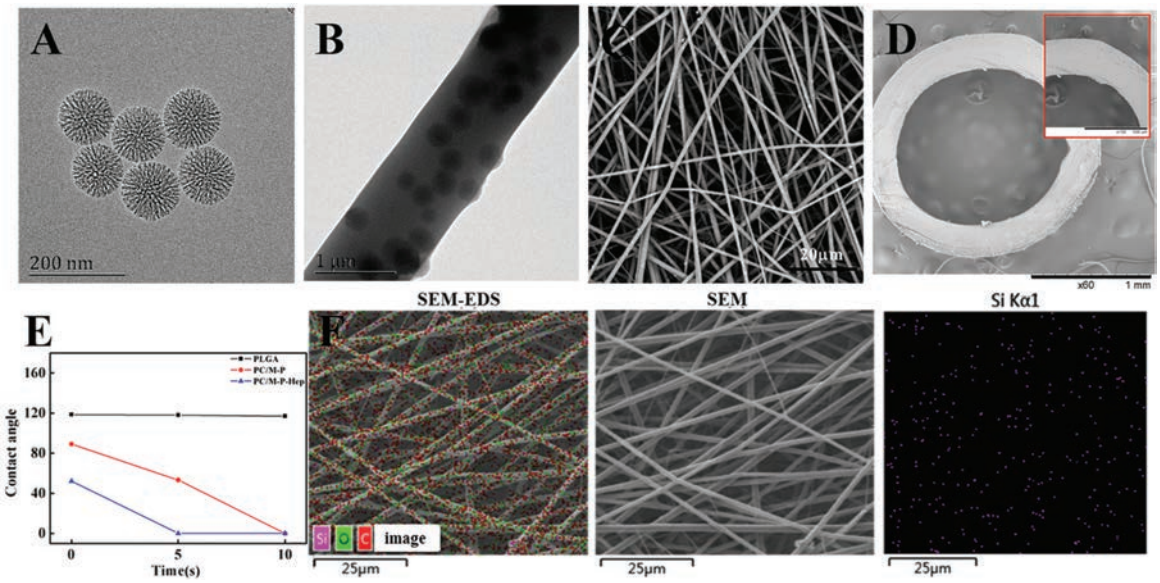

Figure 3. **Characterization of MSN, PC/M-PEG-Hep fibers, the composite graft, the hydrophilicity of PC, PC/M-PEG and PC/M-PEG-Hep, and the distribution of MSN on the fiber membrane: (A) Structure of MSN (B) structure of a PC/M-P-Hep fiber, (C) SEM image of PC/M-PEG-Hep fibers (D) SEM image of the composite graft, (E) contact angle changing of PC, PC/M-P, and PC/M-P-Hep fiber membranes (F) SEM-EDS image of the fiber membrane.**

were white in the dry state and only slightly blue in the wet state. This series of experiments shows that heparin was successfully grafted.

Scaffold Characterization

The scaffold structure was confirmed by TEM. As shown in Figure 3(A), the dendritic structure of MSN was detected, and the diameter of MSN was measured to be approximately 100–200 nm. MSNs were uniformly distributed along the fibers, and some MSNs were exposed on the fiber surface (Fig. 3(B)). The SEM image of the fiber membrane shows that the distribution of fiber diameters was uniform (Fig. 3(C)). Additionally, the composite graft did not exhibit stratification between the two layers (Fig. 3(D)). PC, PC/M-P, and PC/M-P-Hep exhibited different degrees of hydrophilicity. After PEG modification, the hydrophilicity of the fibrous membrane was improved. After further modification with heparin, the hydrophilicity was improved even further due to the fact that both PEG and heparin are hydrophilic (Fig. 3(E)). Additionally, the silicon, oxide, and carbon on the fiber membrane was analyzed by energy dispersive spectrometry (SEM-EDS), confirming that silicon was uniformly distributed on the surface of the fiber membrane (Fig. 3(F)).

