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Photo-cured hyaluronic acid-based hydrogels containing simvastatin as a bone tissue regeneration scaffold

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ABSTRACT

We describe in this study the positive influences on *in vitro* and *in vivo* osteogenesis of photo-cured hyaluronic acid (HA) hydrogels loaded with simvastatin (SIM). Prior to loading SIM, we first characterized the HA hydrogels for their mechanical properties and swelling ratios. The results from this testing indicated that these two factors improved as the substitution degree of 2-aminoethyl methacrylate (AEMA) increased. MTT and live/dead assays showed that the HA hydrogels have good biocompatibility for use as scaffolds for bone tissue regeneration. Moreover, another MTT assay showed that the photo-cured HA hydrogels **III** fabricated with 30% AEMA (300 mg) conjugated HA (**HA-AEMA iii**) loaded with between 0.1 and 1 mg of SIM had a similar cytotoxicity as compared to the HA hydrogel **III** itself. The sustained release of SIM was observed to occur in the HA hydrogel **III** loaded with 1 mg of SIM. *In vitro* and *in vivo* experiments showed that the HA hydrogel **III** loaded with 1 mg of SIM had a significant influence on osteogenesis.

1. Introduction

Hydrogel systems in biomedical applications has attracted increasing attention as tissue engineering scaffolds for cell therapeutics, wound healing, cartilage/bone regeneration and the sustained release of drugs due to similar physical properties to natural tissue [1–6]. Numerous researchers have reported that hydrogel systems, fabricated by several methods [1–7], can provide sufficient space and mechanical stability for the formation of new tissues [2–4]. Among the various fabrication methods, photopolymerization was selected as a means to prepare hydrogels due to its many advantages. It can be used to easily control temporal and spatial reaction kinetics as well as have rapid reaction kinetics under mild conditions. Photopolymerization can also be completed in a single, rapid-step process [7–9]. For these reasons photopolymerization was selected as the means to generate hydrogels for use in drug delivery and scaffolds for tissue engineering [10–12].

Hyaluronic acid (HA) is a hydrophilic and negatively-charged linear D-glucuronic acid and N-acetyl-D-glucosamine copolymer that is widely found in connective, epithelial and neural tissues [13]. It is well known to be a major component of the natural extracellular matrix (ECM) for nearly all mammalian connective tissues [13]. It is a versatile macromolecule that has a great deal of potential for use in biomedical applications due to its hydrodynamic characteristics, viscous properties, and the ability for retaining water [13,14]. However, HA needs to be modified for biomedical applications due to its weak mechanical properties and in natural form is easily dissolved in water and degrades rapidly. The formation of a cross-linked network has been reported as an alternative to overcome the drawbacks [15,16].

Simvastatin (SIM) is an inhibitor of the competitive 3-hydroxy-3-methyl coenzyme A (HMG-CoA) reductase and is a very efficient drug for the induction of osteoblastic differentiation of human adipose-derived stromal cells (hADSCs) [17,18]. Also, it has been known to enhance bone morphogenic proteins (BMPs) in bone cells, alkaline phosphatase (ALP) activity and osteocalcin (OCN) expression level in hypercholesterolemic postmenopausal women [19,20].

In this study, we investigate if a photo-cured HA hydrogel loaded with SIM (Fig. 1) can play an important role as a sufficient

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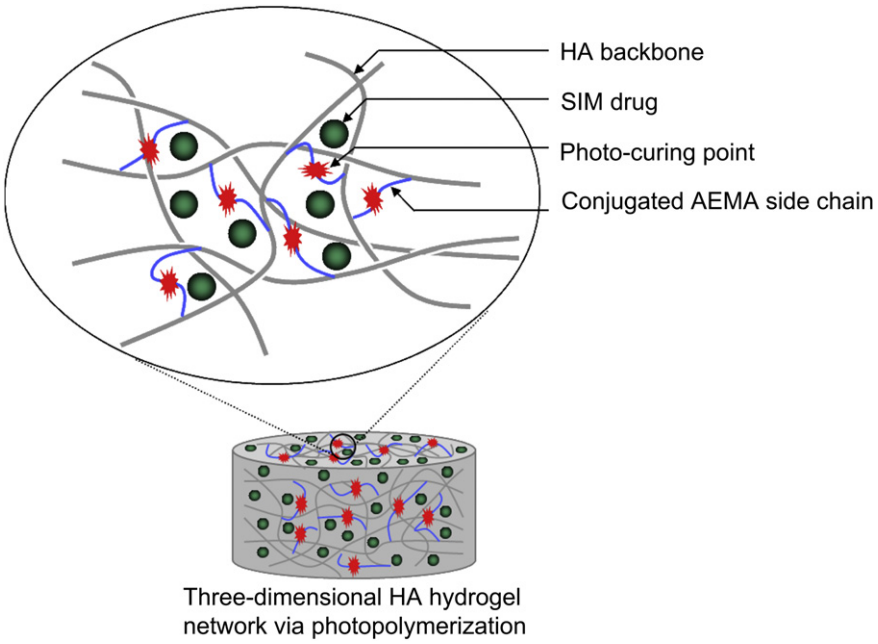

Fig. 1. Schematic of a photo-cured HA hydrogel network loaded with SIM.

and effective scaffold for bone tissue regeneration. Prior to the fabrication of the HA hydrogel, 2-aminoethyl methacrylate (AEMA) was conjugated to HA (**HA-AEMA**) via amide bonds, and then the HA hydrogel was formed by irradiating the mixture of a cytocompatible photoinitiator, 4-(2-hydroxy ethoxy) phenyl-(2-hydroxy-2-propyl) ketone (Irgacure D-2959) and HA-AEMA with 365 nm UV light [21,22]. The reaction was confirmed by measuring ^{1}H nuclear magnetic resonance (NMR) spectroscopy, scanning electron microscope (SEM) observations, and attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR). Constant strain-rate compression and swelling ratio tests were measured to determine whether the HA hydrogel had suitable properties for use as a scaffold for bone tissue regeneration. The biocompatibilities of SIM loaded and non-loaded HA hydrogels were evaluated by MTT and live/dead assays. Their osteogenic properties were evaluated under both *in vitro* and *in vivo* conditions including Alizarin S-red staining, reverse-transcription polymerase chain reaction (RT-PCR) and CBCT/X-ray radiography methods.

