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Research Article

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Crosslinking of hyaluronic acid and human-like collagen with divinyl sulfone

Jingjing Zhang[1,2], Xiaoxuan Ma[1,2], Junfeng Hui[1,2] , Pei Ma[1,2], Jianjun Deng[1,2] and Daidi Fan[1,2*]

[1]Shaanxi Key Laboratory of Degradable Biomedical Materials, Northwest University, 229 Taibai North Road Xi'an, Shaanxi, China
[2]Shaanxi R&D Center of Biomaterials and Fermentation Engineering, School of Chemical Engineering, Northwest University, Xi'an, China

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ABSTRACT

Hyaluronic acid and human-like collagen, both with favorable biocompatibility and biodegradability, were designed as biomaterials with excellent property in tissue engineering. We report an injectable hydrogel particle based on hyaluronic acid and human-like collagen. They were crosslinked with divinyl sulfone to form a three- dimensional network. The divinyl sulfone was removed by dialysis and the content was determined by gas chromatography. The in vitro degradation proved an admirable ability of antienzyme. The cytotoxicity analysis showed the low toxicity to the cell growth. The hydrogel is promised to be applied into medical field.

Key words: hydrogel, hyaluronic acid, human-like collagen, injectable

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INTRODUCTION

Hydrogel is a kind of biomaterials with three-dimensional networks which could absorb and retain a large amount of water [1]. Moreover, hydrogels have a high permeability for nutrients and water-soluble metabolites, allowing cell survival and supporting cellular growth. Hydrogel has got a lot of public attention as soft tissue filler. It is a hydrophobic network polymer which contains abundant water in it. Natural hydrogels derived from cellulose derivatives, xyloglucan, chitosan and glycerophosphate are widely used in drug delivery, cell encapsulation and tissue repair[2]. The macromolecules were crosslinked by chemical or physical methods to enhance their physicochemical and biological performance.

Hyaluronic acid (HA) is a naturally non-sulfated glycosaminoglycan that is widely distributed throughout the extracellular matrix (ECM) of all connective tissues in human and other animals [3,4], result in close attention to its application in cosmetics and pharmaceutical industry. But due to fast biodegradation and low bioavailability,

HA-based material should be modified to provide the desired functional properties [5]. Recently many studies showed that HA could be crosslinked by divinyl sulfone (DVS) which would react with hydroxyl to create intramolecular and intermolecular combination (Unite States Patent, 4636524).

Collagen contributes to the stability and structural of tissues and organs via its characteristic molecular structure, and has been used extensively as a scaffold in skin and bone tissue engineering [6,7]. Human-like collagen (HLC), produced by gene engineering, is a giant recombinant molecule bio-protein with great performance in improving cell growth and cell adhesion. The previous study demonstrated that HLC has several unique characteristics which are significantly different from animal collagen, such as chemically defined structure, virus-free, biocompatibility, water solubility, little immunogenic reaction, and so on [8-10] .

Human-like collagen could crosslink with hyaluronic acid through ether linkage by divinyl sulfone. Then the hydrogel should be washed several times with phosphate buffer in order to remove the redundant divinyl sulfone and adjust pH of the product. Therefore, in this paper, our objective was to study the preparation and evaluate the properties of the HA/HLC composite hydrogel. The results showed that the novel HA/HLC hydrogel would be suitable for soft tissue filling.

EXPERIMENTAL SECTION

Materials

Hyaluronic acid sodium was purchased from Shandong Freda biopharm co.,ltd. Human-like collagen (HLC, China patent number: ZL01106757.8, Mr=97,000) was supplied by our laboratory. Divinyl sulfone was purchased from Sigma–Aldrich. All the other chemical regents used in this work were all analytical pure grade.

