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Hyaluronic acid hydrogel scaffolds with a triple degradation behavior for bone tissue engineering

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ABSTRACT

In this study, in order to better mimick the nature of bone extracellular matrix, hyaluronic acid (HA) hydrogels having a triple degradation behavior were synthesized from 3,3′-dithiodipropionate hydrazide-modified HA (DTPH–HA) and polyethylene glycol dilevulinate (LEV–PEG–LEV) via the reaction of the ketone carbonyl groups of LEV–PEG–LEV with the hydrazide groups of DTPH–HA. The HA hydrogels were characterized by solid state ^{13}C NMR, FT-IR, SEM, and rheological, swelling and degradation tests. The results showed that the HA hydrogels exhibited a highly porous morphology and had pore diameters ranging from 20 to 200 μm. The equilibrium swelling ratio of the HA hydrogels was no less than 37.5. The HA hydrogels could be degraded by hyaluronidase and reducing substances or at acidic pH values. The biocompatibility of the HA hydrogels was evaluated using osteoblast-like MC3T3-E1 cells by live/dead staining and MTT assays. The results revealed that the HA hydrogels had good biocompatibility and could support the attachment and proliferation of MC3T3-E1 cells. All the results indicated that the HA hydrogels synthesized by hydrazone bond crosslinking might have great potential to be used in bone tissue engineering.

1. Introduction

Bone tissue engineering typically involves the combination of scaffold materials and cells and holds great potential in treating and repairing the bone defects in the clinic. The scaffolds used in bone tissue engineering techniques should generally be porous, bioactive, biodegradable and bioresorbable (Wu, Liu, Yeung, Liu, & Yang, 2014; Liu, Lim, & Teoh, 2013; Bose, Roy, & Bandyopadhyay, 2012). Hydrogels, which are just like living tissues when they are highly swollen in water, are in favor of the adhesion, proliferation and differentiation of cells, facilitate nutrient input and waste output, and provide enough space and mechanical stability for new tissue formation (Palumbo et al., 2014; Levengood & Zhang, 2014; Wang & Stegemann, 2010). Up to now, hydrogels have been extensively explored as scaffolds for tissue engineering such as bone (Bae et al., 2011, 2014; Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009), cartilage (Levett et al., 2014), skin (Iannitti, Bingol, Rottigni, & Palmieri, 2013) and adipose (Tan et al., 2009).

Hyaluronic acid (HA) hydrogel scaffolds possess some advantages, including facilitating proliferation of seeded cells and wound healing, good anti-inflammatory ability and promoting intracellular signaling (Oommen et al., 2013; Collins & Birkinshaw, 2013a,b; Collins, 2014). HA hydrogels have been widely applied in the biomedical field because of their good biocompatibility and structural diversity (Collins & Birkinshaw, 2013a,b). On the other side, polyethylene glycol (PEG) has been widely used to synthesize many types of hydrogels for tissue engineering due to its good biocompatibility, ability to promote tissue regeneration and long-term in vivo security (Byeon et al., 2014; Burdick & Anseth, 2002; Yu et al., 2014; Yu, Cao, Zeng, Zhang, & Chen, 2013; Rufaihah et al., 2013). A gentle hydrazone bond crosslinking method was recently reported to synthesize HA hydrogels by reacting HA aldehyde with HA adipic dihydrazide (Oommen et al., 2013). However, macromolecular aldehydes used for the preparation of hydrogels based on hydrazone bond crosslinking still show cytotoxicity and might hinder the migration of cells, although they display better biocompatibility than small molecular aldehydes (Mckinnon et al., 2014). Significantly, a recent study indicates that the hydrogels formed via ketone-hydrazide crosslinking have better biocompatibility than those formed through aldehyde-hydrazide crosslinking (Patenaude, & Hoare, 2014). In our previous work, we have successfully prepared HA hydrogels from crosslinker 2,5-hexanedione and 3,3′-dithiodipropionate hydrazide-modified HA (DTPH–HA) through a hydrazone crosslinking reaction without using additional chemical agents (Cui, Qian, Zhao, & Wang, 2014). However, concerns about the potential cytotoxicity of residual 2,5-hexanedione

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have been raised because it may induce the collapse of neurofilaments (Kim et al., 2008; Zilz, Griffiths, & Coleman, 2007). As far as we know, HA hydrogels containing both hydrazone and disulfide bonds in their crossbridges have not been reported.

In this study, we synthesized a novel kind of HA hydrogels with a triple degradation behavior by directly reacting DTPH–HA with polyethylene glycol dilevulinate (LEV–PEG–LEV). The macromolecular diketone LEV–PEG–LEV was expected to exhibit good biocompatibility. The modification of HA with DTPH could endow the final HA hydrogels with hydrazone and disulfide bonds in their crossbridges. The unreacted pendant DTPH groups onto the HA backbones in HA hydrogels might be utilized for further decoration with bioactive molecules. The HA hydrogels showed a triple-responsive degradation behavior (hyaluronidase, reduction and acidity). The HA hydrogels were characterized by solid state ^{13}C NMR, Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), and rheological, swelling and degradation tests. The biocompatibility was evaluated using live/dead cell staining and MTT assays.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium (HA, molecular weight: 300 kDa), levulinic acid (LEV), *N*-hydroxysuccinimide (NHS), hyaluronidase (HAase) and glutathione (GSH) were purchased from Aladdin Reagent Inc. (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), poly(ethylene glycol) (PEG, 2000 Da) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM, Hyclone) and fetal bovine serum (FBS, Hyclone) were purchased from Gibco. MC3T3-E1 cells were supplied by Medical Center of Xi'an Jiaotong University (Xi'an, China). All other chemicals were of analytical grade and used without further purification. All aqueous solutions were prepared using ultrapure water with a resistance of 18.25 MΩ.

