

Original Article

Biodegradation and biocompatibility of a degradable chitosan vascular prosthesis

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Abstract: An instrument made by ourselves was used to fabricate biodegradable chitosan-heparin artificial vascular prosthesis with small internal diameter (2 mm) and different crosslinking degree from biodegradable chitosan, chitosan derivatives and heparin. *In vivo* and *in vitro* degradation studies, inflammatory analysis and electron microscope scanning of this artificial vascular prosthesis were performed. It was observed that 50% of the prosthesis decomposed *in vivo* and was replaced by natural tissues. The degradation process of the chitosan-heparin artificial vascular prosthesis of small diameter could be controlled by changing the crosslinking degree. This kind of artificial vascular prosthesis shows good biocompatibility that can be controllably designed to achieve desirable in vascular replacement application.

Keywords: Artificial vascular prosthesis, small-diameter, chitosan, biodegradable, degradation

Introduction

There is a great demand of artificial vascular prosthesis in the clinical practice, especially those resemble vascular function, with good biocompatibility, and in small-diameter, are widely used in arterial and venous diseases. The synthetic vessels with the diameter larger than 6 mm have demonstrated a good application in clinical practice, with high success rate and low side effects. However, when the diameter of synthetic vessels smaller than 6 mm, the adverse effects, such as, thrombus formation, and intimal hyperplasia would probably occur [1-5]. It was shown by Brewster *et al.* that the thrombogenicity and intimal hyperplasia were the major causes of graft failure, and both occurred at the junction of luminal interface of vessel and graft. Therefore, to select a biologically compatible material is critical for operation success rate and long-lasting patency [6].

The investigation of artificial vascular prosthesis has been focused on biomaterial that is non-thrombogenic, minimizes the risk of intimal hyperplasia, resembles the physical properties of native vessels, and allows for the regeneration of endogenous arterial tissue. The latest

research on biodegradable polymers has shed a light on the exploration of artificial vascular prosthesis. The biodegradable polymers demonstrated a better bio-compatibility than the other synthetics, especially when used as a small diameter vessel. In addition, the biodegradable polymers can promote the regeneration of vascular, and the endogenous *de novo* synthesized vascular will replace the artificial vessel in the end [2, 5].

Bio-absorbable polymers may provide the ideal solution by providing long-lasting luminal patency, on the other hand, they can be completely absorbed after angiogenesis and vascular remodeling. It was further developed by Zamiri *et al.* and Ignatius *et al.* that COS and NAG can be used as vehicles for drug delivery to inhibit in-stent restenosis [7, 8]. The biodegradable materials is supposed not only be able to promote vascular cell adherence (endothelial cells, vascular smooth muscle cells) but also inhibit the inflammatory response at the implantation site. Chitosan, chitosan derivatives and heparin are good candidates for artificial vascular prosthesis, for they are highly plastic in terms of chemical properties, which enables them good compatibility, favorable biophysical properties,

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and controllable degradation rates, all of these allow the coincidence of physical properties throughout the entire remodeling period [9]. The natural materials like chitosan are highly abundant in the nature, and easily extracted and purified. Through simple separation steps, we can obtain various kinds of chitosan derivatives, which have an even better biocompatibility than chitosan *per se*. Because of favorable physiochemical feature and good biocompatibility, we are able to obtain an ideal type of biodegradable small-diameter artificial vascular prosthesis.

The goal of the current study was to characterize the biodegradable chitosan-heparin small-diameter artificial vascular prosthesis in terms of biodegradation (*in vitro/in vivo*) and to assess its *in vivo* compatibility with subcutaneous tissues. *In vitro* studies were carried out either in the presence of lysozyme or PBS buffer (pH 7.0). For the *in vivo* assessment, the biodegradable chitosan-heparin small-diameter artificial vascular prosthesis were implanted in a subcutaneous rat model, the degree of degradation at different time points was observed and recorded, followed up to 180 days.

Materials and methods

Fabrication of chitosan-heparin small-diameter artificial vascular prosthesis

Chitosan fiber, chitosan derivatives and heparin were used as raw materials to get the artificial vascular prosthesis with 2 mm inner diameter using in-house built instrument. All the materials used were sterile and pyrogen-free. The mixed macromolecules were connected by the crosslinking agent butanediol diglycidyl ether. Chitosan-heparin small-diameter artificial vascular prosthesis with two different crosslinking degrees were prepared (30:1 and 50:1). The crosslinking degree is calculated as the molar ratio of chitosan monosaccharide to the butanediol diglycidyl ether.

