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HPLC detection of loss rate and cell migration of HUVECs in a proanthocyanidin cross-linked recombinant human collagen-peptide (RHC)–chitosan scaffold

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ABSTRACT

Porous scaffolds with appropriate pore structure, biocompatibility, mechanical property and processability play an important role in tissue engineering. In this paper, we fabricated a recombinant human collagen-peptide (RHC)–chitosan scaffold cross-linked by premixing 30% proanthocyanidin (PA) in one-step freeze-drying. To remove the residual acetic acid, optimized 0.2 M phosphate buffer of pH 6.24 with 30% ethanol (PBSE) was selected to neutralize the lyophilized scaffold followed by three times deionized water rinse. Ninhydrin assay was used to characterize the components loss during the fabrication process. To detect the exact RHC loss under optimized neutralization condition, high performance liquid chromatography (HPLC) equipped size exclusion chromatography column was used and the total RHC loss rate through PBSE rinse was 19.5 ± 5.08%. Fourier transform infrared spectroscopy (FT-IR) indicated hydrogen bonding among RHC, chitosan and PA, it also presented a probative but not strong hydrophobic interaction between phenyl rings of polyphenols and pyrrolidine rings of proline in RHC. Further, human umbilical vein endothelial cell (HUVEC) viability analyzed by a scanning electron microscope (SEM) and acridine orange/ethidium bromide (AO/EB) fluorescence staining exhibited that this scaffold could not only promote cell proliferation on scaffold surface but also permit cells migration into the scaffold. qRT-PCR exhibited that the optimized scaffold could stimulate angiogenesis associated genes VEGF and CD31 expression. These characterizations indicated that this scaffold can be considered as an ideal candidate for tissue engineering.

1. Introduction

Constructing "in vitro" biological substitutes can be an alternative to the direct transplantation in tissue engineering [1,2]. Aiming at this approach, much progress has been made in fabricating porous three-dimensional (3-D) scaffolds to promote cell adhesion, migration, proliferation and differentiation [3]. These scaffolds should meet several common characteristics: (1) biocompatible materials would permit cell adhesion, growth and not provoke inflammation or toxicity in vivo; (2) similar microstructure to the extracellular matrix (ECM); (3) high porosity with suitable pore size and open-pore geometry; (4) mechanical properties and proper degradation rate to maintain the predesigned tissue structure; (5) processability of fabricating [2,4].

Various techniques have been reported in the literatures for the fabrication of biomedical porous scaffolds, one of the frequent used methods is the lyophilization. Lyophilization is normally used for solvent removal avoiding the drying stresses and shrinkage that may lead to cracks and warping during normal drying [2,5]. However, during freeze-drying, the final pore structure depends on the underlying freezing process in which ice crystals formulated and followed by sublimation to produce porous structure [6], inappropriate freezing drying condition may result in closed cellular structure [7]. On the other hand, in reduplicative freeze-drying, unorganized ice crystal growth may induce irregular sublimation with recrystallization of water vapor and secondary sublimation in the same areas during freeze-drying procedure [5]. Therefore, numbers of freeze-drying should be reduced for fabrication scaffold with uniform pore structure.

Among the polymeric materials proposed for tissue engineering, natural polymers are widely used as analogs of extracellular matrix (ECM). Collagen is a significant constituent of the natural ECM, collagen based scaffolds have been observed to promote cell attachment and growth [8]. However, most commercially available collagen and gelatin are derived from animal species whose major drawbacks are possible disease transmission, allergic reactions [9]. As a result, recombinant human collagen produced by expression systems in a consistent, efficient, and safe manner has been achieved [10]. In this paper, we used

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a recombinant human collagen-peptide (RHC) to fabricate scaffolds by freeze-drying.

However, the fast biodegrading rate and low mechanical strength of the untreated collagen based scaffolds are crucial that limit the further use of this artificial substitute, especially the recombinant collagen with prominent processability [11]. Reinforcement and cross-linking are two effective strategies to modify the drawbacks [12].

Reinforcement of the original material can be achieved by adding a second material which has higher mechanical properties. Chitosan is a natural polysaccharide that plays an important role in tissue engineering [13]. However, chitosan is normally insoluble in aqueous solutions above pH 7, while in dilute acids (pH < 6), the free amino groups are protonated and the molecule becomes soluble. The high charge density in solution allows chitosan to form insoluble ionic complexes with a wide variety of water soluble polyanionic species [14]. To remove the residual acetic acid, the method such as gradient ethanol immersion has been reported [15], but it is a time-consuming procedure and might be a risk for pore destruction.

To further promote the scaffold properties, it always strives in combining these materials via cross-linking during fabrication. Cross-linkers such as chemical aldehydes, carbodiimide hydrochloride or natural genipin are widely used for their prominent performance [12]. Proanthocyanidin (PA) components are natural products with polyphenolic structures composed of flacan-3-ol subunits linked mainly through C4–C8 and are members of the category of tannins [16–18], they are alternative natural cross-linkers in collagen based scaffold fabrication. Previous reports have shown that PAs are about 120 times less cytotoxic than glutaraldehyde [17] and the grape seed extracts mainly composed of PA presented beneficial effects on skin wound healing [19].

