A Novel Injectable and *In Situ* Crosslinked Hydrogel Based on Hyaluronic Acid and α,β-Polyaspartylhydrazide

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ABSTRACT: A new injectable and *in situ* crosslinked hydrogel, based on a hyaluronic acid (HA) derivative and α,β-polyaspartylhydrazide (PAHy), was produced during the research. This biodegradable and high molecular weight hydrogel can be *in situ* crosslinked in 15 s by the condensation reaction between aldehyde and amine groups. The HA derivative carrying aldehyde (HAALD) was oxidized from HA by sodium periodate, while the synthesis of hydrogel was performed in a phosphate-buffered saline solution (PBS) using HAALD and PAHy without addition of crosslinker or catalyst. The pH and concentration of the reaction solution, considered as the important factors of the amine-aldehyde reaction, were changed to reveal the crosslinking rule. Thereafter, crosslinked hydrogels were characterized by gelation time, gel content, swelling ratio, and *in vitro* degradation. Furthermore, the modified polymers were characterized by Fourier transformed infrared (FTIR) spectroscopy to examine their structures. Results from scanning electron microscope (SEM) observations confirmed that a freeze-dried sample revealed a porous hydrogel structure with interconnected pores. The measurement of the cell adhesion confirmed the application potential of HAALD-PAHy hydrogels. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 125: 1116–1126, 2012

Key words: hyaluronic acid; α,β-polyaspartylhydrazide; hydrogel; injectable; *in situ* crosslink

INTRODUCTION

Hydrogels are crosslinked hydrophilic polymer networks, giving them physical characteristics similar to soft tissues.[1–4] Because of an insoluble network created by the process of crosslinking, swelling, and hydration occur without dissolution of the polymer. Additionally, hydrogels have high permeability, which facilitates exchange of oxygen, nutrients, and other water soluble metabolites.[5] On all accounts, the biocompatible hydrogels can be the optimal choice for tissue engineering applications. Over the past three decades, various hydrogels, produced with chemical or physical methods, have become standard materials for drug delivery, contact lenses, corneal implants, and scaffolds for the regeneration of new skin, encapsulation of cells, and regeneration of tendons, and cartilage.[6–8] Although in the past few years, tissue engineering science has largely focused on *in situ* crosslink structures, there are three main reasons to strongly prefer the use of injectable *in situ* crosslinkable hydrogels. First, *in situ* crosslink hydrogel can be formed into any desired shape: due to the fluidity, the initial materials can be positioned in any complex shape and then crosslinked to conform to the required dimensions. Second, during gel formation, the initial liquid materials more readily adhere to the tissue, resulting in higher tissue-hydrogel interface strength. Finally, injectable *in situ* crosslinkable hydrogel can simplify the invasiveness procedure.[2,9–17]

During the past decade, many natural and synthetic biomacromaterials were introduced to produce injectable *in situ* crosslink hydrogels, and most of them have been examined for clinical applications.

Hyaluronic acid (HA) is an endogenous polysaccharide, present in the vitreous body, synovial fluids, and the extracellular matrix. It consists of repeating disaccharide units composed of b(1-4) linked *N*-acetyl-D-glucosamine, and b(1-3) linked D-glucuronic acid. HA is an attractive biomaterial for biomedical and clinical applications due to its high water adsorption property, excellent biocompatibility, biodegradability, and immunoneutrality.[18–20] Recently, HA is

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used as replacement fluid in joint cavities treatment and eye surgery. Furthermore, HA derivative and unmodified HA are under investigation for drug delivery and tissue engineering applications.[21–23] In 2007, Jia and Yeo[24] synthesized a novel microgel based on HAADH/HAALD to enhance resistance to enzymatic degradation.

PAHy, made from poly (succinimide) (PSI), is a kind of polyaspartic acid derivative. The protein like structure provides PAHy with freely water solubility, biocompatibility, and avirulence, which would be propitious to synthesize macromolecular prodrugs and prepare polymeric hydrogel.[25–28] Cationic PAHy copolymers have been widely used to improve the interaction between materials and cells of various human organisms. For example, Paolino and Cosco[29] prepared gemcitabine-loaded supramolecular vesicular aggregates (SVAs) containing synthesized PAHy derivatives and successfully achieved an improvement of anticancer drug activity.

Recently there has been an increasing interest in HA-based or PASP-based hydrogels, which were mainly used in drug or cell delivery applications. Jian et al. e.g., synthesized a kind of microgel based on HA derivatives carrying aldehyde and hydrazide (HAALD/HAADH) using 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC) as crosslinker,[30] Pitarresi et al.[27] prepared and characterized new hydrogels based on hyaluronic acid and a,b-polyaspartylhydrazide and Jha et al.[31] created a new hyaluronic acid (HA)-based hydrogel materials with HA hydrogel particles (HGPs) embedded in and covalently crosslinked to a secondary network by EDC with PAHy. However, crosslinker, such as EDC, has to be used in the synthesis, and the hydrogels cannot be made injectable. There were also some injectable hydrogles prepared without crosslinker, like a fast-gelling injectable blend made of hyaluronan and methylcellulose (MC) for intrathecal, localized delivery to the injured spinal cord developed by Gupta and Tator.[32] The gel formed as temperature increased because of thermosensitivity of MC, but the gel-forming mechanism is because of the physical entanglement and the shortest gelation time of these gels tested in this experiment, which was almost 2 min. Leach et al.[33] synthesized an *in situ* crosslinkable HA-poly(ethylene glycol) (PEG) polymer. HA was modified by glycidyl methacrylate, then crosslinked with the acrylated 4-arm PEG under UV light and formed into GMHA-PEG hydrogel, which can be used to delivery protein drugs. However, this *in situ* crosslinkable GMHA-PEG hydrogel was not suitable for injection because of the photo-crosslink condition. Shu et al.[34] reported on an *in situ* crosslinkable hydrogel based on thiolated hyaluronan and poly(ethylene glycol) (PEG) derivatives. The synthesis processes of thiolated HA and homobifunctional PEG electrophiles comprize several steps and need careful control to avoid the inactivation of the thiols and double bonds. During the crosslinking step, the gelation time can be controlled by adjusting the molar ratio of thiols and double bonds, whereas the shortest gelation time they reported is 5min.

