Carbohydrate Polymers 85 (2011) 717–725

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Detailed characterization of an injectable hyaluronic acid-polyaspartylhydrazide hydrogel for protein delivery

Rui Zhang, ZhiBing Huang, MingYuan Xue, Jing Yang, TianWei Tan*

Beijing Key Laboratory of Bioprocess, Beijing University of Chemical Technology, Beijing 100029, PR China

ARTICLE INFO

Article history:
Received 6 January 2011
Received in revised form 31 January 2011
Accepted 4 February 2011
Available online 10 February 2011

Keywords:
Hydrogel
Hydrazone
Injectable
Characteristics
Protein delivery

ABSTRACT

The authors previously reported an injectable crosslinking hydrogel system based on the reaction between a hyaluronic acid (HA) derivative and α,β-polyaspartylhydrazide (PAHy). The HA derivative carrying dialdehyde (HAALD) was shown to react fast (30 s) with PAHy under mild conditions to produce a hydrazone conjugated hydrogel, without addition of crosslinker or catalyst. The present paper completes the initial research by examining the properties of the hydrogels in detail. HAALD-PAHy hydrogels were synthesized in PBSA solution and characterized by different methods including gel content and swelling, Fourier transformed infrared spectra, thermogravimetric analysis, in vitro degradation and biocompatibility experiments. A scanning electron microscope viewed the interior morphology of HAALD-PAHy gel whose porous three-dimensional structure enabled it to efficiently encapsulate the proteins. Sustained and stable protein release from the HAALD-PAHy hydrogel was observed during in vitro delivery experiments and exhibited its potentially high application prospect in the field of protein drug delivery.

1. Introduction

Hydrogels are a polymer chain network, producing a colloidal gel containing over 99% water. Hydrogels offer advantages of high adsorbancy and permeability, and their medical and pharmaceutical uses are significant in wound dressing, skin grafts, oxygen-permeable contact lenses and biodegradable delivery of drugs, including proteins.Therapeutically active proteins have been widely used, are well-accepted as important source of medicine, and are considered of high potential in modern bio-therapeutics (Censi et al., 2009; Kobsa & Saltzman, 2008; Ottenbrite, Park, & Okano, 2010; Pavlou & Reichert, 2004; Smith, D, Fears, & Poste, 1993). Proteins with high molecular weight and complex structure are more easily degraded and denatured than traditional small molecule drugs. Because of the instability in acidic gastric fluid, proteins are hard to be absorbed through oral intake (Wang et al., 2010). To enhance the protein delivery, intra-muscular or intravenous injections are recognized as potential (Qiu et al., 2003; Taylor & Amidon, 1995) albeit challenging methods (Censi et al., 2009; Schwoerer, Harling, Menzel, & Daniels, 2008). A high dosage of proteins has to be given during normal injections to compensate the quick inactivation of proteins in vivo, although simultaneously the therapeutic concentration of each protein should be controlled within its safety and efficacy range. Much attention has hence been paid to develop an appropriate system to overcome the above concerns (Censi et al., 2009; Ottenbrite et al., 2010.

Injectable hydrogels, with three dimension network structures, possess the ability to entrap proteins and are considered as important protein delivery systems (Ottenbrite et al., 2010). They can be formed by either physical or chemical crosslinking (Ostrowska-Czubenko & Gierszewska-Druzynska, 2009; Gombotz & Wee, 1998; Janes, Fresneau, Marazuela, Fabra, & Alonso, 2001; Sagle, Ju, Freeman, & Sharma, 2009; Kafedjiiski et al., 2007). Physically cross-linked hydrogels, such as hydrogels combined by ionic interaction, cannot sustain release over a long period due to their relatively poor stability. The hydrogel of the present research was synthesized from a hyaluronic acid (HA) derivative and α,β-polyaspartylhydrazide (PAHy) via chemical combination between aldehyde and amine groups.

Hyaluronic acid (HA), is an endogenous polysaccharide composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine with β(1→4) interglycosidic linkage (Graham, 1998; Graham, 1998; Lee & Mooney, 2001; Nguyen & West, 2002). In view of its good biological activity and biodegradability, HA has been used widely in protein and gene delivery applications. Lee, Chung, and Kurisawa (2009) reported an injectable hyaluronic acid-tyramine hydrogel system that can achieve stable delivery of therapeutic proteins. Nakaji-Hirabayashi, Kato, and Iwata (2009) synthesized a neural cell carrier based on hyaluronic acid, which can incorporate a brain-derived neurotrophic factor and successfully enhances cell survival in the carrier system. The HA derivative carrying aldehyde (HAALD),

* Corresponding author. Tel.: +86 10 64416691; fax: +86 10 64794689.
E-mail address: twtan@mail.buct.edu.cn (T. Tan).

0144-8617/$ – see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.carbpol.2011.02.014

produced by oxidation of HA by sodium periodate, plays an important role in preparing an injectable system due to the functional dialdehyde group. Gurski, Jha, Zhang, Jia, and Farach-Carson (2009) developed a HAALD-HAADH system for poorly adherent bone metastatic prostate cancer cells (C4-2B) for use as an in vitro platform for anti-cancer drug screening.

PAHy, a derivative of polyaspartic acid (PASP), has been related to many biomedical applications attributed to its protein-like structure (Pitarresi, Palumbo, Tripodo, Cavallaro, & Giammona, 2007; Pitarresi et al., 2000; Cao, Ma, Sun, Su, & Tan, 2010). PAHy has a high molecular weight and good biocompatibility: it was previously recommended by Giammona, Cavallaro, Pitarresi, and Pedone (2000); Pedone, Cavallaro, Richardson, Duncan, and Giammona (2001) for preparing PAHy-GTA copolymer for gene delivery, and proposed as polymeric copolymer chains connected with hydrophilic (PEG) or hydrophobic (C16) moieties by Cavallaro (Cavallaro, Licciardi, Mandracchia, Pitarresi, & Giammona, 2008). In 2008, Paolino et al. (2008) prepared gemcitabine-loaded supramolecular vesicular aggregates (SVAs) containing synthesized PAHy derivatives and successfully achieved an improvement of anticancer drug activity.

The present research used a novel fast-gelling HAALD-PAHy hydrogel with different gel precursor concentrations in PBSA solution (pH = 7.4): the advantage is related to its preparation under mild conditions and without catalyst and/or cross linker.

This chemistry of hydrazone conjugation, occurring during the crosslinking step, overcomes the drawbacks of previous techniques such as the carboiimide and maleimido/thiol chemistries. The preparation is straightforward, has a high yield and is of considerable attraction in drug design and delivery due to its biological activity.

To examine its properties and drug release potential, different characteristics were examined and are reported in the present paper. Fourier transformed infrared (FTIR) spectroscopy was used to confirm the cross-linking reaction. Gel content and swelling tests were applied to characterize the cross link density of the HAAALD-PAHy hydrogel. The glass transition temperature will be identified by thermogravimetric analysis. Additional tests of in vitro degradation, SEM imaging, in vitro citotoxicity and release rates of encapsulated bovine serum albumin (BSA) were further used to assess the overall potential and applicability of the HAALD-PAHy hydrogel.

