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Ionically cross-linkable hyaluronate-based hydrogels for injectable cell delivery

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ABSTRACT

Although hyaluronate is an attractive biomaterial for many biomedical applications, hyaluronate hydrogels are generally formed using chemical cross-linking reagents that may cause unwanted side effects, including toxicity. We thus propose to design and prepare ionically cross-linkable hyaluronate compounds that can form gels in the presence of counter-ions. This study is based on the hypothesis that introduction of alginate to hyaluronate backbones (hyaluronate-*g*-alginate) could allow for gel formation in the presence of calcium ions. Here, we demonstrated ease of formation of cross-linked structures with calcium ions without additional chemical cross-linking reagents in hyaluronate-*g*-alginate (HGA) gels. The mechanical properties of HGA gels were regulated through changes in polymer composition and calcium concentration. We also confirmed that HGA gels could be useful in regenerating cartilage in a mouse model following subcutaneous injection into the dorsal region with primary chondrocytes. This finding was supported by histological and immunohistochemical analyses, glycosaminoglycan quantification and chondrogenic marker gene expression. This approach to the design and tailoring of ionically cross-linkable biomedical polymers may be broadly applicable to the development of biomaterials, especially in the drug delivery and tissue engineering fields.

1. Introduction

Natural polymer-based hydrogels have been used in various biomedical applications, due to their superior intrinsic biocompatibility compared to synthetic polymer-based hydrogels. Natural polymers have relatively low potential for toxicity, which is crucial with regard to biomaterial design. Among natural polymers, hyaluronate has been used in many biomedical applications, including in drug delivery [1,2] and tissue engineering [3,4] applications, due to excellent biocompatibility and biological functionality. This polysaccharide is composed of repeating units of β-1,4-D-glucuronic acid-β-1,3-*N*-acetyl-D-glucosamine residues [5], and is abundant in synovial fluid and extracellular matrix. Hydrogels prepared from hyaluronate are particularly attractive, due to their excellent biological function, excellent viscoelastic properties, high water content and biodegradable properties. One typical preparation method of hyaluronate gels is chemical cross-linking [6–9]. Chemical cross-linking reagents, however, can induce acute or chronic side effects, such as immune and inflammatory responses [10]. This may cause potential risk, and limit wide biomedical applications of hyaluronate.

Physical cross-linking is an alternative approach that can be applied in overcoming toxicity issues brought about by chemical cross-linking. Hydrogels can be physically prepared, by varying external environments around stimulus-responsive polymers (e.g., temperature [11,12] and pH [13,14]), or by inducing physical (e.g., ionic [15,16] or hydrophobic interactions [17,18]) to polymers. Alginate forms physically cross-linked structures in the presence of divalent cations (e.g., Ca^{2+}), but in the absence of chemical cross-linkers. Alginate is a linear copolymer composed of blocks of β-D-mannuronate (M) and α-L-guluronate (G) residues [19,20]; it is also a naturally derived biomaterial. It is utilized in biomedical applications owing to its excellent biocompatibility and low toxicity [21,22]. Specifically, alginate modified with cell-adhesive motifs (e.g., RGD peptide) has often been utilized as injectable hydrogels for tissue engineering applications [23,24].

Articular cartilage is important for overall individual well-being. Articular cartilage functions and structures often get interrupted or damaged, due to physical injuries or degenerative diseases, such as osteoarthritis. Because cartilage tissue is both anural and avascular, chondrocyte proliferation is slow, and tissue defects are rarely spontaneously recovered [25,26]. Tissue engineering approaches using hydrogels have demonstrated great potential for treating articular cartilage defects through minimally-invasive chondrocyte delivery. Such minimally-invasive chondrocyte delivery may reduce cost, recovery time, and patient pain [27]. Another benefit of hydrogels is their superior viscoelastic properties. Viscoelastic properties of hydrogels are similar to those of cartilage natural extracellular matrix (ECM) materials [28–30].

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Hyaluronate is a promising candidate for cartilage regeneration, as it is a main component of the ECM of native cartilage. In addition, hyaluronate interacts with CD44 on the chondrocyte surface, which is a molecule that is important in chondrocyte metabolism [31–33]. Hyaluronate has been extensively used in cartilage regeneration applications [34–36].

In this study, we propose to design and prepare ionically cross-linkable, alginate-grafted hyaluronate compounds that can form hydrogels without the addition of chemical cross-linking reagents (Fig. 1). To accomplish these goals, we introduced alginate to the hyaluronate backbone, in order to fabricate hydrogels that physically cross-link in the presence of calcium ions. In this study, RGD peptides were initially coupled to alginate to enhance cellular interactions of resultant hydrogels. Hyaluronate-to-alginate weight ratio was varied, and various characteristics of ionically cross-linked hyaluronate gels were investigated *in vitro*. Additionally, efficacy of cartilage regeneration using injectable gels was evaluated with a mouse model.

