Biologicals 48 (2017) 39–46

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Fabrication of porous scaffolds with decellularized cartilage matrix for tissue engineering application

Bita Nasiri, Shohreh Mashayekhan*

Department of Chemical and Petroleum Engineering, Sharif University of Technology, Tehran, 11365-8639, Iran

ARTICLE INFO

Article history:
Received 2 February 2017
Received in revised form
30 May 2017
Accepted 31 May 2017
Available online 8 June 2017

Keywords:
Cartilage tissue engineering
Extracellular matrix
Hybrid scaffold

ABSTRACT

Due to the avascular nature of articular cartilage, damaged tissue has little capacity for spontaneous healing. Three-dimensional scaffolds have potential for use in tissue engineering approach for cartilage repair. In this study, bovine cartilage tissue was decellularized and chemically crosslinked hybrid chitosan/extracellular matrix (ECM) scaffolds were fabricated with different ECM weight ratios by simple freeze drying method. Various properties of chitosan/ECM scaffolds such as microstructure, mechanical strength, swelling ratio, and biodegradability rate were investigated to confirm improved structural and biological characteristics of chitosan scaffolds in the presence of ECM. The results indicated that by introducing ECM to chitosan, pore sizes in scaffolds with 1% and 2% ECM decreased and thus the mechanical properties were improved. The presence of ECM in the same scaffolds also improved the swelling ratio and biodegradation rate in the hybrid scaffolds. MTT cytotoxicity assays performed on chondrocyte cells cultured on chitosan/ECM scaffolds having various amounts of ECM showed that the greatest cell attachment belongs to the sample with intermediate ECM content (2% ECM). Overall, it can be concluded from all obtained results that the prepared scaffold with intermediate concentration of ECM could be a proper candidate for use in cartilage tissue engineering.

1. Introduction

Articular cartilage is a connective tissue which plays a fundamental role in the growth process and provides sufficient mechanical properties in diarthrodial joints. It is composed of chondrocyte cells and varying amounts of extracellular matrix (ECM) deposited outside the cells. The most abundant proteins found in cartilage ECM are collagen II and large proteoglycans such as aggrecan [1].

Damages to articular cartilage are usually caused by sport-related injuries, aging-related degeneration, disease, trauma and tumor. Due to avascular structure, low cellular metabolic activity, and low supply of progenitor cells, cartilage has limited ability to heal spontaneously, resulting in further chronic problems such as osteoarthritis, malfunction, and extensive pain [2].

Although different treatment protocols such as microfracture, mosaicplasty, autologous chondrocyte implantation (ACI), osteochondral allograft transplantation, and prosthetic joint replacement have been devised to repair cartilage defects over recent decades, there are still several limitations in producing long-lasting cartilage with full biological activity [3,4].

Tissue engineering is an interdisciplinary science that offers promising approaches to cartilage regeneration. Advances in tissue engineering relies on the development of scaffolds, which can provide a microenvironment mimicking the native tissue, provide proper mechanical strength and support the cell's attachment and proliferation [5]. Recently, different scaffolds made of synthesized and natural-based polymers have been investigated in cartilage tissue engineering [6–9].

Chitosan is a natural-based polymer produced from chitin, the second most abundant polysaccharide, found in marine crustacean shells [10]. Because of its attractive properties, chitosan has been widely studied in biomedical applications such as tissue engineering, wound healing and drug delivery [11–13]. Different blends of synthetic and natural polymer with chitosan have been investigated and showed promising results in *in-vivo* and *in-vitro* studies [14,15]. Chitosan's biocompatibility, low immunogenicity, biodegradability [16,17], low cost and similarity to glycosaminoglaycans (GAG) [17] support chondrogenic activities and make it a desirable scaffold for cartilage growth and repair [18].

* Corresponding author.
E-mail address: mashayekhan@sharif.edu (S. Mashayekhan).

http://dx.doi.org/10.1016/j.biologicals.2017.05.008
1045-1056/

Recently, scaffolds made of ECM derived from various decellularized tissue and organs have become more attractive and are used for regeneration or replacement of various damaged tissue and organs including skin [19], skeletal muscle [20], blood vessels [21], nerves [22], tendon [23] and bladder [24]. The ECM is secreted by the cells and provides informational signals and certain unique composition which mimics the natural tissue environment and leads to proper tissue regeneration In articular cartilage [25], chondrocytes are surrounded by a highly hydrated ECM consisting of type-II collagen (Col II), proteoglycans, and various other proteins, which play a crucial role in chondrogenesis [26].

Yang et al. developed a cartilage ECM-derived porous scaffold which showed suitable biocompatibility and 3-D interconnected structure. They also performed *in-vivo* studies to investigate chondrogenesis ability in nude mice. Their results demonstrated appropriate cell repopulation for ECM-derived scaffolds and appropriate neocartilage formation [27].

Chitosan or acellular cartilage ECM-based scaffolds are investigated in different studies [18,28,29], but to the best of our knowledge the crosslinked blend of chitosan/ECM has not been examined yet in cartilage regeneration. We expected that the incorporation of cartilage ECM in chitosan matrix and taking advantage of both polymers' properties would make the hybrid chitosan/ECM scaffold as a suitable candidate for cartilage tissue engineering.

