Materials Science and Engineering C 80 (2017) 532–542

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Fabrication and characterization of hydrothermal cross-linked chitosan porous scaffolds for cartilage tissue engineering applications

Mohammad Amin Shamekhi [a], Ahmad Rabiee [b,*], Hamid Mirzadeh [c,*], Hamid Mahdavi [a], Davod Mohebbi-Kalhori [d], Mohamadreza Baghaban Eslaminejad [e]

[a] Novel Drug Delivery Systems and Biomaterial Department, Science Faculty, Iran Polymer and Petrochemical Institute, Tehran, Iran
[b] Polymer Science Department, Iran Polymer and Petrochemical Institute, Tehran, Iran
[c] Department of Polymer Engineering and Color Technology, Amirkabir University of Technology, 424 Hafez Avenue, Tehran, Iran
[d] Chemical Engineering Department, Faculty of Engineering, University of Sistan and Baluchestan, Zahedan, Iran
[e] Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

ARTICLE INFO

Article history:
Received 30 October 2016
Received in revised form 22 December 2016
Accepted 21 March 2017
Available online 27 March 2017

Keywords:
Cartilage tissue engineering
Hydrothermal cross-linking
Chitosan scaffold
Human articular chondrocyte

ABSTRACT

The use of various chemical cross-linking agents for the improvement of scaffolds physical and mechanical properties is a common practical method, which is limited by cytotoxicity effects. Due to exerting contract type forces, chondrocytes are known to implement shrinkage on the tissue engineered constructs, which can be avoided by the scaffold cross-linking. In the this research, chitosan scaffolds are cross-linked with hydrothermal treatment with autoclave sterilization time of 0, 10, 20 and 30 min, to avoid the application of the traditional chemical toxic materials. The optimization studies with gel content and crosslink density measurements indicate that for 20 min sterilization time, the gel content approaches to ~80%. The scaffolds are fully characterized by the conventional techniques such as SEM, porosity and permeability, XRD, compression, thermal analysis and dynamic mechanical thermal analysis (DMTA). FT-IR studies shows that autoclave inter-chain cross-linking reduces the amine group absorption at 1560 cm^{-1} and increase the absorption of *N*-acetylated groups at 1629 cm^{-1}. It is anticipated, that this observation evidenced by chitosan scaffold browning upon autoclave cross-linking is an indication of the familiar maillard reaction between amine moieties and carbonyl groups. The biodegradation rate analysis shows that chitosan scaffolds with lower concentrations, possess suitable degradation rate for cartilage tissue engineering applications. In addition, cytotoxicity analysis shows that fabricated scaffolds are biocompatible. The human articular chondrocytes seeding into 3D cross-linked scaffolds shows a higher viability and proliferation in comparison with the uncross-linked samples and 2D controls. Investigation of cell morphology on the scaffolds by SEM, shows a more spherical morphology of chondrocytes on the cross-linked scaffolds for 21 days of *in vitro* culture.

1. Introduction

Once damaged, the articular cartilage has very low potential for spontaneous repair due to its avascular and aneural character, low level of chondrocytes density, low proliferative activity and the tendency of chondrocytes to de-differentiate [1–5]. Technically, there are several methods to treat the symptoms amongst scaffold-based procedures are prominent [6–8]. Matrix-induced autologous chondrocyte implantation (MACI) is an alternative method to enhance the capabilities of traditional autologous chondrocyte implantation (ACI) procedure. In MACI, chondrocytes are seeded either on membrane scaffolds or within porous tridimensional scaffolds [9,10]. While, articular cartilage is expected to be one of the first tissues to be successfully engineered, it shows a lot of challenges to rebuild the complex structure and biomechanical properties of the native tissue by the scaffolds. In spite of enormous research works on the issue, only a few entered into the clinical trial stage [11–14].

On the other hand, the scaffolds can be fabricated from a diverse range of materials including natural, synthetic materials or blends of them. Among natural polysaccharides, significant properties of the chitosan including biocompatibility and biodegradability support the use of this polymer in many areas of the biomedical engineering including wound dressing, drug delivery, and tissue engineering [15–19]. Also, the chitosan characteristics support the use of this bio-based polymer in various areas of tissue engineering including skin, cartilage, and bone [20,21]. Chitosan structural similarity to the cartilage specific glycosaminoglycans (GAGs), which are known to have important role in

* Corresponding authors.
E-mail addresses: a.rabbii@ippi.ac.ir (A. Rabiee), Mirzadeh@aut.ac.ir (H. Mirzadeh).

http://dx.doi.org/10.1016/j.msec.2017.03.194
0928-4931/

physicomechanical properties of the cartilage, has provided the versatility of this biopolymer in the cartilage tissue engineering applications [22–26].

Chitosan is able to mimic the specific bioactivities of GAGs such as binding with growth factors and adhesion proteins. In addition, chitosan's amino functional groups can provide reactive sites for attachment of the bioactive factors such as oligopeptides under mild conditions [27–32]. Deacetylated units of chitosan contain a percentage of free amino groups that can become protonated to the cationic amine group ($-NH^{+3}$) making the scaffold surface positively charged. In this regard, these amine groups can then interact with the anionic species likes cells and GAGs. Ionic interactions with such negatively charged species can promote retention and organization of cartilaginous matrix constituents within a chitosan scaffold [33]. Another interesting feature of chitosan is that some of its biodegraded by-products may contribute to or stimulate the synthesis of important extracellular matrix molecules such as chondroitin, chondroitin-sulphate, dermatan-sulphate, keratan-sulphate and hyaluronic acid, which are known to be necessary for the function and nutrition of the cartilage [34,35]. Chitosan also has the ability to maintain chondrocyte round morphology and preserve synthesis of chondrocyte specific extracellular matrix biomolecules [36,37].