Synthesis of hyaluronic acid/human-like collagen hydrogels

Hyaluronic acid/human-like collagen hydrogel was prepared by the method as previous description [11]. Briefly, HA and HLC (total amount 0.1 g), in a mass ratio of 4:1, were dissolved into 4 mL 0.2 M NaOH solution for an hour. Divinyl sulfone was added to the solution as the concentration of 1%, after immediately mixing with a vortex mixer, the mixture was incubated at 45 °C for one hour. After the crosslinking reactions, the resulting gel was purified by dialysis. Dialysis was performed against 0.1 M PBS during 48 h, after which the gel was squeezed through a sieve with pore diameter of 400 μm twice. The hydrogel particles were finally prepared.

Hydrogels prepared with different concentration of DVS, HA and different reaction temperature will be used to search for the best reaction condition. The content of HA in the final hydrogels were detected with carbazole spectrophotometric method.

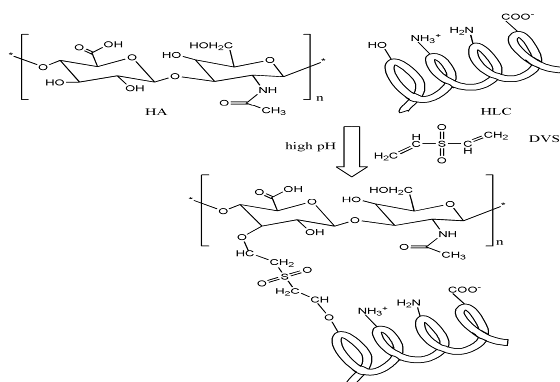

Fig. 1 The Crosslinking Mechanisms

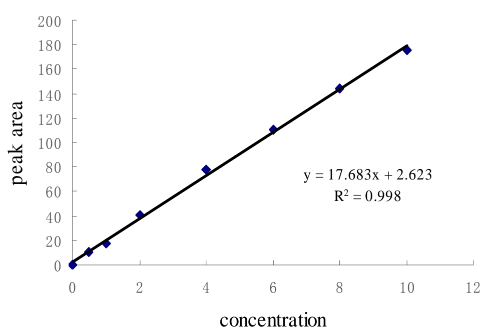

Fig. 2 The linear relation between amount of DVS and peak areas

Residual quantity of DVS

The content of divinyl sulfone in hydrogels was determined by gas chromatography [12], with a FID detector，HP-5 (Agilent 7890A: 30 m×320 μm×0.25 μm), nitrogen as the carrier gas, and multiply column temperature program.

DVS was diluted with absolute ethyl alcohol into 0.5 μg/mL, 1 μg/mL, 2 μg/mL, 4 μg/mL, 6 μg/mL, 8 μg/mL and 10 μg/mL, and 1 μL sample was injected to get a standard curve with the concentration as X-coordinate and the peak area as Y-axis.

The hydrogel particles were weighed and soaked in hyaluronidase solution. The mixture was incubated at 37 °C for 24 h, followed by centrifuging. The supernate was filtered through a membrane with pore size of 0.22 μm. The filtrate was analyzed by gas chromatography. We could get the amount of DVS based on the peak area.

In vitro degradation

Degradation of hydrogel was performed in vitro hyaluronidase (100 U/mL). The lyophilizated samples were weighed (W_0) and sterilized, then immersed in 5 mL enzyme solution. The tubes were kept static at 37.0 ± 0.5 °C in an incubator. After soaking for 1, 2, 4, 6, 8, 10 or 24 hours, the samples were then washed with distilled water three times. After lyophilization, the dry weights were measured (W_1). The rate of weight loss (W_L) was calculated according as

$$W_L=W_1/W_0\times 100\%$$

Cell culture and cytotoxicity analysis

The hydrogel was weighed and sterilized, then placed in a 24-well plate to which fresh culture medium was added at 0.1 g/mL. The cells were cultured at a density of 1.0×10^4 cells/mL on 96-well plates (100 μL/well) in a CO_2 (5%) incubator at 37 °C. After incubation for 24 h, the extracts were added to 96-well plates (100 μL/well) in a CO_2 (5%) incubator at 37 °C. After incubation for 1 d, 3 d, 5 d, and 7 d. 10 μL of cck-8 was added to each well, after which the cultures were incubated at 37 °C for an additional 4 h. Absorbency of the solution was measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) Reader (MODEL550, Bio-Rad, USA). The relative cell growth (%) was calculated as:

Relative cell growth=$[OD]_{test}/[OD]_{control}\times100\%$

RESULTS AND DISCUSSION

Analysis of reaction condition

The HA/HLC hydrogel would be an ideal products used for plastic and reconstructive surgery of soft tissue. The degradation rate of hydrogels is a key factor to its commercial use, the relationship between the degradation rate and concentration of HA (0.5%-3%), DVS (0.25%-2.5%) and reaction temperature (25°C-55°C) was studied. The concentration of HA is very important to the gelling of hydrogels. The figure shows that with the change of the concentration of HA, the property of antienzyme is becoming strong, but when the concentration is higher than 2%, the degradation rate has dropped. That may be attributed to the lack of DVS, there was no enough DVS to crosslinked with HA and HLC so that the structure is looser to resist enzyme.

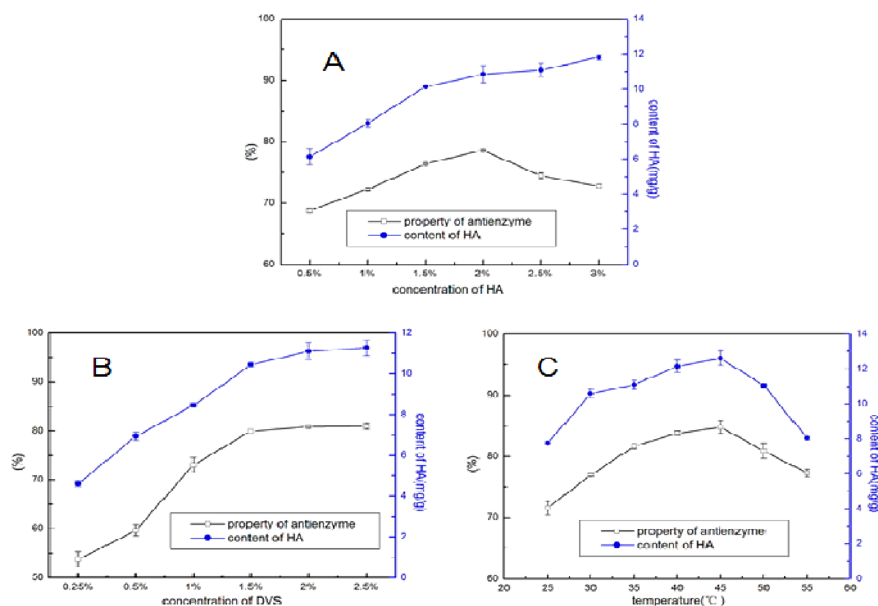

Fig. 3

(A) Effects of HA concentration on the degradation rate

(B) Effects of DVS concentration on the degradation rate

(C) Effects of reaction temperature on the degradation rate

Under a certain concentration of HA, the degradation rate determined by DVS is mainly factor influence on the performance of HA/HLC hydrogel. The data shown in Fig. 2(c) from experiments demonstrated that the higher concentration of DVS, the slower degradation rate of hydrogels. However, the higher concentration of DVS leading to the serious cytotoxicity to hydrogels, it was reported that DVS have serious cytotoxicity and could not promote any cell proliferation. Therefore, the certain concentration of HA (2%), DVS (1.5%) and certain concentration of HLC were used to prepare the hydrogels.