2. Experimental methods and procedures

2.1. Materials

Hyaluronic acid (HA, 1190 kDa) was supplied by Shandong Freda (China). Polysuccinimide (PSI, 200 kDa) was prepared in our laboratory, hyaluronidase was procured from Sigma. Bovine serum albumin (BSA) was purchased from Beijing Xin av Bureau Bioteknologi Co., Ltd., No. 5003-100g. Additional commercially available chemicals, i.e. hydrochloric acid (HCl), sodium hydroxide (NaOH), N′N-dimethylformamide (DMF), sodium periodate ($NaIO_4$), diamid hydrate ($H_2N{\cdot}NH_2{\cdot}H_2O$), sodium chloride (NaCl), potassium dihydrogen phosphate trihydrate ($KH_2PO_4{\cdot}3H_2O$), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were all of analytical grade.

2.2. Preparation of HAALD and PAHy

HA was oxidized to HAALD by $NaIO_4$. Firstly, 0.3 g of HA was dissolved in 30 mL pH = 6.0 deionized water (pH was adjusted using 0.1 M HCl) to form a transparent solution. Subsequently, 3.6 mL of 0.25 mol/L sodium periodate solution was added and continuously stirred for 3 h in the dark at 30 °C. Stirring was continued for 30 min after 30 mL ethylene glycol had been added to terminate the reaction. The mixture was dialyzed (Dialysis sack No. SL07202, Sigma, U.S.A.) against deionized water. The purified product with 59.27% aldehyde content and molecular weight of 3830 kDa was prepared after freeze-drying.

PAHy was synthesized from PSI by diamid hydrate according to the method described by Pitarresi et al. (2007): 1 g of PSI, dissolved in 10 mL DMF, was grafted on hydrazine by adding 0.5 g diamid hydrate into the solution. PAHy appears immediately as a deposit and can be easily separated by filtration through a filter paper (No. 102, Hang Zhou special-paper Co., Ltd., China). The deposit was dissolved in deionized water and the solution was ultrafiltered (Ultrafiltration membrane No. PLBC07610, Millipore Corporation, U.S.A.; Stirred cell No. 8200, Millipore Corporation, U.S.A.) to remove the extra DMF as well as the unreacted diamid hydrate. The purified product was also freeze-dried and kept at 4 °C.

2.3. Preparation of HAALD-PAHy hydrogel

Three solutions were prepared by dissolving respectively 0.015 g, 0.025 g and 0.035 g HAALD in a PBSA solution, consisting of NaCl (7.99 g/L), KCl (0.22 g/L,), $K_2HPO_4{\cdot}3H_2O$ (0.23 g/L) and Na_2HPO_4 (1.15 g/L). The pH was adjusted to 7.40 by 0.1 M HCl. As a result, 3 polymer solutions were obtained, with concentrations of respectively 3%, 5% and 7%. In parallel, the same amounts of PAHy were dissolved in PBSA to produce and PAHy solutions with identical wt% concentrations. All polymer solutions were transferred into 5 mL centrifuge tubes and immediately mixed by vortex mixer. They were subsequently dipped into a water bath at a constant 37 °C to complete the cross linking reaction.

2.4. FTIR-spectroscopic measurement

Fourier transformed infrared (FTIR) spectra of HAALD, PAHy and HAALD-PAHy hydrogel were measured to confirm the expected cross-linking reaction. Various samples were recorded with FTIR spectrometer (3100FTIR, Varian, USA) against a blank KBr pellet background.

2.5. Gel content

The hydrogel was freeze-dried after a reaction period of 24 h at 37 °C. The insoluble part was extracted by dissolving the gel in deionized water for 24 h and then freeze-drying it. The gel content was defined by:

$$\text{Gel content} = \frac{W_1}{W_0} \times 100\%$$

- W_0 is the weight of dry gel before being soaked in deionized water.
- W_1 is the weight of dry gel after soaking in deionized water.

2.6. Swelling test

To measure the equilibrium swelling value of the untreated hydrogel, a swelling test was performed in deionized water or PBSA solution at 37 °C for 24 h. The gel was subsequently weighed again after freeze-drying at −20 °C for 10 h. The equilibrium swelling value of the gel was calculated as follows:

$$\text{SW} = \frac{w_1 - w_2}{w_2}$$

w_1 is the weight of the swollen gel and w_2 is the weight of dry gel.

2.7. Thermogravimetric analysis (TGA)

A Netzsch STA 449C (Germany) thermogravimetric analyzer (TGA) was used to define the thermal properties of PAHy and dry gels. The analysis was carried out on a 5 mg sample in aluminum scales under Ar atmosphere at the flow rate of 24 mL/min. The temperature was raised from 35 to 500 °C at a constant heating rate (temperature ramp) of 10 °C/min.

2.8. In vitro degradation

0.1 g dry gel was incubated in 20 mL PBSA solution (pH = 7.4) or PBSA solution supplemented with HAse (100 U/mL) under continuous shaking (100 rpm) and at a constant temperature (37 °C). The incubation medium was changed daily. At fixed time intervals, i.e. after 4 d, 7 d, 14 d and 28 d of incubation, the medium solution was removed, the gel was washed three times with deionized water, was then lyophilized and weighed. The weight loss was quantified by:

$$W_t\% = \frac{(w_{d0} - w_{dt}) \times 100\%}{w_{d0}}$$

where w_{d0} is the initial dry polymer weight and w_{dt} is the dry polymer weight at the fixed time intervals.

2.9. Morphologies

Morphologies of a pristine hydrogel and degraded hydrogels were characterized by utilizing scanning electron microscopy (SEM). The samples were freeze-dried and then gold-coated. The surface and cross-sectional morphologies were viewed by means of a Hitachi S-570 SEM (Tokyo, Japan).

2.10. Protein loading

HAALD-PAHy hydrogels encapsulated with BSA were immersed into buffer solutions for an in vitro protein release study. BSA was firstly dissolved in a PBSA solution with a concentration of 30 mg/mL. Secondly, adding 0.015 g, 0.025 g or 0.035 g HAALD or PAHy to 0.50 mL BSA solution, polymer solutions of respectively 3%, 5% and 7% concentrations were formed. Thirdly, 0.50 mL HAALD solutions containing protein was added into 5 mL centrifuge tube, then the equal volume PAHy solutions also containing BSA followed and these solutions mixed by vortex mixer immediately. The centrifuge tube was finally dipped into a water bath to keep the reaction temperature constant at 37 °C for 24 h until the cross linking reaction was completed.

2.11. In-vitro protein release

BSA was used as a macromolecular protein model drug to determine the protein release rate from the HAALD-PAHy hydrogel. Three hydrogels of different composition were prepared by adding respectively 30 mg BSA in 1 mL 3% hydrogel; 30 mg BSA in 1 mL 5% hydrogel; and 30 mg BSA in 1 mL 7% hydrogel. They were suspended in PBSA solutions (pH = 7.4), and the maximum release concentration of BSA was 200 μg/mL for each release test. At predetermined intervals, 1.0 mL of release medium was collected for testing and replaced by 1.0 mL of fresh PBSA. The quantification of BSA released from hydrogels was obtained by UV–visible spectrophotometer at absorbance of 595 nm using the coomassie brilliant blue method (Bradford, 1976).

