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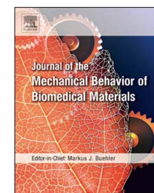

The production of injectable hydrazone crosslinked gellan gum-hyaluronan-hydrogels with tunable mechanical and physical properties

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ABSTRACT

Gellan gum (GG) has been proposed for use in tissue engineering (TE) due to its structural and functional similarities with alginate. The most traditional crosslinking methods of GG, ionical and photocrosslinking, have downsides such as loss of stability or phototoxicity, which can limit their use in certain applications. In this study, an alternative hydrazone crosslinking method is introduced. Hydrazone crosslinking is a simple method that produces no toxic reagents or side-products. The method enables the fabrication of injectable hydrogels. GG was combined with hyaluronan (HA) to improve some properties such as cell attachment. The mechanical and physical properties of GG-HA hydrogels were controlled by changing the molecular weight, the degree of modification, and the ratio of polymer components. GG-HA hydrogels showed ionic nature of deswelling in the presence of cations enabling the control of physical properties in different solution environments. Due to the non-linear elastic behavior of hydrogels and tissues, the stiffness as a function of strain was represented instead of solely giving the second-order elastic constants. The stiffness of GG-HA hydrogels was similar to that of soft tissues at small strains.

1. Introduction

A thorough knowledge of the different properties of materials is important when materials are designed for use in applications such as tissue engineering (TE). The basic principle behind the design of materials for TE is that the material mimics the features of native tissue (Brandl et al., 2007). Hydrogels are materials of particular interest due to their favorable biomimicking properties.

Gellan gum (GG) is an anionic extracellular polysaccharide composed of tetrasaccharide repeating units of 1,3-β-D-glucose, 1,4-β-D-glucuronic acid, 1,4-β-D-glucose and 1,4-$\alpha\beta$-L-rhamnose, containing one carboxyl side group. GG is produced by *Sphingomonas elodea* bacteria (Ferris et al., 2013). In the US, GG has been approved by the Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) in the EU for use in food and medical products as a gelling, stabilizing, and suspending agent (Giavasis et al., 2000; Whiteside and Wallace, 2013). Due to structural and functional similarities with widely used alginate, GG has also been proposed as a scaffold material for TE (Ferris et al., 2015).

At higher temperatures (≥ 40 °C) GG exists as a disordered coil, but on cooling it forms a double-helix structure. This structure is not a true gel, although it has weak gel characteristics. A true gel is formed through the cation-mediated association of helices. Either monovalent or divalent cations can be used. A stronger gel is formed by using divalent cations such as Ca^{2+} and Mg^{2+}, due to bridge formation between pairs of carboxyl groups. Monovalent cations, such as K^{+} or Na^{+}, suppress electrostatic repulsions inducing an aggregation that leads to weaker gels (Ferris et al., 2013). The downside of this crosslinking method is a loss of stability and a weakening of the mechanical properties caused by the exchange of divalent cations with monovalent ones, for example in the physiological environment. An alternative way of producing ionically crosslinked GG hydrogels is by using polyamines such as spermine (SPM^{4+}) and spermidine (SPD^{3+}) (López-Cebral et al., 2013; Soto et al., 2016). In addition, GG hydrogels can also be chemically crosslinked using methacrylate derivatives followed by photocrosslinking with UV-light (Coutinho et al., 2010). The drawbacks of this method are the phototoxicity of UV-light and the reactivity of the photoinitiator.

In order to produce more stable GG hydrogels with an improved gelation environment, a chemical crosslinking method, hydrazone crosslinking, can be used. It is an aldehyde-hydrazide coupling reaction, which belongs to the group of pseudo click chemistry reactions (pseudo

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is characterized by moderate orthogonality). These reactions are versatile, simple and reversible and there are no toxic reagents or side-products. The reactions also have high reactivity and yield (Jiang et al., 2014) The properties of hydrazone crosslinked hydrogels can be controlled by altering the number of crosslinkable groups available, the molecular weight of the polymer, the ratio of gel components, and water content. The fabrication of injectable hydrogels is also possible. The injectability of hydrogels would be especially beneficial for TE or drug delivery applications.