In vitro degradation analysis

In vitro degradation studies of chitosan-heparin small-diameter artificial vascular prosthesis with different crosslinking degrees were carried out in the presence of 50 units/ml of lysozyme in phosphate buffer saline (PBS) at 37°C, pH 7.2 for 15, 30, 60, 90, 120, 150, 180 and 210 days (n=6) and followed protocols similar to previous *in vitro* enzyme studies [10-12]. The

degradation profile of chitosan-heparin small-diameter artificial vascular prosthesis in lysozyme-free PBS was also collected. Enzyme activity was measured as described previously [13]. A quantitative assessment of the degradation of chitosan-heparin small-diameter artificial vascular prosthesis with different crosslinking degrees was obtained.

Determination of releasing rate of heparin from the artificial vascular prosthesis

Heparin was determined by the method reported by Park et al. To obtain the standard curve, various concentrations (5-35 mg/ml) of heparin solution (2.0 ml) were prepared in separate 10 ml conical flasks. 3 ml of toluidine blue solution (25 mg toluidine blue dissolved in 500 ml 0.01 M HCl containing 0.2% NaCl) was added to each flask, which was then shaken to ensure complete reaction. After 30 min, 3 ml n-hexane was added to the solution, mixed, and allowed to phase-separate. The heparin/toluidine blue complex formed was extracted in the n-hexane and its concentration was determined using a spectrophotometer at 631 nm. The amount of heparin present was then calculated. To measure the heparin releasing rate in the chitosan-artificial vascular, 2 ml of distilled water and 3 ml of toluidine blue solution were added into an empty 10 ml conical flask. A sample, which had been calculated for the total amount of heparin, was then immersed into the solution and the system was allowed to react for 30 min. After that, 3 ml of n-hexane was added into the flask, which was shaken to accelerate the extraction of heparin/toluidine blue complex by n-hexane. By measuring the absorbance of the aqueous solution at 631 nm, the amount of heparin that had been released from the chitosan-artificial vascular prosthesis could be determined from the standard curve. This experiment was repeated three times and the average results were reported [14].

In vivo implantation

Rats (250-300 g) were anesthetized with 3% pentobarbital sodium prior to the intramuscular implantation of the biodegradable chitosan-heparin small-diameter artificial vascular prosthesis with different crosslinking degrees. Artificial vascular prosthesis with 1 cm long was put into the incision in one leg. As contrast, a section of suture line with 5-8 cm long was put into the incision in the other leg. From then on,

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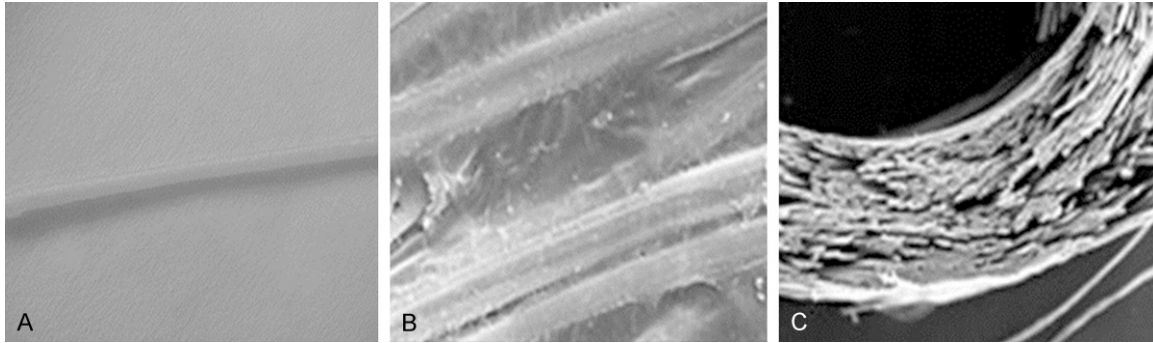


Figure 1. The appearance of chitosan-heparin small-diameter artificial vascular prosthesis (A) visual observation; (B) The SEM observation of the inner layer; (C) SEM observation of the cross section.