In this study, chemically cross-linked chitosan/ECM scaffolds with different weight ratios of ECM were prepared and evaluated in terms of pore sizes, mechanical properties, swelling properties, in-vitro enzymatic biodegradation and proliferation of chondrocyte cells to provide evidence to prove the potential applicability of the prepared blends for cartilage tissue engineering.

2. Materials and method

2.1. Materials

Medium molecular weight chitosan, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydro-chloride (EDC), hydroxysulfosuccinimide (NHS), 2-(N-morpholino) ethanesulfonic acid (MES), tris (hydroxymethyl) amino methane and lysozyme from chicken egg white were purchased from Sigma-Aldrich. Pepsin was purchased from Merck. Sodium dodecyl sulfate (SDS) and Triton X-100 were purchased from Dae Gung. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, phosphate buffer saline (PBS) and Trypsin/EDTA were purchased from GIBCO. All reagents were used as received. Fresh articular cartilage of bovine femur bones was obtained immediately after slaughter. Then the samples were cut to 2×2 mm slices, washed with PBS immediately, and transferred to the freezer at $-20\ ^{\circ}C$ prior to use.

2.2. Decellularization of cartilage

The decellularization method was done according to previous report [30]. Briefly, the cartilage slices were freeze-dried for 24 h at $-56\ ^{\circ}C$ in a lyophilizer (Operon, Korea). The samples were then ground into a fine powder for 20 min with Mixer/Mill device. The cartilage powder was agitated with 0.25% (w/v) trypsin + EDTA for 24 h and trypsin + EDTA solution was changed every 8 h. After washing the samples with PBS for 30 min, the samples were treated with 10 mM hypotonic Tris–HCl solution for 24 h followed by incubation in 1% Triton X-100 (v/v, in PBS) for another 24 h. Finally, the samples were washed in PBS for 24 h and the PBS changed every 8 h. The decellularized cartilage powder was stored at $-20\ ^{\circ}C$ prior to use. All above solutions contained 1% penicillin/streptomycin during the decellularization process.

2.3. Scaffold fabrication

Various hybrid scaffolds were fabricated using chitosan and ECM solution as presented in Table 1. Chitosan solution was prepared by dissolving chitosan in 2% (v/v) acetic acid and stirring overnight. ECM was prepared by enzymatic digestion according to the previous report [31]. Decellularized cartilage powder was treated with 1 mg/ml pepsin in 0.1 M HCl. The suspension was under constant stirring for about 60 h at room temperature to complete the digestion process. The resultant was brought to pH 7.4 by the addition of 2 M NaOH to form a viscous gel solution.

Amide bonds are created between the primary amino group of the chitosan and a carboxylic group of ECM existed in collagen II [32] and glycosaminoglycan(GAG) [33] by using crosslinking agents, EDC (1-ethyl-3-[3 dimethylaminopropyl] carbodiimide) and NHS (N-hydroxysuccinimide), as shown in Fig. 1. Briefly, for activating the carboxyl group presented in ECM, the ECM solution was reacted with crosslinking reagents with a final concentration of 50 mM MES, 30 mM EDC, and NHS for 15 min. Then, the chitosan solution was added and stirred for 24 h at room temperature. Following this, the chitosan/ECM blend was poured into 24-well plates, frozen at $-20\ ^{\circ}C$ and lyophilized overnight. To neutralize the remaining acetic acid and improve the mechanical properties, the scaffolds were immersed in 1 M NaOH for 15 min, then washed three times by distilled water, frozen again at $-20\ ^{\circ}C$, and lyophilized in freeze-drier for another 24 h.

2.4. Evaluation of decellularization process

2.4.1. Histological evaluation

Parts of cartilage samples (native and decellularized) were fixed for 24 h in 10% neutral buffered formalin solution in PBS (pH 7.4) at room temperature. Subsequently the samples were washed, dehydrated in graded series of ethanol, embedded in paraffin, and sectioned with a thickness of 5 μm with microtome. Hematoxylin and eosin (H&E) and alcian blue staining was performed after deparaffinization by xylene and rehydrating to validate the chondrocyte's removal from decellularized cartilage tissue.

2.5. Scaffold characterization

2.5.1. Morphological characterization

The morphology of lyophilized scaffolds and its interior pore structure were investigated by scanning electron microscopy (SEM, Seron Technology, AIS-2100, Korea). The scaffolds were cut by sharp blade and the cross sectional specimens were coated with gold before observation. ImageJ analysis software was used for measuring the pore sizes from the obtained data.

2.5.2. Fourier transform infrared spectroscopy

The chemical composition of hybrid scaffolds, decellularized ECM, and chitosan, were examined by Fourier transform infrared spectroscopy (FTIR) using ABB Bomem (MB series(spectrophotometer in the range of 4000-400 cm^{-1} at room temperature.

2.5.3. Mechanical properties

The mechanical properties of scaffolds were measured by compressing the hydrated scaffolds up to 70% strain at room temperature by using Hounsfield H10KS mechanical tester with a load cell of 0.5 kN. The loading rate was 0.5 mm/min. To measure the compressive properties, the lyophilized scaffolds were immersed in phosphate buffer saline (PBS) for 1 h at room temperature. The Young's modulus was determined from the slope of the initial linear section of stress-strain diagram (less than 10% strain).