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Hyperbranched phosphoramidate-hyaluronan hybrid: A reduction-sensitive injectable hydrogel for controlled protein release

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ABSTRACT

In the study, an injectable hybrid hydrogel (HPPAE–HA) was fabricated by in situ polymerization between acrylated hyperbranched polyphosphoramidate (HPPAE–AC) and thiolated hyaluronan (HA-SH) via Michael-addition reaction. Gelation times ranged from 4 to 360 min, depending on the stoichiometric ratio of HA-SH to HPPAE–AC, HA concentration and solution pH. In addition, due to the readily oxidation of thiol groups, the HPPAE–HA hydrogel bears disulfide bond. The DTT triggered disulfide bond cleavage and the subsequent cross-linking between thiol and abundant acryloyl groups presented in HPPAE–HA hydrogel contribute to the controlled release of bovine serum albumin (BSA) from the hydrogels. The burst release of BSA from HPPAE–HA hydrogel decreased from 23.7 to 4.0% during the first 3 h, and the 80% cumulative release of BSA was retarded for 12 h to nearly 96 h in 10 mM DTT. The facile synthesis of Michael-additional, disulfide containing HPPAE–HA hydrogel may enable further development of hydrogel matrices potentially suitable for tissue engineering and drug delivery applications.

1. Introduction

Successful design of a hydrogel for a specific application, although challenging, can be realized via understanding of the application and environment that the hydrogels is intended to serve (Hoffman, 2002; Van Vlierberghe, Dubruel,& Schacht, 2011; Kharkar, Kiick, & Kloxin, 2013). Presently, hydrogels are widely used for drug delivery and targeting due to their tunable chemical and physical structure, high water content and biocompatibility that closely resemble to our extracellular matrix (Censi, Martino Di, Vermonden, & Hennink, 2012; Park & Lee, 2011), while the incorporation of drugs by sorption is time-consuming and limits the loading level. A delivery formulation where gelation and drug loading can be achieved simultaneously with a high loading in an aqueous environment is attractive. Injectable hydrogel is such a kind of hydrogel with the distinguish feature of minimally invasive delivery procedure, providing reduced healing time, reduced scarring, decreased risk of infection, and ease of delivery compared with surgically implanted materials (Li, Rodrigues, & Tomas, 2012; Wang et al., 2010; Hou, De Bank, & Shakesheff, 2004).

Yet many hydrogel systems quickly release the majority of the encapsulated drugs during the initial release stage, which restricts the practical use of injectable hydrogels for therapeutic drug delivery. To modulate the release of proteins entrapped in hydrogels, the degradation rate and cross-linking density of the network can be controlled through tuning the composition and macromolecular chemistry of the hybrid hydrogel. Moreover, to further enhance the desired delivery features in the potential application of complex biological compartments, it became particularly attractive to use the reduction-sensitivity polymer to regulate the drug release profile in extracellular milieus and normal tissue. With respect to the molecular level, the disulfide linkage is a typical character of reduction-sensitive polymers, and usually locates in main chains, side chains or cross-linkers (Zarrabi, Adeli, Vossoughi, & Shokrgozar, 2011; Fleige, Quadir, & Haag, 2012; Bauhuber, Hozsa, Breunig, & Gopferich, 2009; Kinnane et al., 2009). A notable fact has been found that the cleavage of disulfide linkages, due to the thiol-disulfide exchange reaction, is sensitive to the reduction conditions in the human body, namely the disulfide bonds show enough stability to a low concentration of glutathione tripeptide (GSH), while the disulfide linkage quickly cleaves under high GSH concentration (Meng, Hennink, & Zhong, 2009).

Despite the extensive use of hyperbranched polymer, fundamental synthetic studies aimed toward developing novel hydrogel materials based on hyperbranched polymer are lacking. While it has been showed that, as a gel macromolecular precursor, the

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hyperbranched structure allows considerable degradation before the cross-linked network breaks down, and maintains mechanical integrity during degradation. In addition, the presence of a number of functional groups of hyperbranched polymer also allows higher crosslink densities at low concentrations compared to that of linear functionalized polymers, which is desirable to obtain a hydrogel with high swelling ratio. For these reasons it would be highly necessary to develop novel hydrogels which combine the best features of traditional material with an increased level of chemical control and diversity of hyperbranched polymer.

While the synthetic hydrogel allow structural and compositional variations in the design of hydrogels, but they mostly lack the necessary biofunctionality in comparison to naturally derived materials and this may limit their use in targeted and specific biological activities. Hence, it has been a motivation to develop hybrid hydrogels with tightly defined physical, chemical, and biological properties by the combination of synthetic polymers with natural polymers or peptide/protein sequences. Hyaluronan (HA), a naturally occurring polysaccharide, is widely used in numerous important processes such as water homeostasis of tissues and joint lubrication, treatment of osteoarthritis, and cell motility (Serban, Yang, & Prestwich, 2008). When applied as drug carriers, comparing to traditional polymeric substances such as poly(ethylene glycol), poly(lactic acid), and chitosan, HA provides greater flexibility when designing controlled drug release profiles, more stable drug formulations, enhancement of bioavailability and biocompatibility of drugs, and reduction of drug cytotoxicity in healthy tissues (Esposito, Menegatti, & Cortesi, 2005; Collins & Birkinshaw, 2013).

In the study, hyperbranched polyphosphoramidate (HPPAE) (Liu, Yan, Li, & Chen, 2013), which is a degradable synthetic hydrophilic hyperbranched polymer was chosen as a model hyperbranched polymer. To provide an additional avenue to tune the release behavior, hyaluronan with disulfide moiety (HA) was chosen as a biodegradable component to fabricate HPPAE–HA hydrogel. The release profiles of model proteins from HPPAE–HA hydrogel under DL-dithiothreitol (DTT) reductive condition were evaluated. There are no current hydrogels available based on the hybrid of synthetic hyperbranched polymer and natural polymer possessing disulfide moiety, and thus, the novelty to this work is the development of a new hydrogel release system via considering the individual benefit of macromonomer and the strategy of controlled release.