To assess the rate-controlling mechanism of protein release, both the Weber–Morris (Hall, Tang, Baeyens, & Dewil, 2009) and Ritger–Peppas models (Ritger & Peppas, 1987) were used to evaluate the BSA release. Calculate the in vitro protein release results as following equations.

$$\text{Weber-Morris Model}: \ q_t = kt^{0.5} + C$$

where q_t is the concentration of BSA released from hydrogel to the PBSA solution, $t^{0.5}$ is the square root of releasing time, k is the intraparticle diffusion rate constant (μg/(mL $\text{min}^{0,5}$)) and C is a constant.

$$\text{Ritger-Peppas Model}: \ Q = k_{RP}t^n$$

where Q denotes the concentration of BSA in the PBSA solution at time t, when it moves from hydrogel into the PBSA solution, k_{RP} is the Ritger–Peppas release rate constant characteristic of the controlled release mode and n is the diffusional exponent, indicative of the mechanism of drug release.

2.12. Biocompatibility

To evaluate the biocompatibility of the HAALD-PAHy hydrogel, use was made of WST-8 dye (Beyotime Inst Biotech, China) to investigate the in vitro cytotoxicity of the gel against mouse fibroblasts (L-929). 2.0 g sterile hydrogel were added to 10.0 mL growth medium, and kept in a 37 °C incubator containing 5% CO_2 in the air for 24 h in preparation of extraction. The concentration of original extract was 200 mg/mL, and diluted with growth medium to 100 mg/mL, 50 mg/mL and 25 mg/mL after centrifugation and filtration. L-929 cells were seeded into 96-well cell culture plates with a same density of 1000 cells per well. Subsequently, the culture medium in the 96-well plate, which had been incubated again to allow the cells to grow to confluence during 24 h, was replaced by 0.20 mL extract of different concentrations. After 3 d, 5 d and 7 d of incubation (37 °C, 5% CO_2), 20.0 μL of CCK-8 solution with WST-8 dye were added to each well and the plate was returned to the incubator and incubated for a further 4 h. Finally, the optical density (O.D.) of the resulting solution in each well was determined at 450 nm using a micro plate reader, and a relative growth rate (RGR) was used to identify the cytotoxicity.

$$\text{RGR} = \frac{(\text{average O.D. of experimental group}) \times 100}{(\text{average O.D. of negative control})}$$

3. Results and discussion

3.1. Characterisation by physico-chemical analyses methods

3.1.1. HAALD-PAHy hydrogel formation and gelation time

The diol structure of HA was transformed by $NaIO_4$ into a dialdehyde group, which is more active than diol. A PAHy containing hydrazide group was prepared from PSI. As shown in Fig. 1, the generated dialdehyde of HAALD can react spontaneously with hydrazide of PAHy to yield a hydrazone conjugated hydrogel (Fig. 1) in a PBSA solution without addition of cross linker or catalyst (Hermanson, 2008; Tan, Chu, Payne, & Marra, 2009), water being the only by-product.

A short gelation time is a crucial qualification for an in situ cross linked injectable hydrogel. The gel precursor with slow gelation rate leads to fluids diffusion away from the injection site and causes undesired gel formation (Gupta, Tator, & Shoichet, 2006; Hou, De Bank, & Shakesheff, 2004; Lee, Chung, & Kurisawa, 2008; Lee et al., 2009).

Because of the high gelation rate, it is impossible to assess the gelation point of HAALD-PHAy hydrogel by rheology. An inversion method was therefore used to observe the sol–gel phase transition and test the gelation time of HAALD-PAHy hydrogel at 37 °C in a

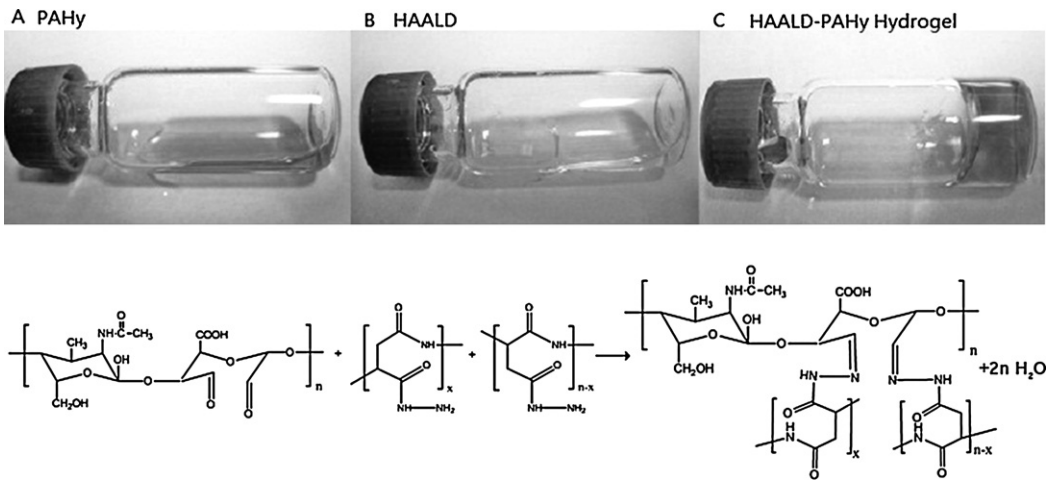

Fig. 1. Illustration of the state of gel precursors (A) and (B) and hydrogel (C) and the corresponding reaction equation.

water bath (Jeong, Bae, & Kim, 1999). On the basis of the above-mentioned hydrogel preparation steps, the time was counted from the mixing point. A gel state formed in 30 s and no fluidity can be visually observed by inverting the tube. By varying the concentrations of HAALD and PAHy solutions, the gelation time did not alter significantly, the gelation time of 5% hydrogels being 25 s.

3.1.2. FTIR characterization of HAALD, PAHy and HAALD-PAHy hydrogel

After dialysis and freeze-drying steps, the FTIR spectrum (Fig. 2) was used to confirm the hydrazide-dialdehyde reaction. We found that dialdehyde absorption peak (2879.82 cm^{-1}) and dialdehyde stretch peak (1732.71 cm^{-1}) which associate with the C=O group of HAALD, disappeared in the IR curve of HAALD-PAHy hydrogel. At the same time, compared with the IR curve of PAHy, the amide absorption peaks of hydrogel shifted from 1665.39 cm^{-1} and 1521.52 cm^{-1} to respectively 1639.44 cm^{-1} and 1556.83 cm^{-1}, corresponding to the consumption of hydrazide and dialdehyde group to form the hydrazone bond between HAALD and PAHy. The IR curve details proved the cross-linking reaction as Fig. 1 showed.

Complementary to FTIR-analysis, the modified functionalities of raw materials and hydrogel product can also be determined by solids nuclear magnetic resonance, where each specific molecular environment of the molecules is identified according to a specific proton chemical shift. The NMR-analysis is currently performed and results will be reported in a follow-up paper, together with a more detailed investigation of the protein release studies.

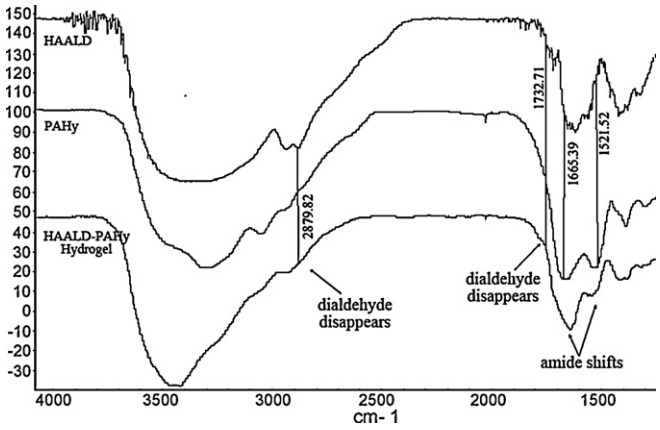

Fig. 2. FTIR spectra of HAALD, PAHy and freeze-dried HAALD-PAHy hydrogel. (HAALD: concentration of $NaIO_4$ solution: 0.25 mol/L, volume of $NaIO_4$ solution: 3.6 mL, HA: 0.3 g, reaction time: 3 h, reaction temperature: 30 °C, pH of HA solution: 6; PAHy: FTIR spectra of PAHy, which produced at room temperature after 4 h reaction; HAALD-PAHy hydrogel: reaction solution: PBSA, pH of reaction solution: 7.4, concentration of gel precursor was 5%, reaction temperature: 37 °C).