To create an injectable and *in situ* crosslinkable hydrogel system, with a short gelation time and straightforward synthesis sequence, the present research relates to a HAALD-PAHy-based hydrogel, which can be *in situ* crosslinked in 15 s by condensation reaction between aldehyde of modified HA and amine of PAHy, without the crosslinker or catalyst used. The best condition for preparing HAALD was considered carefully from several respects, such as dosage of oxidants, reaction pH, reaction time, and so on. The *in situ* crosslink reaction happened in phosphate buffered salines with different pH and concentration to determine the influence of pH and ionic strength to aldehyde-amine reaction. Whereafter, gelation time, gel content, and swelling ratio of crosslinked hydrogels were measured, and the modified polymers were characterized by FT-IR spectroscopy to ensure their structures. Results from scanning electron microscope (SEM) observations confirm a porous hydrogel structure with interconnected pores after freeze-drying. HAALD-PAHy hydrogels is a noncell adhesion biomaterials and can be used in a large field.

EXPERIMENTAL INVESTIGATIONS

Materials

Totally, 1.19×10^3 kDa hyaluronic acid (HA) was purchased from Shandong Freda (China), polysuccinimide (PSI) of 200kDa was prepared in our laboratories. Hyaluronidase was procured from Sigma, *N′N*-Dimethylformamide (DMF), sodium periodate ($NaIO_4$), diamid hydrate, hydrochloric acid (HCl), 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 of analytical grade, and used as purchased.

Change of conditions to prepare HAALD

HA was modified into HAALD by $NaIO_4$ oxidation, at different reaction conditions (according to details of Table I) to prepare the optimal HAALD. Briefly, 0.3 g of 1.19×10^3 kDa HA was dissolved in 30 mL deionized water (pH was adjusted by 0.1*M* HCl) to form a transparent solution. Then sodium periodate solution (different volume with different concentration) was added and constantly stirred for several hours in the dark. The stir continued for 30 min after adding 30 mL ethylene glycol to terminate the

TABLE I
Details of Oxidation Experiments Conditions

Ref.	Concentration of $NaIO_4$(mol/L)	Volume of $NaIO_4$ solution (mL)	HA repeating unit to $NaIO_4$	Reaction time (h)	Reaction temperature (°C)	Reaction pH
EXP.1	0.10	4.5	2:1	2	30	6
EXP.2	0.25	3.6	1:1	2	30	6
EXP.3	0.25	3.6	1:1	3	30	6
EXP.4	0.25	3.6	1:1	4	30	6
EXP.5	0.25	7.2	1:2	2	30	6
EXP.6	0.25	3.6	1:1	2	25	6
EXP.7	0.25	3.6	1:1	2	35	6
EXP.8	0.25	3.6	1:1	2	30	5
EXP.9	0.25	3.6	1:1	2	30	4

reaction. The mixture was dialyzed against deionized water. The purified product was freeze-dried and kept at 4°C. FT-IR analysis confirmed the successful oxidation of HA. The residual $NaIO_4$ in the reaction mixture was quantified using the iodometric method.[35] Totally, 0.01mol/L NaOH was used to analyze the concentration of formic acid byproduct.

The aldehyde content of HAALD was measured by the hydroxylamine-based method[36] and the molecular weight of HAALD was determined by Shimadzu high-performance liquid chromatography (HPLC) operating in size exclusion chromatography mode (SEC), using a RID-10A refractive index detector and a Shodex gel column (GF-510HQ) calibrated by agarose molecular weight standards. Sodium phosphate buffer (pH 7.2, 0.02*M*) was used as the mobile phase, and the flow rate was 0.5 mL/min. The sample concentration was 2 mg/mL.

Preparation of PAHy

The 200 kDa PSI was modified into PAHy with diamid hydrate,[28] through dissolving 1g of 200 kDa PSI in 10 mL DMF, whereafter 0.500 g diamid hydrate was added dropwise to the solution and deposition appeared immediately. The mixture was stirred for 4 h at room temperature to ensure the completion of the reaction. DMF containing the leach-out products was subsequently filtered through a filter paper. The deposition was dissolved in deionized water and the solution was filtered again by using ultrafiltration to remove the extra DMF as well as the unreacted diamid hydrate. The purified product was freeze-dried and kept at 4°C. The PAHy structure was confirmed by FT-IR.

Preparation of HAALD-PAHy hydrogel

Choice of reaction solution pH

HAALD and PAHy were separately dissolved in 0.20*M* PBS solutions with different pH ranging from 5.8 to 7.4 and formed 5% (w/v) (weight/volume) solutions. The HAALD and PAHy solutions with equal pH value were both injected into a 5 mL centrifuge tube and mixed by vortex mixer immediately. Then all the tubes were dipped into a water bath to keep the reaction temperature constant at 37°C for 24 h to form crosslinked composite hydrogels.

Choice of reaction solution concentration

The 5% HAALD and 5% PAHy solutions were prepared in pH = 7.4 PBS with different concentration (0.01*M*/0.05*M*/0.10*M*/0.20*M*). The HAALD and PAHy solutions, dissolved in the same PBS, were both injected into a 5mL centrifuge tube and treated in an identical set of steps as described in the above section.

Gelation time

The inversion method was used in the experiments to determine the gelation time of HAALD-PAHy at 37°C in a water bath.[37] On the basis of the abovementioned preparation steps, the time was counted from the mixing point. Inverting the tube determined a gel state when no fluidity was visually observed after a certain reaction period, and this period was defined as the gelation time.

Gel content

The hydrogel was removed from the tubes after having reacted for 24 h at 37°C and freeze-drying. The uncrosslinked polymer was removed by dissolving the gel in de-ionized water for 24 h and then freeze-drying it. The gel content is given by:

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

W_o is the weight of dry gel before soaking in de-ionized water;

W_1 is the weight of dry gel after soaking in de-ionized water

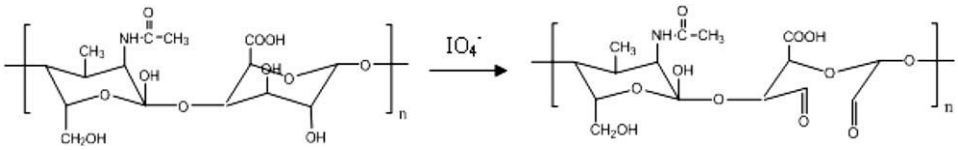

Figure 1 Reaction equation of oxidization reaction between HA and $NaIO_4$.