2. Materials and methods

2.1. Synthesis of hyaluronate-g-alginate

All alginate samples were originally modified with RGD peptides to enhance cellular interactions. Briefly, 1 g of sodium alginate (molecular weight = 200,000–300,000; FMC Biopolymer; Philadelphia, PA, US) was dissolved in 100 ml of 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES; Sigma-Aldrich; St. Louis, MO, US) buffer solution (pH 6.5, 0.3 M NaCl). Then, a peptide (16.7 mg) with the sequence of (glycine) 4-arginine-glycine-aspartic acid-serine-proline (G_4RGDSP; Anygen; Korea) was added to the alginate solution in the presence of 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC; Sigma-Aldrich) and *N*-hydroxysulfosuccinimide (Sulfo-NHS; Thermo; Waltham, MA, US), in conjunction with vigorous solution stirring. Reaction was allowed to take place for 20 h at room temperature. Resultant solution was purified by dialysis against distilled water for four days (molecular weight cut off = 3500), followed by treatment with charcoal. After filtration with a 0.22-μm filter for sterilization, solution was frozen at −20 °C and lyophilized. Hyaluronate (molecular weight = 600,000–850,000; Lifecore Biomedical; Chaska, MN, US) was first reacted with ethylenediamine (Sigma-Aldrich), prior to conjugation with alginate. Synthesis of NH_2-hyaluronate was carried out with the same procedure with EDC and NHS, as described above. A 10-fold molar ratio excess of ethylenediamine was added to inhibit intermolecular cross-linking between hyaluronate chains during the reaction. The reaction was allowed to proceed for 20 h at room temperature. The solution was then dialyzed, treated with charcoal, filtered with a 0.22-μm filter for sterilization, and lyophilized. Alginate was coupled to NH_2-hyaluronate via carbodiimide chemistry according to the above-described procedure (Fig. 1).

2.2. Nuclear magnetic resonance spectroscopy

Hyaluronate, alginate and hyaluronate-g-alginate were analyzed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Bruker Avance 500 MHz; Billerica, MA, US) at 70 °C. Samples were dissolved in D_2O at 3 mg/ml.

2.3. Dimethyl methylene blue (DMMB) assay

DMMB assays were performed to quantify alginate/hyaluronate content in hyaluronate-g-alginate. Briefly, 16 mg of DMMB was dissolved in 25 ml of ethanol and filtered with filter paper, and 100 ml of 1 M guanidine hydrochloride containing 0.17 M of sodium formate and 1 ml of formic acid was mixed with filtered DMMB. The solution was mixed with deionized water to a total volume of 500 ml. Each sample was diluted with deionized water, to yield a solution of 0.1 wt.%. One milliliter of DMMB solution was added to 100 μl of each sample and mixed vigorously for 30 min. Incubated samples were centrifuged at 12,000 *g* for 10 min to precipitate the complex. Supernatant was removed and dried for 30 min at room temperature. Pellets were dissolved with 1 ml of decomplexation solution. Decomplexation solution was prepared with 50 mM of sodium acetate buffer (pH 6.8), containing 10% 1-propanol and 4 M guanidine hydrochloride. After 30 min of mixing, 100 μl of each sample was transferred to a 96-well plate. Absorbance was measured at 656 nm using a spectrophotometer (SpectraMax M2^e, Molecular Devices; Sunnyvale, CA, US).

2.4. Rheological measurement

Viscoelastic properties of ionically cross-linked hyaluronate-g-alginate gels were measured using a rotational rheometer with a cone-and-plate (20 mm diameter plate, 4 degree cone angle) fixture (Bohlin Gemini 150; Malvern, Worcestershire, UK). A 150-μm gap opening was set at the apex of the cone and plate, and operating temperature was set to be constant at 37 ± 0.1 °C.

2.5. Cell isolation and culture

Primary chondrocytes were isolated from the articular cartilage of New Zealand white rabbits (four-week-old; Samtako; Korea). The rabbits were sacrificed, and cartilage tissue fragments were obtained from hind leg knee joints. After fragments were washed with cold PBS, minced and digested with 4.5 mg/ml collagenase type II (Worthington) in DMEM/F-12 containing 10% FBS and 1% penicillin–streptomycin over a 6-h period. Digested cell suspension was passed through a cell strainer (40 μm; SPL Life Science) to remove undigested tissue fragments. Cells were collected using a centrifuge, washed twice with PBS, and suspended in DMEM/F-12 containing 10% FBS and 1% penicillin–streptomycin. Isolated cells were cultured using standard culture procedures,

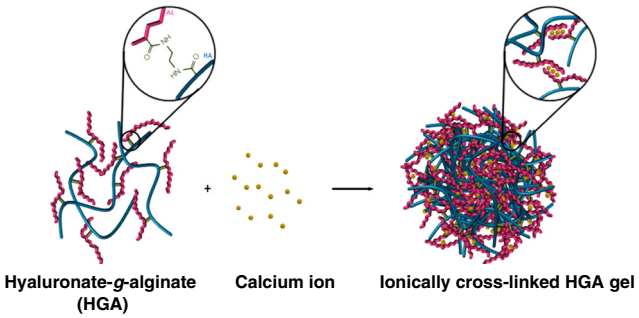

Fig. 1. Schematic description for hyaluronate-g-alginate (HGA) and its hydrogel formation in the presence of calcium ions. Hyaluronate (HA) was initially modified with ethylenediamine (NH_2-HA). Then, NH_2-HA was reacted with alginate (AL) via carbodiimide chemistry.