3.1.3. Properties of the hydrogel

HAALD-PAHy hydrogels were characterized by their gel content test, their glass transition temperature and their swelling behavior.

The gel content of each sample was determined by measuring its insoluble part after extraction in distilled water for 24 h at room temperature. The gel content is a basic parameter to characterize the three-dimensional network structure of a gel. Table 1 shows that the gel contents of HAALD-PAHy are a function of the concentration of gel precursor solutions. The highest gel content was 78.55%, for the sample with 5% concentration of gel precursor solution. The gel content of 3% gel and 7% gel were 72.38% and 54.42% respectively. The gel content of 5% gel exceeds that of 3% gel since the higher active group density of a more concentrated gel precursor solution facilitates the creation of a more complete gel net structure. The 7% gel had the lowest gel content due to the high viscosity of HAALD, a well-known property of HA derivatives with applied biological functions in tissue engineering. The high viscosity however blocks the conjugation between the active groups of HA derivative and other polymers, with a low gel content as result. Within the experimental range and concentrations, the 5% gel has the most complete three-dimensional network structure.

The correspondent values of gel glass transition temperature (T_g) were also tested and presented in Table 1. The T_g trend of samples was consistent with their gel content. Differences in T_g are mainly dependent on the cross link density of gels. The high crosslink density, represented by multiple cross linking points, restricts the motion of chains and enhances the thermal property of gels. The 5% gel has the highest cross link density as well as the most complete three-dimensional network structure among all the gel samples.

Table 1
Characterization of hydrogels (HAALD-PAHy hydrogels: reaction solution: PBSA, reaction temperature: 37 °C, concentrations of gel precursors were 3%, 5% and 7% respectively).

Gel content (%)		T_g (°C)	Equilibrium swelling values	
			In deionized water	In PBSA solution
3 gel	72.38	99.4	71.815	35.792
5 gel	78.55	107.6	56.567	19.839
7 gel	54.42	96.1	159.246	40.431

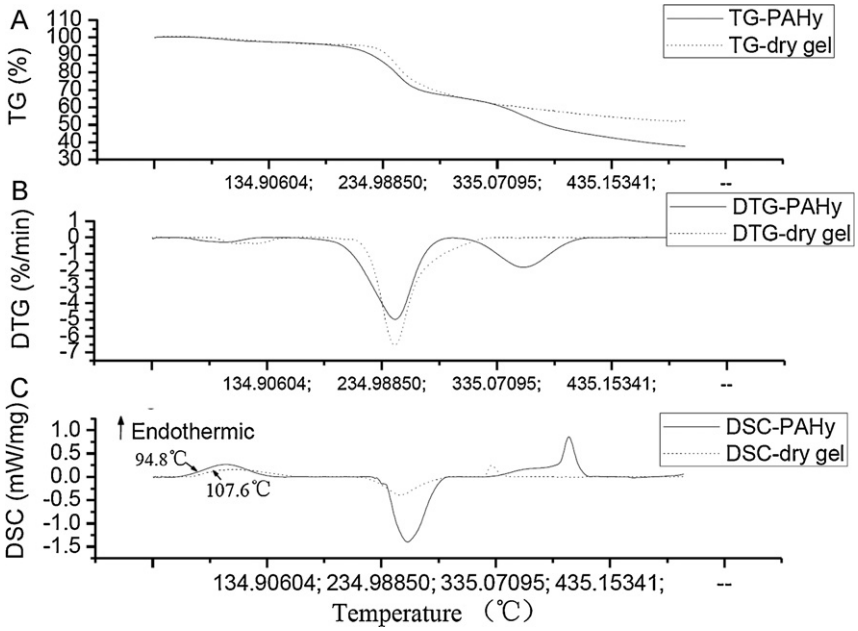

Fig. 3. Thermogravimetric analysis of PAHy and hydrogel. (A) TG curve of PAHy and HAALD-PAHy gel. (B) DTG curve of PAHy and HAALD-PAHy gel. (C) DSC curve of PAHy and HAALD-PAHy gel (PAHy: produced at room temperature after 4 h reaction; HAALD-PAHy hydrogel: concentration of gel precursor was 5%, reaction solution: PBSA, reaction temperature: 37 °C).

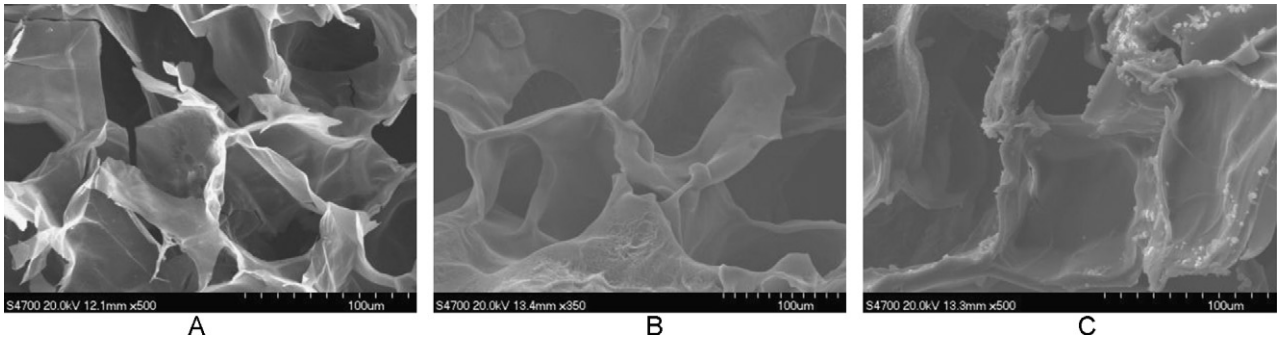

Fig. 4. SEM photographs of freeze-dried hydrogel samples. (A) SEM photograph of a pristine hydrogel sample. (B) SEM photograph of a sample degraded in PBSA solution. (C) SEM photograph of a sample degraded in PBSA solution containing HAse (100 U/mL). (HAALD-PAHy hydrogel: concentration of gel precursor was 5%, reaction solution: PBSA, reaction temperature: 37 °C).