Mass swelling test

Untreated hydrogels, produced by the above-mentioned method, were separately immersed in distilled water at 37°C for 24 h. After removing from the distilled water, they were hung over a table during 5 min until no dripping water was observed and then weighed. They were then freeze-dried at −20°C to weigh the dry gel. The content of the distilled water in the swollen gels was calculated by the following equation:

$$W = \frac{w_1 - w_2}{w_2}$$

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

The content of the artificial tears in the swollen gels was also tested by this way.

The mass swelling ratio of hydrogels in Pbsa solution can be tested using the same method and equation.

In vitro degradation

Totally, 0.1 g dry gels were swollen in 20 mL PBSA solution (pH = 7.4) or in a PBSA solution containing HAse (100 U/mL) keeping shaken at the constant temperature (37°C) and at 100 rpm for a desired period, while the PBSA solution was changed daily. At specified time intervals, i.e., after 4, 7, 14, and 28 days of incubation, the PBSA solution was removed, the gels were lyophilized, and then weighed. The weight loss was quantified using the following equation:

$$W_{1???}\ \% = \frac{(w_{d0} - w_{dt})}{w_{d0}} \times 100\%$$

Where w_{d0} is the initial dry polymer mass and w_{dt} is the dry polymer mass at the specified time.

Infrared (IR) spectroscopic measurement

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

Morphologies

Morphologies of hydrogels were characterized by utilizing scanning electron microscopy (SEM) after gelation. The hydrogels 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).

Cell adhesion assessment

Mouse fibroblast (L-929) cells were used in a cell adhesion assessment experiment. First of all, cells were harvested in DEME media with L-glutamaine, 10% fetal bovine serum and 1% penicillin at 37°C, in a humidified and 5% CO_2 environmental incubator. To test the cell adhesive properties of HAALD-PAHy hydrogel, 3 × 1 mL HAALD-PAHy hydrogels with PBSA as their solvents were prepared and

TABLE II
Results of Corresponding Experiments Condition

Ref.	Concentration of residual $NaIO_4$(mol/L)	Concentration of formic acid(mol/L)	Aldehyde content of HAALD(%)	Molecular weight of HAALD (kDa)
EXP.1	3.39×10^{-3}	5.0×10^{-7}	33.65	5.52×10^{2}
EXP.2	8.08×10^{-3}	4.9×10^{-6}	55.48	4.28×10^{2}
EXP.3	7.49×10^{-3}	4.6×10^{-6}	59.27	3.83×10^{2}
EXP.4	6.35×10^{-3}	3.9×10^{-6}	67.55	2.40×10^{2}
EXP.5	1.13×10^{-2}	8.8×10^{-6}	65.66	1.42×10^{1}
EXP.6	5.48×10^{-3}	5.9×10^{-6}	50.32	4.98×10^{2}
EXP.7	6.67×10^{-3}	4.6×10^{-6}	62.54	2.39×10^{2}
EXP.8	7.89×10^{-3}	6.3×10^{-6}	50.75	$<1.0 \times 10^{1}$
EXP.9	7.76×10^{-3}	6.7×10^{-6}	47.67	$<1.0 \times 10^{1}$

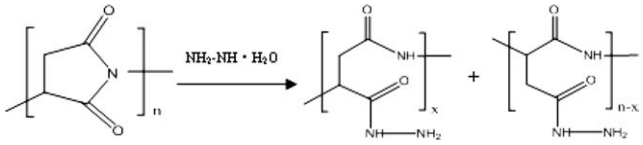

Figure 2 Reaction equation of ring-opening between PSI and hydrazine.

sterilized in 35 mm I.D. petri dishes (35 mm × 10 mm, Corning, USA). Thereafter, 0.2mL cell suspensions with density of 1×10^5 cells/cm^2 was added onto the hydrogel samples and incubated for five days. The final step involved washing the samples with PBS solution for three times to remove the non-adherent cells after incubation. SEM (SUPRA 55, ZEISS, Germany) was used to observe cells morphology on the samples.

Statistical analysis

All the data presented in the paper are average results of three repeated experiments. The maximum deviation for the individual experimental data was less than 5% in comparison with the reported average value.

RESULTS AND DISCUSSION

Change of condition to prepare HAALD

As stated before, polysaccharides with diol-functions can be selectively oxidized or even ruptured by sodium periodate, and thereby modified to form dialdehyde, formaldehyde, or formic acid according to the structure of the sugar ring.

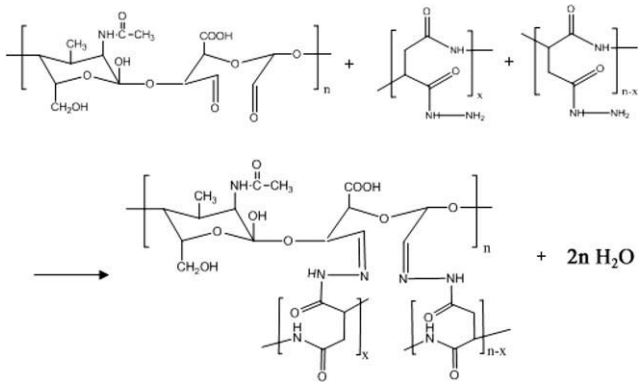

Figure 4 Reaction equation of HAALD and PAHy.

This oxidation follows specific principles. If diols are present at the 1→2 bond, or at the 1→4 bond, one molecule adjacent to diols will be oxidized into the same molar amount of dialdehyde by one molecule of $NaIO_4$ without forming formic acid; while two molecules of $NaIO_4$ would be consumed by one molecule of 1→6 diol with one molecule of formic acid being formed. Oxidizition does not occur when diol-functions are present at the 1→3 bond.