2. Materials and methods

2.1. Materials

HA (Mw:143,000 g/mol) was purchased from Lifecore Biomedical Co. (Chaska, MN, USA). AEMA and 2-morpholinoethanesulfonic acid (MES), cetylpyridinium chloride, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Sigma (St. Louis, MO, USA). Cellulose membrane (MWCO:3500) for dialysis was purchased from Spectrum Laboratories Inc., (Rancho Dominguez, CA, USA). A cytocompatible photoinitiator (Irgacure D-2959) for phtopolymerization was purchased from Ciba Specialty Chemicals (Basel, Switzerland). Fetal bovine serum (FBS) and penicillin–streptomycin were purchased from Gibco BRL (Gaithersburg, MD).

2.2. Synthesis of HA-AEMAs

Three kinds of **HA-AEMAs** were synthesized according to a previously reported method [21]. Briefly, to a solution of 1 g of HA and 0.5 M of sodium chloride (NaCl) in 50 mM MES a varying amounts of NHS and EDC were added to activate the carboxylic acid groups of HA. There were three sets made with NHS and EDC amounts for each as indicated: **i** - NHS (0.53 g, 0.05 mol) and EDC (1.75 g, 0.11 mol), **ii** - NHS (0.80 g, 0.07 mol) and EDC (2.63 g, 0.17 mol) and **iii** - NHS (1.06 g, 0.09 mol) and EDC (3.50 g, 0.22 mol). After stirring for 1 h, 20% (w/w) AEMA (200 mg), 30% AEMA (300 mg), and 40% AEMA (400 mg) were added to each of the HA solution containing **i**, **ii**, and **iii** respectively. The moles of NaCl, EDC and NHS were determined in proportion to the mole of AEMA to activate the carboxylic acid groups existing to HA. In this study, these are designated as **HA-AEMAs i**, **ii** and **iii** for each reaction level. These mixtures were continuously stirred at room temperature for 24 h, and then purified by dialysis (MWCO: 3500) for 3 days against distilled water. Following this the aqueous solutions were filtered, evaporated and lyophilized. The reaction was confirmed by ^{1}H NMR (Varian Unity Plus 300, Varian Inc., Palo Alto, CA, USA) spectrometer (300 MHz) and ATR-FTIR (TENSOR 37, Bruker, USA) (Scheme 1A).

2.3. Photopolymerizations of HA-AEMAs [23]

Each of the three kinds of **HA-AEMAs i–iii** were dissolved as 1 g in 10 mL of distilled water. To this solution 0.05 w/v% of Irgacure D-2959 was added, and then exposed to UV light (365 nm, CL-1000 UV-crosslinker, UVP) at 100 μJ/cm^2 for 5 min. This resulted in the formation of three kinds of photo-cured and transparent HA hydrogels (Scheme 1B). These HA hydrogels were analyzed by SEM (S-2300, Hitachi, Japan) and ^{1}H NMR measurements. In this study, the three kinds of HA hydrogels fabricated with **HA-AEMA i**, **ii**, and **iii** were designated as photo-cured HA hydrogels **I**, **II** and **III**, respectively.

2.4. Rheological analyses of photo-cured HA hydrogels *I–III*

The elastic modulus of the photo-cured HA hydrogels **I–III** was measured by constant strain-rate compression tests using a Rheometrics Solid analyzer (Rotational Rheometer, Gemini 150, Malvern, England) equipped with a 10 N load cell, followed by measurements of their elastic (storage, G′) and viscous (loss, G″) modulus.

2.5. Swelling ratios (Q) of photo-cured HA hydrogels *I–III* [24]

In order to investigate the swelling ratios of the photo-cured HA hydrogels **I–III**, they were weighed under both dry and wet conditions. Briefly, the dry HA hydrogels **I–III** were prepared by lyophilization for 1 week, and then weighed (*Wd*). The dry HA hydrogels **I–III** were then immersed in 50 mL of distilled water and then incubated at room temperature for 1 h, 1 day and 1 week. At each time point the mass of the wet hydrogel was recorded. The swelling ratios were calculated by using the equation $Q = Ws/Wd$.

2.6. Cytotoxicities and cell viabilities of photo-cured HA hydrogels *I–III*

Fibroblasts were trapped into three kinds of HA hydrogels **I–III** during photopolymerization. The cytotoxicities of the HA hydrogels **I–III** were evaluated by using MTT assay. The fibroblasts were seeded onto a 96 well tissue culture plate at a density of 1×10^4 cells per well in 200 μL of Dulbecco's modified eagle medium (DMEM) containing 10% of FBS and 1% of penicillin–streptomycin. These cells were then cultured at 37 °C for 24 h in an atmosphere of 5% CO_2. Each of the sterile **HA-AEMAs i–iii** solutions were then added into each of the cell-cultured wells with