Comparative Properties of Hyaluronic Acid Hydrogel Cross-linked with 1, 4-Butanediol Diglycidyl Ether Assayed Using a Marine Hyaluronidase

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Comparative Properties of Hyaluronic Acid Hydrogel Cross-linked with 1, 4-Butanediol Diglycidyl Ether Assayed Using a Marine Hyaluronidase

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Abstract. Hyaluronic acid (HA) hydrogels for injection were fabricated using 1, 4-butanediol diglycidyl ether (BDDE) as a cross-linker. We used hyaluronidase fermented by the marine bacteria *Arthrobacter globiformis* A152, to comprehensively investigate the effects of BDDE concentration on swelling degree, modification degree, morphology, HA content, anti-enzyme ability, and residual free cross-linker. The results showed that within a certain range, the measured modification degrees of the HA hydrogels and HA contents changed linearly with increased concentration of the cross-linker. However, the change in anti-enzyme ability was not linear, potentially reflecting the influence of the spatial network structure.

1. Introduction

Hyaluronic acid (HA) is a polysaccharide with high molecular weight that consists of linear, repeating units of β-D-glucuronic acid (GlcA) and β-D-N-acetyl glucosamine (GlcNAc) that are linked by β-1, 4 and β-1, 3 glycosidic bonds. [1, 2] the excellent biocompatibility and high water retention capacity of HA makes it of major interest as a biomaterial for many medical and cosmetic applications. [3].

However, studies have shown that free HA is easily digested by enzymes in vivo, and the half-life of HA is less than 24 hours after subcutaneous injection. [1] Therefore, in order to improve the mechanical properties of HA and prolong its retention time in vivo, HA is often modified by cross-linkers. [4] Currently used methods to crosslink HA are the amino-crosslink method and the hydroxyl-crosslink method. A widely used diepoxide cross-linker is 1, 4-butanediol diglycidyl ether (BDDE). Due to its low sensitization and good biocompatibility, BDDE is widely used in many dermal filler products. BDDE promotes stable covalent ether linkages between HA and BDDE in an alkaline environment. [5] In the reaction, nucleophilic groups of HA react with the epoxide groups of BDDE. In theory, there are six available sites in every HA–disaccharide unit that can react with BDDE. However, the deprotonated hydroxyls are the strongest, so these are the most likely reaction sites. Stable ether bonds can be formed between HA and the cross-linker. [6] Linear HA molecules can connect with BDDE in two ways, pendent modification, in which one ether linkage forms between one side of a BDDE molecule and a disaccharide unit, and cross-link modification, in which two ether linkages form between the sides of a

BDDE molecule and two disaccharide units. [7] However, despite its widespread use as a cross-linker, only few reports have examined the effect of BDDE concentration on the properties of HA hydrogels.

The purpose of cross-linking is to prolong the retention time of HA in vivo. HA hydrogel that is more highly modified exhibits stronger and better resistance to enzymes, resulting in a longer retention time in vivo. However, with increased cross-link degree, the other properties of the HA hydrogel may also change. [8] For example, the hardness of the HA hydrogel will increase, which can increase the difficulty of injection. [9] Thus, obviously, it is not advisable to merely increase the cross-link degree as a strategy to prolong the retention time. During the preparation of HA hydrogels for a specific purpose, the different properties of the HA hydrogel must be considered comprehensively. [10] Although many studies have examined how to improve the analytical and measurement methods to assess cross-linked HA hydrogel properties, [6,11] there has been little comprehensive evaluation of HA hydrogel properties using a single hyaluronidase. Recently, the HA-degrading Arthrobacter globiformis strain A152 was isolated from intertidal sludge collected from Jading in Qingdao, China, and the hyaluronidase from this strain was isolated and purified. [12] The goal of this study was to prepare a series of HA hydrogel samples cross-linked with different concentrations of BDDE, and then explore the effect of BDDE concentration on the properties of the prepared hydrogels using the marine hyaluronidase from Arthrobacter globiformis strain A 152. This work will provide the reference and guidance to customize the preparation of HA hydrogels for different applications.

2. Experimental

2.1. Materials

HA (medical grade) with a weight average molecular mass of 1.55×10^6 Da was purchased from Top Science Biological Technology Co. (Shandong, China); BDDE (1,4-butanediol diglycidylether) with a purity of 95% and ammonium acetate were purchased from Sigma-Aldrich; NaOH, KOH, KCl, HCl, H_2SO_4, nicotinamide, formic acid, ethanol, hypnone, and carbazole were purchased from Sinopharm Chemical Reagent Co., Ltd. Ammonium acetate was of chromatographic grade, and all other reagents used in this study were of analytical grade. Hyaluronidase freeze-dried powder (fermented by *Arthrobacter globiformis* A152) was provided by the Marine Biomedical Research Institute of Qingdao (Shandong, China). Deionized water was purified using a Milli-Q system.