Like many other polysaccharides, GG is a relatively inert biomaterial. In order to make GG less inert, hyaluronan (HA) can be added. HA is a naturally occurring anionic polysaccharide composed of β-1,4-linked D-glucuronic acid and β-1,3 N-acetyl-D-glucosamine disaccharide units. HA is a major glucosaminoglucan component of the extracellular matrix (ECM) found in different mammalian tissues. It can be enzymatically degraded with hyaluronidase enzyme. HA has a fairly simple repetitive chemical structure with groups suitable for specific modification (Burdick and Prestwich, 2011).

GG and HA can be modified with complementary reactive aldehyde and hydrazide groups to enable hydrazone crosslinking using, for example, oxidation and adipic acid dihydrazide (ADH) coupling reactions. To date, there have only been a few studies carried out on GG-HA hydrogels (Cencetti et al., 2011; Cerqueira et al., 2014), and no studies were found on using hydrazone crosslinking.

In this study, we have modified GG and HA polymers using aldehyde and hydrazide groups and produced injectable hydrazone crosslinked GG-HA-hydrogels with controllable swelling, biodegradation, rheological and mechanical properties. The aim of the study was to replace the traditional crosslinking methods with hydrazone crosslinking in order to produce GG-HA hydrogels with tunable mechanical and physical properties suitable, for example, for soft tissue applications.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt from streptococcus equi (HA, $M_w = 1.5–1.8 \times 10^6$ g/mol), 1-hydroxybentzotriazole (HOBt), adipic acid dihydrazide (ADH), hydroxylamine hydrochloride, sodium periodate, 1-Ethyl-3-[3-(dimethylamino) propyl]carbodiimide (EDC), hyaluronidase from bovine testes (Type I-S, 400-1000 units/mg solid), sucrose, gellan gum (GG, Gelzan^{TM}, $M_w = 1$ kg/mol), ethylene glycol, dimethyl sulphoxide (DMSO) and deuterium oxide (99.9 atom % D, contains 0.05 wt. % 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hyaluronic acid sodium salt (HA, $M_w = 1.5 \times 10^5$ g/mol) was purchased from Lifecore (Chaska, MN, USA). All solvents used were of analytical quality. Milli-Q water was used in synthesis and determinations. Dialysis membranes (Spectra/Por® cut-off 1000 and 12-14,000 g/mol) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA).

2.2. Synthesis of aldehyde-modified gellan gum

GG was modified with aldehyde groups using periodate oxidation according to a previously reported method (Gong et al., 2009) that was slightly modified. The reaction scheme is shown in Fig. 1. Briefly, GG (0.500 g) was dissolved in deionized water (50 mL) in 60 °C for a few hours. Sodium periodate (0.05 M; 0.022, 0.038, 0.044 or 0.048 g) was added dropwise and stirred for 4 h at room temperature under nitrogen. Ethylene glycol (4 equivalents) was added to inactivate any unreacted periodate and the reaction was stirred for 1 h. Derivatized GG was dialyzed with MW cutoff 1000 membranes against deionized water for a few days. Purified polymers were lyophilized to obtain white cotton-like products (GGALD1, 2, 3 and 4).

All Nuclear Magnetic Resonance (NMR) spectroscopy experiments were measured with a Varian Mercury 300 MHz NMR Spectrometer (Palo Alto, USA). Samples (5 mg) were dissolved in deuterium oxide (600 µL) containing an internal standard (0.05 wt.% 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt). ^{1}H NMR 300 MHz (D_2O, ppm, Fig. 1): δ 5.15 (s, 1H, CH-1 of rhamnose unit), 4.72 (s, 1H, CH-1 of glucose unit), 4.56 (s, 1H, CH-1 of glucuronic acid unit), 4.07-3.43 (m, 5H, CH-2-5 of units), 1.3 (s, 3H, CH_3 of rhamnose unit).