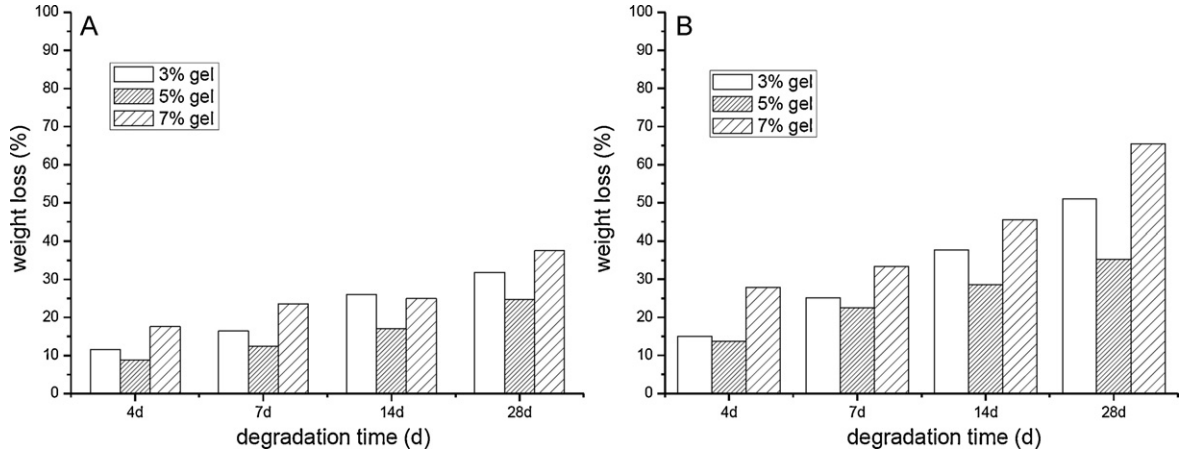

Fig. 5. Relationship between weight loss and degradation time in a degradation medium. (A) In vitro degradation of hydrogel in PBSA solution. (B) In vitro degradation of hydrogel in PBSA solution containing HAse (100 U/mL). (HAALD-PAHy hydrogels: concentrations of gel precursors were 3%/5%/7%, reaction solution: PBSA, reaction temperature: 37 °C).

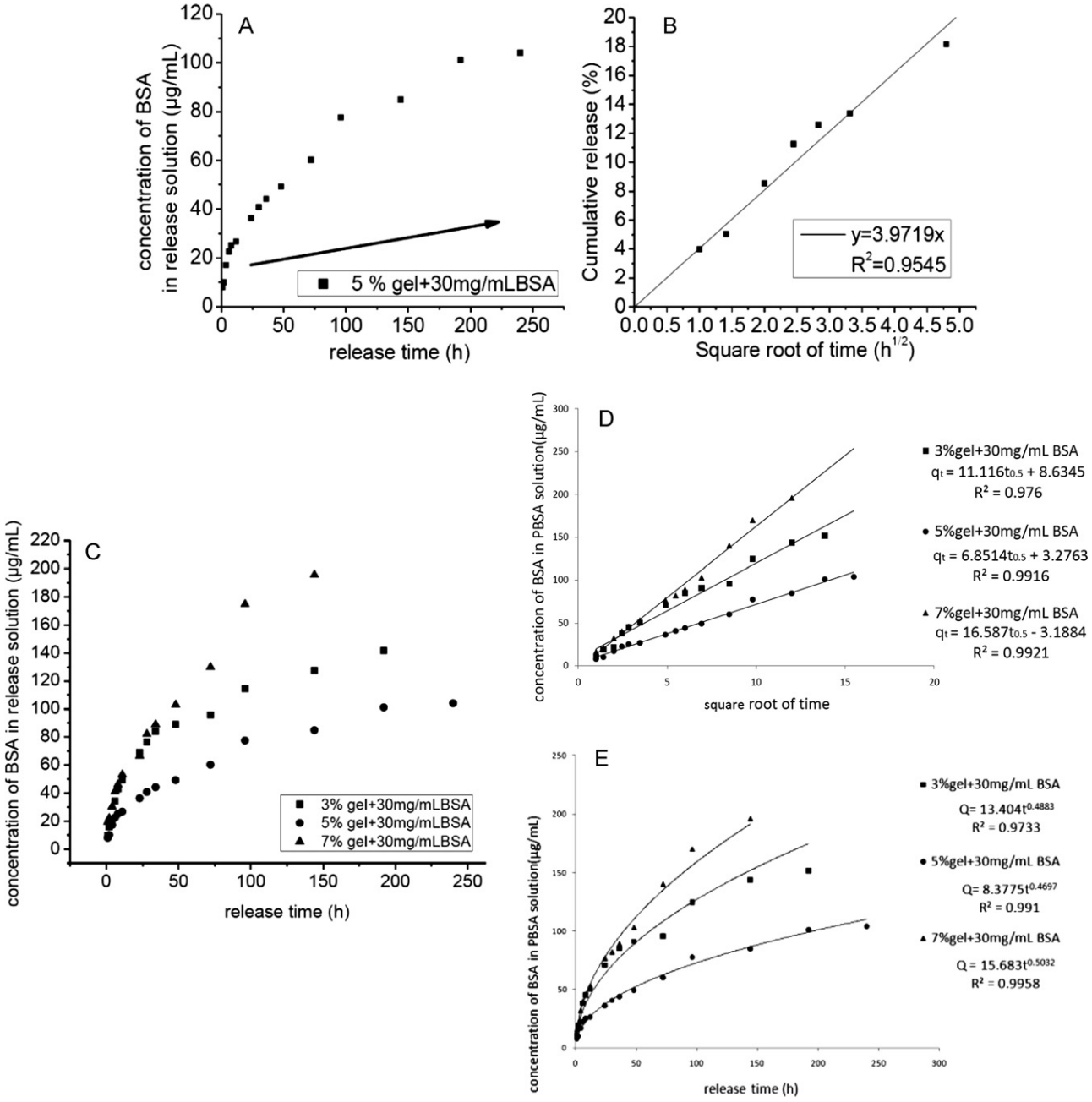

Fig. 6. In vitro protein release of HAALD-PAHy hydrogels. (A) and (B) In vitro protein release of 5% HAALD-PAHy hydrogel. (C) Relationship between protein release rate and hydrogel concentrations. (D) Weber–Morris analysis. (E) Ritger–Peppas analysis. (HAALD-PAHy hydrogels: reaction solution: PBSA, reaction temperature: 37 °C, concentrations of gel precursors were 3%, 5%, and 7% separately, concentration of BSA was 30 mg/mL, release solution was PBSA, pH = 7.4).

The equilibrium swelling values of Table 1 confirm the relationship between cross link density and the concentration of gel precursor solutions. A high swelling value is one of the basic requirements for hydrogels used in biomedical and tissue engineering (Liu, Liu, Wang, Du, & Chen, 2007). The swelling value is correlated with the gel cross link density and can be used to evaluate it (Lee et al., 2009; Cai & Kim, 2010). The equilibrium swelling value will usually increase with the decrease of cross link density, thus being inverse to the T_g criterion: the high equilibrium swelling value of 7% gel disclosed its low cross link density and the low equilibrium swelling value of 5% gel indicated its high cross link density.

Clearly, the 5% gel is preferred due to the highest crosslink density and the most complete three-dimensional network structure of all the samples. The 3% gel ranked second while the 7% gel was the worst.

