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In situ forming hydrogels of hyaluronic acid and inulin derivatives for cartilage regeneration

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ABSTRACT

An in situ forming hydrogel obtained by crosslinking of amino functionalized hyaluronic acid derivatives with divinylsulfone functionalized inulin (INU-DV) has been here designed and characterized. In particular two hyaluronic acid derivatives bearing respectively a pendant ethylenediamino (EDA) portion (HA-EDA) and both EDA and octadecyl pendant groups (HA-EDA-C_{18}) were crosslinked through an aza-Michael reaction with INU-DV. Gelation time and consumption of DV portions have been evaluated on hydrogel obtained using HA-EDA and HA-EDA-C_{18} derivatives with a concentration of 3% w/v and a ratio 80/20 w/w respect to the crosslinker INU-DV. The presence of pendant C_{18} chains improves mechanical performances of hydrogels and decreases the susceptibility to hyaluronidase hydrolysis. Bovine chondrocytes, encapsulated during crosslinking, sufficiently survive and efficiently proliferate until 28 days of analysis.

1. Introduction

Hydrogels based on hyaluronic acid are very promising for tissue regeneration, like cartilage, thanks to their biomimetic potential, biocompatibility and biodegradability (Kang et al., 2009; Soo-Hong & Heungsoo, 2007). In addition, if these hydrogels are in situ forming, additional advantages are showed, such as: the gel forming solution can fill up the articular defect; its administration is minimally invasive requiring the use of a syringe without surgery, with a consequent increase in patient compliance, and cells (chondrocytes) can be loaded in the polymer solution and then uniformly entrapped in the hydrogel during gelation process (Kretlow, Klouda, & Mikos, 2007).

Generally in situ forming hydrogels are obtained from polymer solutions that are injected in fluid form into the body and undergo gelation in vivo, triggered by a solvent removal precipitation, variation in temperature, pH or ionic strength or through the formation of chemical bonds between the polymer chains (Chung & Park, 2009; Tan & Marra, 2010).

In this latter case, if the chemical reaction between polymer chains occurs in physiological conditions, without the use of toxic initiators and catalysts, and with an appropriate rate, it is possible to load cells in polymer solution and then to obtain a homogenous cellular scaffold, after in vivo gelation.

In this context, chemical crosslinking via Michael addition between amine containing polymers and vinyl sulfone groups is a valid approach. For this reason, in this work appropriate derivatives of two polysaccharides, such as hyaluronic acid and inulin, they have been synthesized for obtaining in situ forming hydrogels in physiological conditions. Hyaluronic acid and inulin have been chosen as starting materials because the first is a natural component of extracellular matrix of articular cartilage, where it interacts with proteoglycan and collagen type II and promotes chondrocyte differentiation thanks to the interaction with CD44 and RHAMM receptors (Bedi, Feeley, & Williams, 2010; Wu, Chang, Wang, Wang, & Ho, 2010); the second one, is a biocompatible, water soluble and FDA approved polymer, with great potentiality in biomedicals

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(Licciardi, Scialabba, Sardo, Cavallaro, & Giammona, 2014; Palumbo et al., 2014; Pitarresi, Tripodo, Cavallaro, Palumbo, & Giammona, 2008). In particular, ethylenediamine derivatives of hyaluronic acid bearing or not octadecylamine chains have been prepared as well as a divinylsulfone derivative of inulin; these polymers are able to react in situ and in mild conditions with production of hydrogels. These latter have been characterized with regard to their gelation time, mechanical properties and stability/degradation in physiological medium or in the presence of hyaluronidase. Viability of bovine chondrocytes entrapped during hydrogel formation has been confirmed by MTS test, DAPI assay and SEM analysis.

2. Experimental

2.1. Materials

Low molecular weight hyaluronic acid (HA) (Mw 240 kDa, polydispersity index 1.9) and its tetrabutylammonium salt (HA-TBA) were produced as reported elsewhere (Palumbo et al., 2012). Inulin (INU) from Dahlia Tubers (Mw 5 kDa, polydispersity index 1.1, as detected by SEC measurement using pullulan standard as a reference, amount of glucose about 5% respect to fructose), divinyl sulfone ≥98.0% (DV), ethylenediamine (EDA), anhydrous dimetylsulfoxide (DMSO) and dichloromethane (DCM) were obtained from Fluka (Milano, Italy).

Octadecylamine (C_{18}—NH_2), tetrabutylammonium hydroxide (TBA-OH), testicular hyaluronidase (1040 U/mg), bis(4-nitrophenyl) carbonate (4-NPBC), picrylsulfonic acid solution (2,4,6-trinitrobenzenesulfonic acid-TNBS), Dulbecco's Phosphate Buffered Solution (DPBS), Dulbecco's Modified Eagle Medium (DMEM), 4′,6-diamidino-2-phenylindole (DAPI), Sirius Red F3B and hexamethyldisilazane were purchased from Sigma-Aldrich (Milano, Italy). Collagen sponges (Antema®) were a generous gift of Opocrin Spa (Modena, Italy).

Inulin-divinylsulfone derivative (INU-DV) was prepared and characterized as previously reported (Pitarresi, Tripodo, Triolo, Fiorica, & Giammona, 2009). The degree of derivatization in DV (DD_{DV}%), determined by ^{1}HNMR in D_2O was 28 ± 3 mol%.