2. Materials and methods

2.1. Materials

HPPAE (M_n 19,800 g/mol, 58 hydroxyl groups per molecule) was synthesized as previous (Liu et al., 2013). Hyaluronic acid with M_w of 80 kDa and purity of 98.5% was purchased from Xi'an Rongsheng Biotechnology Company, China. Dimethyl sulfoxide (DMSO, $H_2O < 0.005\%$), acryloyl choloride (AC), 4-(*N*,*N*-dimethylamino)pyridine, (DMAP), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 99%)), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), *N*-hydroxysuccinimide (NHS), D,L-dithiothreitol (98%), 1-cysteine hydrochloride (98%), coomassie brilliant blue (CBB) and bovine serum albumin (BSA,98%) were purchased from J&K Chemical Technology, Beijing, China. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC, 98%) was purchased from Energy chemical, China. All chemicals were used as received.

2.2. Synthesis of acrylated HPPAE (HPPAE–AC)

For the synthesis of the acrylated HPPAE derivative (HPPAE–AC), HPPAE (2.00 g, 0.1 mmol, 5.8 mmol OH equivalent) was dissolved in the mixture solution of DMSO/acetone (1 mL/20 mL) at room temperature under a nitrogen atmosphere. After dissolution of DMAP (0.10 g, 0.82 mmol), a cold solution of acryloyl chloride (7.1 mL, 9.0 mmol) in dry DMSO (4 mL) was injected dropwise. The reaction was then carried out at ice-water bath under a nitrogen atmosphere for 12 h. Then, the reaction was allowed to run at room temperature for 12 h. After the reaction, the solvent was removed by vacuum distillation, and the mixture was precipitated and washed with diethyl ether (100 mL) for three times. The final product was dried overnight at room temperature and subsequently stored under nitrogen at −20 °C. The substitution degree (SD, the percentage of hydroxyl derived acrylated groups) of HPPAE–AC was determined by ^{1}H-NMR spectroscopy (section 2.5). ^{1}H-NMR: δ_H (500 MHz, D_2O) 5.70–5.82 ((multiplet, double bond of acryloyl groups), 6.12–6.25 (multiplet, double bond of acryloyl groups), 6.71–6.85 (multiplet, double bond of acryloyl groups) were used to determine the percentage of modification of OH into acryloyl groups. Determined by ^{1}H-NMR, 8.3% hydroxyl groups of HPPAE macromolecule were functionalized with acryloyl groups. IR (cm^{-1}): 3370 cm^{-1} (OH of HPPAE), 1722 cm^{-1} (C═C acryloyl), 1614 cm^{-1} (C—O of HPPAE), 1234 cm^{-1} (P═O of HPPAE), 1015 cm^{-1} (P═O—C of HPPAE), 746 cm^{-1} (P—N of HPPAE).

2.3. Synthesis of thiol-derived HA (HA-SH)

The thiol functionalized hyaluronan was carried out using a one-pot synthesis procedure (Jin et al., 2010; Lee, Choi, & Park, 2006). Typically, hyaluronan (200 mg) was dissolved in 50 mL de-ionized water (0.4% w/v solution). The pH of the solution was adjusted to 5.5 with 0.1 M HCl. EDAC (290 mg, 1.5 mmol) and NHS (177 mg, 1.5 mmol) were added to the solution and the pH was adjusted back to 5.5 with 0.1 M HCl. After 2 h, cysteamine hydrochloride (114 mg, 1.5 mmol) was added and the pH was adjusted to 6.0 with 0.1 M HCl. The reaction was then carried out at room temperature under stirring for 6 h, after which the solution was dialyzed (M_w cut-off 3.5 kDa) against de-ionized water for 24 h. After dialysis, a 5-fold molar excess of DTT was added, and the pH of the solution was raised to 7.5 with 1 M NaOH. The reaction was stirred for 24 h at room temperature, after which the pH of the reaction mixture was decreased to 5 with 1.0 M HCl. The acidified solution was first dialyzed (M_w cut-off 10 kDa) against 0.2 mM HCl at 4 °C in the dark, followed by dialysis against 0.2 mM HCl containing 1% NaCl. After dialysis, pH of the solution was readjusted to 4 by addition of 0.2 mM HCl. Finally, the solution was lyophilized and the conjugated HA containing free thiol groups, was obtained as a white foam (yield, 85%). The degree of thiolation was characterized by NMR and the content of free thiol group was determined by Ellman (1958) method.

2.4. Hydrogel formation

Thiol-acrylate hydrogels were formed by in situ polymerization between HPPAE–AC and HA-SH. PBS solution of HA-SH (1 mL, 2% (w/v)) and HPPAE–AC (100 μL, 15% (w/v)) were injected into a disposable glass vial. Then, the pH of the solution was adjusted to 7.4 using 0.1 M NaOH. The resulting solution was gently mixed by vortex and allowed to cross-link in air until the gel has no fluidity. To achieve optimal cross-linking efficiency, the cross-linking reaction was carried out for additional 1–2 h for the resulting characterization of hydrogel properties.

2.5. Characterization

^{1}H NMR spectrum of HPPAE–AC was recorded in DMSO-δ6 on a Bruker Avance 300 MHz spectrometer. The reference line of DMSO-δ6 was set at 2.47 ppm. ^{1}H NMR spectra of HA-SH were recorded

in D_2O. The content of disulfide moiety and the number of free thiol groups of HA-SH were determined by ^1H NMR (cysteamine signal at 2.74 ppm (—CH_2 in —CH_2SH) vs. HA signal at 1.85 ppm (*N*-acetyl methyl group)) and Ellman's assay, respectively. Storage and loss moduli (G' and G'') on swollen gels were obtained in small strain oscillatory shear by using an Anton Paar MCR 302 rheometer. Swollen gels were placed between the two plates of the rheometer. An amplitude sweep (G' measured as a function of strain) was performed in order to confirm that measurements were conducted within the linear viscoelastic regime. Oscillatory frequency sweeps was performed at 37 °C with constant strain (10%) in the frequency of 0.1–10 Hz, and the storage (G') and loss (G'') moduli were recorded. Raman spectra were recorded on a ALMEGA Dispersive Raman spectrometer equipped with a 514 nm model 70 Argon ion laser excitation. Spectrum was collected at room temperature with 1 cm^{-1} resolution at 30 s exposure time and 40 scans.

