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Research paper

Injectable hydrogels based on the hyaluronic acid and poly (γ-glutamic acid) for controlled protein delivery

Xuebin Ma[a], Tingting Xu[b], Wei Chen[b], Hongye Qin[b], Bo Chi[b,*], Zhiwen Ye[a,**]

[a] School of Chemical Engineering, Nanjing University of Science and Technology, Nanjing, China
[b] State Key Laboratory of Materials-Oriented Chemical Engineering, Nanjing Tech University, Nanjing, China

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ABSTRACT

Injectable hydrogels have great potential in minimally invasive delivery. In this work, novel injectable hydrogels were prepared *via* self-crosslinking of aldehyde hyaluronic acid (HA-CHO) and hydrazide-modified poly (γ-glutamic acid) (γ-PGA-ADH) for proteins delivery. The HA/γ-PGA hydrogels could be formed *in situ* as fast as 9s with high swelling ratios. Rheological properties illustrated a wide processing range and good mechanical properties, which were reflected by broad linear viscoelastic region and higher threshold shear stress (σ_c) and storage modulus (G'). Meanwhile, the gelation time, swelling ratio, rheological properties, as well as the protein release behavior could be modulated conveniently. Bovine serum albumin (BSA) was designed as a model drug to study the release behavior. We found that the release mechanisms were either diffusion or Case-II relaxation depending on the different hydrogel components. The HA/γ-PGA hydrogels also showed good biocompatibility. Therefore, the HA/γ-PGA hydrogels have great potential as promising injectable biomaterials for controlled protein delivery.

1. Introduction

In recent years, various native and genetically engineered proteins, including enzymes, antibodies, hormones, and cytokines, have been used as biopharmaceuticals. However, protein drugs often have poor bioavailability due to their short half-lives and rapid clearance from the body (Alves et al., 2017; Koyamatsu et al., 2014). In order to achieve therapeutic effects, a common strategy is bolus injection of proteins, but this approach often cause some side effects, such as hematoma, increased postoperative morbidity, risk of tumor formation and so on (Hariawala et al., 1996; James et al., 2016). One of the most effective methods to overcome this problem is pharmaceutical formulation of proteins. Injectable hydrogels are considered as an ideal material for protein delivery, because of remarkable advantages such as easy incorporation of therapeutic drugs *via* simple mixing, minimally invasive surgical procedure, convenience of filling irregular surgical defects completely, and the tunability properties (Burdick & Murphy, 2012).

Previous studies have confirmed that the injectable hydrogels prepared by natural polysaccharides showed good biocompatibility than many synthetic polymers (Matsumura, Nakajima, Sugai, & Hyon, 2014). Hyaluronic acid (HA) is a naturally non-sulfated glycosaminoglycan, and possesses good biocompatibility, biodegradability, non-immunogenicity, as well as excellent gel-forming properties. It has gained great attention and interest in drug delivery (Fang, Chen, Leu, & Hu, 2008; Zawko & Schmidt, 2010). Thermosensitive injectable HA hydrogels were concerned by many teams (Fang et al., 2008; Jung, Park, Park, Lee, & Na, 2017) because of its sensitivity to body temperature and superior biocompatibility, but low stability and poor mechanical properties were also observed due to the sensitivity of some non-covalent interactions to physiological environments. Other injectable HA hydrogels have also been prepared by various methods, such as UV crosslinking (Leach, Bivens, Collins, & Schmidt, 2004), "click" chemistry (Hu, Li, Zhou, & Gao, 2011) and enzymatic crosslinking (Kim et al., 2011). However, some potential cytotoxic molecules, such as photosensitizers, catalytic agents and oxidizing agents, which may cause protein degeneration during the crosslinking process, are inevitably introduced. Michael addition reaction is considered to be a suitable method to prepare injectable HA hydrogels. The resultant hydrogels exhibited good biocompatibility, whereas, the crosslinking reaction was too slow (~30 min or longer) (Hahn, Oh, Miyamoto, & Shimobouji, 2006). In our work, the injectable hydrogels were prepared *via* Schiff base reactions because of the prominent advantages such as avoidance of crosslinking agents and easy control of reaction rate.