Hyaluronic acid-ethylenediamine derivative (HA-EDA) was prepared and characterized as previously reported (Palumbo et al., 2012). The degree of derivatization in EDA (DD_{EDA} %), determined by ^{1}HNMR in D_2O was 50 ± 2 mol%.

2.2. Apparatus

Proton nuclear magnetic resonance (^{1}HNMR) was performed using a Brucker AC-300 instrument of 300 MHz.

FT-IR spectra were carried out by using a Brucker Alpha instrument.

Ultraviolet (UV) measurements for TNBS assay were carried out by using a Shimadzu UV-2401PC spectrophotometer.

Scanning electron microscopy (SEM) images were recorded by using a scanning electron microscope Philips XL30 ESEM; samples were freeze-dried, freeze fractured, gold coated and finally observed.

Fluorescence measurements were carried out by using a Zeis Axio Vert microscope.

Compression tests were performed using an Instron apparatus (Model 3345, Instron, Norwood, MA).

2.3. Synthesis of hyaluronic acid-ethylenediamine-octadecylamine (HA-EDA-C_{18}) derivatives

One gram of HA-TBA dissolved in 90 ml of anhydrous DMSO was mixed with 10 ml of anhydrous DMSO containing the appropriate amount of 4-NPBC to have a molar ratio between 4-NPBC and HA-TBA repeating unit equal to 0.7. The reaction was left at 40 °C for 4 h. After this time, temperature was increased to 70 °C and an appropriate amount of octadecylamine (C_{18}—NH_2) was added to have a molar ratio between C_{18}—NH_2 and HA-TBA repeating unit equal to 0.7, 0.35 and 0.175. The reaction was left at 60 °C for 16 h, after this time and temperature was decreased to 40 °C and an appropriate amount of ethylenediamine (EDA) was added to have a molar ratio between EDA and 4-NPBC equal to 10. According to the different molar ratio between C_{18}—NH_2 and HA-TBA repeating unit, as above reported, HA-EDA-C_{18}a, HA-EDA-C_{18}b and HA-EDA-C_{18}c derivatives have been obtained. The work-out was accomplished by adding 1 ml of aqueous NaCl saturated solution under stirring for 30 min to change tetrabutylammonium with sodium, then each reaction product was precipitated into a mixture diethylether/chloroform 1:1 v/v, further washing with the same mixture and then with a mixture ethanol/water 8:2 v/v and finally with ethanol. Each product was recovered after freeze-drying.

^{1}HNMR spectrum (in D_2O/CD_3OD 1:2) of each HA-EDA-C_{18} derivative showed main peaks at δ 0.99 (—C**H$_3$** of octadecylamine), δ 1.5 (—CH_2—(C**H$_2$**)$_{16}$—CH_3 of octadecylamine), δ 1.9 (—NH—CO—C**H$_3$** of hyaluronic acid), δ 3.3–4.0 (pyranosyl C**H** of hyaluronic acid).

The derivatization degree in C_{18} portions linked to HA was calculated comparing the peak at δ 0.99 and δ 1.5 attributable to methyl and methylene groups of C_{18} chain with the peak at δ 1.9 attributable to acetamido group of HA.

The derivatization degree in EDA portion linked to HA was calculated by the colorimetric assay with TNBS (Kirker & Prestwich, 2004; Matricardi et al., 2011). Functionalizations degrees in C_{18} and EDA were then finally expressed as mol of portions linked per moles of HA-EDA-C_{18} repetitive units % (mol%).

2.4. Preparation of hydrogels

HA-EDA-C_{18}c, HA-EDA and INU-DV were dissolved singly in DPBS pH 7.4 at a concentration of 3% w/v for each polymer. Then, an appropriate volume of INU-DV solution was added to HA-EDA-C_{18}c or HA-EDA solution, in order to obtain a weight ratio between HA-EDA-C_{18}c (or HA-EDA) and INU-DV equal to 80:20. Polymer dispersion were left at 37 °C in orbital shaker until complete gelation. The hydrogel formation was qualitatively monitored by the inversion tube test (Jeong, Bae, & Kim, 1999) and confirmed by FT-IR analysis. In particular FT-IR spectra of starting polymers and obtained hydrogels were compared to qualitatively detect the consumption of DV portions during crosslinking.

2.5. Chemical and enzymatic degradation studies

For the swelling studies, aliquots of freeze dried HA-EDA-C_{18}c/INU-DV and HA-EDA/INU-DV hydrogels prepared as above described were weighed and introduced in a 48 well plate. One milliliter of DPBS pH 7.4 was added in each well to allow swelling of the sample. After 24 h, the initial swollen weight of each sample was determined (Wi), then another ml of DPBS pH 7.4 plus 0.02% NaN_3 was added in the absence or in the presence of hyaluronidase (HAase) with a concentration of 30 U/ml. Samples were left under stirring (100 rpm) in an orbital shaker at 37 °C until 28 days. During this period, at pre-determined times, samples were weighed after removing the excess of liquid with a blotting paper, then weighed again to determine the swollen weight in the specific time (Wt) after treatment with DPBS pH 7.4 alone or in the presence of enzyme. The swelling behavior was expressed as the swelling weight ratio (q) between Wt and Wi. Each experiment was performed in triplicate.

Hydrogels recovered after incubation with DPBS pH 7.4 alone or in the presence of HAase were washed several times with

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