At temperatures lower than 45 °C, the degradation rate were shortened with increasing temperature; around 45 °C, the degradation rate were minimum, while at temperatures higher than 45 °C, the degradation rate increased gradually with increasing temperature. Because of the reaction condition with high temperature, HA were damaged in the crosslinking process, which made that the hydrogels were with faster degradation rate. So, we studied the functional performance of the HA/HLC hydrogel crosslinked by DVS between 40 °C and 45 °C.

Residual quantity of DVS
Divinyl sulfone, with high toxicity, is harmful to our body. Also it is the main reason of initiating adverse reaction while the hydrogel being injected, so we have to ensure that the content of DVS in the particles is at a relatively low level. The results show that the residual quantity of DVS is 1.67 μg/g, which is lower than that provided by industrial standard. The hydrogel could be a kind of harmless biomaterial.

In vitro hydrolysis degradation behavior
The resistance to degradation of the hydrogels was studied *in vitro* by examining weight loss with time in 10% (w/v) hyaluronidase at 37 °C (Fig. 4). The weight loss was greatest in six hours, followed by that, the rate of weight loss was reduced slowly. The hyaluronidase is specialized to hyaluronic acid, which made the speed of biodegradation faster in earlier stage.

Hyaluronidase, commonly found in human bodies, was able to degrade the matrix to D-glucuronic acid and N-acetyl glucosamine residues. Overall, the rate of weight loss increased with time after degradation by hyaluronidase. HLC chains can effectively prevent hyaluronidase from completely destroying the original structure, and also can slow the degradation of the hyaluronic acid chain. The results indicated that the HA/HLC hydrogel would be degraded slowly with time, indicating its suitability as a body filler.

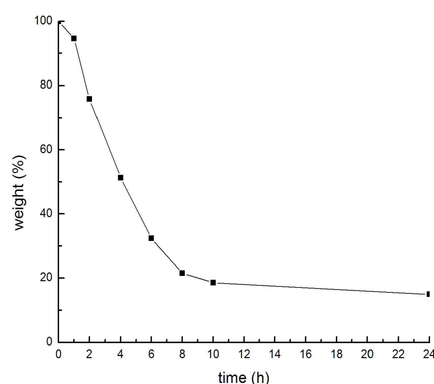

Fig. 4 Weight Loss of HA/HLC Hydrogel After Degradation by Hyaluronidase

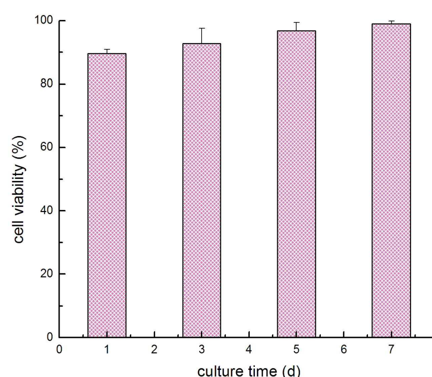

Fig. 5 Cell viability in hydrogels after culture for 1, 3, 5 and 7 Days

CCK-8 Assay
The ideal biomedical material should not release toxic products or produce adverse reactions, which can be evaluated through *in vitro* cytotoxic tests. The cck-8 assay is a routine method for detecting the toxicity of tissue-filling materials. The study indicated that the hydrogel had a little influence on the BHK proliferation after 1, 3, 5 and 7 days. According to ISO standards (ISO10993.12-2005), the toxicity of this hydrogel was classified as grade , therefore, the HA/HLC hydrogel will not restrain the proliferation of BHK, indicating its potential as a biomaterial for some specific uses such as soft tissue augmentation.

CONCLUSION

The HA/HLC hydrogel were successfully prepared by crosslinking with DVS and the hydrogels showed low residual of crosslinker. In vitro degradation indicated that the materials showed excellent property of antienzyme. The cytotoxicity analysis in vitro demonstrated this hydrogel particles were without cytotoxicity. There is a great potential for this hydrogel to be used as biomaterials for tissue engineering.

Acknowledgments
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