On the basis of the structure of HA, it should be oxidized into HAALD as presented in Figure 1. However, byproducts can be detected, such as formic acid, which means the structure of HAALD changed due to the strong oxidative capability of sodium periodate. The concentration of formic acid has hence to be tested to ensure the minimum transformation of HAALD.

The results, including residual $NaIO_4$ in the reaction mixture, the concentration of byproduct formic acid, the aldehyde content of HAALD, and the

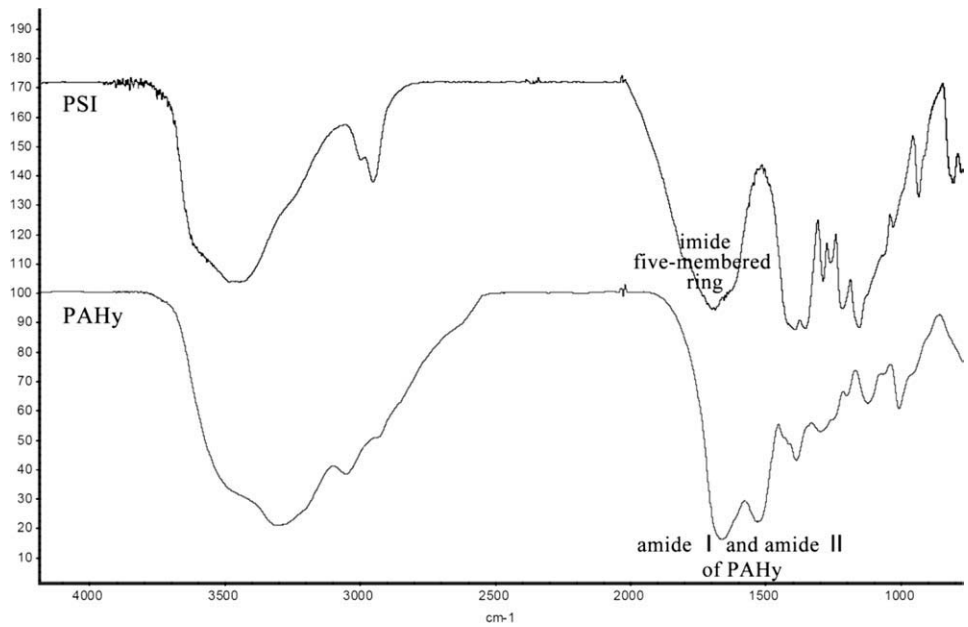

Figure 3 FTIR spectra of PSI and PAHy (PAHy: produced at room temperature after 4-h reaction).

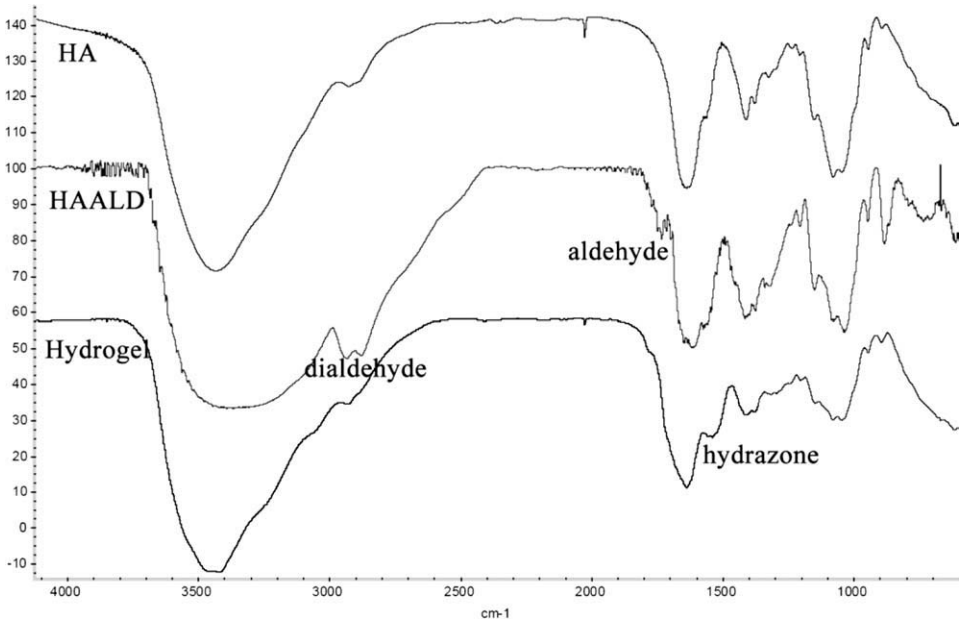

Figure 5 FTIR spectra of HA, HAALD, and HAALD-PAHy hydrogel (HAALD: concentration of $NaIO_4$ solution: 0.25 mol/L, volume of $NaIO_4$ solution: 3.6 mL, HA: 0.3 g, reaction time: 3 h, reaction temperature: 30°C, pH of $NaIO_4$ solution: 6; Hydrogel: reaction solution: PBS, pH of reaction solution: 7.4, concentration of reaction solution: 0.05*M*, concentration of HAALD and PAHy both are 5%, reaction temperature: 37°C).

molecular weight of HAALD, are shown in Table III. The concentration of residual $NaIO_4$ in the reaction mixture and the aldehyde content of HAALD, as well as the concentration of byproduct increases when the dosage of $NaIO_4$ increased, while adversely the molecular weight of HAALD decreased. These findings illustrate that the excess sodium periodate oxidated not only the glycosidic bond of hyaluronic acid, but also the main chain of HA. The strong oxidizing power enhances the destruction of HA and HAALD, and reduces their molecular weight, thus seriously affecting the viscoelastic properties if the dosage of sodium periodate is too high. These experiments were designed to examine the best oxidation conditions of hyaluronic acid.

The reaction time was compared from 2 to 4 h for equal amount of sodium periodate. With the extension of the reaction time, the concentration of residual $NaIO_4$ and the molecular weight of products decreased, the aldehyde content of the products were conversely increased as expected, whereas the formic acid concentration remained fairly unchanged, as illustrated in Table II.

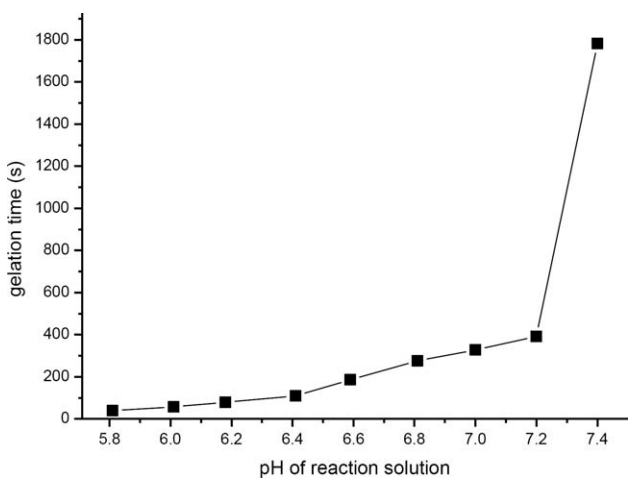

Figure 6 Relationship between gelation time and pH of reaction solution (reaction solution: PBS, concentration of reaction solution: 0.2*M*, concentration of HAALD and PAHy both 5%, reaction temperature: 37°C).

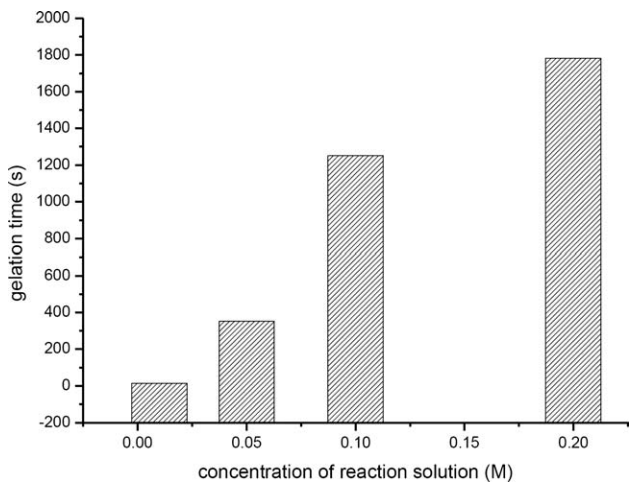

Figure 7 Relationship between gelation time and concentration of reaction solution (reaction solution: PBS, pH of reaction solution: 7.4, concentration of HAALD and PAHy both 5%, reaction temperature: 37°C).

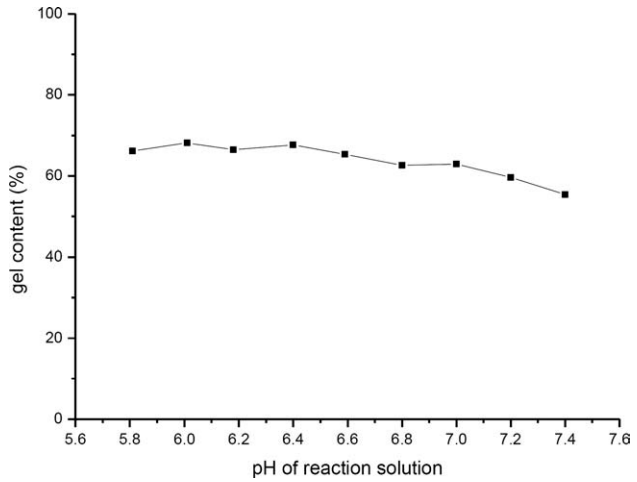

Figure 8 Relationship between the gel content and pH of the reaction solution (reaction solution: PBS, concentration of reaction solution: 0.2*M*, concentration of HAALD and PAHy both 5%, reaction temperature: 37°C).

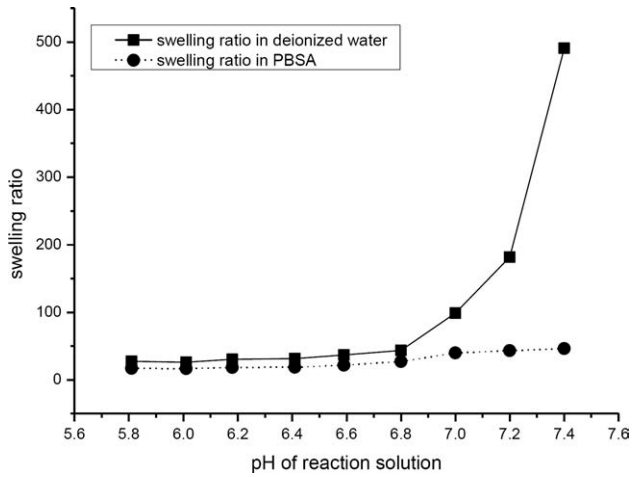

Figure 10 Relationship between swelling ratio and pH of reaction solution (reaction solution: PBS, concentration of reaction solution: 0.2*M*, concentration of HAALD and PAHy both 5%, reaction temperature: 37°C).

The conditions of 3.6 mL 0.25 mol/L of sodium periodate provided a stable oxidization effect on the 1→4 glycosidic bond of 0.3 g HA, and did not cause a rupture of the 1→6 glycosidic bond of HA by reaction time extension. The significant reduction in molecular weight of HA led authors to believe that sodium periodate played an important oxidization effect on the main chain of HA as the reaction time increased. The present research suggested that a more satisfactory HAALD could be obtained in 3 h when the appropriate amount of sodium periodate was added.

There were no significant differences of the results among several different reaction temperatures below 35°C. As reported by Lei et al.,[36] the best reaction conditions imply pH = 3 in the cellulose oxidization. However, HA is extremely sensitive to acids and alkalis, thus necessitating a careful control of the pH of the oxidization reaction to avoid degradation. The data clearly illustrate that for a pH of 4 or 5, the molecular weight of products were both less than 10kDa, and the aldehyde content was only 47.67% and 50.75%, respectively, thus not matching the high results of products obtained at pH = 6.

In summary, the best oxidization conditions include: 0.3 g of 1.19×10^3 kDa HA was dissolved in 30 mL pH = 6.0 deionized water (pH was adjusted by HCl) to form transparent solution, then 3.6 mL 0.25 mol/L sodium periodate solution was added and constantly stirred for 3 h in the dark at 30°C. Under continued stirring during 30 min after adding 30 mL ethylene glycol to terminate the reaction, the mixture is then dialyzed against deionized water. The purified product was freeze-dried and kept at 4°C.

