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Self-reinforcement and protein sustained delivery of hyaluronan hydrogel by tailoring a dually cross-linked network

Chunhong Luo [a], Guoguang Xu [a], Xinghui Wang [a], Mei Tu [a,b], Rong Zeng [a,b], Jianhua Rong [a,b], Jianhao Zhao [a,b,*]

[a] Department of Materials Science and Engineering, College of Science and Engineering, Jinan University, Guangzhou 510632, China
[b] Engineering Research Center of Artificial Organs and Materials, Ministry of Education, Guangzhou 510632, China

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ABSTRACT

A series of self-reinforcing hyaluronan hydrogels were developed to improve mechanical properties and protein sustained delivery thanks to a dually cross-linked network. Hyaluronan gel particles (HGPs, 1–5 μm in diameter) with different cross-linking densities, i.e. HGPs-1.5, HGPs-3 and HGPs-15, were prepared in an inverse emulsion system and used as the reinforcing phase after glycidyl methacrylation, while glycidyl methacrylated hyaluronan with a substitution degree of 45.2% was synthesized as the matrix phase. These two phases were cross-linked under ultraviolet irradiation to form self-reinforcing hyaluronan hydrogels (srHAs) that showed typical cross-linked structure of HGPs connecting the matrix phase by cross-section observation. In comparison to hyaluronan bulk gels and their blends with HGPs, srHAs distinctly enhanced the mechanical properties and BSA long-term sustained delivery, especially srHA-1.5 showed the highest compressive modulus of 220 ± 15 kPa and the slowest BSA delivery (67% release at 14 d). The 3T3 fibroblast cell culture showed that all the srHAs had no cytotoxicity.

1. Introduction

Hyaluronan (HA), a linear polysaccharide composed of alternating D-glucuronic acid and N-acetyl-D-glucosamine units and present in most natural tissues, has attracted most attentions in soft tissue engineering for its high water adsorption, excellent cytocompatibility and key role in cellular response [1–3]. However, traditional HA bulk gels (BGs) with a single cross-linked network lack structural integrity that makes it less competitive in many fields where good mechanical properties are required. Meanwhile, the fast delivery of bioactive molecules that are essential for tissue regeneration, is another drawback of this kind of hydrogel [2,4–6]. If HA hydrogel can be modified to improve the mechanical properties and sustained delivery of bioactive molecules, it will be a good candidate material for promoting would healing and tissue regeneration. To strengthen HA hydrogel, filling rigid inorganic or polymeric nano-particles is a conventional method, but it may also lead to poor miscibility, non-degradability and cytotoxicity [7–10]. The fabrication of interpenetrating network is another attempt for reinforcement, but the sustained release of bioactive molecules is still challenging [11–13]. Recently, Jia's group has developed a dually cross-linked HA hydrogel produced by connecting oxidized HA microgels by converting aldehyde groups to hydrazides via Schiff base formation, and found some improvement in mechanical properties [14]. This idea provides an opportunity for both strengthening and releasing bioactive molecules. Unfortunately, this method only provided the hydrogel an elastic modulus of about 1000 Pa that is poor for tissue engineering; meanwhile the newly formed C═N bond by Schiff base formation is also not stable with the risk of hydrolysis in acid conditions [15,16]. Furthermore, the reinforcing mechanism of HA microgels and their effect on the protein sustained delivery are still unknown. Hence, this dually cross-linked HA hydrogel needs more investigation and improvement before application in tissue engineering.