Furthermore, chitosan shows antibacterial and antifungal properties that could reduce the risk of bacterial and fungal infections when used as a scaffold [38–40]. Chitosan can be processed into various forms such as hydrogel, sponge, micro and nano-particles full filing diverse forms of its applications. Still, the other features of chitosan are its tailor-made mechanical properties which are controlled by adjusting some parameters such as shape, concentration, Mw, deacetylation, crystallinity, cross-linking and freezing and heating rate [41].

However it is worth to mention that, sterilization of chitosan scaffolds in the forms of solid sponges or hydrogels is required prior to implantation or injection in the body. In this respect, the autoclave saturated steam sterilization is an effective, easily available method which is assumed as the most practical means of sterilization for the medical devices and fluids [42–48]. Thermal cross-linking *via* autoclave has shown to be an effective method of cross-linking which improve the physical and mechanical properties of the chitosan scaffolds, simultaneously. In the present study, the chitosan scaffolds are sterilized by autoclave and simultaneously cross-linked which prevents the application of traditional toxic materials like glutaraldehyde [49,50]. Further stabilization step is conducted by treatment of the lyophilized scaffolds with ethanol series before the autoclave sterilization process [51]. In addition, the methodology of pre-freezing at the determined temperature (*i.e.* - 20 °C) is under taken to assure maximum pore size suitable for cartilage tissue engineering. The optimum processing parameters including curing time, chitosan concentration and molecular weight are determined *via* gel content measurements. The prepared porous scaffolds are comprehensively characterized by the conventional techniques and the effect of autoclave sterilization and simultaneous cross-linking on the physical and mechanical properties are investigated. Based on the experiences on the chitosans for diverse biomedical applications, it is believed that this system is suitable for many tissue engineering areas including cartilage tissue engineering scaffolds [52–54]. To the best of our knowledge, there is no documented information regarding the use of such scaffolds for the cartilage tissue engineering applications. In addition, the morphology of the human articular chondrocytes on the scaffolds is assessed.

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan (Catalog No. 448877, deacetylation degree in the range 75–85%), low molecular weight chitosan with (Catalog No. 448869, and deacetylation degree of ≥75%) and Dulbecco's modified eagle medium (DMEM), are prepared from Sigma-Aldrich. Ninhydrin reagent is prepared from Merck. Acetic acid is diluted to 0.2 M from glacial acetic acid (Merck) with the double distilled water.

2.2. Scaffold fabrication

Porous chitosan scaffolds are prepared *via* the freeze-drying method. Briefly, chitosan solutions with concentrations of (1.5 and 3% (w/v)) are prepared by dissolution of chitosan in 0.2 M acetic acid after filtration to remove the impurities. The solutions are poured into 24-well tissue culture polystyrene dishes (TCPS) and frozen at −20 °C for 24 h in a freezer. The frozen samples are then transferred into the freeze-dryer (Gamma (Christ), Germany) and lyophilized at −20 °C until they are completely dried.

Subsequent treatments are conducted for all scaffolds. Briefly, fresh lyophilized scaffolds are rehydrated in ethanol series (from 96 to 70% each for 30 min) and then completely washed with PBS to remove the acidic remainder and freeze dried again in the same condition. The cylindrical porous scaffolds (~10 mm diameter and ~5 mm thickness) are then exposed to simultaneous autoclave sterilization and cross-linking for different sterilization times (0, 10, 20, and 30 min) by a homemade autoclave (121 °C and 1.2 bar).

2.3. Investigation of physico-chemical properties

2.3.1. Gel content and cross-link density assessment

For gel content measurements, specimen of scaffolds are dried by vacuum oven (50 °C, 48 h) and weighed (W_d). The dry scaffolds are then completely washed with 6 ml of a 0.2 M acetic acid solution at room temperature for three days to dissolve non-cross-linked portions. The solution is renewed for every day. Finally, after washing three times with deionized water, the samples become dried with vacuum oven and assigned as (W_g) [55].The gel content is calculated with Eq. (1):

$$\text{Gel content } (\%) = W_g/W_d \times 100 \quad (1)$$

Cross-link density is accessed *via* ninhydrin assay as follows. Ninhydrin (2, 2-dihydroxy-1, 3-indanedione) reagent is used to calculate the amount of free amino groups in each chitosan sample. Different chitosan samples are pulverized and then swelled in distilled water. The samples are boiled for 30 min and then absorbance of the test samples is recorded by a spectrophotometer (Shimadzu, Japan) at 570 nm. Different specified concentrations of glycine (Sigma-Aldrich) are used as internal standard. The absorbance of samples with the ninhydrin is related to the concentration of free amino groups before (C_1) and after (C_2) cross-linking. Cross-link density of the thermally cured chitosan scaffolds is calculated from Eq. (2) [56,57].

$$\text{Cross}-\text{linking density } (\%) = [(C_1 - C_2)/C_2] \times 100 \quad (2)$$

2.3.2. Fourier transform infrared spectroscopy (FT-IR)

Grinded chitosan scaffolds were exposed to FT-IR spectroscopy (BRUKER IFS 484) according to ASTM E1252. Briefly, chitosan powders obtained from pulverization of the scaffolds, were mixed with KBr powder and the spectra were recorded from 4000 to 500 cm^{-1}.

2.3.3. Measurement of the porosity

The porosity of different thermal cross-linked scaffolds is calculated by liquid displacement procedure by using ethanol as solvent. The scaffolds (dry weight, W_d) are dipped in the ethanol for 30 min and the weight of the scaffolds in ethanol is assigned as W_1. The excess amount of ethanol is blotted from the scaffold by a tissue paper, and weighed