Carbohydrate Polymers xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Incorporation of chitosan in biomimetic gelatin/chondroitin-6-sulfate/hyaluronan cryogel for cartilage tissue engineering

Chang-Yi Kuo [a,1], Chih-Hao Chen [a,b,1], Chien-Yu Hsiao [c], Jyh-Ping Chen [a,c,*]

[a] Department of Chemical and Materials Engineering, Chang Gung University, Kwei-San, Taoyuan 333, Taiwan, ROC
[b] Craniofacial Research Center, Department of Plastic and Reconstructive Surgery, Chang Gung Memorial Hospital, Chang Gung University, College of Medicine, Kwei-San, Taoyuan 333, Taiwan, ROC
[c] Research Center for Industry of Human Ecology, Chang Gung University of Science and Technology, Kwei-San, Tao-Yuan 333, Taiwan, ROC

ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form 7 October 2014
Accepted 21 October 2014
Available online xxx

Keywords:
Chitosan
Chondroitin sulfate
Hyaluronan
Cryogel
Cartilage tissue engineering
Gelatin

ABSTRACT

We prepare an elastic macroporous gelatin/chondoitin-6-sulfate/hyaluronan (GCH) cryogel scaffold mimic the composition of cartilage extracellular matrix for cartilage tissue engineering. By incorporating chitosan in the cryogel to replace 20% gelatin, a GCH-chitosan cryogel was also synthesized and compared with GCH cryogel for scaffold mechanical properties and chondrocytes response. The GCH-chitosan cryogel has larger pores, higher ultimate strain (stress) and elastic modulus, and lower stress relaxation percentage than the GCH cryogel. Both cryogels show a highly elastic property with a loss tangent around 0.1, but chitosan incorporation increases the storage modulus (elasticity). Chondrocytes proliferate and redifferentiate in cryogels; chitosan diminishes cell proliferation but up-regulates glycosaminoglycans (GAGs) and type II collagen (COL II) secretion. Implantation of a chondrocytes/GCH-chitosan cryogel construct in a full-thickness articular cartilage defect regenerates cartilage with positive stainings for GAGs and COL II and an elastic modulus similar to the native cartilage.

1. Introduction

Functional tissue constructs for cartilage defects can be prepared by isolating viable chondrocytes from the patient, expanding the cell population, culturing cells in vitro on a scaffold, and then implanting the resulted tissue engineered construct back into the patient (Vacanti, 2003). In this approach, the biodegradable three-dimensional scaffold acts like an artificial extracellular matrix (ECM) while temporarily serves as a template to guide cell adhesion, proliferation and tissue development. For tissue engineering purpose, the scaffold should have an interconnected open macroporous network to allow unobstructed cell penetration and transport of nutrients, oxygen and waste products. In addition to these essential properties, for engineering soft tissues like cartilage that exhibits elastic properties, elasticity of scaffolds is being looked upon as an important design parameter (Yang, Webb, Pickerill, Hageman, & Ameer, 2006). A scaffold that can maintain its mechanical integrity during cyclic mechanical strains without permanent deformation may be another important criterion.

Cryogels are gel matrices that are synthesized at subzero temperatures using monomeric or polymeric precursors (Lozinsky et al., 2003). These gels can be obtained through the formation of both physically and covalently crosslinked homogeneous or heterogeneous polymer networks. At subzero temperature, most of the solvent gets frozen while part of the solvent is left unfrozen in the liquid microphase where dissolved substances concentrate and undergo chemical reactions (Lozinsky, Plieva, Galaev, & Mattiasson, 2001). These chemical reactions in the liquid microphase lead to gel formation with the crystals of frozen solvent acting as porogens. After thawing the ice crystals, a system of large interconnected pores is formed within the cryogel. Cryogels have some important characteristics including interconnected macroporous structure, mechanical stability and elasticity (Lozinsky et al., 2003). Elastic poly(vinyl alcohol) cryogel has been suggested for cartilage repair in arthritic shoulder replacement (Swieszkowski, Ku, Bersee, & Kurzydlowski, 2006).