2.6. Swelling properties of HPPAE–HA hydrogels

Each hydrogel was prepared from 500 μL of precursor solution. Freshly prepared gels were dried under vacuum and weighed to obtain dried polymer weights (W_d). The dried polymers were then incubated in 20 mL of PBS at 37 °C for 3 days. At predetermined time intervals, gels were removed from the medium, and the gel surface was blotted with filter paper before obtaining swollen weights (W_s). Hydrogel mass swelling ratios were calculated from the equation W_s/W_d. All experiments were performed in triplicate.

2.7. Degradation properties of HPPAE–HA hydrogels

The hydrogel samples (diameter = 10 mm, height = 2 mm) were immersed in phosphate buffer solution (25 mL, pH 7.4) at 37 °C with a shaking speed of 50 rpm. The buffer solution was renewed twice a week. At regular time intervals, the samples were taken from the buffer solution, and after lyophilization the weight was measured. All experiments were performed in triplicate.

2.8. In vitro release of BSA from HPPAE–HA hydrogel

Gel precursor solution (2 mL) prepared as described above was mixed with PBS solution of BSA (200 μL, 5 mg mL^{-1}) in a disposable glass vial. For the in vitro release studies, 10 mL of PBS release solution was added in the glass vial. The glass vial was then incubated in an incubator at 37 °C with the shaken speed of 50 r min^{-1}. 4 mL of sampled release buffer was pipetted and 4 mL fresh PBS solution was added into the glass vial. The protein content was quantified by using UV-vis spectrophotometer at the maximum absorbance wavelength of BSA at 595 nm with the presence of coomassie brilliant blue.

3. Results and discussion

3.1. Synthesis of HPPAE–HA hydrogel

3.1.1. Synthesis of HPPAE–AC

Hydroxyl groups of HPPAE were modified with acryloyl chloride (AC) to introduce acryloyl groups via an esterification reaction. The chemical structure of HPPAE–AC was confirmed by ^1H NMR spectra both in DMSO-δ6 and after exchange with deuteroxide water (D_2O) (Fig. 1). The substituted hydroxyl groups of HPPAE were identified by the signals of the acryloyl group of acryloyl chloride at 5.70–5.82, 6.12–6.25 and 6.71–6.85 ppm (protons of de double bond H_a and H_b, respectively), having a ratio of 1:1:1 as expected. The substitution degree (SD) can be determined by the integrated peak areas between acryloyl and hydroxyl groups. Due to the uncertain position of hydroxyl group in H NMR, the methylene next to hydroxyl

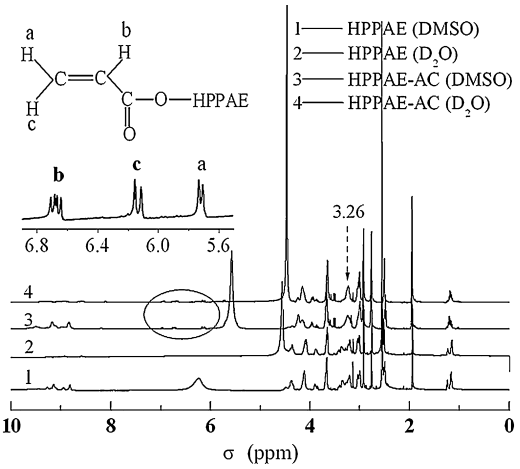

Fig. 1. ^1H NMR of HPPAE and HPPAE–AC.

group (CH_2CH_2—OH), was utilized as standard peak to calculate the SD of hydroxyl groups. Other signals at 9.0 and 1.29 ppm represent the protons of —NH— and CH_3 groups of HPPAE, respectively (Liu et al., 2013). ^1H NMR analysis of the obtained products demonstrated that at room temperature under a nitrogen atmosphere with DMSO as solvent and DMAP as catalyst, the acryloyl groups are directly linked to HPPAE, resulting in HPPAE–AC with a SD of 8.3% (415 μmol g^{-1}).

3.1.2. Synthesis of HA-SH

Thiolated HA (HA-SH) was prepared as shown in Fig. 2. The HA were modified by reacting the carboxylic groups of HA with the amine groups of cystamine dihydrochloride using EDAC/NHS activation at an optimal pH of 4–5 at room temperature. The ^1H NMR spectrum of HA-SH showed the characteristic peak of *N*-acetyl methyl protons of HA. In addition, HA-SH exhibits two signals at 2.86 and 2.65 ppm (Fig. 2, peak 2 and peak 3), respectively, belonging to two side chain methylene protons of cysteamine moieties (CH_2CH_2SH). SD of HA-SH was calculated as the difference between the methylene proton signal of cysteamine relative to the *N*-acetyl methyl proton signal of HA. The content of free thiol (S—H) groups was further determined by Ellman's method. Depending on the preparation condition, SD of HA-SH is 23.8% of the glucuronates, and the content of free thiol groups is 222 μmol g^{-1}.

