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Three dimensional cell printing with sulfated alginate for improved bone morphogenetic protein-2 delivery and osteogenesis in bone tissue engineering

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ABSTRACT

Three-dimensional (3D) cell printing is a unique technique that enables free-form fabrication of cell-laden hydrogel scaffolds with controllable features and interconnected pores for tissue engineering applications. To this end, bioink materials able to offer good printability and favorable cellular interaction are highly required. Herein, we synthesized alginate sulfate, which is a structural mimic of heparin that can strongly bind with growth factors to prolong their activities, and studied its feasibility for cell printing applications. Several bio-inks composed of alginate and alginate-sulfate were studied to characterize their material properties and their utilities in 3D printing. The inclusion of alginate-sulfate in bio-inks (alginate/alginate-sulfate) did not significantly influence their rheological properties and allowed for a good 3D printing processibility with distinct pores and features. Moreover, alginate/alginate-sulfate bio-inks exhibited an improved retention of bone morphogenetic protein 2 in 3D-printed scaffolds. Osteoblastic proliferation and differentiation *in vitro* were promoted by alginate/alginate-sulfate 3D-printed constructs with an optimal composition of 3% alginate and 2% alginate-sulfate. We envision that bio-inks displaying prolonged interactions with growth factors will be useful for tissue engineering applications including bone regeneration.

1. Introduction

Ideal tissue engineering approaches involve the three-dimensional (3D) encapsulation of appropriate cells in bioactive scaffolds that stably present instructive cues for functional tissue regeneration (Chan & Leong, 2008). 3D bio-printing has enabled the fabrication of well-designed 3D constructs for use as transplants with specific shapes and features using various biomaterials and cells (Murphy & Atala, 2014). Importantly, 3D printing of cell-laden hydrogels can provide appropriate cellular microenvironments with interconnected pores allowing for the sufficient transportation of oxygen and nutrients and thereby affect cellular behaviors including viability, proliferation, and differentiation (Loo & Hauser, 2015; Yang, Zhang, Yue, & Khademhosseini, 2017). Compared to other conventional scaffolds, such as electrospun mats or microbeads, 3D printed scaffolds therefore can enable the production of free-form 3D macrofeatures containing cells to produce variously shaped tissue constructs. Still, it is required to develop hydrogel bioinks that exhibit proper printability and biocompatibility for 3D cell printing and tissue engineering applications (Chia & Wu, 2015; Park, Jang, Lee, & Cho, 2017).

Hydrogels have attracted significant attention for their potential use as bio-inks enabling controlled drug delivery and the construction of artificial 3D tissue scaffolds owing to their good biocompatibility and native tissue-like high water content (Guan et al., 2017; Hoffman, 2002). Alginate (alg) is one of the most studied natural biopolymers used as a bio-ink for 3D cell printing. Alginate readily forms a stable hydrogel in the presence of divalent cations such as calcium by ionic crosslinking (Andersen, Auk-Emblem, & Dornish, 2015; Hoffman, 2002). These unique properties have made alginate-based bio-inks useful for applications including the 3D printing of constructs containing various cell types and the delivery of growth factors for the regeneration of damaged or injured bone tissue (Gu, Amsden, & Neufeld, 2004; Hong, Yang, Lee, & Kim, 2017; Priddy et al., 2014; Wee & Gombotz, 1998). Due to relatively low moduli of alginate-based bioinks, typical applications of alginate-based cell printing include cell delivery for non-load bearing applications for bone tissues and production of mineralized bone tissues *in vitro* for implantation (Heo et al., 2017; Puppi, Chiellini, Piras, & Chiellini, 2010). However, alginate-

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based bio-inks have some drawbacks that limit their applicability, such as the lack of biological activities to stimulate bone formation (e.g., osteoconductivity and osteoinductivity). Thus, the development of bioactive alginate-based bio-inks with prolonged osteogenic activities is highly desired. To imbue alginate with enhanced bioactivities, growth factors such as bone morphogenetic protein-2 (BMP-2) can be incorporated into alginate hydrogels. However, growth factors embedded in alginate hydrogels rapidly diffuse out of the alginate scaffolds and become unable to sustain long-term activity due to the poor interaction between the growth factors and alginate (Arlov, Aachmann, Feyzi, Sundan, & Skjak-Braek, 2015; Chinen et al., 2003). Another approach that has been reported to achieve a sustained presentation of growth factors is the use of hybrid hydrogels of alginate and heparin that bind strongly with various growth factors (Jeon, Powell, Solorio, Krebs, & Alsberg, 2011; Tanihara, Suzuki, Yamamoto, Noguchi, & Mizushima, 2001). These composite materials unfortunately raise a concern about the retention of heparin in the hybrid biomaterials because heparin is not crosslinked with the alginate strands. As an alternative, alginate can be chemically sulfated to produce alginate-sulfate (alg-s), which is a structural and functional mimic of heparin (Arlov, Aachmann, Sundan, Espevik, & Skjak-Braek, 2014; Freeman, Kedem, & Cohen, 2008). Due to its structural and chemical similarity to heparin, alginate-sulfate interacts strongly with various growth factors (e.g., BMP-2) (Arlov et al., 2015, 2014; Freeman et al., 2008). This strong binding affinity of alginate-sulfate with growth factors can be useful for the long-term activities of growth factors and thus for applications in tissue engineering. Mhanna et al. found that the proliferation and phenotype maintenance of bovine chondrocytes were significantly improved in alginate-sulfate containing hydrogels as compared to alginate hydrogel controls (Mhanna et al., 2014). Consequently, alginate-sulfate-containing bio-inks appear to be beneficial for 3D cell printing with prolonged bioactivities of growth factors. Recently, Müller et al. prepared and used nanocellulose and alginate-sulfate composites to investigate 3D printing conditions with chondrocytes for potential artificial cartilage construction (Müller, Öztürk, ArlovPaul, & Zenobi-Wong, 2017). For cell printing application of alginate-sulfate for bone tissue engineering, their effects on printability and cellular activities have to be carefully investigated together. To this end, we produced various bio-inks using alginate and alginate-sulfate with BMP-2 and evaluated the feasibility of 3D printing with osteoblasts and their osteogenesis. BMP-2 is a multifunctional cytokine belonging to the transforming growth factor superfamily of proteins that plays a critical role in bone formation; thus, it has been widely used in bone tissue engineering (Amini, Laurencin, & Nukavarapu, 2012; Chen, Zhao, & Mundy, 2004). The long-term delivery and presentation of BMP-2 have been suggested to be important for the induction of bone regeneration (Ko, Yang, Shin, & Cho, 2013; Lee, Silva, & Mooney, 2011). BMP-2 was reported to contain a heparin-binding site and to strongly bind heparin with a dissociation constant of approximately 20 nM (Ruppert, Hoffmann, & Sebald, 1996). In the present study, we prepared various bio-inks using alginate and alginate-sulfate with BMP-2 and utilized them for 3D cell printing for potential bone tissue engineering applications. To this end, the physicochemical characteristics of the construct and the cellular responses of the printed osteoblasts were studied by *in vitro* cell culture experiments.