2.2. Preparation of HA hydrogels

Different volumes of BDDE (25, 50, 75, 100, 125, 150, and 175μL) were separately dissolved in 5mL of NaOH (1%, w/w), then 1g of HA powder was added for the hydrogel synthesis reaction. The concentration of BDDE was 5, 10, 15, 20, 25, 30, and 35μL/mL in the reactions, which were defined as Reaction1-7, respectively. The reactions were incubated at 42°C for 4h. The products of Reaction1-7 were swelled in 100 mL deionized water for 12h, allowing the formation of transparent hydrogels, and then were passed through a 50-mesh sieve to granulate. Next, 10g of the differently prepared hydrogel granules were separately suspended in 10mL of deionized water and the pH was adjusted to 6.8-7.2 by addition of 0.1mol/L HCl. The mixture of hydrogel granules and water was centrifuged at 4000r/min for 5min, and then the supernatant was discarded. The water from the surface of the hydrogel granules was then absorbed using filter paper.

The hydrogel granules prepared as above were then washed as follows. The hydrogels were soaked in 100 mL deionized water, and stirred with a magnetic stirrer at 200 r/min for one hour. The mixture was then centrifuged at 4000r/min for 5min, and the water was absorbed from the surface of the hydrogel granules by filter paper. This washing process followed by absorbing the superficial water was performed five times for samples were defined as Sample1-7, or eight times for samples defined as Sample1'-7'.

2.3. Determination of the swelling degrees

For each Sample1-7, 1 g was removed and dried in a 60◦C oven for 12 h until the weight did not change. The constant weights were recorded as the W0. Next, 10mL deionized water was added to the dried hydrogel samples and the mixture was incubated at 25◦C for 12h. The water attached to the hydrogel was removed by a filter paper and the weight of the hydrogel was determined and recorded as W1. The swelling ratio was calculated by Eq. (1):

$$\text{Swelling degree} = (W_1 - W_0)/ W_0 \quad (1)$$

2.4. Determination of the modification degrees

Size exclusion chromatography (SEC) was used to determine the total modification degree (t-MOD), and SEC with mass spectrometry (SEC-MS) to determine the cross-link modification degree (c-MOD) and the pendent modification degree (p-MOD). [7].

Hyaluronidase (HAase) at a concentration of 5×10^4 U was added to 1g Sample1-7 and 1g 2% HA solution, and the volumes of each reaction mixture were then adjusted to 5 mL by the addition of deionized water. After incubation for 24h at 42°C, the digestion mixture was boiled in a boiling water bath for 5 minutes to inactivate the enzyme, and then was centrifuged for 20min at 1×10^4 r/min to remove the enzyme. The supernatant was lyophilized.

To determine the total modification degree (t-MOD), the digestion products of Sample 1-7 and 2% HA solution were analysed by SEC using a Superdex 30 Increase 10/300 GL column (GE Healthcare) on an Agilent 1260 Infinity system. Lyophilized solid samples were dissolved in deionized water at a concentration of 3mg/mL. The solution samples and the mobile phase (0.5mL/min) of 10mmol/L ammonium acetate were filtered through a 0.2 μm Millipore filter. The injection volume was 20μL and the UV detection was set at 232nm. To confirm the composition of the mixtures, peaks were collected by SEC, and then were analysed using an Agilent MS 6410B system (negative mode, and the decluttering potential and entrance potential were set to −50 and −10, respectively).

To determine the cross-link modification degree (c-MOD) and the pendent modification degree (p-MOD), the digestion products were analysed by SEC-MS on an Agilent 1260 Infinity/6410B system. The mobile phase (0.3 mL/min) consisted of 10 mM ammonium acetate and chromatographic grade acetonitrile at a ratio of 4:1 (v/v), filtered through a 0.2μm Millipore filter. The other chromatographic conditions and mass spectrometry conditions used were the same as above. The ions (m/z) of the modified fragments in the total ions chromatogram (TIC) were selected as quantitative ions, and the peak areas of these ions were used to quantify the relative amounts.

2.5. Micro morphology

The morphologies were characterized by scanning electron microscopy (SEM). Sample1-7 and 2% HA solution were treated by freeze-drying, and the samples became white sponge-like solids. The solids were covered with a thin layer of gold before observation of the local morphologies of the samples using a scanning electron microscope (Hitachi S-4800, Japan).

2.6. Anti-enzymatic ability analysis

The degradation step was performed via depolymerization of the cross-linked hydrogel into oligosaccharide fragments using HAase fermented by *Arthrobacter globiformis* A152 (prepared in-house). The enzyme cleaves the β-1, 4 linkages between N-acetyl-D-glucosamine (GlcNAc) and glucuronic acid (GlcA). [12].

The rate of residual after enzyme-degradation was used to indicate the anti-enzymatic ability. Under the same conditions, the higher the hydrogel residual rate, the better the anti-enzymatic ability. Approximately 300 U of HAase were added to 1g Sample1-7 and 1g 2%HA solution, with each reaction in duplicate, and the volumes of systems were then adjusted to 2mL by the addition of deionized water. After incubation for 30 min and 60 min at 42°C, the enzymatic reactions were stopped by the addition of 3 mL of absolute ethanol. The mixtures were centrifuged at 12000r/min for 20min, and then the

residual hydrogels were collected. The HA content of residual solids and original hydrogels or solution samples were determined according to the carbazole assay, [13] using D-glucuronic acid lactone as the standard. The hydrogel residual rate can be calculated via Eq. (2).

$$\text{Hydrogel residual rate} = [HA] / [HA]0 * 100\% \quad (2)$$

Where [HA] (μg) represents the HA content in the residues, $[HA]_0$ (μg) is the HA content in 1g of the original hydrogel or the 2% HA solution samples.

3. