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Short communication

Dual layer collagen-GAG conduit that mimic vascular scaffold and promote blood vessel cells adhesion, proliferation and elongation

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ABSTRACT

A dual layer conduit, which consists of collagen I-hyaluronic acid (Col-HA, external layer) and collagen I-heparin (Col-HP, inner layer), has been developed. This conduit not only meets the structural requirement of nature blood vessel, but also presents favourable mechanical performance after crosslinking *via* EDC. In addition, its biological properties are evaluated through culturing with fibroblast cell (Cos-7) on Col-HA surface and vascular endothelial cell (HMEC) on Col-HP layer. Results demonstrate that the conduit can support Cos-7 and HMEC adhesion, proliferation and elongation on respective side. This dual layer conduit would be a new choice of artificial vascular scaffold and improve blood vessel regeneration for tissue engineering.

1. Introduction

Vascular injury caused by various cardiovascular diseases is one of the most serious diseases threatening human health [1, 2]. Autotransplant is regarded as the most perfect treatment for veins and arteries replacement, since the substitutes own similar properties to the native blood vessel [3]. However, in the process of vascular clinical therapy, this method faces various limitations, especially mismatched length, inconsistent size and high morbidity. At present, artificial vascular scaffold gradually become promising substitutions of repairing damaged blood vessel, owing to the controllable shape, size, length and components [4, 5]. An ideal artificial vascular graft firstly should have the similar structure composition of blood vessel *in vivo*: the inner most layer consisting of the endothelial cell lining with basement membrane and sub-endothelial connective tissue layer; the middle layer for populating smooth muscle cells; the outer most layer for embedding fibroblasts [6–8]. Secondly, it should be non-thrombogenic, non-immunogenic and biocompatible, as well as conductive to the rapid formation of new-born layers [9]. In addition, a certain degree of strength, viscoelasticity and biodegradability are also the necessary conditions [10, 11].

Classical artificial vascular scaffold is primarily based on either synthetic polymers, such as polytetrafluoroethylene, or natural polymers like cellulose, silk fibroin [12–15]. Synthetic polymers always perform well for large-diameter blood vessels in clinical settings, but they are not suitable for the replacement of small-caliber blood vessels due to severe thrombosis. Natural polymers possess the advantage of good biocompatibility, while how to enhance mechanical properties is still a challenge [16]. Collagen and glycosaminoglycans (GAGs) comprise the majority of the extracellular matrix (ECM) and form a highly organized, three-dimensional scaffold that surrounds the cells [17, 18]. They play dominant roles in maintaining the biological and structural integrity of ECM and proceeding to manipulate cellular behavior and tissue function [19, 20]. GAGs, such as heparin and hyaluronic acid, are more biocompatible than other natural polymers. Additionally, they are biodegradable, nontoxic and non-antigenic [21–23]. In particular, heparin has been proved to be anticoagulant. The adding of heparin into the preparation of artificial blood vessels can bring the antithrombotic effects [24].

Herein, we manufacture a dual layer conduit consisting of collagen-heparin (Col-HP, inner layer) and collagen-hyaluronic acid (Col-HA, external layer) to mimic vascular scaffold, which was expected to facilitate the proliferation of vascular endothelial cell and fibroblast separately, bringing the regeneration of blood vessel (Scheme 1). To enhance the mechanical properties, the conduit was crosslinked with collagen and GAGs *via* EDC reaction. For all we know, this is one of the few work related to double layer vascular scaffold which is built up with ECM components collagen, heparin and hyaluronic acid together.

2. Materials and methods

2.1. Materials

Collagen I was purchased from BIOT Biology (Wuxi, China).

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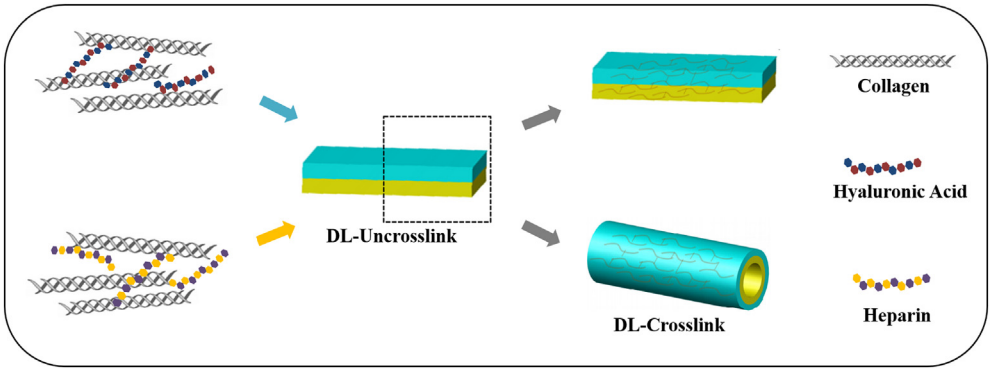

Scheme 1. Schematic description of dual layer collagen-GAG conduit.