3.1.4. Thermogravimetric analysis (TGA)

The TG and the corresponding DTG curves of PAHy and HAALD-PAHy dry gel are shown in Fig. 3. The TG and DTG curves of the gel exhibit two thermal degradation steps only, whereas PAHy, shows three stages. The first thermal event, for both the PAHy and HAALD-PAHy gel degradation curves, occurs in the temperature range 35–150 °C with weight loss ranging from 2.71% to 3.64% as a result of the evaporation of water and low molecular products, although the temperature (107.1 °C) when the HAALD-PAHy gel mass loss reaches a maximum, is higher than that of PAHy (99.1 °C) due to the good hydrophilic property of HAALD. When cross linked to hydrophilic HAALD, the interaction force between the water and the backbone of HAALD-PAHy gel was strengthened, and the water was harder to remove. The weight loss of gel was higher than that of PAHy since more water and low molecular products were intro-

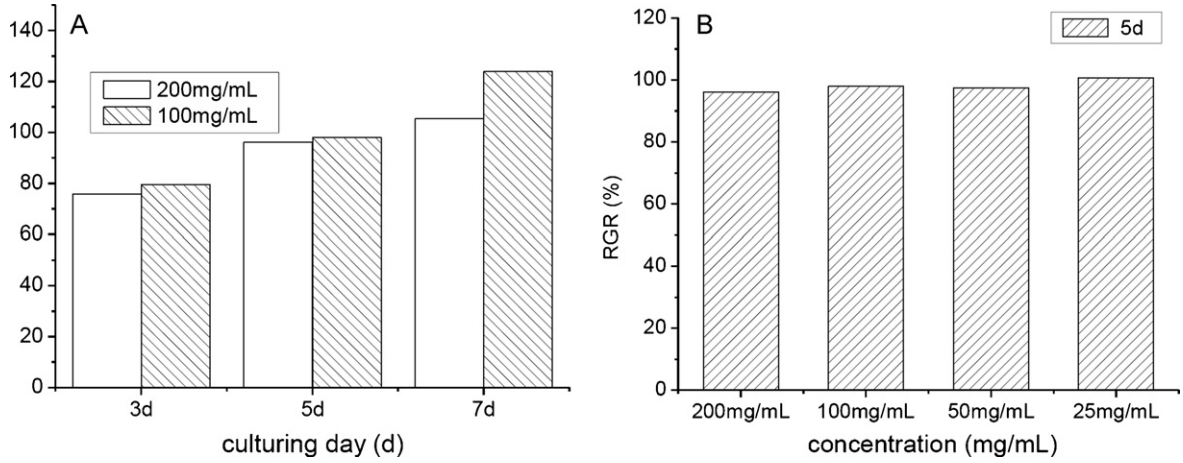

Fig. 7. The relative growth rate of L-929 cells proliferated in different concentrations of HAALD-PAHy hydrogel extracts for several days. (A) RGR% of L-929 cells proliferated in 200 mg/mL and 100 mg/mL extracts for 3d/5d/7d. (B) RGR% of L-929 cells proliferated in 200/100/50/25 mg/mL extractions for 5d. (HAALD-PAHy hydrogel: reaction solution: PBSA, reaction temperature: 37 °C, concentration of gel precursor was 5%).

duced into the gel system via the cross linking reaction. The second thermal stage is attributed to the chemical bond breakdown of the polymer chain. The second thermal degradation step of HAALD-PAHy gel occurs between 200 and 350 °C with a weight loss of 47.76%, slightly higher than that of PAHy, and associated with the poor heat-resistant performance of HAALD. The third degradation stage, which corresponded to the decomposition of samples, was only present in PAHy with a maximum weight loss at 358.2 °C. These results demonstrated that the crosslinked HAALD-PAHy gel had good thermal properties and was able to resist decomposition at high temperature.

Differential scanning calorimetry (DSC) is convenient to measure the glass transition temperature (T_g), and DSC results are illustrated in Fig. 3C, showing T_g of PAHy (94.8 °C) and HAALD-PAHy dry gel (107.6 °C), respectively. The glass transition temperature (T_g) is an important characteristic parameter of materials, and affected by numerous factors, such as structure of the main chain, its flexibility and molecular mass, cross-linking of the chains, polarizability and crystallinity of the materials. (Ignjatović, Liu, Czernuszka, & Uskoković, 2007; Ward & Sweeney, 2004). The different T_g-values of gel and PAHy are due to the chemical cross linking points formed between HAALD and PAHy, thus increasing the chain entanglement with limiting the motion of polymer chains and increasing the glass transition temperature of gel.

3.1.5. SEM

The interior morphology of the HAALD-PAHy gel (5%) before and after 28 days of in vitro degradation was observed by SEM. According to Fig. 4A, a pristine hydrogel sample showed a porous three-dimension structure with pore diameters in the range of 80–120 μm, that can effectively encapsulate a protein drug. Fig. 4B and C shows the microstructures of hydrogel samples degraded in PBSA solution (pH = 7.4) and PBSA solution containing HAse (100 U/mL). On day 28 of the degradation in PBSA solution without HAse, the hydrogel (Fig. 4B) maintained its porous structure to a large extent. However, the hydrogel sample (Fig. 4C) was eroded by HAse and presented inhomogeneous pores and cracks on the surface.

3.2. Functional characterization

3.2.1. In-vitro degradation

The hydrazone bond, formed between aldehyde of HAALD and hydrazide of PAHy is a stable Schiff base (Hermanson, 2008). Nevertheless, HAALD, a kind of polyasccharide derivative, has biodegradable properties even after cross linking. So the in vitro degradation behaviors of HAALD-PAHy hydrogels were conducted at 37 °C for 28 days in PBSA or PBSA containing HAse (100 U/mL). Comparing the weight loss data of Fig. 5A with Fig. 5B, differences were observed between the degradation rates in PBSA and in PBSA containing HAse. In Fig. 5A, all the gel samples had very slow degradation rates and maintained at least 60% of the initial weight after 28 days of incubation in PBSA. The highest weight loss of all the gels was only 37.55%, for the 7% gel. The 5% gel has the lowest weight loss only 24.66%.

As expected, the addition of HAse in PBSA accelerated the degradation rate of HAALD-PAHy hydrogels as shown in Fig. 5B. For example, the weight loss of 3% gel was increased from 31.84% to 51.04% with HAse powder. The 5% gel, with high cross link density and complete network structure, presented good resistance to HAse under the same conditions and lost only 35.27% of its initial weight in 28 days.

A high cross link density and complete network structure helps gels to maintain their weight during in vitro degradation.

3.2.2. In vitro protein release and activity of released proteins from HAALD-PAHy hydrogels

BSA was used as a model drug in the release studies. As shown in Fig. 6A and B, the release of BSA from HAALD-PAHy hydrogel presents a short period of fast release, followed however by a sustained and stable release for a long period. This release pattern can be divided into three phases and explained as follows. Firstly, the BSA near the surface of hydrogel diffused rapidly in the first few hours, due to the BSA concentration gradient inside and outside of the hydrogel. With swelling in PBSA solution, the mesh sizes of hydrogel enlarge gradually and the concentration of BSA in the release solution increases simultaneously. The linear relationship between the release concentration of BSA during the initial 24 h and the square root of the time, stresses that the release of BSA from HAALD-PAHy hydrogel is of typical Fick diffusion during the second step. Because of the interaction between BSA and HAALD-PAHy hydrogel, BSA can combine with hydrogel system and exhibit a reduced release rate at a longer time scale, typical for the third phase, where the drug delivery behavior from hydrogel is dominated both by the interaction of BSA with the hydrogel system, and by the hydrogel degradation (Bromberg & Ron, 1998; Gong et al., 2009). The sustained and stable release behavior is considered to be more suitable for achieving a desirable controlled release of protein drugs

To assess the effect of the hydrogel concentration on the protein release rate, three hydrogels, with different concentrations of 3%, 5% and 7% were used. Based on the precious findings that the higher cross linking density leads to a lower swelling ratio and slower in vitro degradation rate, the cross linking density was considered as one of the key parameters in affecting the protein delivery through diffusion and degradation. Table 1 illustrated the highest cross linking density of 5% hydrogel: it can therefore be expected that the BSA release rate of 5% hydrogel would be the lowest and the residual BSA content the highest. Fig. 6C shows the in vitro release of BSA from 3%, 5% and 7% hydrogels and demonstrates that the controlled release of BSA was achieved for 192 h, 240 h and 144 h respectively. The residual BSA contents were 50%, 95% and 5%. All the data clearly reflect the relationship between BSA release rates of different hydrogels and validated our hypothesis.