Biodegradability

The morphology of fiber membranes was observed via SEM after 15 days of degraded *in vitro* (Fig. 4). Fibers exhibited a swelling phenomenon, and the uniform distribution of the MSNs embedded on the fiber surface was observed. The weight loss rate of the fiber membrane

Table I. **The weight loss rate of PC/M-P-Hep fiber membrane.**

Days	15	30	45	60
Weight loss rate (%)	10.75%±1.7	58.64%±2.75	77.8%±5.43	92.4%±4.89

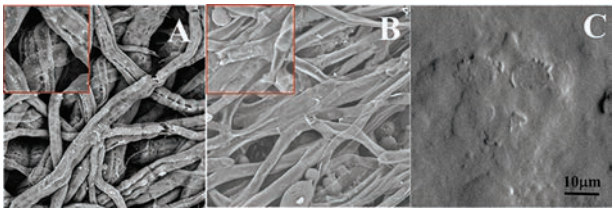

Figure 4. **Scanning electron micrographs of PC/M-P-Hep fiber membranes after biodegradation for different periods; (A) 15 days, (B) 30 days, (C) 45 days.**

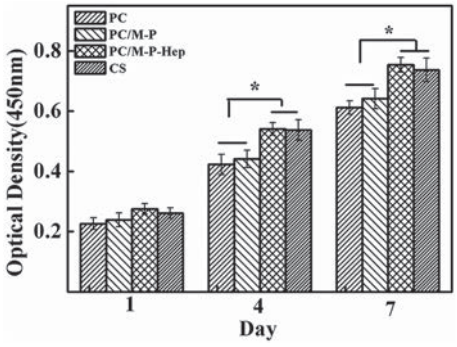

Figure 5. **Proliferation after culturing for 1, 4, and 7 days on PC, PC/M-P, PC/M-P-Hep fiber membranes and CS.**

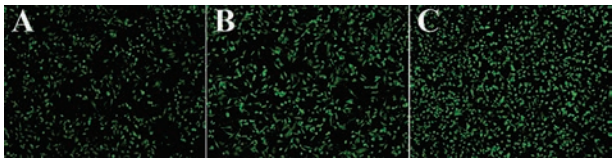

Figure 6. **Fluorescence images of HUVECs on different fiber membranes after culturing for 4 days. (A) PC fiber membrane (B) PC/M-P fiber membrane (C) PC/M-P-Hep fiber membrane.**

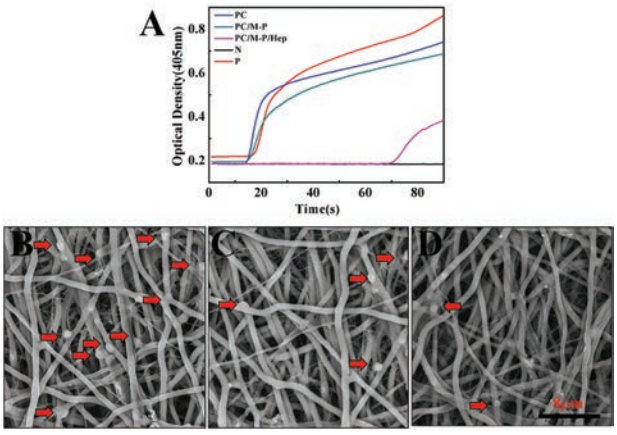

Figure 7. **(A) Plasma recalcification profiles and SEM images of platelet adhesion on (B) PC, (C) PC/M-P, and (D) PC/M-P-Hep. Positive control (P), negative control (N).**

was 10.75%. Fiber morphology also was observed after 30 days, at which point the weight loss rate had increased to 58.64%. The structure of fiber was altered, and fibers tended to merge together. There was no observable fiber morphology after 45 days when the weight loss rate was 77.8%. After 60 days, the weight loss rate was 92.4%. The weight loss rate of PC/M-P-Hep fiber membrane see Table I.

Cell Culture

Figure 5 shows HUVEC proliferation on PC, PC/M-P, and PC/M-P-Hep fiber membranes, and on cover slips (CS). After one day, HUVEC proliferation showed no significant differences among the different groups. However, when cultured for 4 days and 7 days, the proliferation of HUVECs on the PC/M-P-Hep fiber membrane was significantly higher than that on the PC or PC/M-P fiber membranes. Fluorescence micrographs of PC, PC/M-P, and PC/M-P-Hep fiber membranes 4 days after HUVECs were seeded are depicted in Figure 6. We observed that the number of HUVECs on the PC/M-P-Hep fiber membranes was greater than that on the PC and PC/M-P fiber membranes. These results indicate that the PLGA and collagen fiber membranes are suitable for the growth of HUVECs. Furthermore, modifying the fiber membrane had a significant effect on HUVEC proliferation.