2.2. Preparation of HA hydrogels

DTPH–HA (about 40% degree of substitution) was synthesized according to our previously reported procedure (Xu et al., 2013). Briefly, HA (300 mg, 0.75 mmol repeating units) and DTPH (802.4 mg, 3.37 mmol) were first dissolved in deionized water (60 ml), and the pH of the solution was adjusted to 4.8–5.0 with 1 M HCl. After EDC·HCl (43 mg, 0.23 mmol) and NHS (26 mg, 0.23 mmol) were added, the mixture solution was stirred for 24 h at room temperature. The resulting solution was dialyzed (MWCO 3500) against distilled water for 3 days and lyophilized, obtaining the product DTPH–HA.

To synthesize crosslinker LEV–PEG–LEV, a mixture of PEG (5 g, 2.5 mmol), LEV (2.88 g, 12.5 mmol) and *p*-toluenesulfonic acid (9.5 mg, 0.05 mmol) was dissolved in 50 ml of toluene in a 250-ml three-neck flask, and the solution was heated under reflux for 12 h. After the reaction had been completed, the majority of the solvent was removed under vacuum, and the residue was poured into an excess amount of cold diethyl ether. The product was obtained as a faint yellow solid.

HA hydrogels were prepared as follows: DTPH–HA (60 mg, 129 μmol) was dissolved in water (1.6 ml), and then 0.4 ml of solution containing LEV–PEG–LEV (6.45, 12.9 or 19.35 μmol) was added to the solution. The mixture was vigorously vortexed, defoamed and left to stand. A light yellow gel was formed within 30 min. According to the percentages (6.45, 12.9 and 19.35 μmol) of LEV–PEG–LEV relative to the repeating units of DTPH–HA (129 μmol), the formed HA hydrogels were designated as HA–PEG10, HA–PEG20 and HA–PEG30, respectively, where the numbers defined the nominal degrees of crosslinking of the HA hydrogels (10%, 20% and 30%).

2.3. Characterization of HA hydrogels

^{1}H NMR and ^{13}C NMR spectra of LEV–PEG–LEV were recorded on a Bruker 400 MHz spectrometer (Bruker, Germany), using deuterated chloroform ($CDCl_3$) as the solvent. Solid state ^{13}C NMR spectra of DTPH–HA and HA hydrogel were recorded on a Bruker AVANCE III HD 400 MHz spectrometer (Bruker, Germany). FT-IR spectra were recorded on a Shimadzu IR Prestige-21 FT-IR spectrometer in transmittance mode in the frequency range of 1200–1800 cm^{-1} with a 2 cm^{-1} resolution. The freeze-dried HA hydrogel samples were sputter coated with gold and observed by scanning electron microscope (SEM, S-3400 N, Hitachi, Japan) at 10 kV. Rheological characterization was performed using a TA instrument DHR-2 rheometer in the frequency range of 0.1–10 Hz. Degradation tests were carried out in either PBS with different pH values or PBS (pH 7.4) containing HAase (50 U/ml) or GSH (5 mM). Swelling tests were carried out in PBS (pH 7.4) at 37 °C. The swelling ratio was calculated according to the following equation:

$$\text{swelling ratio}\,(\%) = \left[\frac{(W_1 - W_0)}{W_0}\right] \times 100$$

where W_0 and W_1 are the mass of dried and swollen HA hydrogels, respectively.

2.4. Cell culture

MC3T3-E1 cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. After sterilization with ethylene oxide, lyophilizated HA hydrogel samples were placed in 48-well plates. A single cell suspension containing 2×10^3 MC3T3-E1 cells was dropped onto the surface of each HA hydrogel sample. After 4 h of culture, 1 ml of culture medium was added to each well, and the hydrogel/cell constructs were cultivated at 37 °C in 5% CO_2 for 1 day and 3 days. The culture medium was replaced every day.

2.5. Live/dead cell staining

Live/dead cell assay was employed to evaluate the viability of MC3T3-E1 cells cultivated on the HA hydrogel samples for 1 day and 3 days. The concentrations of calcein AM and ethidium homodimer (ethD-1) were fixed at 10 μM. Calcein AM and ethD-1 were used to stain the live and dead cells, respectively. The samples were observed and imaged by a confocal laser scanning microscope (Leica, TCS SP5 II, Germany).

2.6. MTT assay

The viability and proliferation of MC3T3-E1 cells on the HA hydrogels were quantitatively evaluated by the MTT assay. After 1 day or 3 days of cultivation, the proliferative capacity of MC3T3-E1 cells was measured. The optical absorbance value of the solution in each well was measured at 490 nm using a microplate reader (PerkinElmer, EnSpire, America). The wells without HA hydrogel samples were used as a control group.

2.7. Statistical analysis

Experimental data were expressed as mean ± standard deviation. The statistical analysis for significance between the groups