To further assess the rate-controlling step in the BSA-release, both a Weber–Morris and Ritger–Peppas approach were used, as introduced in Section 2.11. Applying Weber–Morris as q_t versus $t^{0.5}$ produces a linear relationship (Fig. 6D), indicating that intra-peptide diffusion controls the BSA release. The Ritger–Peppas correlates Q in terms of t^n, with controlling mechanisms being a function of exponent n as follows:

I. $n < 0.45$, corresponding to Fick diffusion;
II. $0.45 < n < 0.89$, corresponding to diffusion through the gel layers and gel matrix erosion;
III. $n > 0.89$, corresponding to matrix erosion.

In Fig. 6E, plot of q versus t^n provide n values of 0.47–0.5, with regression coefficient of 97.3–99.6%. According to Ritger–Peppas, this is a controlling mechanism of Type II, although the n value is very close to the Weber–Morris result, representative of Fick-diffusion control.

Comparing k_{RP} and n values among these gel systems, the 7% gel has the highest values of k and n, meaning that gel matrix erosion is more significant in it than in gel systems with lower wt%.

Further release tests are ingoing and will be used to better evaluate both model approaches.

3.2.3. Biocompatibility

The cytotoxicity of gel was quantitatively assessed and can be classified within 5 grades according to the China National Standard GB/T 16886 documents (Hao, 2001). The relationship between relative growth rate (RGR) and grade is given as following: Grade 0 (RGR ≥ 100%) and 1 (RGR = 75–99%) are considered to correspond with no cytotoxicity, Grade 2 (RGR = 50–74%) is mildly cytotoxic, whereas Grades 3, 4 and 5 (all of RGR < 50%) show moderate to significant cytotoxicity.

The results of RGR from L-929 cells, cultivated in different concentrations of HAALD-PAHy hydrogel extracts for 3d/5d/7d, are presented in Fig. 7 showing that all of RGR data exceeded 75%, indicating that the cytotoxicity of HAALD-PAHy hydrogel belongs to Grade 1 or 0. For all concentrations studied, ranging from 25 mg/mL to 200 mg/mL, the RGR were similar to each other after 5d of incubation. The highest RGR was 100.72%, cultivated in 25 mg/mL, while the lowest was 96.15% with a 200 mg/mL concentration of extract. There is no significant increase of RGR among extracts with different concentrations. In 3 d culturing, the RGR of original extracts and for a 1 time dilution were 75.82% and 79.58% respectively, and the data increased to 96.15% and 97.9%. All data were below 100%, possibly because the cells were in a new environment. Beyond 7 days, the RGR of these two extracts were 105.41% and 123.94%, indicating that the L-929 cells proliferated quicker in extracts than in the negative control solution. In conclusion, we can confirm that the HAALD-PAHy hydrogel shows no cytotoxicity, possesses a better cell growth property than the negative control, and was a biocompatible material.

4. Conclusion

A fast-gelling HAALD-PAHy hydrogel was prepared in PBSA solution (pH = 7.4). The HA derived dialdehyde (HAALD), oxidized from HA by sodium periodate, can reacted spontaneously with PAHy under mild condition and yields a hydrazone conjugated hydrogel in 30 s without addition of cross linker or catalyst. The fast gelation provides this system with injectable properties. The concentrations of gel precursors influenced the cross linking density of hydrogel which was analyzed by gel content, swelling tests, Fourier Transformed Infrared (FTIR) spectroscopy and thermogravimetric analysis. The results indicated that the 5% gel has the highest cross link density and the most complete three-dimensional network structure of all hydrogel samples. The preference for the 5% gel was further confirmed by in vitro degradation during 28 days in PBSA or PBSA containing HAse (100 U/mL), and the SEM-imaging confirmed the porous three-dimension structure. The biocompatibility and the in vitro cytotoxicity results confirm that the HAALD-PAHy hydrogel can be used to encapsulate BSA and achieve a stable and sustained release for a long time, which provides a vast application prospect in the field of protein drug delivery. The protein release rate can be controlled by adjusting the cross link density of HAALD-PAHy hydrogels. Experiments to determine the possible pH-sensitivity of the drug release are on going and will be reported in a following paper.

Acknowledgments

Contract grant sponsor: National High Technology Research and Development Program of China (863 Program); contract grant number: 2007AA100404, 2007AA10Z360. National Key Development Program for Basic Research of China (973 Program); contract grant number: 2007CB714304, National Natural Science Foundation of China; contract grant numbers: 20636010, 20876011, 20806006; National transgenic major projects: 2008ZX08012-001.

References

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, *72*, 248–254.
Bromberg, L. E., & Ron, E. S. (1998). Temperature-responsive gels and thermogelling polymer matrices for protein and peptide delivery. *Advanced Drug Delivery Reviews*, *31*, 197–221.
Cai, Z. J., & Kim, J. (2010). Preparation and characterization of novel bacterial cellulose/gelatin scaffold for tissue regeneration using bacterial cellulose hydrogel. *Journal of Nanotechnology in Engineering and Medicine*, *1*, 021002, 6 pages.
Cao, H., Ma, X. Y., Sun, S. H., Su, H. J., & Tan, T. W. (2010). A new photocrosslinkable hydrogel based on a derivative of polyaspartic acid for the controlled release of ketoprofen. *Polymer Bulletin*, *64*, 623–632.
Cavallaro, G., Licciardi, M., Mandracchia, D., Pitarresi, G., & Giammona, G. (2008). Hydrophilic and hydrophobic copolymers of a polyaspartylhydrazide bearing positive charges as vector for gene therapy. *Polymer International*, *57*, 708–713.
Censi, R., Vermonden, T., Van Steenbergen, M. J., Deschout, H., Braeckmans, K., De Smedt, S. C., et al. (2009). Photopolymerized thermosensitive hydrogels for tailorable diffusion-controlled protein delivery. *Journal of Controlled Release*, *140*, 230–236.
Giammona, G., Cavallaro, G., Pitarresi, G., & Pedone, E. (2000). Novel polyaminoacidic copolymers as nonviral gene vectors. *Colloid and Polymer Science*, *278*, 69–73.
Gombotz, W. R., & Wee, S. F. (1998). Protein release from alginate matrices. *Advanced Drug Delivery Reviews*, *31*, 267–285.
Gong, C. Y., Shi, S., Wu, L., Gou, M. L., Yin, Q. Q., Guo, Q. F., et al. (2009). Biodegradable in situ gel-forming controlled drug delivery system based on thermosensitive PCL-PEG-PCL hydrogel. Part 2: Sol–gel–sol transition and drug delivery behavior. *Acta Biomaterialia*, *5*, 3358–3370.
Graham, N. B. (1998). Hydrogels: their future, Part I. *Medical Device Technology*, *9*, 18–22.

Gupta, D., Tator, C. H., & Shoichet, M. S. (2006). Fast-gelling injectable blend of hyaluronan and methylcellulose for intrathecal, localized delivery to the injured spinal cord. *Biomaterials*, *27*, 2370–2379.