All Fourier Transform Infrared (FTIR) spectroscopy experiments were collected on a Perkin Elmer Spectrum One FT-IR Spectrometer (Waltham, MA, USA) in the spectral range of 400 cm^{-1} to 4000 cm^{-1}. Samples (1 mg to 2 mg) were pressed into KBr (200 mg) tablets. FTIR (KBr, cm^{-1}, Fig. 2): 1733 (ν(C=O) of –C(O)H), 1615 (ν(C=O) of –C(O)OH).

The degree of substitution (DS%) of GGALD was determined using a previously reported method (Tang et al., 2012). Briefly, GGALD (0.1 g) was dissolved in deionized water (20 mL) and the pH was adjusted to 5 by using 0.1 M HCl. The pH of 0.1 M hydroxylamine hydrochloride solution was also adjusted to 5 by using 0.5 M NaOH. These solutions were mixed together and allowed to react for 15 min. The reacted solution was titrated with 0.5 M NaOH to pH 5. A blank titration was done to serve as a control. The DS% was calculated from

$$DS\% = \frac{645(V_1 - V_2) \times c \times 10^{-3}}{2\,M} \tag{1}$$

where V_1 is the volume (mL) of NAOH reacted with GGALD, V_2 is the volume (mL) of NAOH reacted with a blank sample, c is the concentration (mol/L) of NAOH, M is the mass (g) of the sample, and 645 is the monomer M_w of GG residues.

2.3. Synthesis of hydrazide-modified hyaluronic acid

HA was modified with hydrazide groups (Fig. 1) according to a previously reported method (Bulpitt and Aeschlimann, 1999; Jia et al., 2006). Briefly, HA (200 mg, high $M_w = 1.5–1.8 \times 10^6$ Da or low $M_w = 1.5 \times 10^5$ Da) was dissolved in deionized water (66.66 mL) in a three-necked flask. A 30-fold molar excess of ADH (2.614 g) was added, and the pH was adjusted to 6.8 with 0.1 M HCl or 0.1 M NaOH. EDC (387 mg) and HOBt (271 mg) were dissolved in 2 mL of DMSO/H_2O (vol 1:1) and little by little, this mixture was added to the flask using a dropping funnel. The pH was maintained at 6.8 by adding 0.1 M NaOH or 0.1 M HCl. The reaction was allowed to proceed overnight. Next day, the pH was adjusted to 7 with 0.1 M NaOH. Derivatized HA was dialyzed with MW cutoff 12–14,000 membranes against deionized water for few days. The solution was taken out from dialyzing membrane and NaCl was added to obtain a 5% w/v solution. Derivatized HA was precipitated by adding ethanol (3 vol equivalents). The precipitate was weighted, dissolved in deionized water (5 mg/mL) and dialyzed against deionized water. Purified polymers were lyophilized to obtain white cotton-like products (high molecular weight HAADH1 and low molecular weight HAADH2).

^{1}H NMR 300 MHz (D_2O, ppm, Fig. 1): δ 2.42 (m, 2H, $NHNHCOCH_2$), 2.26 (m, 2H, $CH_2CONHNH_2$), 1.66(m, 4H, $CH_2CH_2CH_2CH_2$).

FTIR (KBr, cm^{-1}, Fig. 2): 1706 (ν(C=O) of sec. amide), 1652 (d (N–H) of prim. amine and amide), 1559 (d(N-H) of sec. amine and amide), 1410 (d(CH_2–C=O) of –CH_2), 1078 (ν(C–N) of amine).

The DS% of HAADH components were determined from integration of the ^{1}H-NMR peaks (Oh et al., 2008). The methyl resonance ($\delta = 2.03$ ppm) of the acetamido moiety of the N-acetyl-D-glucosamine was used as an internal standard. The degree of ADH modification of HA at the carboxyl group was determined by the peak area of methylenes of ADH.

2.4. Formation of hydrazone crosslinked gellan gum-hyaluronan hydrogels

GG-HA hydrogels were prepared by combining two complementary

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