In order to fabricate stable self-reinforcing HA hydrogels with the features of dually cross-linked network and protein sustained release, stable covalent bonds between reinforcing phase and matrix phase as well as adjustable cross-linked network, are expected to be built. Glycidyl methacrylate (GMA) is one widely used functional monomer with a highly reactive epoxy group that can easily react with nucleophilic groups such as COO^- of HA via ring opening [17,18]. Simultaneously, the carbon–carbon double bond in GMA offers an opportunity of crosslink by photo-polymerization under ultraviolet radiation [19,20]. Therefore, GMA is an ideal connector between HA microgels and HA matrix for the fabrication of self-reinforcing HA hydrogels. Additionally, with respect to the sustained release of bioactive molecules, our previous work has realized the sustained delivery of bovine serum albumin (BSA) by tailoring the cross-linking density of HA microgels [21]. Hence, by using GMA connector and controlling the cross-linking density of HA microgels, it is expected to achieve both self-reinforcement and protein sustained release for dually cross-linked HA hydrogels. In this work, glycidyl methacrylated HA (HAGMA) was synthesized as the

* Corresponding author at: Department of Materials Science and Engineering, College of Science and Engineering, Jinan University, Guangzhou 510632, China.
E-mail address: jhzhao@jnu.edu.cn (J. Zhao).

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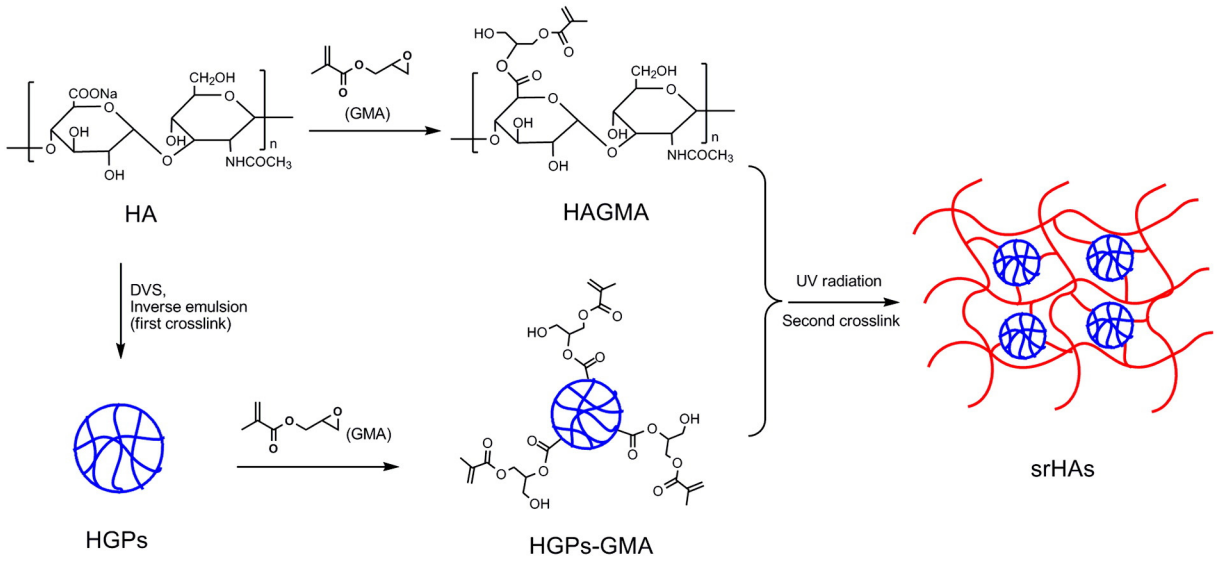

Scheme 1. Schematic diagram for the fabrication of srHAs with a dually cross-linked network.

matrix phase, while HA gel particles prepared in an inverse emulsion system (defined as the first crosslink) were used as the reinforcing phase after glycidyl methacrylation, i.e. HGPs-GMA. These two phases were then chemically connected by photo-crosslinking (defined as the second crosslink) under ultraviolet irradiation to engineer self-reinforcing HA hydrogels (srHAs). The effects of HA microgels with different cross-linking densities on the morphology, water swelling ratio, mechanical properties, in vitro degradation, BSA loading and delivery, and cytotoxicity of srHAs, were systematically studied.