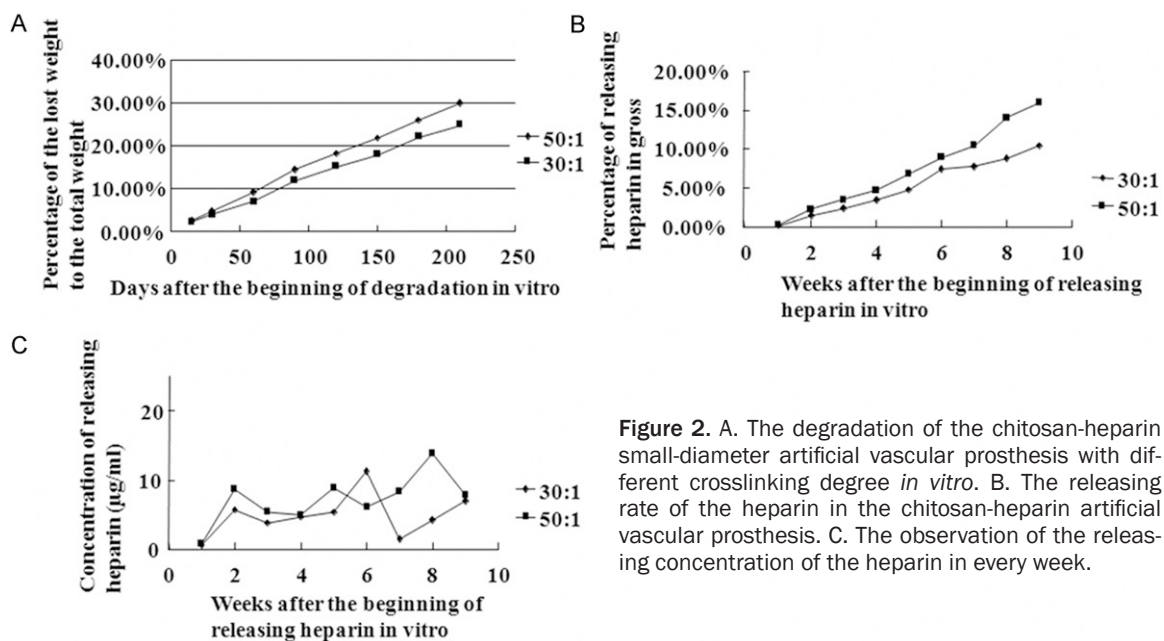


Figure 2. A. The degradation of the chitosan-heparin small-diameter artificial vascular prosthesis with different crosslinking degree *in vitro*. B. The releasing rate of the heparin in the chitosan-heparin artificial vascular prosthesis. C. The observation of the releasing concentration of the heparin in every week.

the artificial vascular prosthesis and suture line were got from the rats once a month until the 6th month. At each given time point, the rats were initially anesthetized with 3% pentobarbital sodium, and samples were explanted with a small amount of surrounding tissue being removed. The rats were sacrificed by cervical dislocation post explantation. All animal experiments were conducted according to the ethical guidelines of Ocean University of China.

Explanted tissue digestion

Explanted chitosan-heparin small-diameter artificial vascular prosthesis not used for histology or scanning electron microscopy (SEM) studies were treated using the methods mentioned previously [15]. After digestion of the

explanted tissue, the remaining artificial vascular prosthesis were then dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 90%, 95%, and 100% for 1 h each) and left to air-dry for three days in a fume hood prior to weighing using an analytical balance (± 0.0001 g) and calculating the percent mass loss.

Scanning electron microscopy

Whole chitosan-heparin small-diameter artificial vascular prosthesis were washed in PBS (pH 7.2) and fixed in 2.5% glutaraldehyde overnight prior to dehydration in increasing concentrations of ethanol (30%, 50%, 70%, 90%, 95% and 100%, 1 h each), which may depend on the initial conditions [16]. Then the samples were dried through critical point drying. The dried