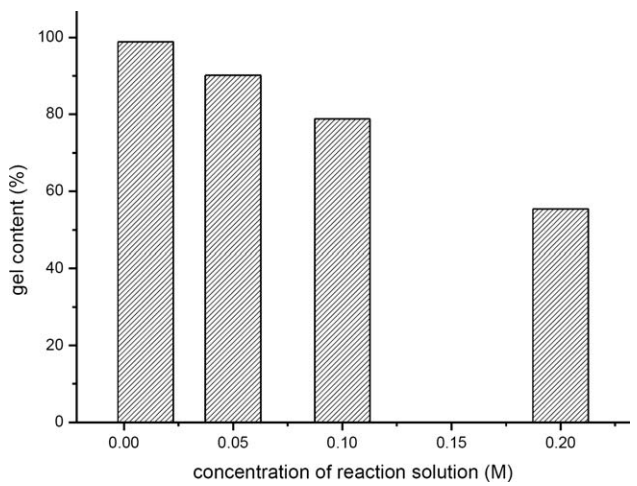

Figure 9 Relationship between the gel content and the concentration of reaction solution (reaction solution: PBS, pH of reaction solution:7.4, concentration of HAALD and PAHy both 5%, reaction temperature: 37°C).

Structure of polysaccharide derivatives

The ring-opening reaction occurred between the imide five-membered ring of poly (succinimide) (PSI) and hydrazine, with the formation of an hydrazide group, as shown in Figure 2. The chemical structure of PSI and PAHy are shown in Figure 3, with displayed differences from 1700 to 1500 cm^{-1}. As shown in Figure 3, the absorption peak at around 1700 cm^{-1} of PSI stood for the imide five-membered ring, whereas in the spectrum of PAHy, it was separated into two peaks at 1665 cm^{-1} and 1521 cm^{-1} related to the amide I and amide II of PAHy.

HAALD was obtained by the introduction of aldehyde groups into HA reacting with sodium periodate, which oxidized adjacent diols to dialdehyde by opening the sugar ring of the glucosamine units (Fig. 4). Compared with HA, the spectrum of HAALD showed two absorption peaks at 2930 to

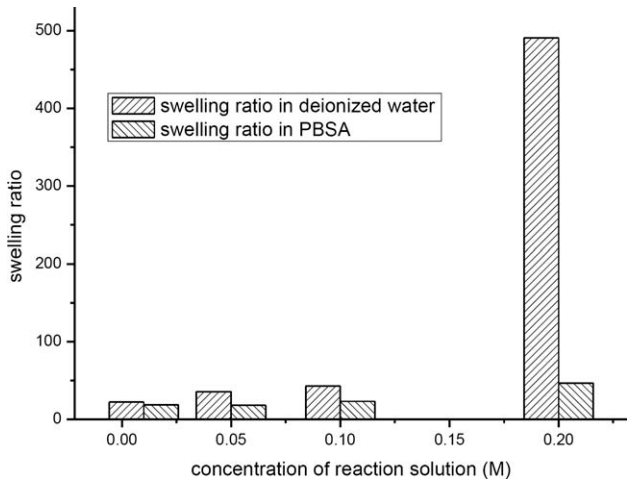

Figure 11 Relationship between the swelling ratio and the concentration of reaction solution (reaction solution: PBS, pH of reaction solution:7.4, concentration of HAALD and PAHy both 5%, reaction temperature: 37°C).

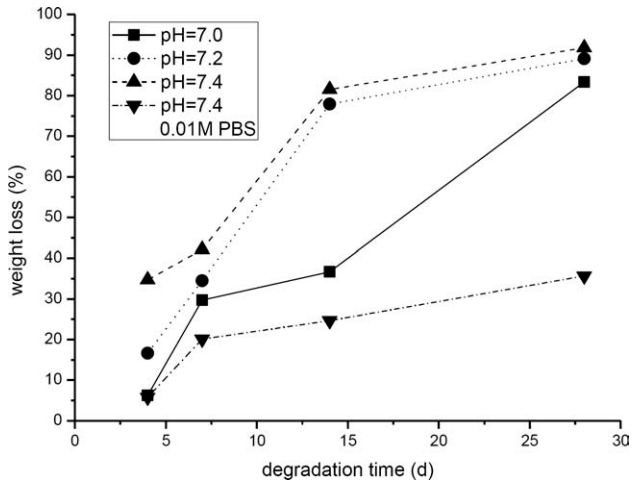

Figure 13 Relationship between weight loss and vitro degradation days in PBSA solution containing Hase (reaction solution: 0.2*M* PBS with pH ranging from 7.0 to 7.4 and 0.01*M* PBS with pH = 7.4, concentration of HAALD and PAHy both are 5%, reaction temperature: 37°C).

2880 cm^{-1}, which corresponds to the generation of the dialdehyde group. The peak appearing at 1732 cm^{-1} is associated with the C=O group of HAALD. In the spectrum of hydrogel (Fig. 5), the peak of aldehyde is not present and a new characteristic peak of the hydrazone structure is generated at 1548 cm^{-1} showing that the coupling reaction was followed between aldehyde groups of HAALD and hydrazide of PAHy.

Gelation time

The gelation time, characteristic of the gel–sol transition rate of *in situ* crosslinkable hydrogel, was determined carefully to evaluate the influence of crosslinking conditions.