Blood Compatibility

Due to the reaction of calcium ions with platelets, PRP will gradually coagulate, causing a change in the absorbance value. However, the PRP solution will not coagulate if the anticoagulant properties of the fiber membranes are adequate. Experiments depicted in Figure 7(A) show that PC/M-P-Hep fiber membranes had a significantly better anticoagulant effect than that of the PC and PC/M-P fiber membranes. Additionally, the anticoagulant effect of PC/M-P fiber membranes was inferior to that of the PC/M-P-Hep fiber membranes. This is most likely due to the fact that PEG has no biological anticoagulant effect. The result of the platelet adhesion test is shown in Figures 7(B)–(D). SEM images show that the amount of platelets on PC/M-P fiber membranes was less than that on PC fiber membranes. Furthermore, by grafting heparin, platelet adhesion was decreased on the PC/M-P-Hep fiber membranes compared with the PC/M-P fiber membrane. These results demonstrate that the PC/M-P-Hep fiber membrane is blood-compatible.

Biocompatibility *In Vivo*

Figure 8 shows Masson staining images of grafts that were subcutaneously embedded into rats for 7, 14, and 28 days. There was no inflammatory reaction detected on

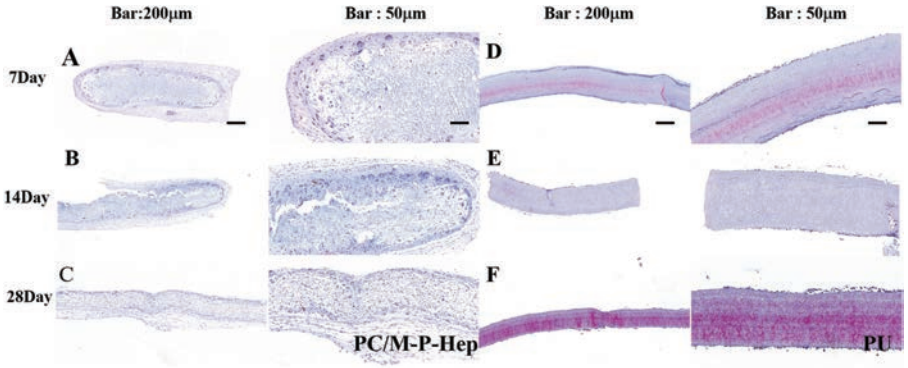

Figure 8. **Masson staining images of PC/M-P-Hep (A–C) and PU (D–F) fiber membranes, embedded subcutaneously into rats subcutaneous for 7, 14, and 28 days.**