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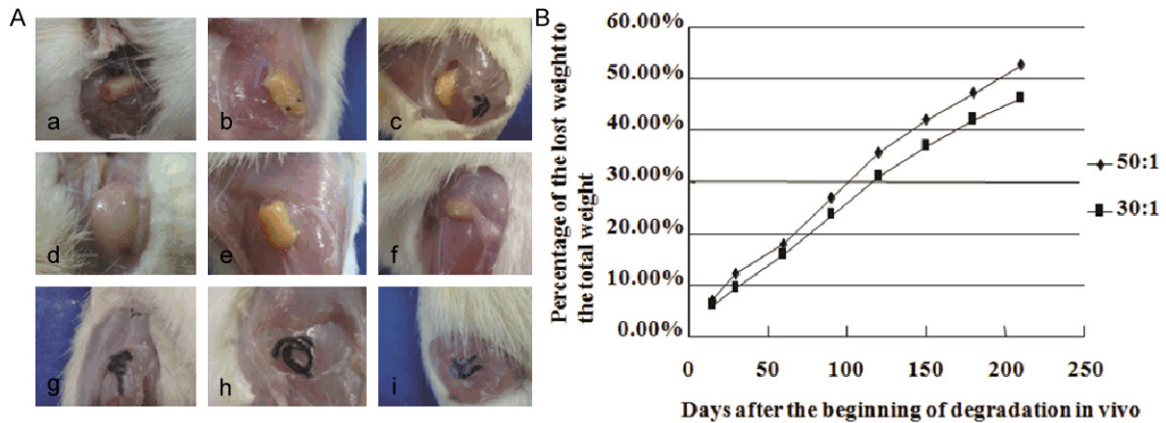


Figure 3. A. The observation of the degradation of the chitosan-heparin small-diameter artificial vascular prosthesis with 30:1 (a-c) and 50:1 (d-f) crosslinking degree and the control group (g-i) at 30th, 90th, 180th days, respectively. B. The degradation of the chitosan-heparin small-diameter artificial vascular prosthesis with different crosslinking degree *in vivo*.

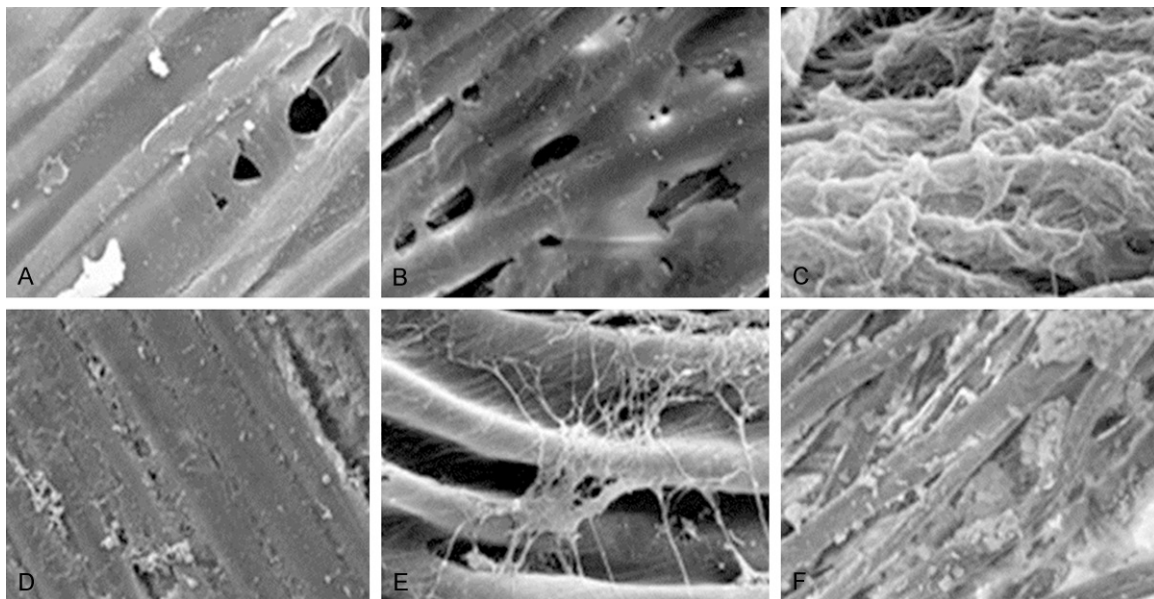


Figure 4. SEM observation of the artificial vascular prosthesis with 30:1 crosslinking degree at 30th, 90th, 120th days (A-C) after intramuscular implantation and the artificial vascular prosthesis with 50:1 crosslinking degree at 30th, 90th, 180th days (D-F) after intramuscular implantation.

specimens were sputter coated with gold before being observed under a scanning electron microscope to investigate the surface morphology at the lumen of the prosthesis.

