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Bilayered vascular grafts based on silk proteins

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ABSTRACT

A major block in the development of small diameter vascular grafts is achieving suitable blood vessel regeneration while minimizing the risk of thrombosis, intimal hyperplasia, suture retention, and mechanical failure. Silk-based tubular vessels for tissue engineering have been prepared by molding, dipping, electrospinning, or gel spinning, however, further studies are needed to improve the mechanical and blood compatibility properties. In the present study a bilayered vascular graft based on silk fibroin (SF) was developed. The graft was composed of an inner silk fiber-reinforced SF tube containing heparin and a highly porous SF external layer. Compared with previously fabricated SF tubes the fiber-reinforcement provided a comparable or higher mechanical strength, burst pressure, and suture retention strength, as well as mechanical compliance, to saphenous veins for vascular grafts. Heparin release was sustained for at least 1 month, affording blood compatibility to the grafts. The outer layer of the grafts prepared through lyophilization had a highly porous structure in which the macropore walls were composed of nanofibers similar to extracellular matrix, which offered an excellent environment for cell growth. In vitro studies showed good cytocompatibility and hemocompatibility.

1. Introduction

Despite the increasing incidence of cardiovascular disease there are limited options for vascular grafts, with autologous saphenous veins and mammary arteries serving as the gold standards for bypass surgery and to treat vascular disease [1]. Synthetic grafts made of polyethylene terephthalate (Dacron) and expanded polytetrafluoroethylene (ePTEE) are options for large diameter vascular grafts, but the foreign surfaces and compliance mismatches of these materials result in unfavorable immune responses and anastomotic intimal hyperplasia that lead to re-occlusion in smaller caliber vessels [2]. Developing vascular grafts for small diameter vessel replacement (2–5 mm diameter) remains the clinical challenge.

Recent research suggests that small diameter grafts should have the following properties: biocompatibility to promote optimal tissue regeneration, low blood leakage, very low thrombogenicity and adequate mechanical properties, including a compliance matching that of native arteries and resistance to aneurysm formation [3]. Generally, natural biomaterials have good biocompatibility and low thrombogenicity, while synthetic biomaterials such as polyurethanes can provide suitable mechanical strength. Unfortunately, the combination of biocompatibility, antithrombogenicity, and mechanical properties is difficult to achieve with a homogeneous construct [4–6]. To address this issue multilayered grafts composed of different synthetic polymers and natural biomaterials have been pursued [3,5,7–11]. Different types of multilayered grafts have been designed to mimic the extracellular matrix (ECM) and the mechanical properties of native arteries and these systems have been evaluated in vitro and in vivo [8–10,12,13]. However, further studies are warranted to continue to improve the applicability of these small diameter vessel grafts to avoid inflammation and to match the mechanical properties.

Tissue engineered small blood vessel grafts based on entrapment of human dermal fibroblasts in different degradable scaffolds are another attractive way to yield completely biological vascular grafts that possess the circumferential alignment characteristic of native arteries, essential to their mechanical properties [14–19]. However, there are still major challenges to clinical application, including long-term culture of cells and equipment costs. More recently rapid remodeling of a cell-free synthetic graft to a neo-artery was achieved with a fast degrading elastomer, providing a promising method to rebuild blood vessels with multiple advantages, such as ready availability and potentially faster clinical adoption [18]. Designing grafts with suitable biocompatibility, blood

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compatibility, mechanical properties, degradation behavior and porous structure is critical for successful rapid host remodeling of blood vessels. Silk, one of the toughest and most versatile biomaterials known, is a biological protein fiber with exceptional mechanical properties and biocompatibility [4]. In recent years silk-based materials have been used in high technology applications, with a promising future in tissue engineering, drug release systems, and optical applications [20,21]. Considering the ability to support the attachment, proliferation, and differentiation of vascular cells, as well as the exceptional mechanical properties with a high modulus and tensile strength, silks have the potential to be used in the design of small diameter vessel grafts with adequate biocompatibility and mechanical strength [4,22]. Several electrospun silk-based blood grafts have been prepared which achieved better mechanical strength than those prepared with collagen or other commonly used natural biomaterials [4,22–25]. However, these silk-based grafts do not have the mechanical properties of natural silk fibers due to the regeneration process required for electrospinning [26]. Additional silk-based vascular grafts, generated by gel spinning, meet the required mechanical requirements, yet could be further improved for functional use, biocompatibility, and related clinical needs [1,27]. Further study is needed to continue to improve the properties of silk-based grafts to reach the gold standard of saphenous veins.