2. Materials and methods

2.1. Synthesis of alg-s and bio-inks

Sodium alginate (A2033, Sigma-Aldrich) was used as bioinks and a starting substance for the production of alg sulfate. Alginate was reported to have a mannuronic acid to guluronic acid ratio (M/G) of 1.56. Furthermore, we analyzed its molecular weight (Mw) using a gel permeation chromatography (GPC) and was determined to be 758,400 g/mol) (Supplementary Information Fig. S1). The detailed experimental conditions for GPC are described in the following parts. The sulfation of sodium alginate was performed according to the previous report (Freeman et al., 2008). Prior to sulfation, sodium alginate (Sigma-Aldrich, St. Louis, MO, USA) was converted to a tertiary amine salt. In brief, 0.6 g of sodium alginate was completely dissolved in 300 mL of double-deionized (DDI) water for 24 h in a 4 °C refrigerator. Then, 40 g of protonated Dowex 50W-X8 (ion-exchange resin with 20–50 mesh size, Sigma-Aldrich) was added to the sodium alginate solution and mixed for 5 min. Tributylamine (TBA, Sigma-Aldrich) was added dropwise into the solution until the pH reached the range of 6.0–6.5. The solution was then freeze-dried and stored at −20 °C until use. Usually, 0.7 g of the TBA-alginate was obtained. For the sulfation of TBA-alg, 6 g of sulfuric acid (Duksan Chemical Co., Korea) was added to 200 mL of dimethyl formamide (Duksan Chemical Co) and the solution was cooled to room temperature. Then, 0.6 g of TBA-alg was added into the solution and completely dissolved by magnetic stirring, followed by the addition of 12 g of *N,N′*-dicyclohexylcarbodiimide (Alfa Aesar, Haverhill, MA, USA) to the solution. The reaction was performed at ambient temperature with stirring for 2 h. The precipitate was removed several times by filtration using a filter paper and a glass filter to obtain a clear solution. Then, 600 mL of dichloromethane (Duksan Chemical Co) was added to the filtered solution and stirred for several minutes to form a second precipitate. This precipitate was collected by decantation and dissolved in 200 mL of 0.5 N sodium hydroxide solution (Sigma-Aldrich). The solution was dialyzed using a dialysis membrane (MWCO 12 kDa; Spectrum, Houston, TX) against DDI water for 3 days while exchanging the DDI every day and then freeze-dried. Note that we did not add extra salts during the dialysis.

2.2. Preparation of bio-inks

Alginate solution was prepared according to our optimized protocols as reported previously (Park, Lee et al., 2017; Park, Lee, & Kim, 2011). Various solutions were prepared with differing ratios of alginate to alginate-sulfate, which were completely dissolved in Dulbecco's modified Eagle's medium (DMEM, Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) containing 1% (w/v) $CaCl_2$ for rheological and printing experiments. The nomenclatures and compositions of the alginate and alginate-sulfate bio-inks are shown in Table 1.

2.3. Characterization

The elemental composition of the synthesized alginate-sulfate was analyzed to determine the sulfur content by inductively coupled plasma atomic emission spectroscopy (GeovisION-EA Isoprime; Elementar, Langenselbold, Germany). Relative atomic molar ratios of the synthesized alginate-sulfate were 2.80 ± 0.04, 4.82 ± 0.89, 3.65 ± 0.07, and 0.10 ± 0.01 for carbon, hydrogen, oxygen, and sulfur, respectively (Supplementary Information Table S1). The atomic ratios of sulfur and carbon were compared to determine the degree of sulfation as sulfur atoms per uronic acid unit in alginate (Xie et al., 2016). The degree of sulfation was calculated to be approximately 0.21 sulfate group per uronic acid unit. Note that there was no substantial detection of sulfur atoms in pristine alginate. Fourier transform infrared (FTIR) spectroscopy was performed using a Spectrum 2000 FTIR spectrometer (PerkinElmer, Waltham, MA, USA). Molecular weights and polydispersities (Mw/Mn) of alginate and alginate-sulfate were examined by gel

Table 1
Bio-ink names and compositions.

	alg (mg/mL)	alg-s (mg/mL)
alg	30	0
alg/alg-s1	30	5
alg/alg-s2	30	10
alg/alg-s3	30	30