JOURNAL OF THE MECHANICAL BEHAVIOR OF BIOMEDICAL MATERIALS 37 (2014) 69–77

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Research Paper

Effects of alginate hydrogel cross-linking density on mechanical and biological behaviors for tissue engineering

Jinah Jang[a], *Young-Joon Seol*[1,b], *Hyeon Ji Kim*[b], *Joydip Kundu*[b,2], *Sung Won Kim*[c,d], *Dong-Woo Cho*[b,*]

[a]*Division of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk, 790-784, South Korea*
[b]*Department of Mechanical Engineering, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk, 790-784, South Korea*
[c]*Department of Otolaryngology-Head and Neck Surgery, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 137-701, South Korea*
[d]*Department of Biomedical Science, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 137-701, South Korea*

ARTICLE INFO

Article history:
Received 7 February 2014
Received in revised form
1 May 2014
Accepted 4 May 2014
Available online 14 May 2014

Keywords:
Alginate
Tissue engineering
Hydrogel
Structure-properties relations
Cross-linking

ABSTRACT

An effective cross-linking of alginate gel was made through reaction with calcium carbonate ($CaCO_3$). We used human chondrocytes as a model cell to study the effects of cross-linking density. Three different pore size ranges of cross-linked alginate hydrogels were fabricated. The morphological, mechanical, and rheological properties of various alginate hydrogels were characterized and responses of biosynthesis of cells encapsulated in each gel to the variation in cross-linking density were investigated. Desired outer shape of structure was maintained when the alginate solution was cross-linked with the applied method. The properties of alginate hydrogel could be tailored through applying various concentrations of $CaCO_3$. The rate of synthesized GAGs and collagens was significantly higher in human chondrocytes encapsulated in the smaller pore structure than that in the larger pore structure. The expression of chondrogenic markers, including collagen type II and aggrecan, was enhanced in the smaller pore structure. It was found that proper structural morphology is a critical factor to enhance the performance and tissue regeneration.

*Corresponding author. Tel.: +82 54 279 5889.
E-mail addresses: jinahjang@postech.ac.kr (J. Jang), yseol@wakehealth.edu (Y.-J. Seol), ghj901017@postech.ac.kr (H.J. Kim), joydip15@gmail.com (J. Kundu), kswent@catholic.ac.kr (S.W. Kim), dwcho@postech.ac.kr (D.-W. Cho).
[1]Present address: Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA.
[2]Present address: Department of Chemical Engineering, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA.

http://dx.doi.org/10.1016/j.jmbbm.2014.05.004
1751-6161/

1. Introduction

Alginate, naturally derived from brown algae polysaccharides, is composed of β-D-mannuronic acid and α-L-guluronic acid (Martinsen et al., 1989). Sodium alginate is soluble in aqueous solutions and forms the hydrogel with multivalent cations, such as Ca^{2+}, Ba^{2+}, and Fe^{3+} (Smidsrød, 1990). It has been widely used in numerous biomedical applications, including the encapsulation of transplanted cells for tissue engineering due to the resemblance of fibrous structure to the natural extracellular matrix (Iozzo and Murdoch, 1996). Moreover, alginate provides an immunoisolation barrier; therefore it could be used as bioactive drug carrier (De Vos et al., 2006). Specifically, alginate hydrogel has been widely utilized in cartilage tissue engineering to facilitate maintenance of the chondrocyte phenotype (Benya and Shaffer, 1982; Kimura et al., 1980; Li and Zhang, 2005; Solursh et al., 1982; Thonar et al., 1983). Chondrocyte encapsulation with alginate hydrogel does not require any specific ligand–receptor binding for the cells to maintain their original phenotype. Cell-encapsulated alginate gel is usually composed of two compartments: a thin rim of cell-associated matrix that corresponds to the pericellular matrix of articular cartilage and a more abundant further matrix that is the equivalent of the territorial matrix in the tissue. This means that the alginate gel forms a physical 3D carrier to maintain the spherical shape of cells (Häuselmann et al., 1996). Encapsulation of the dedifferentiated chondrocytes into the alginate hydrogel has recovered their chondrocytic functions and most of the stem cells, including induced pluripotent and mesenchymal stem cells that have been differentiated to chondrocytes in alginate matrix (Domm et al., 2002; Wei et al., 2012; Xu et al., 2008).