Chondroitin-6-sulfate is a sulfated glycosaminoglycan (GAG), composed of a chain of alternating units of glucuronic acid and N-acetylgalactosamine, the latter being sulfated at O6. It is usually

* Corresponding author at: Department of Chemical and Materials Engineering, Chang Gung University, Kwei-San, Taoyuan 333, Taiwan, ROC. Tel.: +886 3 2118800; fax: +886 3 2118668.
E-mail address: jpchen@mail.cgu.edu.tw (J.-P. Chen).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.carbpol.2014.10.056
0144-8617/

found attached to proteins as part of a proteoglycan. It is an important structural component of cartilage and provides much of its resistance to compression. Inclusion of chondroitin sulfate in scaffold may promote the secretion of proteoglycan and type II collagen (Sechriest et al., 2000). Hyaluronan (hyaluronic acid) is an anionic, nonsulfated GAG distributed widely distributed throughout connective, epithelial, and neural tissues. As one of the chief components of the ECM, hyaluronan contributes to cell proliferation and migration, and facilitate the integration of engineered cartilage during embryonic cartilage development (Solchaga, Goldberg, & Caplan, 2001). Gelatin is considered as denatured collagen, the major protein component of cartilage, but with relatively low antigenicity and cost than collagen. Crosslinked gelatin and hyaluronan/gelatin scaffolds were shown to be suitable for application in cartilage tissue engineering (Angele et al., 2009; Lien, Li, & Huang, 2008). Chitosan is the cationic (1-4)-2-amino-2-deoxy-β-D-glucan with typical degree of acetylation close to 0.20. Chitosan has recently attracted interests as a candidate for cartilage repair because of its biocompatibility and structural similarity with the GAG present in the ECM of cartilage (VandeVord et al., 2002). Incorporating chitosan in cartilage tissue engineering scaffolds could provide a friendly environment for chondrocytes to propagate, produce typical ECM and maintain the correct phenotype (Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012). Chitosan-based porous scaffold was demonstrated to sustain chondrogenesis (Nettles, Elder, & Gilbert, 2002).

The objective of this study is to fabricate a macroporous, elastic cryogel scaffold from gelatin, chondoitin-6-sulfate and hyaluronan, whose chemical environment mimicking that of cartilage ECM (Chang, Liu, Lin, Chou, & Lin, 2003). Since chitosan has the potential to fulfill many of the requirements of a cartilage tissue engineering scaffold material, we further incorporate this GAG analog in the cryogel and study its effect on matrix properties, cell proliferation and differentiation.

2. Materials and methods

2.1. Materials

Sodium hyaluronan (HA, average molecular weight = 1.3 MDa) was obtained from Bloomage Freda Biopharm Co. Ltd. Gelatin (GEL, type A from porcine skin, 300 bloom, average molecular weight = 60 kDa) and chondroitin-6-sulfate (C6S, sodium salt from shark cartilage), chitosan (from shrimp shells, average molecular weight = 150 kDa, degree of deacetylation = 85%) were obtained from Sigma–Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was obtained from Acros. 2-(N-morpholino)ethanesulfonic acid (MES), 2,4,6-trinitrobenzene sulfonic acid (TNBS), antibiotics and trypsin-EDTA were all purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12, Sigma) and fetal bovine serum (FBS, HyClone) were used for cell culture. The 4′,6-diamidino-2-phenylindole (DAPI) solution for cell staining was obtained from Life Technologies.