Histological staining

Following fixation for 18 h in formalin (10%; PBS at 37°C, pH 7.2), samples were embedded in paraffin and sectioned at 5 μm using a rotary microtome, following the method used by Trantina et al. [17]. The sections were stained

with hematoxylin-eosin for the identification of cells. Sections were analyzed and inflammation was assessed using bright field light microscopy, and images were acquired using a digital color camera [18].

Results

Morphology

The inner diameter of the chitosan-heparin small-diameter artificial vascular prosthesis we

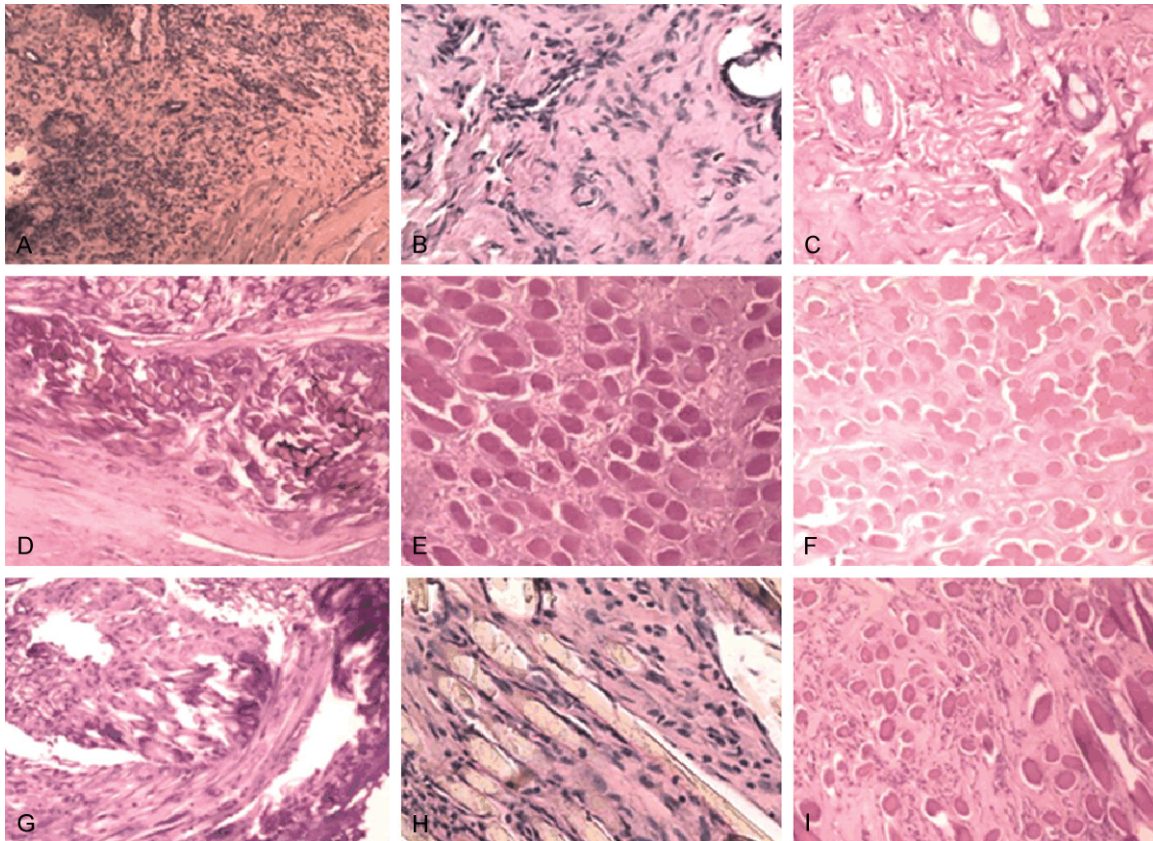


Figure 5. Hematoxylin-eosin staining of the specimens and suture lines being put into the back's subcutaneous (100 \times). A-C were the control group with suture lines at 30th, 90th and 180th days, respectively; D-F is the experimental group with 30:1 crosslinking degree at 30th, 90th and 180th days, respectively; G-I is the experimental group with 50:1 crosslinking degree at 30th, 90th and 180th days, respectively.

have prepared is 2 mm. The length of the artificial vascular prosthesis varies from 10 cm to 20 cm and the thickness of the wall of the artificial vascular prosthesis is about 0.5 mm. **Figure 1** shows the smooth inner layer and the cross section of the chitosan-heparin small-diameter artificial vascular prosthesis observed by scanning electron microscopy.