Gurski, L. A., Jha, A. K., Zhang, C., Jia, X. Q., & Farach-Carson, M. C. (2009). Hyaluronic acid-based hydrogels as 3D matrices for in vitro evaluation of chemotherapeutic drugs using poorly adherent prostate cancer cells. *Biomaterials*, *30*, 6076–6085.

Hall, S., Tang, R., Baeyens, J., & Dewil, R. (2009). Removing polycyclic aromatic hydrocarbons from water by adsorption on silicagel. *Polycyclic Aromatic Compounds*, *29*, 160–183.

Hao, H. P. (2001). (1st ed.). *GB/T16886-ISO10993* China: Standards Press of China. ISBN 756622920.

Hermanson, G. T. (2008). (2nd ed.). *Bioconjugate Techniques* Elservier. ISBN 9780123705013, 1202 pages.

Hou, Q. P., De Bank, P. A., & Shakesheff, K. M. (2004). Injectable scaffolds for tissue regeneration. *Journal of Materials Chemistry*, *14*, 1915–1923.

Ignjatović, N. L., Liu, C. Z., Czernuszka, J. T., & Uskoković, D. P. (2007). Micro- and nano-injectable composite biomaterials containing calcium phosphate coated with poly(DL-lactide-co-glycolide). *Acta Biomaterialia*, *3*, 927–935.

Janes, K. A., Fresneau, M. P., Marazuela, A., Fabra, A., & Alonso, M. J. (2001). Chitosan nanoparticles as delivery systems for doxorubicin. *Journal of Controlled Release*, *73*, 255–267.

Jeong, B., Bae, Y. H., & Kim, S. W. (1999). Thermoreversible gelation of PEG-PLGA-PEG triblock copolymer aqueous solutions. *Macromolecules*, *32*, 7064–7069.

Kafedjiiski, K., Jetti, R. K. R., Foger, F., Hoyer, H., Werle, M., Hoffer, M., et al. (2007). Synthesis and in vitro evaluation of thiolated hyaluronic. *International Journal of Pharmaceutics*, *343*, 48–58.

Kobsa, S., & Saltzman, W. M. (2008). Bioengineering approaches to controlled protein delivery. *Bioengineering Approaches to Controlled Protein Deliver*, *63*, 513–519.

Lee, K. Y., & Mooney, D. J. (2001). Hydrogels for tissue engineering. *Chemical Reviews*, *101*, 1869–1879.

Lee, F., Chung, J. E., & Kurisawa, M. (2008). An injectable enzymatically crosslinked hyaluronic acid–tyramine hydrogel system with independent tuning of mechanical strength and gelation rate. *Soft Matter*, *4*, 880–887.

Lee, F., Chung, J. E., & Kurisawa, M. (2009). An injectable hyaluronic acid–tyramine hydrogel system for protein delivery. *Journal of Controlled Release*, *134*, 186–193.

Liu, L., Liu, D. R., Wang, M., Du, G., & Chen, Ch. J. (2007). Preparation and characterization of sponge-like composites by cross-linking hyaluronic acid and carboxymethylcellulose sodium with adipic dihydrazide. *European Polymer Journal*, *43*, 2672–2681.

Nakaji-Hirabayashi, T., Kato, K., & Iwata, H. (2009). Hyaluronic acid hydrogel loaded with genetically-engineered brain-derived neurotrophic factor as a neural cell carrier. *Biomaterials*, *30*, 4581–4589.

Nguyen, K. T., & West, J. L. (2002). Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials*, *23*, 4307–4314.

Ostrowska-Czubenko, J., & Gierszewska-Druzynska, M. (2009). Effect of ionic crosslinking on the water state in hydrogel chitosan membranes. *Carbohydrate Polymers*, *77*, 590–598.

Ottenbrite, R. M., Park, K., & Okano, T. (2010). *Biomedical application of hydrogels hand-book* Springer. ISBN 1441959181. 432 pages.

Paolino, D., Cosco, D., Licciardi, M., Giammona, G., Fresta, M., & Cavallaro, G. (2008). Polyaspartylhydrazide copolymer-based supramolecular vesicular aggregates as delivery devices for anticancer drugs. *Biomacromolecules*, *9*, 1117–1130.

Pavlou, A. K., & Reichert, J. M. (2004). Recombinant protein therapeutics success rates. *Nature Biotechnology*, *22*, 1513–1519.

Pedone, E., Cavallaro, G., Richardson, S. C. W., Duncan, R., & Giammona, G. (2001). α,β-poly(asparthylhydrazide)–glycidyltrimethylammonium chloride copolymers (PAHy–GTA): novel polymers with potential for DNA delivery. *Journal of Controlled Release*, *77*, 139–153.

Pitarresi, G., Cavallaro, G., Carlisi, B., Giammona, G., Bulone, D., & San Biagio, P. L. (2000). Novel hydrogels based on a polyasparthydrazide, synthesis and characterization. *Macromolecular Chemistry and Physics*, *201*, 2542–2549.

Pitarresi, G., Palumbo, F. S., Tripodo, G., Cavallaro, G., & Giammona, G. (2007). Preparation and characterization of new hydrogels based on hyaluronic acid and α,β-polyaspartylhydrazide. *Euro Polymer Journal*, *43*, 3953–3962.

Qiu, B., Stefanos, S., Ma, J. L., Lalloo, A., Perry, B. A., Leibowitz, M. J., et al. (2003). A hydrogel prepared by in situ cross-linking of a thiol-containing poly(ethylene glycol)-based copolymer: a new biomaterial for protein drug delivery. *Biomaterials*, *24*, 11–18.

Ritger, P. L., & Peppas, N. A. (1987). A simple equation for description of solute release. I. Fickian and non-Fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs. *Journal of Controlled Release*, *5*, 23–36.

Sagle, A. C., Ju, H., Freeman, B. D., & Sharma, M. M. (2009). PEG-based hydrogel membrane coatings. *Polymer*, *50*, 756–766.

Schwoerer, A., Harling, S., Menzel, H., & Daniels, R. (2008). Release behaviour of hydrogel microparticles based on hydroxyl-ethyl-starch as a drug delivery system for proteins. *Journal of Controlled Release*, *132*, 1–18.

Smith, A. G., Dewdney, Fears, M., & Poste, R. G. (1993). Chemical derivatization of therapeutic proteins. *Trends in Biotechnology*, *11*, 397–403.

Tan, H. P., Chu, C. R., Payne, K., & Marra, K. G. (2009). Injectable in situ forming biodegradable chitosan-hyaluronic, acid based hydrogels for cartilage tissue engineering. *Biomaterials*, *30*, 2499–2506.

Taylor, M. D., & Amidon, G. (1995). *Peptide-based drug design: controlling transport and metabolism* A.C.S. ISBN 9780841230583, 567 paper.

Wang, K., Xu, X., Liu, T. T., Fu, S. Zh., Guo, G., Gu, Y. Ch., et al. (2010). Synthesis and characterization of biodegradable pH-sensitive hydrogel based on poly(e-caprolactone), methacrylic acid, and pluronic (L35). *Carbohydrate Polymers*, *79*, 755–761.

Ward, I. M., & Sweeney, J. (2004). (2nd ed.). *An introduction to the mechanical properties of solid polymers* John Wiley & Sons Ltd. ISBN 047149626X, 384 pages.