2.2. Preparation of GCH and GCH-C cryogels

Cryogels were prepared by a modified method reported previously (Chang, Liao, & Chen, 2013). For GCH cryogel, 10% (w/w) GEL and 2% (w/v) C6S/0.1% (w/v) HA solutions were prepared separately at 70 °C and 37 °C in 0.5 M MES buffer (pH 6.0). EDC was added to the C6S/HA solution to a final concentration of 4% (w/v) and mixed for 30 min to obtain the C6S/HA/EDC solution. Two milliliter each of GEL and C6S/HA/EDC solution was added to a 5 ml disposable syringe (13 mm internal diameter), shaken at 70 °C for 30 s and placed in −16 °C ethanol in a deep freezer for 16 h in to complete the crosslinking reaction. After thawing at room temperature, the formed cryogel was pushed out from the syringe and washed by incubating in 1000 ml of phosphate buffered saline (PBS) for 2 h to remove residual EDC and reaction byproducts. Disks (2 mm in thickness and 10 mm in diameter) were cut from the cryogel with a sharp blade and a biopsy puncher and washed again by incubating in 500 ml of 70 °C 0.1 M MES buffer for 4 h to remove non-reacting substances. A final wash by incubating in 1000 ml de-ionized distilled (DDI) water for 12 h removed MES buffer from the cryogel. The preparation of GCH-chitosan cryogel followed the same procedure as GCH cryogel by replacing the 10% (w/w) GEL solution with an 8% (w/w) GEL/2% (w/v) chitosan solution. The final weight percentage of each component is 82.6% GEL, 16.5% C6S and 0.83% HA in the GCH cryogel; 66.1% GEL, 16.5% chitosan, 16.5% C6S and 0.83% HA in the GCH-chitosan cryogel.

2.3. Characteristics of cryogels

Pore size measurement was carried out with capillary flow porometry (PMI CFP-1100-AI, Porous Materials Inc., USA) using ethanol as the wetting agent. Porosity was determined using the ethanol displacement method (Zhang and Ma, 1999). The density of scaffold was calculated by the mass of the dry or wet scaffold over its volume. The microstructure of scaffold was observed by scanning electron microscope (SEM) (Philips XL-30) after sputter coating for 60 s.

Compression behavior of cryogel scaffolds were investigated by unconfined compression tests using an ElectroForce 5200 BioDynamic Test Instrument (Bose) in PBS at 37 °C. Wet samples were used for the tests after soaking in PBS for 24 h prior to testing. The compression load was applied with a 250 N load cell at a crosshead speed of 0.02 mm/s. A stress (σ)–strain (ε) curve was recorded with an uniaxial stress. The point where failure of the cryogel occurred gave the ultimate stress and ultimate strain values. The stress–strain data up to failure were fitted by a non-linear equation (Woo, Gomez, & Akeson, 1981),

$$\sigma = Ae^{(B\varepsilon - 1)} \quad (1)$$

A and B are elastic constants. Elastic modulus at 10% and 30% strain was calculated from this non-linear elastic model from the slope of the tangent to the stress–strain curve. Toughness (compressive strain energy to failure) of a cryogel, defined as the energy necessary to deform a specimen to failure, was obtained from the area under the stress–strain curve.

For evaluating the stress-relaxation behavior, the samples were compressed to 30% strain in 1 s followed by 500 s relaxation to reach steady state. The stress relaxation curve was plotted as remaining stress percentage and curve-fitted according to the non-linear equation (Wan, Campbell, Zhang, Hui, & Boughner, 2002),

$$\sigma_t/\sigma_o = \sigma_R + \alpha e^{-k_1 t} + \beta e^{-k_2 t} \quad (2)$$

σ_t is the stress at time t, σ_o is the initial stress, σ_R is the relative remaining stress at t = 500 s, α and β are proportional constants, k_1 and k_2 are relaxation-rate fitting parameters for the initial and final regions, respectively.

The cyclic compression test was similarly performed by loading the sample to 30% strain with 3200 cycles at a frequency of 1 Hz. The energy absorption in cryogel was derived from the stress–strain relation. A hysteresis loop, bounded by the loading and unloading curves, could be found, indicating dissipation of energy or energy absorbed due to the viscous properties of the cryogel. The dissipation energy (kJ/m^3) loss during the hysteresis cycle was calculated from the area bounded within the hysteresis loop. The percentage of energy dissipation (%) was calculated by dividing the dissipative energy with the area bounded between the loading curve and

Please cite this article in press as: Kuo, C. -Y., et al. Incorporation of chitosan in biomimetic gelatin/chondroitin-6-sulfate/hyaluronan cryogel for cartilage tissue engineering. *Carbohydrate Polymers* (2014), http://dx.doi.org/10.1016/j.carbpol.2014.10.056