In our previous study silk fibroin (SF) films containing heparin were prepared and achieved slow delivery of heparin to maintain blood compatibility for about 1 month [28]. Endothelial cells grew on the films to form a dense layer within 2 weeks, implying the feasibility of using these films for vessel grafts. However, the mechanical properties in the wet and dry states required improvement. Recently porous silk scaffolds composed of nanofibers were achieved through lyophilization to further improve biocompatibility and to facilitate tissue regeneration [29]. In the present study the objective was to design multilayer grafts from silk protein and to assess their potential as small diameter vessel prostheses. The outer layer of the grafts is composed of nanofibrous silk scaffolds that provide a suitable environment for the growth of smooth muscle cells and fibroblasts while the internal layer consists of silk fiber-reinforced films containing heparin for low thrombogenicity. The mechanical properties matched those of the mammary artery, including burst pressure, suture retention strength, and compliance. The thrombo-resistance of the internal layer was assessed through a series of blood tests in vitro. Finally, amniotic fluid-derived stem cells, with the capacity to differentiate along multiple lineages and avoiding the ethical concerns associated with embryonic stem cells, were studied to assess cell compatibility of the bilayered grafts. Cumulatively these studies indicate that the proposed bilayer design has potential for vascular prosthesis, thus follow on in vivo studies will be pursued.

2. Materials and methods

The preparation of materials for experiments presented in this paper was carried out under protocols approved by the Soochew University Ethical Committee.

2.1. Preparation of aqueous silk fibroin solutions

Bombyx mori SF solutions were prepared according to our previous published procedures [24,30]. Cocoons were boiled for 20 min in an aqueous solution of 0.02 M Na_2CO_3 and then rinsed thoroughly with distilled water to extract the sericin proteins. After drying the extracted SF was dissolved in 9.3 M LiBr solution at 60 °C for 4 h, yielding a 20% (w/v) solution. This solution was dialyzed against distilled water using Slide-a-Lyzer dialysis cassettes (Pierce, molecular weight cut-off 3500 Da) for 72 h to remove the salt. The solution was optically clear after dialysis and was centrifuged to remove the small amount of silk aggregates that formed during the process. The final concentration of silk in water was about 6%. To determine the concentration of the silk solution the weight of a small weigh boat was first measured. Then 1 ml of the silk solution was added to the boat and allowed to dry at 60 °C for >24 h. Once the silk was dry the weight of the silk was determined, yielding the weight per volume percentage.

2.2. Preparation of SF bilayered vascular grafts

In previous studies SF tubes were prepared through gel spinning, dipping or electrospinning [1,2,4,22–25,27]. Some of the tubes were brittle in the dry state and lost mechanical strength in the wet state, resulting in practical difficulties in further fabrication and application. Recently gel spun silk tubes with improved mechanical properties have been successfully implanted in rats, showing promising potential as vascular graft materials [1], however, further modifications are needed to optimize the tube mechanics, blood compatibility and vascular cell responses. In order to address the problems with some of these systems degummed silk fibers were used to improve the mechanical properties of the silk tubes. The mesh tubes were produced with silk yarn (20–22 denier, Hekang Medical Instrument Co. Ltd, Jiaxing, People's Republic of China) on a high-speed rope braiding machine (JC 2-24, Zhangjiagang Textile Machinery Co., Jiangsu, People's Republic of China) under controlled process conditions. The yarns were twisted around a stainless steel mold (Fig. 1, mold A) with a 2/2 pattern at a take-up rate of 1.2 m min^{-1} to achieve mesh tubes with a braid angle of 60° and an interval between adjacent yarns of about 0.6 mm. The mesh tubes were boiled for 20 min in an aqueous solution of 0.02 M Na_2CO_3 and rinsed thoroughly with distilled water to remove the sericin proteins and impurities. Then the mold with the mesh tube was nested tightly inside silk solutions vertically at 60 °C. Following evaporation of the silk solution a silk fiber-reinforced tube was formed without any post-treatment. The reinforced tubes had good flexibility in the dry state, making it easy to prepare bilayered grafts. Different amounts of heparin (Ruitai Bio Co., Beijing, People's Republic of China) were added to the silk solutions to prepare tubes containing heparin via the same process. The tubes containing 1% and 3% heparin were termed SF-H1 and SF-H3, respectively, while the pure silk tube was termed SF.

In order to facilitate porous structure formation in the outer layer fresh silk solution was treated by a slow concentration process [28]. The concentration was increased at 60 °C to form a 20% silk solution after 24 h. Then the concentrated solution was placed at 4 °C for 1 week to further facilitate the formation of nanofibrils in solution. After dilution to 2% the treated silk solution containing silk nanofibrils was poured into a stainless steel mold (mold B) in the middle of which was fixed the sealed fiber-reinforced silk tube. The mold was subsequently frozen at −20 °C for 12 h, and then lyophilized for about 72 h, to prepare bilayered grafts. Finally, the grafts were treated with 70 vol.% ethanol for 30 min to induce crystallization and insolubility in water (Fig. 1) [31].

2.3. Structural analysis

The morphology of the vascular grafts was observed by scanning electron microscopy (SEM) (Hitachi S-4800, Tokyo, Japan) at 3.0 kV. Samples were mounted on a copper plate and sputter-coated with a 20–30 nm thick gold layer prior to imaging.

The secondary structure of the samples was analyzed with a Nicolet 5700 FT-IR spectrometer (Thermo Electron Corp, Waltham, MA) equipped with a MIRacle™ attenuated total reflection (ATR)

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