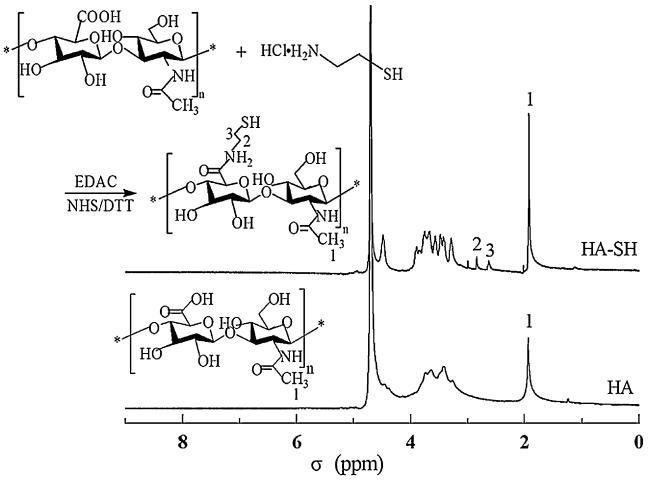

Fig. 2. ^1H NMR of HA-SH.

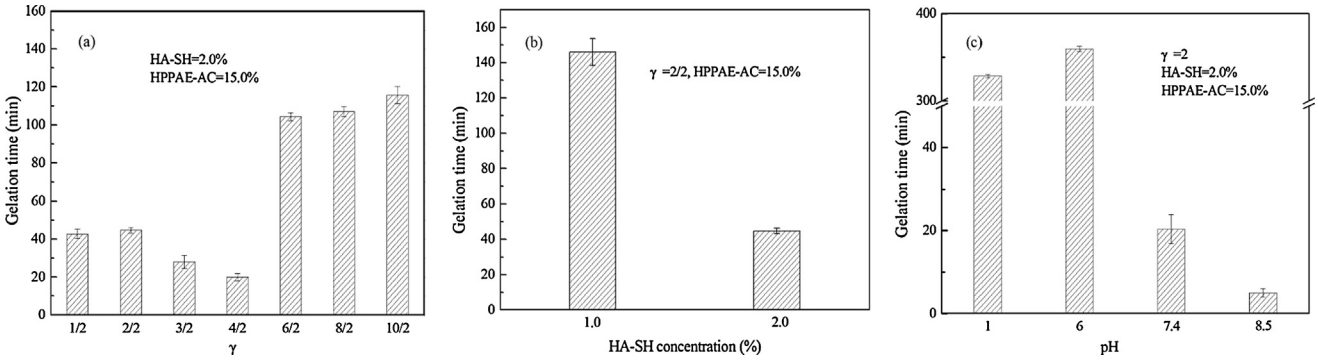

Fig. 3. Gelation time as a function of (a) γ, (b) HA-SH concentration, and (c) pH value of gel precursor in 0.1 M PBS.

3.1.3. Hydrogel formation

HPPAE–HA hydrogels were fabricated via thiol Michael addition reaction with HPPAE–AC and HA-SH in PBS. It was demonstrated that deprotonated thiol (thiolate) is often the active species in Michael additions involving thiols ((Ellman, 1958)). Therefore, the gelation time of the HPPAE–AC and HA-SH hybrid system is determined by the thiolate concentration. Factors affecting the thiolate concentration are SD of HA-SH, HA-SH concentration and pH of the gel precursor (Nuttelman et al., 2008). This section addresses the influence of each factor on the gelation time.

Michael-addition reaction between thiol and acryloyl groups provides an avenue to tune the network structure via simply changing stoichiometric ratio of thiol to acryloyl groups (γ). As a result, the stoichiometry of reactive groups (*r*) plays a key role on the final properties of the hydrogels, including their mechanical stiffness and swelling ratio. While, the alkaline conditions required for Michael-type reactions promote disulfide formation that has been implicated in the off-stoichiometric reaction of monomers during hydrogel formation, and the content of thiol group was lower than expected. In addition, the macromonomer generally has a low mobility due to the high solution viscocity and entanglement between macromonomers, could led to incomplete reaction (Sperinde & Griffith, 1997). In the study, networks were obtained with 2% (w/v) HA-SH and 15% (w/v) HPPAE–AC at seven *r* varied from 0.5 to 5. Fig. 3a shows the gelation time as a function of γ. In the case of HPPAE–HA hydrogel, the gelation time decreased from 45 to 20 min with increasing γ from 1/2 to 4/2. When increase γ from 4/2 to 6/2, gelation time increases and shows little change on further increasing *r* to 8/2 and 10/2. With the increasing of γ, the content of thiolate anions increased, which is benefit for the fast primary cross-linking by Michael-type addition. However, after passing an optimum *r* of about 4/2, disulfide bond formation may prevail, which requires prolonged reaction time on the scale of hours. Together with sterically hindered entanglements between macromonomers, some thiol groups can not contribute to network formation.

Fig. 3b illustrates the relationship between gelation time and HA-SH concentration with a comparable SD with HPPAE–AC concentration kept at 15%. Gelation time decreased from 145 to 48 min by increasing the HA-SH concentration from 1 to 2%. This is attributed to an increase in the number of functional groups per volume of gel precursor that may create more polymer entanglements during gel formation. Fig. 3c shows the gelation time as a function of the solution pH. As expected, the gelation time decreased with increasing solution pH from 6.5 to 8.0. For example, the gelation time decreased from 357 to 4 min by increasing the pH from 6 to 8.5 as for γ of 1 obtained with 2% (w/v) HA-SH and 15% (w/v) HPPAE–AC. The faster gelation at higher pH value is due to the presence of higher concentration of reactive thiolate anions that are responsible for the rapid Michael addition reaction. In addition, the rates of Michael addition reaction and oxidation, that is the formation of disulfide products, were both sensitive to pH. With an increase in pH value, oxidization was accelerated and the content of disulfide bond was improved. Therefore, pH 7.4 phosphate buffer saline was selected as the solvent for synthesis of HPPAE–HA hydrogel to minimise the disulfide linkage. In summary, all the experiment observations described above consistently demonstrated that gelation time was significantly affected by γ, HA-SH concentration and gel precursor pH and HPPAE–HA hydrogel with the gelation time ranged from minutes to few hours can be achieved, which is advantage for the desired application.

3.2. Properties of HPPAE–HA hydrogel

3.2.1. Rheological characterization

In order to fully analyze the mechanical behavior of HPPAE–HA hydrogels, HPPAE–HA hydrogels were analyzed by equilibrium welling in PBS solution and by subject to dynamic frequency sweep of rheometry. The storage modulus (G') and loss modulus (G'') were monitored over frequency ranges from 0.1 to 10 Hz at 25 °C. In all cases, G' value of HPPAE–HA hydrogels was independent of the tested frequencies, suggesting the characteristics of the covalently cross-linked hydrogel network (Fig. 4). This stability indicated that the permanent chemical cross-links were not destroyed by the increasing frequency at a constant strain (0.1%). Results also showed significantly higher storage modulus (G') than loss modulus (G''), approximately two orders of magnitude higher than the

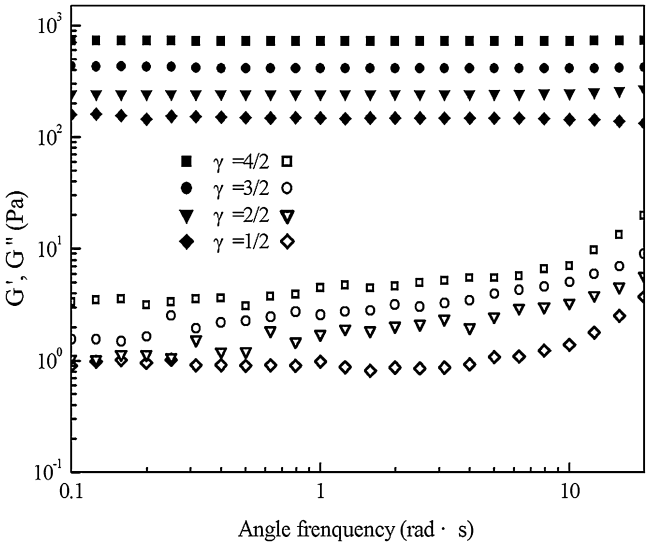

Fig. 4. Rheological characterization of HPPAE–HA hydrogels against various γ.

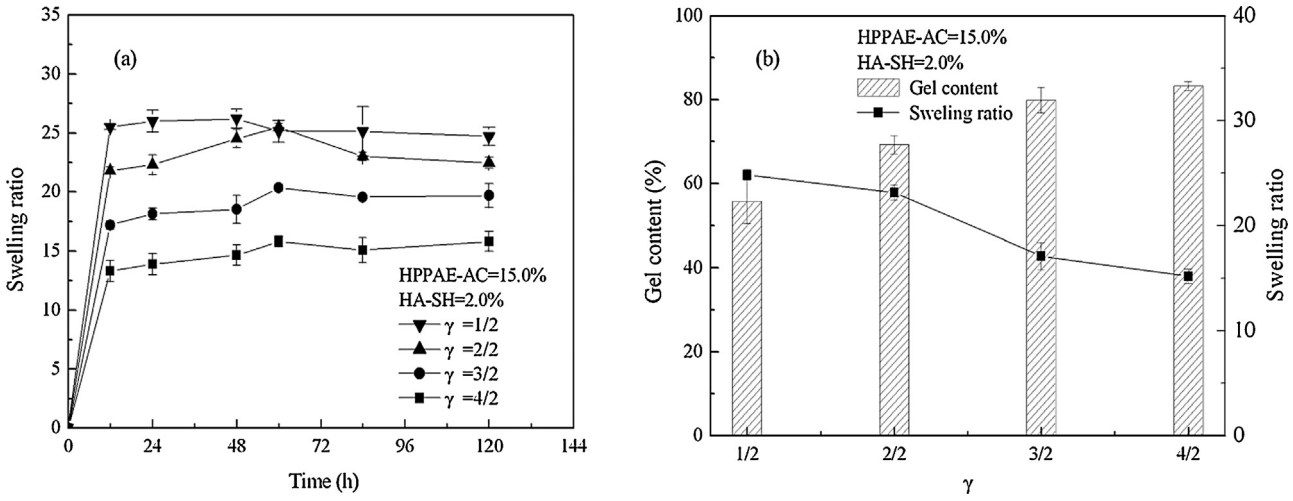

Fig. 5. Equilibrium swelling with respect to time for HPPAE–HA hydrogels.

corresponding G'' values for all formulations, indicating that these hydrogels are highly elastic. For example, the average G' were 724 Pa for HAAE-HA with γ of 4/2, whereas, the G'' value of it was 7.5 Pa. In addition, G' of HPPAE–HA hydrogel increases with γ. HPPAE–HA hydrogel with γ of 1/2 and 4/2 shows G' of 724 Pa and 149 Pa, respectively, indicating higher γ corresponds to higher storage modulus. Therefore, the storage modulus of HPPAE–HA hydrogels can be adjusted by varying stoichiometric ratio of functional groups.

3.2.2. Equilibrium swelling

The swelling behavior of HPPAE–HA hydrogels was monitored by mass after incubation in PBS at 37 °C. Swelling ratios reached a plateau after 60 h (Fig. 5a), and equilibrium swelling values come to equilibrium and revealed swelling ratio between 10 to 30 as shown in Fig. 5b. In addition, as r increases, gel content increases due to the improvement of cross-linking density. For instance, gel content was improved from 54 to 82% as r was increased from 1/2 to 4/2, which means there is amount of sol fraction along with the formation of hydrogel. Due to the steric hindrance of macromonomer and the inter- and intra chain entanglement, the conversion of functional groups cannot reach complete. Swelling characteristics of hydrogels are also strongly influenced by the degree of cross-linking. The swelling ratio was found to be inversely proportional to the modulus as hydrogel networks with low cross-linking density should have correspondingly larger pores or cavities, resulting in greater water adsorption. Therefore, r of the hybrid hydrogels plays an important role in controlling swelling data, as a higher swelling in those HPPAE–HA hybrid hydrogels possesses lower r (e.g., r = 1/2, 2/2) in all cases. This is due to the lower cross-linking density with a lower HA feed ratio in the gel precursor. The hydrogel with r of 4/2, having the highest content of HA, demonstrates the lowest swelling ratio of 14.5. HPPAE is a hydrolytic degradable polymer, therefore, HPPAE–HA hydrogel with the highest swelling ratio may show the fastest degradation. To verify the speculation, degradation behaviors of HPPAE–HA hydrogels under different media were studied.

3.2.3. In vitro degradation

In vitro degradation of HPPAE–HA hydrogels were conducted at 37 °C for up to 40 days in pH 7.4 phosphate buffer. Fig. 6a shows that r significantly affects the degradation rate of the hydrogels. For instance, after 15 days of incubation in PBS, HPPAE–HA hydrogel with r of 4/2 showed 48.4% weight loss, while HPPAE–HA hydrogels with r of 3/2, 2/2 and 1/2 showed 60.6%, 67.1% and 68.2% weight loss, respectively. In addition, HPPAE–HA hydrogels with

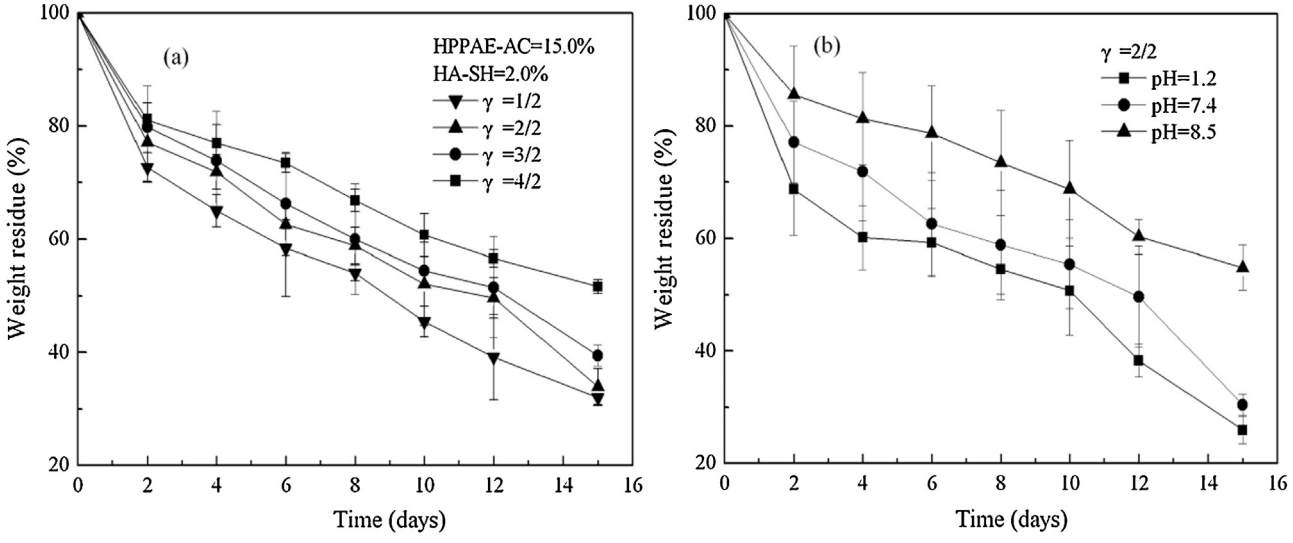

Fig. 6. Degradation of HPPAE–HA hybrid hydrogels against (a) r and (b) pH of PBS solution.

r of 1/2, 2/2, 3/2 and 4/2 lost 50% of their weight at day of 9, 12, 13 and 15, respectively. The mass loss during early portion of degradation (1–3 days) is attributed to the sol fraction of incomplete reaction. As degradation proceeds, the network structure and molecular weight of the kinetic chains are the main factors that influence the mass loss profiles. Networks with low cross-linking density (e.g., *r* = 1/2, 2/2) degrade faster as for the low content of thiol groups, and the data supports the conclusion derived from the rheological analysis that HPPAE–HA hydrogel with the lowest degradation (*r* = 4/2) possesses the highest cross-link density. The lower cross-linking density of HPPAE–HA hydrogels permits fragments to be facilely released into solution at a faster rate. In addition, the slow degradation of all HPPAE–HA hydrogels in PBS buffer revealed that the disulfide bonds comprising both inter- and intra-molecular cross-links between HA-SH macromonomers were quite stable and rendered the hydrogels resistant to hydrolysis. A remarkable example is the hydrogel with *r* of 4/2, more than 50% weight is retained after hydrolyzed at 37 °C for up to 15 days in pH 7.4 phosphate buffer. As expectedly, the degradation profiles were dependent on the composition and cross-linking densities. Therefore, the mass loss profile can be controlled through alterations in initial thiol-acryloyl macromonomer composition. Additionally, the degradation behavior of HPPAE–HA hydrogels with *r* of 2/2 under different pH was also investigated (Fig. 6b). At pH of 1.2, HPPAE composed with phosphate and phosphamide bond can readily degraded. The relatively rapid degradation in acid solution is due to the chemistry of HPPAE. At alkalic condition, like pH 8.5, thiolate anion is readily formed, and the Michael addition reaction between thiol and acryloyl groups thereby facilitated, contributing to the cross-linking density. As a result, the degradation of HPPAE–HA is suppressed. At acid (pH = 1.2) and neutral solution (pH = 7.4), the degradation of HPPAE–HA is the result of fast hydrolysis of HPPAE (Liu et al., 2013).