In vitro degradation

In vitro degradation of the chitosan-heparin small-diameter artificial vascular prosthesis with different crosslinking degree is shown in **Figure 2A**. During the whole degradation time, a statistically greater mass loss of the chitosan-heparin small-diameter artificial vascular prosthesis with 50:1 crosslinking degree in the presence of CE was observed when compared with samples with 30:1 crosslinking degree ($P < 0.05$). And both of the chitosan-heparin small-diameter artificial vascular prosthesis with 50:1 and 30:1 crosslinking degrees sho-

wed a stable and controlled degradation *in vitro*.

Releasing rate of heparin from the artificial vascular prosthesis

The gross releasing rate of heparin and releasing concentration of heparin every week from the artificial vascular prosthesis with different crosslinking degree are shown in **Figure 2B** and **2C**, respectively. The result reveals that after degradation of more than 2 months, the release of heparin with different crosslinking degree was sustained and stable, and there was no burst release in the whole process of degradation. The releasing rate of heparin with 50:1 crosslinking rate is much faster than the releasing rate of heparin with 30:1.

In vivo implantation

At the earliest degradation time point (30 days), the chitosan-heparin small-diameter artificial

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vascular prosthesis with 30:1 crosslinking degree showed less tissue adhesion than the samples with 50:1 crosslinking degree (**Figure 3Aa, 3Ad**). The degradation of the two samples by 90 days showed no obvious difference, however, by 180 days the degradation of the sample with 50:1 crosslinking degree was higher than the sample with 30:1 crosslinking degree (**Figure 3Ab, 3Ac, 3Ae, 3Af**). **Figure 3B** revealed *in vivo* degradation of the chitosan-heparin small-diameter artificial vascular prosthesis with 50:1 and 30:1 crosslinking degree. The result of the *in vivo* degradation is similar with the result of the *in vitro* degradation. Both of them showed stable and controlled degradation, and the samples with 50:1 crosslinking degree showed higher degradation percentage than the samples with 30:1 crosslinking degree during the whole degradation time. The control group has no evident degradation showed as the **Figure 3Ag-i**. Both of the chitosan-heparin small-diameter artificial vascular prosthesis with 50:1 crosslinking degree and 30:1 crosslinking degree caused no evident inflammatory response in the lateral tissues.

Scanning electron microscopy

SEM observation results showed that after six months of implantation, the artificial vascular prosthesis itself (**Figure 4A-F**) has degraded to a great degree, and similar tissues have grown in the inside of the implants (**Figure 4C-F**). The part of the artificial vascular prosthesis that degrades first is the inner membrane and then is the chitosan fiber. Degradation of the chitosan-heparin small-diameter artificial vascular prosthesis with 30:1 crosslinking degree (**Figure 4A-C**) is similar with the samples with 50:1 crosslinking degree (**Figure 4D-F**), and the difference of them lies in the amount of tissues grow in the inner structure of the sample.

Histological staining

Histological evaluation of the explanted grafts revealed that the negative control has serious inflammatory response during the whole time of implantation (**Figure 5A-C**). Both of the experimental groups with 30:1 crosslinking degree (**Figure 5D-F**) and 50:1 crosslinking degree (**Figure 5G-I**) have serious inflammatory response at the early time of the implantation, but the inflammatory response alleviated to a great extent at the 90th and 180th day after

implantation compared with the control group. The inflammatory response of the samples with 30:1 crosslinking degree is less serious than that of the samples with 50:1 crosslinking degree. Ingrowths of the nature tissues can be observed obviously at 90th and 180th day after implantation.