Cross-linking density of hydrogel usually determines the pore size of polymerized network (Antonietti et al., 1999; Drury and Mooney, 2003). Even when the same materials or experimental conditions (e.g., the concentration of gels or types of cross-linker) were used for cross-linking of hydrogel, the final pore structure of hydrogel could be different depending on the cross-linking method. There are various methods to cross-link alginate gel for 3D cell culture, including direct treatment with calcium chloride ($CaCl_2$), $CaCO_3$, and calcium sulfate ($CaSO_4$) solutions onto the mold filled with cell/alginate solution mixture, internal gelation via mixing cell/gel/$CaCO_3$ solution mixture, and stirring cell/gel mixture with $CaCl_2$ (Atala et al., 1994; Jang et al., 2012; Kuo and Ma, 2001; Paige et al., 1996; Seol et al., 2012; Skjåk-Bræk et al., 1989; Sugiura et al., 2005). In the case of making alginate hydrogel via addition of cell–alginate mixture to 100 mM (almost 1%) $CaCl_2$ bath, the cell-laden 1% and 2% alginate provide microstructures with a pore size of 3.4 ± 0.8 and 5.0 ± 0.5 μm, respectively, whereas the cross-linked gel using a stirring method provides the pore size of 50–200 μm (Wang et al., 2009). This observation is due to the difference of crosslinking speed at outside and inside of alginate. The cations are randomly distributed inside the alginate gels that penetrate into the alginate through diffusion mechanism.

The pore size of hydrogel plays an important role in cell adhesion, metabolism, migration, and proliferation (Bhardwaj et al., 2001; Griffon et al., 2006; Lim et al., 2009). In general, small pores enhance cell attachment and intracellular signaling, whereas large pores can allow effective nutrient supply and oxygen diffusion (Lien et al., 2009). However, the appropriate pore size varies in different target tissues. The other reason for importance of pore size in tissue regeneration is that the certain pore range facilitates the enhancement of inherent function of cells. For instance, in the bone tissue regeneration, the pore volume fraction and the pore size of ceramic scaffold provide critical effects on the growth and function of human osteoblasts (Bose et al., 2003). The specific pore size range (290–310 μm) in the polycaprolactone (PCL) scaffold with pore size gradient showed faster new bone formation than those of other pore sizes (Oh et al., 2007). Moreover, the pore size of the gelatin scaffold also affects cell growth, proliferation, and ECM production of rat chondrocytes. Regardless of what a target tissue is, the pore size is a critical factor for cellular activity (Lien et al., 2009).

Kuo and Ma, 2001 showed the possibility of crosslinking alginate by using $CaCO_3$, and investigated how the concentration affected the characteristics of the gel (Kuo and Ma, 2008). These studies are used as adjuvant references to determine the concentration of calcium ion; whereas there is no standard condition for encapsulating cells into the alginate by using $CaCO_3$. Many researchers have chosen the concentration of $CaCO_3$ arbitrarily: Draget et al. (1989) used 15 mM $CaCO_3$ to crosslink the alginate gel for plant tissue culture, and Morimoto et al. (2009) used 75 mM $CaCO_3$ to trap mammalian cells in alginate gel. Thus, we have demonstrated the effect of various $CaCO_3$ concentrations on the mechanical and biological behaviors of chondrocytes encapsulated in alginate gel. The morphological, mechanical and rheological characteristics of the alginate gel were characterized, and the influences of those conditions on cellular functions were verified, especially the ECM synthesis of encapsulated chondrocytes in the alginate gel.

2. Materials and methods

2.1. Chemicals and preparation of hydrogel

Sodium alginate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in double distilled water (DDW) at a final concentration of 2 wt%. Calcium carbonate powder ($CaCO_3$, Junsei Chemical, 99.5%, Japan) was added to sodium alginate solution samples at final molarities of 36, 72, and 144 mM. The alginate/$CaCO_3$ solution was thoroughly mixed via vortexing for 10 s. To initiate ionization of $CaCO_3$, D-(+)-gluconic acid δ-lactone (GDL, Sigma-Aldrich, St. Louis, MO, USA) solution was added to the alginate/$CaCO_3$ mixture solution. The concentrations of $CaCO_3$ and GDL were varied to reach calcium levels of 36, 72, and 144 mM. A $CaCO_3$ to GDL molar ratio of 0.5 was always maintained to achieve neutral pH. The fabricated structures were maintained in a 37 °C humidified incubator with 5% CO_2 for 12 h to create a tight gel network.

The gel without cells was utilized to verify the mechanical behaviors including compressive testing, morphological analysis, rheological analysis and degradation test. The gel encapsulating cells were used for examining biological behavior such as synthesized ECM quantification and gene expression analysis. Each hydrogel sample was designed as a cylinder with 8 mm