European Polymer Journal 72 (2015) 717–725

Contents lists available at ScienceDirect

European Polymer Journal

journal homepage: www.elsevier.com/locate/europolj

Tough biohydrogels with interpenetrating network structure by bienzymatic crosslinking approach

Yemin Zhang, Zhiping Fan, Chen Xu, Shuo Fang, Xiaojiu Liu, Xinsong Li *

School of Chemistry and Chemical Engineering, Southeast University, Nanjing 210018, China

ARTICLE INFO

Article history:
Received 25 October 2014
Received in revised form 14 December 2014
Accepted 29 December 2014
Available online 3 January 2015

Keywords:
IPN hydrogel
Chitosan–phloretic acid
Bienzymatic crosslinking
Gelatin
Mechanical strength

ABSTRACT

Enzymatically crosslinked biohydrogels have increasingly attracted attention mainly due to the mildness of this type of reaction. However, their mechanical strength is usually so weak that their applications are limited in the field of medicine. In order to improve their mechanical properties and degradation resistance, bienzymatic crosslinking approach was utilized to prepare biohydrogels of gelatin and chitosan with an interpenetrating polymer network (IPN) structure in this report. First of all, chitosan was grafted with phloretic acid (chitosan-PA) used as a substrate of horseradish peroxidase (HRP). After that, the gelation process of gelatin/chitosan-PA IPN hydrogels was monitored by a rheometer. The results indicated the formation of dual networks: one gelatin network crosslinked by transglutaminase (TG) and another chitosan-PA network crosslinked by HRP in the presence of a low concentration of H_2O_2. In addition, the mechanical performances of the hydrogels were measured by a universal testing machine. It was found that the mechanical properties of the IPN gels were significantly improved compared with gelatin hydrogel crosslinked by TG. Moreover, the swelling ratio, degradation behavior, and cytocompatibility of the IPN hydrogels were investigated in detail. The preliminary biological evaluation indicated that the IPN hydrogels can support L929 cell adhesion and proliferation. Overall, the gelatin/chitosan IPN hydrogels prepared by bienzymatic crosslinking approach have excellent biocompatibility and mechanical properties. Therefore, dual enzyme-mediated crosslinking of natural polymer hydrogels is promising for the development of tissue engineering scaffolds and wound dressing.

1. Introduction

Biohydrogels which are composed of natural polymers have attracted widespread attention in the field of tissue engineering and regenerative medicine. They have physical properties emulating native extracellular matrix (ECM), biocompatibility, biodegradability, and biomimetic features [1,2]. However, the mechanical strength of biohydrogels is usually weak, limiting their applications as tissue engineering scaffolds and wound dressing [3,4]. Therefore, the modification of biohydrogels for the enhancement of mechanical properties becomes one of the most important research areas.

In the past decades, several approaches have been developed to tackle this problem. Chemical crosslinking is a common method to improve the mechanical strength of biohydrogels, but the residues of crosslinkers or initiators may bring about cytotoxic side-effects even after thorough purification, restricting its application in biomedical area [5–7]. Physical crosslinking is an alternative method to enhance mechanical strength of hydrogels by hydrophobic interaction [8], hydrogen bonding [9], ionic crosslinking [10], etc., without using chemical substances. But, the

* Corresponding author.
E-mail address: lixs@seu.edu.cn (X. Li).

http://dx.doi.org/10.1016/j.eurpolymj.2014.12.038
0014-3057/

mechanical properties of the hydrogels crosslinked by physical methods are insufficient, and the crosslinking points are easy to disintegrate in biological fluids [11]. Introducing interpenetrating polymer network (IPN) structure is an effective way to improve the mechanical properties of hydrogels, in which each polymer forms a crosslinked network that is entangled with another polymer network [12–15]. Double network (DN) hydrogels possessing IPN structure have already achieved great progress [16]. Inspired by the DN principle, several groups have also designed and developed several IPN systems such as poly (ethylene glycol)/poly (acrylic acid) (PEG/PAAc) [17–19], but chemical crosslinkers were used in these systems [11].

Enzymes can effectively catalyze biochemical reactions and have been applied to synthesize polymers as reported in the literature [20]. Microbial transglutaminase (mTG) can catalyze the chemical reaction between glutamine and lysine residues on adjacent gelatin chains, thus providing amide bonds that contribute to form three-dimensional gelatin networks [15,21]. Horseradish peroxidase (HRP) can catalyze the crosslinking of phenol groups in the presence of hydrogen peroxide (H_2O_2), and it has gradually been employed to prepare polymer hydrogels with excellent biocompatibility [22,23]. Apparently, enzymatic crosslinking, completely devoid of potentially cytotoxic small molecule crosslinkers, is a biocompatible approach in the development of hydrogels. Unfortunately, the hydrogels crosslinked by single enzyme-mediated reaction forming one network structure have poor mechanical strength and rapid degradation, which have potential as injectable biomaterials [24–26].