2. Materials and methods

2.1. Materials

Sodium hyaluronan with a weight average molecular weight (M_w) of 1.5×10^6 Da was provided by Shandong Institute of Medical Instruments (China). Dioctyl sulfosuccinate sodium salt (AOT, 98%), 2,2,4-trimethyl-pentane (isooctane, anhydrous), divinyl sulfone (DVS), and 1-heptanol (1-HP), glycidyl methacrylate (GMA), triethylamine (TEA), tetrabutyl ammonium bromide (TBAB), photo-initiator Irgacure 2959 (I2959) and hyaluronidase from bovine testes (Type I-S, lyophilized powder, 400–1000 units/mg solid) were purchased from Sigma-Aldrich (USA). All other reagents and solvents used were of analytical grade.

2.2. Synthesis of HAGMA

HAGMA was synthesized according to the previous method [11]. Briefly, 0.5 g HA was dissolved in deionized water to get a 2 mg ml^{-1} solution. Relative to the total hydroxyls on the repeating unit of HA, 20 mol% TEA, 20 mol% TBAB, and 50-fold excess of GMA were in sequence added into the above solution under stirring at room temperature. After reaction for 24 h, the mixture was dialyzed against 0.1 M sodium chloride solution followed by deionized water for 5 days each. HAGMA was finally obtained after lyophilization at 0.6 mbar for 48 h at −80 °C.

2.3. Preparation of HGPs-GMA

HA gel particles (HGPs, 1–5 μm in diameter) with different cross-linking densities were firstly prepared in a reverse emulsion system as our previous work [21]. Briefly, after mixing 0.27 ml HA (4 mg ml^{-1} in 0.2 M sodium hydroxide solution) and 7.5 ml organic solution composing of 0.2 M AOT and 0.04 M 1-HP in isooctane, DVS as the crosslinker was added at a varying molar ratio of DVS to repeating unit of HA, i.e. 1.5, 3 and 15, respectively. By vigorous stirring for 30 min at room temperature, the mixture was precipitated using a large excess of acetone. The precipitate was then thoroughly washed with deionized water, ethanol and acetone in sequence. Various HGPs were obtained after being dried at vacuum and named as HGPs-1.5, HGPs-3 and HGPs-15, respectively. Finally, these HGPs performed similar GMA modification as above. The modified particles were correspondingly named as HGPs-1.5-GMA, HGPs-3-GMA and HGPs-15-GMA, respectively.

2.4. Fabrication of srHAs

The fabrication of srHAs was shown in Scheme 1 and the compositions of various HA-based hydrogels were summarized in Table 1. Briefly, each dried HGPs-GMA of 30 mg were swollen in 1 ml HAGMA aqueous solution (30 mg ml^{-1} in deionized water) containing 1 mg I2959 as the photo-initiator. After vortex, each mixture of 200 μl was then injected into a cylindrical mode (diameter × height = 8 mm × 5 mm). srHAs were formed by exposing the mixture to ultraviolet radiation (365 nm, 15 kJ) for 15 min. The obtained srHAs were soaked in deionized water for 2 days by refreshing the water twice per day to remove the photo-initiators and unreacted monomers. The as-synthesized srHAs were dehydrated by passing them through graded ethanol solutions (ethanol/water(v/v) = 25%, 50% and 100%) and drying them under vacuum overnight at room temperature. These dried srHAs produced from HGPs-1.5-GMA, HGPs-3-GMA and HGPs-15-GMA were named as srHA-1.5, srHA-3 and srHA-15 correspondingly. By comparison, bulk gels from 60 mg ml^{-1} HAGMA without HGPs, and blending hydrogel from 30 mg ml^{-1} HAGMA and 30 mg ml^{-1} HGPs-1.5, were together used as the controls and named as BGs and BGs/HGPs-1.5 respectively.

2.5. Characterizations

2.5.1. Proton nuclear magnetic resonance (^1H NMR) analysis

The ^1H NMR spectra of HA before and after GMA modification were recorded in D_2O with a polymer concentration of 20 mg ml^{-1} using a 500 MHz Bruker AVANCE III (Bruker, Germany) spectrometer at room temperature.