In commonly, the lyophilized scaffold should immerse in cross-linking solution followed by cross-linker rinse, after which the scaffolds need to be stored in buffer [12] or treated with a second freeze-drying for long-term storage [20]. Meanwhile, considering that both residual acid and cross-linkers need to be removed before application, it will be efficient (time saving) to produce the scaffold in a one-step cross-linking method. In addition, the interaction between polyphenols and collagen is proposed to be primarily driven by hydrophobic effect between phenyl rings of polyphenol and pyrrolidine rings of proline without pH-sensitive groups [21], so the interactions will not be compromised by an acidic environment, this mechanism is distinctively different from that of common chemical cross-linkers such as glutaraldehyde.

In this paper, we attempted to fabricate a PA cross-linked RHC–chitosan scaffold by a one-step premixing method, in consideration of the interference of chitosan and PA to several protein characterizations, ninhydrin assay was used as a cursory analysis method during fabrication process, meanwhile, HPLC was attempted as a more accurate measurement to detect RHC loss.

It is also known that angiogenesis is one of the major issues in wound repair, which is indispensable for the delivery of oxygen and nutrients to cells on the wound sites. Angiogenesis requires a dynamic and temporally and spatially regulated interaction between endothelial cells, angiogenic factors and surrounding extracellular matrix proteins since vascularization is important in skin substitute fabrication [22,23]. In this case, for biocompatibility, HUVECs were selected to detect if this scaffold could support cell proliferation, migration and further angiogenesis related gene secretion.

2. Materials and methods

2.1. Materials

Recombinant human collagen-peptide (RHC) with high hydrophility owing to collagen gene modification was obtained in our laboratory as described previously [24,25]. Chitosan (deacetylation > 90.0%, viscosity < 100 cps) was purchased from Lanji (Shanghai, China). Primary HUVECs were kindly supplied by Prof. Liang (School of Pharmacy, Wenzhou Medical University, Zhejiang, China), and maintained at 37 °C in an 5% CO_2 humidified atmosphere (Thermo Scientific Series 8000 WJ, USA), using Dulbecco's Modified Eagle's Medium-high glucose (DMEM-H, HyCLSone, Logan, USA) with 1% penicillin/streptomycin and 10% fetal calf serum (Sijiqing, Hangzhou, China), and the cells were used in the fifth–seventh passages. Grape seed-extracted PA (with a purity of over 85%) was obtained from Jianfeng Natural Product R&D Co., Ltd., (Tianjin, China). Ninhydrin was obtained from Bio Basic Inc. (Boston, USA). Acridine Orange/Ethidium Bromide (AO/EB), cetyl trimethylammonium bromide (CTAB), polyvinylpyrrolidone (PVP), ethylenediaminetetraacetic acid (EDTA) and other chemicals of analytical quality or HPLC grade were purchased from Sinopharm Chemical Reagent (Shanghai, China). Deionized water was used throughout this study.

2.2. Fabrication of PA cross-linked RHCC scaffold

RHC and chitosan were dissolved in 0.1 M acetic acid and mixed by gentle stirring for 1 h at room temperature (20 °C). PA acetic acid solution was slowly added to the blending suspension to a final concentration of 0.5% (wt.%) RHC and 0.5% (wt.%) chitosan while PA was ranging from 10%, 20%, 30%, 40%, to 50% (mass ratio of the blend suspension). Each mixture was further detected by ninhydrin assay.

The mixture with 30% PA was stirred for another 1 h and degassed in a vacuum evaporator (Christ, RVC 2–18, Germany) to remove air bubbles. 48 wells polystyrene plate was used as a mold for freeze-drying to fabricate the cross-linked scaffolds. The temperature of shelf and chamber of the freeze-dryer (LGJ-10D, Sihuan, China) was pre-cooled and maintained at −60 °C for freezing process. Then the plate was placed into the shelf to freeze the suspension for 6 h. The solid phase was then sublimated under vacuum to obtain porous scaffolds. RHC–chitosan blend without PA was freeze-dried in the same way as control scaffold.

2.3. Optimizing of scaffold neutralization

These lyophilized 30% PA cross-linked RHC–chitosan scaffolds were neutralized by 0.1 M or 0.2 M phosphate buffer with various pH levels (5.5–7.0) to determine the suitable pH, and phosphate buffer with settled pH ranging from 0.01 M to 0.3 M to determine the best concentration. The neutralized scaffold under optimized condition as well as the one neutralized by same solution with 30% ethanol were washed by deionized water for three times. Each scaffold was immersed in solution with a mass/vehicle ratio of 1 mg/ml at room temperature for 0.5 h in each step and the soaking solution was collected for further loss rate detection.

2.4. Loss rate and cross-linking degree detection

Solution specimens for loss rate detection were collected as described in Section 2.3 and filtered with 0.22 μm filtration membrane for HPLC and ninhydrin assays.

2.4.1. High performance liquid chromatography (HPLC)

Analytical runs were performed on an Agilent 1260 infinity HPLC system. Chromatograms were obtained under isocratic conditions at a nominal flow rate of 0.5 ml/min. Size exclusion chromatography column (Ultimate® SEC-300, Yuexu, China) was equilibrated with phosphate-buffered saline (0.01 M phosphate buffer, pH 7.4, 30% acetonitrile) and the mobile phase was sterilized by filtration through a 0.22 μm filter at first. 10 μl filtered samples were injected onto the column automatically; the amount of protein was determined by measuring the optical density at 220 nm under 25 °C.

Quantitative analysis of RHC by HPLC was performed with prepared calibrators. Filtered calibrators of definite concentration were injected in graded volumes automatically. A linear calibration curve of amplitude