The aim of our research is to develop a new method – bienzymatic crosslinking to prepare biopolymer based IPN hydrogels with improved mechanical strength and favorable biocompatibility. The hydrogels based on gelatin and chitosan with IPN structure were crosslinked by a combination of mTG and HRP mediated reactions. The rheological behaviors of the hydrogels were investigated to confirm the formation of the IPN structure. The gel mechanical properties, swelling ratio, degradation behaviors and cytocompatibility were studied further. To the best of authors' knowledge, this is the first report that uses dual enzymes to prepare biopolymer based IPN hydrogels.

2. Materials and methods

2.1. Materials

Water-soluble chitosan (8 kDa, 90% DD) was purchased from Shunbo Biological Technology Research Institute Co., Ltd. (Qingdao, China). Gelatin (type A), phloretic acid (PA), N,N-dimethylformamide (DMF), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), horseradish peroxidase (HRP, 250 U mg^{-1}), hydrogen peroxide (H_2O_2, 30 wt.%) and papain (6000 U mg^{-1}) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Microbial transglutaminase (1000 U g^{-1}) was a gift from Dongsheng Food Science and Technology Co., Ltd. (Taixin, China).

Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin (180 U mg^{-1}) and penicillin–streptomycin solution were purchased from Invitrogen Co. (Carlsbad, CA). L929 mouse fibroblast cells were a gift from the Public Health of Southeast University. All reagents were of analytical grade.

2.2. Synthesis of chitosan-PA conjugate

Chitosan-PA conjugate was synthesized by a general carbodiimide/active ester-mediated coupling reaction (Scheme 1) [27,28]. Phloretic acid (0.882 g, 5.3 mmol), EDC HCl (1.528 g, 8 mmol) and NHS (0.92 g, 8 mmol) were dissolved in 100 mL mixture of water and DMF (3/2, *v*/*v*). The reaction was conducted at room temperature for 4 h. After that, 1.288 g water-soluble chitosan in 100 mL distilled water was dropped into the reaction mixture, and stirred at room temperature for another 12 h. The solution was then dialyzed against distilled water (MWCO 3500 Da) for 3 days, and lyophilized. Finally, chitosan-PA conjugate was obtained with the yield 79.5%, named CPA1.

The structure of the chitosan-PA conjugate CPA1 was characterized by ^{1}H NMR (D_2O) spectrum recorded on a VARIAN Mercury apparatus (Varian, 300 MHz, Palo Alto, CA) as follows (Fig. S1 in supporting information): δ 6.85 ppm and 7.18 ppm (aromatic protons of the phenol groups), 4.87 ppm (anomeric proton of chitosan), 3.18 ppm and 3.75–3.92 ppm (glucopyranose ring protons of chitosan). The degree of substitution (DS) of phloretic acid in the chitosan, defined as the number of substituted phloretic acid per 100 glucopyranose rings of chitosan, was determined using ^{1}H NMR by comparing the integrals of signals at δ 4.87 ppm (anomeric proton of chitosan) and δ 6.85–7.18 ppm (aromatic protons of the phenol groups) to be 29.3% [28].

Using the same procedure as above, other chitosan-PA conjugates named CPA2 and CPA3 with DS of 15.1% and 41.8% were prepared (^{1}H NMR spectra were shown in Figs. S2 and S3 in the supporting information) by changing the molar ratio of carboxylic groups of PA to chitosan amino groups from 1:6 to 2:1. The yield of chitosan-PA varied from 74% to 82%.

2.3. Rheological test

Rheology measurements were performed with a rotational rheometer (HAAKE RS600, Thermo-Fisher, Hampton, VA) using a parallel plate geometry (25 mm diameter, 0°) in the oscillatory mode, and the elastic modulus G′ and viscous modulus G″ were recorded as a function of time at 37 °C with a strain of 5% at a frequency of 1 Hz. Briefly, the gelatin/chitosan-PA (CPA1) gel mixture with the composition as shown in Table 1 was quickly loaded on the bottom plate, the upper plate was lowered down to a measuring gap size of 0.28 mm and the test was immediately started. H_2O_2 with the concentration 0.2 mmol L^{-1} was used for rheological testing. The low concentration 0.2 mmol L^{-1} of H_2O_2 is preferred, resulting in a lower rate of chitosan-PA crosslinking in order to have enough time to monitor gelation process [23,29].