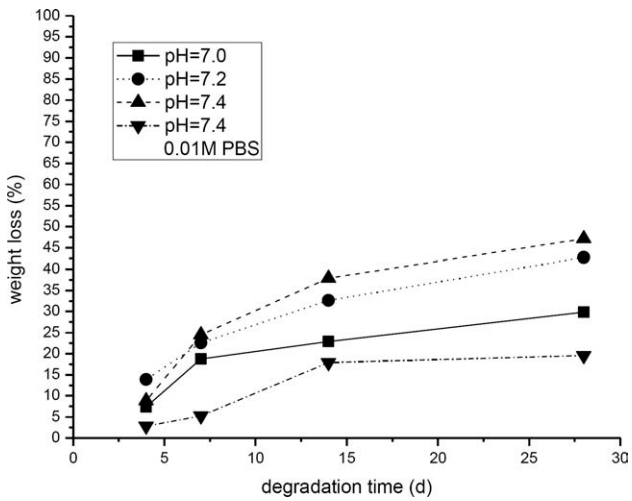

Figure 12 Relationship between weight loss and vitro degradation days in PBSA solution (reaction solution: 0.2*M* PBS with pH ranging from 7.0 to 7.4 and 0.01*M* PBS with pH = 7.4, concentration of HAALD and PAHy both are 5%, reaction temperature: 37°C).

The hydrazone bond, which is a conformation of a Schiff base, formed spontaneously from the interaction between aldehyde of HAALD and hydrazide of PAHy (Fig. 4). According to the principle point of organic chemistry, this reaction could be accelerated in acid conditions, since the positive change of the carbonyl carbon is enhanced after the proton reaction of the carbonyl oxygen atom. However, the nucleophile H_2N:-Y would be protonated to H_3N^+-Y in a harsh acidic condition, and the nucleophilic addition reaction with aldehyde cannot occur due to a loss of pro-nuclear capability.

Figure 6 summarizes the data collected during the experiment of gelation time, and demonstrates from that the pH of the reaction solution is one of the most important factors in the gelation time because a sharp increase of the gelation time is seen as the pH of the reaction increases. The acidity of the solution should be carefully controlled to ensure a smooth progress of the reaction.

Figure 7 presents the relationship between gelation time and the concentration of PBS, for a constant pH of PBS solution = 7.4: by increasing the concentration of PBS solution from 0.01*M* to 0.2*M*, the gelation time significantly increased from 15.51 s to 1782.36 s, the reason being that a low ionic strength, reflected by the low PBS concentration, benefits polymer expansibility and active sites extendibility, thus leading to a high HAALD-PAHy gel formation speed.

As a result, the gelation time of Haald-PAHy gel can be controlled by adjusting the PBS concentration as well as the pH.

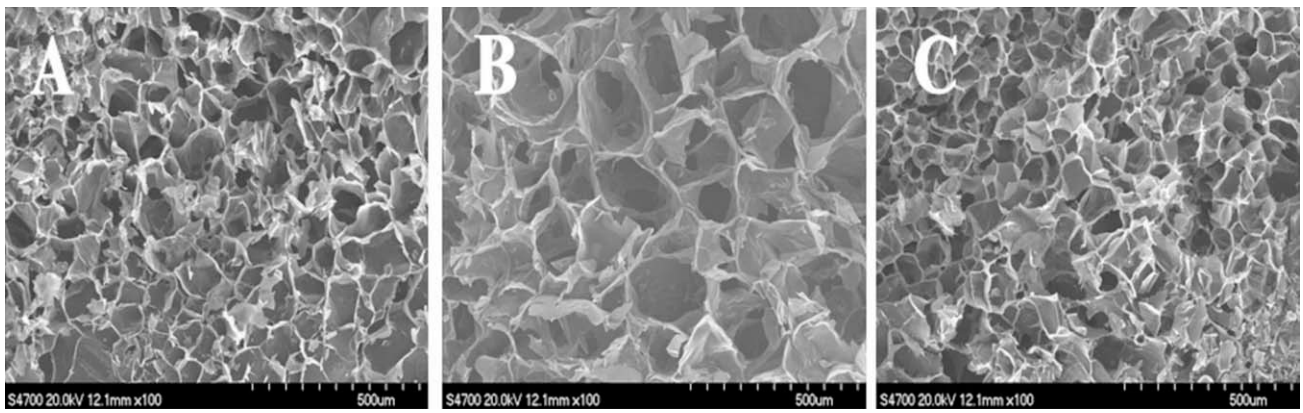

Figure 14 SEM photographs of freeze-dried HAALD-PAHy hydrogels. A–C: Surface morphologies of hydrogels crosslinked (HAALD-PAHy hydrogel A: 0.2*M* PBS solution pH = 5.81; HAALD-PAHy hydrogel B: 0.2*M* PBS solution pH = 7.40; HAALD-PAHy hydrogel C: 0.01*M* PBS solution pH = 7.40).

Gel content

The gel content is a basic parameter to calibrate gel formation due to crosslinking, because not all the macromonomers eventually join the gel network.[38]

As noted in Figure 8, the gel content, reflecting the integrity and strength of the three-dimensional network structure, decreased gradually as the pH of PBS solution increased. For example, the gel content was 68.55% when the gel was formed at pH = 6.0, while that dropped to 55.39% when the pH of reaction solution was 7.4. The results of gel content experiments indicated that a better three-dimensional network structure was achieved by controlling the pH of reaction from 5.8 to 6.4.

To confirm the influence of PBS concentration on gel formation, the differences among gel contents are reported in Figure 9. The gel contents ranged from 98.87% to 55.39% and went down with PBS concentrations enhanced. This phenomenon was also attributed to the effect of ionic strength.

In short, lower pH and concentration of reaction solution leaded to higher gel content and more completed 3D structure of HAALD-PAHy hydrogel.

The relation ship between swelling ratio and pH of reaction solution

The swelling ratios of these HAALD-PAHy gels prepared at different pH or concentration conditions were tested in deionized water and PBSA solution at 37°C, respectively. The pH and concentration of reaction solution largely affected the swelling ratio of HAALD-PAHy hydrogels, as illustrated in Figures 10 and 11.

The swelling ratios of gels in deionized water and in PBSA solution are shown: the swelling ratios of gels enhanced as the pH or concentration of reaction solution increased. Especially when the reaction solution pH reached to 7.4 and the concentration of PBS equaled 0.2*M*, the swelling ratio in deionized water rose sharply to 490.65. This implies that the network structure of the gel was more compact as the pH and concentration of PBS solution decreased. It is hence concluded that the crosslinking degree of the gel shifted to higher values as pH and concentration of PBS solution decreased.