* Corresponding author at: State Key Laboratory of Materials-Oriented Chemical Engineering, Nanjing Tech University, No. 30 South Puzhu Road, Nanjing 211800, Jiangsu, China.
** Corresponding author at: School of Chemical Engineering, Nanjing University of Science and Technology, Xiaolingwei 200, Nanjing 210094, Jiangsu, China.
E-mail addresses: chibo@njtech.edu.cn (B. Chi), yezw@njust.edu.cn (Z. Ye).

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Another biomaterials widely used in protein delivery systems are polypeptides, which are poly (amino acid)s linked by peptide bonds with structures mimicking natural proteins (Deming, 2007). Previous studies showed that the polypeptide-based drug carriers could enhance protein drugs stability, bioactivity and improve drugs bioavailability (Lee et al., 2009; Sun, Huang, Shi, Chen, & Jing, 2009). However, polypeptides are usually expensive and their synthesis procedures are too complex. In recent years, poly (γ-glutamic acid) (γ-PGA) has attracted considerable attention due to its good biocompatibility and commercial scale availability through microbial fermentation (Zhang, Feng, Zhou, Zhang, & Xu, 2012). It contains lots of carboxyl side group that can be functionalized easily (Gentilini et al., 2012) and is water-soluble, biodegradable, edible and nontoxic towards human (Shih, Wu, & Shieh, 2005). Moreover, the amine group in the N-terminal γ-glutamic unit can be identified by γ-glutamyl transpeptidase in the cell membrane and thus promoting drug delivery (Peng et al., 2011; Sung, Sonaje, Liao, Hsu, & Chuang, 2012). Therefore, γ-PGA is a desirable natural polymer for proteins delivery.

Here, the HA/γ-PGA hydrogels prepared by Schiff base reaction can be formed fastly and possess good biocompatibility because of the excellent biological properties of HA and γ-PGA. Moreover the gelation time and mechanical properties as well as the protein release behavior can be tailored. In this work, the modification of HA and γ-PGA were described. The gelation time, swelling behavior, rheological properties were investigated at various conditions. The release kinetics and mechanism of protein drugs from HA/γ-PGA hydrogels were also analyzed using different mathematical models. Finally, the cell biocompatibility of HA/γ-PGA hydrogels was examined.

2. Materials and methods

2.1. Materials

HA (1000 kDa), Sodium periodate ($NaIO_4$), Hydroxylamine hydrochloride and Ethylene glycol were obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). γ-PGA (700 kDa) was obtained from Shineking Biotechnology Co., Ltd. (Nanjing, China). Fluorescein isothiocyanate conjugated bovine serum albumin (FITC-BSA), Acridine orange (AO) and Ethidium bromide (EB) were purchased from Solarbio Life Sciences (Beijing, China). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), Adipic acid dihydrazide (ADH) and N-hydroxy-succinimide (NHS) were obtained from Aladdin Industrial Corporation (Shanghai, China). All chemicals are analytical and not further purified.

2.2. Synthesis of ploymers

HA-CHO with different oxidation degree (OD) were synthesized by oxidizing the proximal −OH groups of HA using $NaIO_4$ (Ito et al., 2007). γ-PGA-ADH with different degree of substitution (DS) were synthesized *via* a facile carbodiimide coupling reaction of γ-PGA and ADH using EDC and NHS (Yan et al., 2014). The details were in the supplementary data.

2.3. Characterization of polymers

The modification of HA and γ-PGA were confirmed by ^{1}H NMR (Bruck Avance 400 MHz) and FTIR spectrophotometer (Nicolet-6700 spectrometer from Thermo Electron). ^{1}H NMR spectra were used to characterize the DS of ADH to the γ-PGA side chains. The DS was defined as the number of substituents per 100 carboxyl groups in γ-PGA. The functional groups of γ-PGA, ADH, γ-PGA-ADH, HA and HA-CHO were identified in the region of 2000–700 cm^{-1} by FTIR spectroscopy at room temperature.