Hyaluronic acid (Mw: 30,000) was purchased from Freda Biopharm Co. (Shandong, China). Heparin sodium (Mw: 20,000) was from Sangon Biotech (Shanghai, China). Collagenase was from Aladdin. Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco. Penicillin-streptomycin was obtained from Life Technologies. Human Microvascular Endothelial cells (HMEC) originated from the French Institute of Health and Medical Research (Paris, France). Cos-7 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Calcein AM and Propidium Iodide (PI) was purchased from Fanbo Co. (Beijing, China). All chemicals were of analytical grade and used without further purification.

2.2. Preparation of DL-Uncrosslink and DL-Crosslink membranes

Collagen I (200 mg) and hyaluronic acid (10 mg), collagen I (200 mg) and heparin (10 mg) were dissolved in ionic liquid (2 mL) respectively for stirring overnight at 37 °C. Afterwards, the collagen-hyaluronic acid mixture (300 μL) was poured into 6-well plate to form Col-HA layer, then collagen-heparin mixture (300 μL) was cover on it to form Col-HP layer. Ethanol and water (v/v = 1:1) was added into the well for extracting ionic liquid several times. After drying 48 h in air, the DL-Uncrosslink membrane was obtained. One part of DL-Uncrosslink membrane was immersed in EDC solution (5 mM) for crosslink reaction. 4 h later, the extra EDC solution was removed, and the membrane was washed with ethanol and water (v/v = 1:1) three times. After drying 48 h in air, DL-Crosslink membrane was prepared.

2.3. Fabrication of DL-Uncrosslink and DL-Crosslink conduits

DL-Crosslink conduit was made through Teflon model which consists of three hollow cylinders in 3 mm, 3.5 mm and 4 mm inner diameter. Col-HP mixture was added into the space of 3 mm–4 mm hollow cylinder, then 4 mm hollow cylinder was removed and the 5 mm one was anchored when Col-HP layer was in stable state. Col-HA mixture was added to fill the space. After extraction of ionic liquid and crosslinking with EDC, the DL-Crosslink conduit was formed.

2.4. Degradation test

The biodegradation properties of DL-Uncrosslink and DL-Crosslink conduits were tested *in vitro* with collagenase I. Same amount of conduits was immersed into PBS buffer with collagenase I (1 U/mL) and kept in a constant temperature shaker at 100 rpm/min, 37 °C. Then, the samples were centrifuged to remove PBS with enzyme at predetermined points (1 h, 6 h, 12 h, 24 h, 2 d, 3 d, and 7 d). The precipitate was lyophilized to measure the remaining weight. The biodegradation ratio was tested with the mean ± standard deviation (n = 3).

2.5. Cell culture

HMEC was maintained in Endothelial Cell Medium (ECM) supplemented with fetal bovine serum (FBS, 10%), endothelial cell growth supplement (ECGS, 1%), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cos-7 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified incubator at 37 °C under CO_2 (5%) and air atmosphere (95%). The culture media was changed every 2 days.

2.6. Cell viability through CLSM

The DL-Crosslink membrane was placed into cell culture dish. Firstly, HMEC were cultured on the Col-HP side at an initial cell seeding density of 7.5 × 105 cells. 12 h later, the membrane was turned over and inoculated with Cos-7 on the Col-HA layer with the same initial cell density. After culturing at 37 °C for another 1 day or 3 days, cells were stained with calcein AM (4 μg/mL) and PI (20 μg/mL) for 30 min. Finally, the stained samples were washed with PBS buffer and fixed with paraformaldehyde (4%). Cell viabilities were observed through CLSM.

2.7. Morphological characterization through SEM

The morphologies of cells culturing on DL-Crosslink conduit were observed by SEM 3 days later. Herein, Cos-7 and HMEC cells were seeded on Col-HA or Col-HP layer separately. Conduits were washed with PBS, and then fixed with paraformaldehyde (4%) for 15 min. The resulting samples were washed with ultrapure water and lyophilized.

3. Results and discussion

3.1. Preparation and characterization of collagen-GAGs conduits

The complete synthetic procedures of dual collagen-GAGs layer conduit were represented in Supporting information in details. Herein, ionic liquid was used to dissolve collagen, heparin and hyaluronic acid, since ionic liquid is not only an environmental friendly solvent, but also able to increase the solubility of collagen. The mixture of collagen and hyaluronic acid was poured into module as the bottom layer (Col-HA), shortly afterwards, collagen and heparin solution was added as the upper layer (Col-HP). After exchanging ionic liquid with ethanol and drying, half part of the membrane was left for control experiment, named as DL-Uncrosslink membrane, the other part was immersed into EDC solution for crosslinking to form DL-Crosslink membrane.

The morphologies and structure of individual layer and DL-Crosslink conduit were first analyzed. The thickness of Col-HA and Col-HP layer was similar, about 80 μm (Fig. 1a, d), what's more, they both presented porous structure as shown in Fig. 1b and e. The pore size of