3.3. Reduction triggered de-cross-linking and BSA release

To investigate the controlled release behaviors from the hydrogels with building blocks of different *r*, BSA was uniformly encapsulated into gel precursor as a model protein. The mass of protein released over time was monitored using UV–vis spectrometer at 585 nm. While a small amount of degradation occurs over this timeframe, the release profile appears to be largely diffusion-mediated and the release of BSA is dependent on *r*. The release of BSA from HPPAE–HA hydrogels showed very different release profiles (Fig. 7). For hydrogel with *r* of 4/2, the limited release of BSA was observed after a relatively fast release of 61.3% at the time point of 12 h. The cumulative drug releases from hydrogels with *r* of 3/2 and 2/2 were 70.0% and 77.5%, respectively. The burst release of BSA is ascribed to BSA located near the hydrogel surface which can be released immediately from the hydrogel to the medium as soon as the hydrogel are immersed in the buffer solution. After the initial burst release, the following release seemed to be a diffusion process, and the crosslinking density is in the reverse order with the release rate owning to the lower degradation of the dense network. With the increase of *r*, the release of BSA was retarded owning to the improved crosslinking density. For example, the recovered release of BSA from hydrogels with *r* of 4/2, 3/2 and 2/2 exhibited a value of 82.0%, 92.5% and 98.8%, respectively, after 3 days. The proteins were not completely released and recovered, possibly owing to precipitation of the protein. In addition, the strengthened hydrophibic interaction between HA and BSA with the increased content of HPPAE in the precursor gel may be another aspect that contributes to the controlled release.

When gels forms in situ and entrap biomolecules and cells, the oxidation of two free thiolates by air most are inevitable, which results in disulfide cross-linked hydrogels. Such oxidation reactions are intrinsically difficult to control during the progression of gelation. While, disulfide cross-linked HA hydrogels are of particular interest as protein or dug carrier because of the potential dual degradability of the system. The disulfide bond presented in HA-SH and the disulfide bond formed through the oxidation of two thiols during the gelation can easily be cleaved enzymatically by HAase and chemically by biological such reductants as glutathione (GSH) and cysteine. Therefore, HPPAE–HA hydrogels fabricated from HPPAE–AC and HA-SH hybrid system exhibit reduction-sensitive rapid de-cross-linking, which is desired for the controlled drug release in intracellular fluids. In vitro BSA release studies of HPPAE–HA hydrogels were carried out in the presence of DTT, a mild organic reducing regent commonly used to reduce DNA and protein. Unexpectedly, under all *r* examined, similar trends were observed, the release rates of BSA were significantly slowed down in the presence of DTT (Fig. 7). For instance, as a *r* of 3/2, in the absence of DTT, more than 70% BSA were released from hydrogels within 12 h, while the same hydrogel showed a much less release of 10.7% under 10 mM DTT within the same time. Likely, the total release of the hydrogel after 120 h was retarded with the cumulative release of 82.7% as for *r* of 3/2. Consistently, after 120 h, the cumulative release of BSA was even less with a value of 76.5% as for *r* of 4/2 because of their highly cross-linked structure as compared with that of *r* of 3/2. In the presence of 10 mM DTT, sustained and virtually quantitative release of BSA was observed under otherwise identical conditions, what's more important is that there is no burst release as for all the formulations. To reveal the origin of the controlled release in DTT solution and the effects of DTT concentrations on the BSA release, HPPE-HA hydrogel with *r* of 2/2 was investigated in detail.

The release profiles of BSA from the macromolecular hydrogels against various molar concentration of DTT in pH 7.4 PBS solution were investigated (Fig. 8a). The release profile of BSA-loaded hydrogel without DTT showed a prominent burst release characteristic, and the accumulative release ratio was about 99.0% until 3 days, owning to the severe degradation of HPPAE–HA. When the DTT concentrations were increased from 0 to 10 mM, the cumulative release of BSA in the first 3 h was decreased from 23.7 to 4.0%, and the 80% cumulative release of BSA was retarded for 12 h to nearly 96 h. Further increase the concentrations of DTT to 20 mM, the cumulative release of BSA after 120 h was improved from 80.0 to 86.9%. These results indicate that DTT triggered de-cross-linking

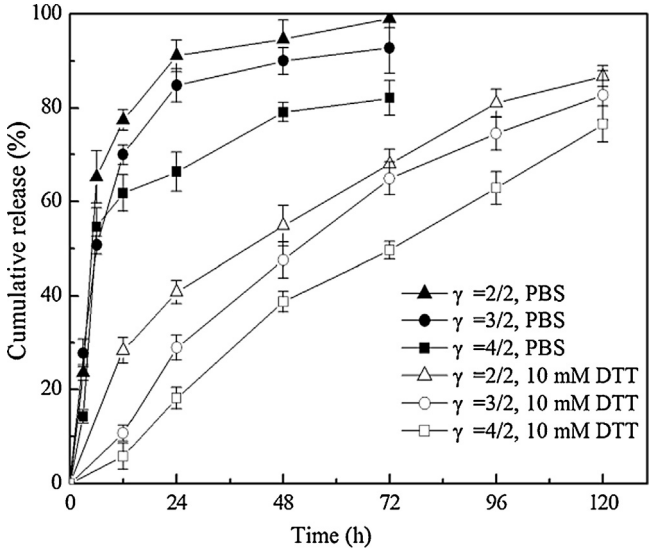

Fig. 7. In vitro release profiles of BSA from HPPAE–HA hybrid hydrogels with various r against PBS buffer and 10 mM DTT.