Table 1
Compositions and rheological parameters of the γ-PGA/HA hydrogel samples.

Sample		DS of γ-PGA-ADH/(%)	OD of HA-CHO/(%)	Solid content/(%)	Molar ratio of $-NH_2/-CHO$	σ_c/(Pa)	tan δ
Change the solid content	A_1	36.2	19.2	*1*	1:1	–	–
	A_2	36.2	19.2	*3*	1:1	450	0.020
	[a]A_3	36.2	19.2	*5*	1:1	660	0.025
	A_4	36.2	19.2	*7*	1:1	700	0.025
Change the molar ration of $-NH_2/-CHO$	B_1	36.2	19.2	5	*2:1*	1080	0.0076
	[a]B_2	36.2	19.2	5	*1:1*	660	0.025
	B_3	36.2	19.2	5	*1:2*	619	0.029
	B_4	36.2	19.2	5	*1:3*	405	0.053
Change the DS	C_1	*24.3*	19.2	5	1:1	790	0.025
	C_2	*30.9*	19.2	5	1:1	630	0.015
	[a]C_3	*36.2*	19.2	5	1:1	660	0.025
	C_4	*42.4*	19.2	5	1:1	380	0.017
Change the OD	D_1	36.2	*11.5*	5	1:1	234	0.017
	D_2	36.2	*15.7*	5	1:1	314	0.020
	[a]D_3	36.2	*19.2*	5	1:1	660	0.025
	D_4	36.2	*22.1*	5	1:1	670	0.022

[a] The samples A_3, B_2, C_3 and D_3 are the same sample.

2.4. Preparation and characterization of hydrogels

HA-CHO was dissolved in PBS solution (0.01 mol/L, pH = 7.4) overnight in the 4 °C refrigerator and γ-PGA-ADH was dissolved in PBS at room temperature. Then, the two solutions were rapidly mixed by the oscillator (1500 rpm for 5–10 s) to prepare hydrogels. The compositions of all hydrogels are listed in Table 1.

Gelation time was measured by the vial tilting method (Wang et al., 2016). According to the compositions in Table 1, HA-CHO and γ-PGA-ADH were mixed in a vial rapidly. The time until the mixtures don't flow was considered as the gelation time. This test was carried out in triplicate.

Microporous morphologies of hydrogels were observed by scanning electron microscopy (SEM, Philips-FEI, Holland). Firstly, the HA/γ-PGA hydrogels were freeze-dried for 3 days, and then the cross-sectional were treated with gold coating and viewed by SEM.

Rheological measurements were carried out with an Anton Paar rheometer (MCR302) using a 25 mm plate–plate sensor with a proper gap. The hydrogel samples were prepared directly on the plate at room temperature. The linear viscoelastic region of all hydrogel samples were determined by the strain amplitude sweep within the range of 0.1–1000% at 1.0 Hz. Frequency sweep tests were carried out at a constant strain obtained from the linear viscoelastic domain. The frequency was varied from 0.6 to 100 Hz. These two tests were performed at 37 °C.

For the swelling test, the hydrogel samples with different components were immersed in PBS at 37 °C for 48 h until the hydrogels reach steady state. The swollen hydrogels were taken out and removed the surface water gently, and then weighed (W_s) immediately. After that, the hydrogels were lyophilized for 3 days and weighed (W_d). This test was carried out in triplicate and the swelling ratio was defined as: $(Ws - W_d)/W_d$

2.5. Preparation of BSA-loaded hydrogels and BSA release study

An *in situ* polymerization method was used to incorporate FITC-BSA molecules into hydrogel (Leacha & Schmidt, 2005). Briefly, FITC-BSA was initially dissolved in PBS to get a 0.02 wt% solution, and then HA-CHO and γ-PGA-ADH were dissolved in 0.02 wt% FITC-BSA solution, respectively. After that, these two solutions were mixed to prepare the BSA-loaded hydrogels at room temperature.