In addition, since saline solutions have a great influence on ionic gels,[39] the swelling ratio of gels in the PBSA solution decreased generally in contrast to

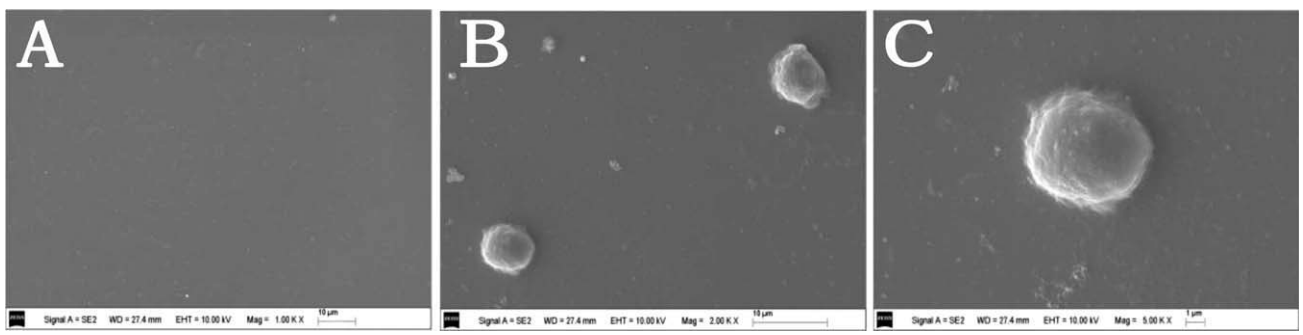

Figure 15 SEM photographs of morphology on HAALD-PAHy hydrogels. (HAALD-PAHy hydrogel: PBSA solution, pH = 7.4, concentration of HAALD and PAHy both are 5%, reaction temperature: 37°C).

those in deionized water. For instance, the swelling ratio of the gel, crosslinked in 0.2*M* PBS (pH = 5.8), was 27.52 in deionized water and 16.86 in PBSA; the swelling ratio of the gel, crosslinked in 0.1*M* PBS (pH = 7.4), was 42.54 in deionized water and 22.94 in PBSA. In accordance with the Flory-Huggins theory, the interplay of ionogens in the ionic gel and saline ions in the saline solution resist the swelling of the ionic gel network,[40] the ionic strength of PBSA solution affected the osmotic pressure within gel, and the diffusion and expansion ability of the polymer chain were reduced, thereby reducing the swelling ratio in PBSA.

In vitro degradation

In vitro degradation of HAALD-PAHy hydrogels was conducted at 37°C for up to 28 days in PBSA, or PBSA containing HAse (100U/mL). All the gels had very slow degradation rates in PBSA solution, but there were significant differences in the rate of degradation among gels prepared in different reaction solutions (Fig. 12). After 28 days of incubation in PBSA, the highest weight loss of all the gels was 47.16%, which corresponded to the gel formed at pH = 7.4 0.02*M* PBS solution. The weight loss of gel formed under pH = 7.0 was 29.86% only and the gel prepared in 0.01*M* PBS solution (pH = 7.4) had even a lower weight loss, only 19.58%.

The addition of HAse in PBSA significantly accelerated the degradation of HAALD-PAHy hydrogels as expected (Fig. 13). Gels, produced in PBS solution with pH = 7.2 and 7.4, lost 89.08% and 91.82% of their initial weights after 28 days of incubation. However, the gel prepared in 0.01*M* PBS solution (pH = 7.4) presented good resistance to HAse under the same conditions, losing only 35.68% of its initial weight in 28 days.

In summary, gels, crosslinked at lower pH and ionic strength condition, had a higher gel content and more complete three-dimensional network structure, and they were able to maintain their weight in the degradation solution.

Morphologies of hydrogels

The microstructure morphologies of HAALD-PAHy hydrogels were characterized by SEM. The surface images of freeze-dried hydrogels are presented in Figure 14(A–C). On the basis of the cross-sectional SEM images (Fig. 14), the hydrogels exhibited a continuous and porous structure in virtue of the freeze-drying step, with pore diameters in the range of 100–300 μm, which was reported as the pores being the result of ice crystal formation and resembling other natural macromolecular hydrogel system structures.[41] The internal morphology depended on the pH and concentration of reaction solution. Higher pH and ionic strength resulted in bigger pore size, which means that the higher pH and strength of reaction solution, the looser the space structure of HAALD-PAHy hydrogels formed.

Cell adhesion

As reported earlier,[32] biomaterials, with resisting cell adhesion ability, can restrain cell invasion, scar formation, and avoid serious clinical complications during the healing process.

Figure 15 presents the cells morphology on HAALD-PAHy samples. The figure shows that most cells are removed by washing, and there are only few spread cells left on the HAALD-PAHy samples. These morphology indicates that the HAALD-PAHy hydrogel is noncell adhesive, which is desirable for the injectable hydrogel, especially to prevent adhesion in the treatment of e.g., tendons, nerves, or joint peridural and peritoneal affections.[42–44]

CONCLUSIONS

The *in situ* forming HAALD and PAHy composite hydrogels were prepared via amine-aldehyde reaction, according to an optimum and selected succession of treatment steps. The gelation time, gel content, swelling ratio, and *in vitro* degradation were tested to evaluate the properties of hydrogels. The gelation time could be controlled in the range of 15 s to tens of minutes via adjusting the pH and the concentration of reaction solution: the hydrogel, crosslinked under low pH and ionic strength condition, showed a short gelation time, high gel content, low swelling ratio, and slow degradation rate. The HAALD-PAHy hydrogel, prepared in 0.01*M* PBS solution (pH = 7.4), can be gelled in 15.51 s, its gel content was 98.87% and its swelling ratio was lower than the others. The experiments revealed that 0.01*M* PBS solution (pH = 7.4), used as reaction solution, provided the hydrogel with a more complete three-dimensional network structure and stronger antidegradation ability. Finally, the modified polymers were characterized by FT-IR spectroscopy to ensure their structures. Results from scanning electron microscope observations confirm a porous hydrogel structure with interconnected pores after freeze-drying. HAALD-PAHy hydrogel is a noncell adhesion biomaterial, and its gelation behavior and porous structure make it extremely promising and attractive to biomedical applications such as drug delivery, adhesion prevention, or tissue engineering.

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