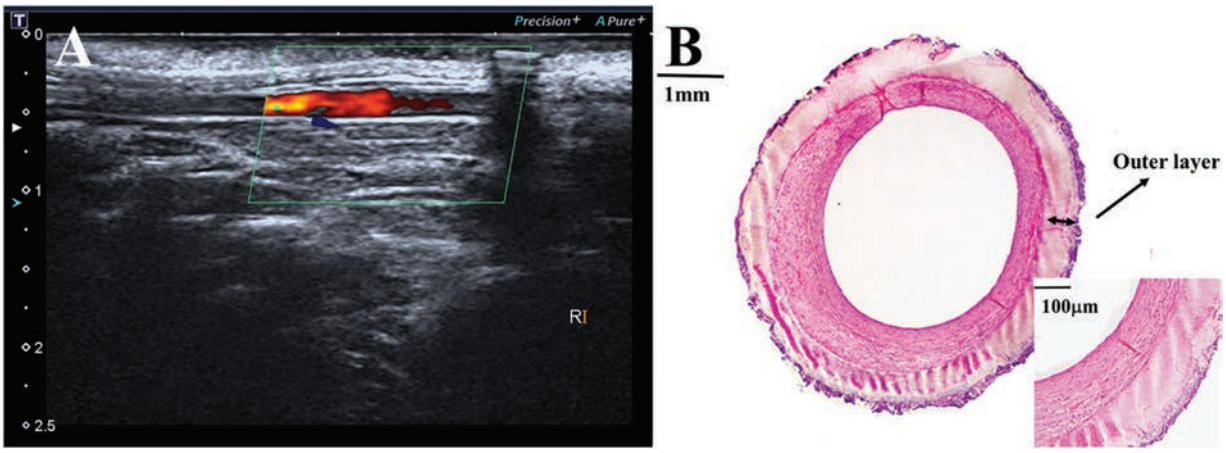

Figure 9. **(A) Color doppler flow imaging (CDFI), and (B) histological analysis of a composite graft that was implanted *in vivo* after 8 weeks.**

the PC/M-P-Hep and PU fiber membranes. These materials have good biocompatibility. PC/M-P-Hep fiber membranes were continuously degraded, and cells had fully grown into the material after 28 days. These cells only grew on the surface of the PU fiber membrane because PU does not degrade. PC/M-P-Hep fiber membranes were not detectable *in vivo* after 8 weeks.

Composite Grafts Evaluation *In Vivo*

Before evaluation of the grafts *in vivo*, we investigated the patency of the composite grafts using color doppler flow imaging (CDFI), which shows patency in the lumen of the graft for 2 months of implantation (Fig. 9(A)). Due to the synergistic effect of PEG and heparin, no thrombi were observed and blood was able to flow through the graft. Histological analysis of transverse sections was performed following H&E staining (Fig. 9(B)). The inner layer successfully regenerated endothelial tissue. The composite graft maintained its integrity and exhibited good mechanical support properties. Fluorescence micrographs of longitudinal graft sections with labeled endothelium and smooth muscle are shown in Figure 10, and nascent cells on the inner layer can be observed. CD-31 and α-SMA were used to label the endothelium and smooth muscle to detect their regeneration (Fig. 10). The endothelial cells formed a continuous layer after 8 weeks of implantation, and at this time point smooth muscle also formed a continuous intimal layer. The *in vivo* biocompatibility test showed that the PC/M-P-Hep fiber membrane was completely degraded after 8 weeks (Fig. 9), and that the endodermal regeneration processes matched the inner layer degradation time. Furthermore, analysis of the longitudinal sections demonstrated that endothelial cells and smooth muscle cells successfully grew into the lumen of composite graft. All of the above results indicate that the blood vessel lumen was well regenerated and functionalized after 8 weeks.

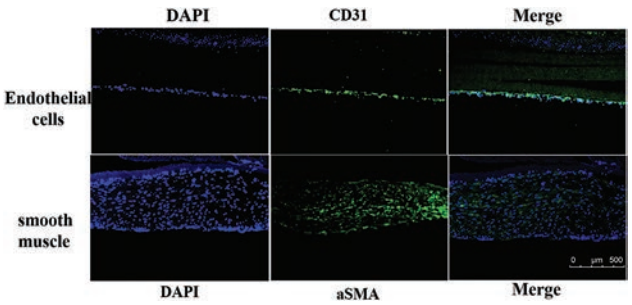

Figure 10. **Immunohistochemistry of endothelial cells and smooth muscle cells. DAPI (blue), CD-31 and αSMA (green).**

CONCLUSION

We have developed a composite scaffold with an inner layer that can be rapidly degraded within two months. The inner layer, modified with MSN-PEG-Hep, promotes the proliferation of endothelial cells and has good blood compatibility. In addition, MSN-PEG-Hep is exposed gradually as the fiber membrane is degraded. The inner layer did not evoke an inflammatory reaction *in vivo*, and cells grew into the fiber membrane over the course of 4 weeks. The composite graft exhibited patency and the structure remained intact *in vivo* up to 8 weeks of implantation. Fluorescence microscopy demonstrated that both the endothelium and smooth muscle successfully regenerated and had formed a continuous neointimal layer. Thus, we have developed a composite graft with a bilayer structure that provides a feasible method of vascular tissue engineering to maintain long-term vascular patency.

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