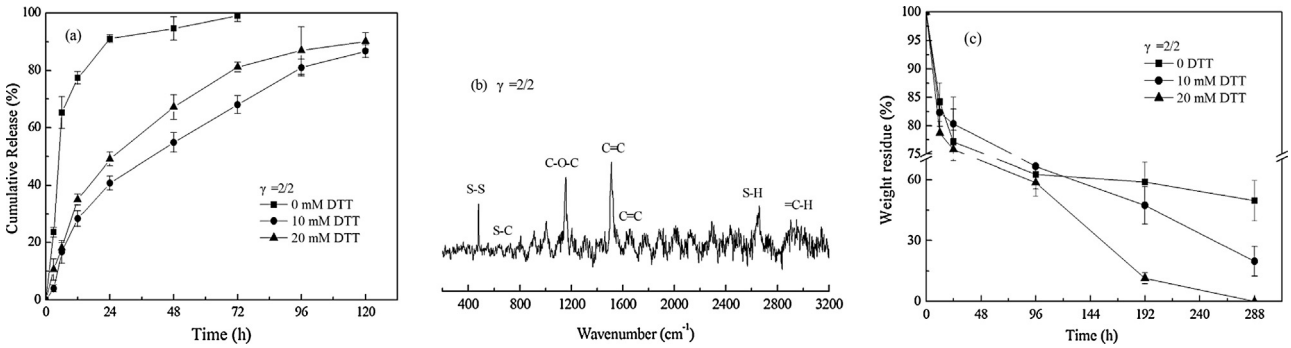

Fig. 8. HPPAE–HA hydrogel: (a) In vitro BSA release profiles against various content of DTT in PBS buffer; (b) Raman spectrum; (c) degradation behavior against various content of DTT in PBS buffer.

of hydrogel is rapid and prevail over the controlled release in DTT solution. Remarkably a higher molar ratio of DTT vs. disulfide bond resulted in a higher release rate of BSA, possibly due to more disulfide bonds cleavaged. In all cases, the molar ratio of DTT vs. disulfide bond was about 10, the rapid erosion of the disulfide bond containing HPPAE–HA hydrogel under high reductant concentration is inevitable. In addition, due to the inevitable disulfide bond formation during the gelation and thereby consuming functional groups that impair the equimolar stoichiometry, the thiol groups that take part in Michael-addition reaction is decreased. To confirm this speculation, the presence of both S—S and the S—C bonds of HPPAE–HA hydrogel was investigated by Raman spectrum.

Fig. 8b presents Raman spectra of the cross-linked hydrogel with *r* of 2/2. Weak peaks originated for C—S—C stretching vibrations were identified at 720 and 644 cm^{-1} in the spectrum of HPPAE–HA hydrogel (Liu et al., 2013). In addition, the bands observed at 1652 and 2972 cm^{-1} indicate the presence of acrylic C=C group and acrylic C—H stretch, respectively (Colthrup, Daly, & Wiberley, 1990). The presence of acrylic groups indicates that only part of the acryloyl groups react with thiol groups, which is also correspondenced to the weak intensity of S—C band. As for the hydrogel with *r* of 2/2, the content of thiol group and acryloyl group is equivalent, and the conversion of acryloyl group can reach a high value under an ideal condition. While, the sharp peak at 487–506 cm^{-1} is attributed to S-S group, which means the presence of a large amount of disulfide bond (S—S) (Poole, Lyons, & Church, 2011). Due to the steric hindrance of macromonomer and the inevitable formation of disulfide bond between thiol groups during the gelation, the conversion of acryloyl group is low. Therefore, there are abundant acryloyl groups in HPPAE–HA hydrogel in addition to the presence of the sol fraction (about 44% of total acryloyl groups of HPPAE–AC estimated from Fig. 8b). The thiol groups produced by the reduction of the disulfide bonds are capable of forming cross-linked gel with the abundant free acryloyl polymer chains, which contribute to the network formation of HPPAE–HA hydrogel. Therefore, the DTT-triggered partially de-cross-linking and the subsequent Michael type cross-linking is the main reason that results in the controlled release of BAS release in 10 mM DTT solution. As the DTT content increased to 20 mM, the reduction reaction increased, more disulfide linkage may be cleavaged, which is benefit for the BAS release as compared with that in 10 mM DTT solution.

Degradation of HPPAE–HA hydrogel can give further support for the enhanced cross-linking against DTT solution (Fig. 8c). During the initial 12 h, the degradation of HPPAE–HA hydrogel is accelerated in DTT solution, especially in 20 mM DTT solution. Whereas with the time point increased to 24 and 96 h, HPPAE–HA hydrogel exhibits the slowest degradation in 10 mM DTT solution, and HPPAE–HA hydrogel in 20 mM DTT and in 7.4 PBS, respectively, shows similar weight residue. The initial degradation is largely depends on the gel content of hydrogel, and the weight loss is the result of sol fraction from macromonomers. Meanwhile, HPPAE–HA hydrogels exhibited a faster disulfide cleavage under a high DTT solution, which contributes to the degradation. When the disulfide bond is cleaved, macromonomers with thiol groups formed. The free thiol groups can crosslink with neighboring free acryloyl groups of HPPAE–AC, leading to further cross-linking. As a result, the weight loss is lowed. These observed changes in weight loss are directly related to the disulfide cleavage and the subsequent Michael-addition reaction contributing to the network.

Whilst disulfide containing polymers are commonly known for breaking down and contribute to the release due to the reductive capacity in reduction medium, in the research we demonstrate the opposite effect. Since the incorporation of proper DTT loading-level could still produce supramolecular hydrogel and show the ability to regulating the drug release as designed, this strategy might extend the application of such reduction-sensitive macromonomer for fabricating hydrogel in controlled release.

4. Conclusions

Hyperbranched polyphosphoramidate-hyaluronan hybrid hydrogels (HPPAE–HA) were formed from thiol functional HA and acrylated hyperbranched polyphosphoramidate macromonomer via Michael-addition reaction. The gelation time, mechanical and degradation properties are readily controlled by varying in concentration of HA-SH, stoichiometric ratio of thiol to acryloyl groups and solution pH. Free thiol groups were produced with the redox triggered partially de-cross-linking due to the presence of disulfide bond in the backbone of HPPAE–HA hydrogel, and the subsequent Michael addition reaction between these thiol groups and neighboring free acryloyl groups present in the hydrogel contributes to the network. As a result, the degradation behavior and release rates of BSA from the HPPAE–HA hydrogel were significantly controlled with the presence of DTT and abundant acryloyl groups presented. These results indicate that the injectable HPPAE–HA hydrogels have a high potential for drug delivery applications.

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