Discussion

There is an urgent need for improved coating strategies in vascular prosthesis design, both to enhance endothelial cell (EC) adhesion and function, thereby providing an anti-thrombogenic luminal surface with vasoactive properties, and to limit smooth muscle cell (SMC) migration to the luminal surface and subsequent neointimal formation. Chitosan and its derivatives such as carboxymethyl chitosan have a good compatibility and mechanical property, and heparin is known as a kind of good anticoagulant. So it is potentially feasible to prepare an ideal biodegradable small-diameter artificial vascular prosthesis using chitosan, chitosan derivatives and heparin.

The degradation rate is especially important to the biodegradable artificial vascular prosthesis, and it influenced the ultimate success of failure of artificial vascular prosthesis implantation. If the degradation is too fast, the artificial vascular prosthesis would have been degraded totally or could not remain its original shape or function, which could induce the failure of the implantation. If the degradation is too slow, the growth of the autologous tissue would be restrained, and there would not be enough space for the cells to grow inside the artificial vascular prosthesis. In which case, the autologous tissue would grow outside the structure of the artificial vascular prosthesis, which would cause intimal hyperplasia and clinical failure. The degradation rate of the artificial vascular prosthesis is determined by the material itself and crosslinking degree. Therefore it is necessary to evaluate the degradation and compatibility of the artificial vascular prosthesis with different crosslinking degrees. Previously, we have demonstrated that chitosan artificial vascular prosthesis with 30:1 and 50:1 crosslinking degree had good mechanical property, so the degradation and biocompatibility of the chitosan artificial vascular prosthesis with different crosslinking degrees was evaluated.

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In this study, the percentage of degradation of the chitosan-heparin artificial vascular prosthesis after six months of implantation is about 50%, and when the crosslinking degree increased, the degradation was slowed down to some degree. Both of the artificial vascular prosthesis with 30:1 and 50:1 crosslinking degrees showed stable and controlled degradation, and the integrity of the samples was favorable until 6 months after the implantation. Considering the importance of heparin in the whole process of anticoagulation, it is essential to evaluate the releasing rate and amount of heparin from the artificial vascular prosthesis. The heparin and chitosan were crosslinked by butyl glycol double glycidyl ether. Heparin will be released from the artificial vascular prosthesis by the interaction with various kinds of enzymes, and then enter into the blood. After degradation for a while, there must be enough heparin left in the artificial vascular to inhibit coagulation during the interaction of the blood and the artificial vascular prosthesis. In this study, after degradation of more than 2 months, the release of the heparin with different crosslinking degrees was sustained and stable, and there was no burst release during the whole time of degradation, and the amount of heparin released every week is always 5-15 ug/ml. The good release performance ensures the sustained anticoagulation function of the artificial vascular prosthesis, and provides enough time for the ingrowths of the autologous tissues.

The physical dimensions of open spaces through which the inner and outer surfaces of the graft directly communicate determines the porosity of a scaffold or synthetic graft through which cellular ingrowths can occur while the permeability of a graft is defined by its ability to permit passage of a substance (i.e. growth factor) through itself. Still, the rate of tissue ingrowths can be improved by optimizing graft porosity or permeability. Clowes et al. have demonstrated enhanced tissue ingrowths and complete reendothelialization of ePTFE grafts in a baboon model [19].

The SEM observation results revealed that, during the degradation process, the part of artificial vascular prosthesis that degraded first is the inner membrane, which degraded completely 3 months after implantation, and at the same time, the chitosan fiber degraded to some extent too. After 6 months of implanta-

tion, the remaining structure of the artificial vascular prosthesis were coated with autologous tissues. HE staining revealed that, at the earliest time of the implantation, the inflammatory response was rather serious, 90 days after the implantation, there have been a number of cells growing in the inner space of the artificial vascular prosthesis although there are still some inflammatory cells. And 180 days after the implantation, the inflammatory cells disappeared and more autologous cells grew in the inner space regularly.

In this study, the degradation of the chitosan-heparin small-diameter artificial vascular prosthesis could be controlled by changing the crosslinking degree. Both of the implantation and degradation test *in vivo* and *in vitro* demonstrated good biocompatibility of the biodegradable chitosan-heparin small-diameter artificial vascular prosthesis, and this study may provide a theoretical and experimental support for its potential application in the medical field.

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Disclosure of conflict of interest

None.

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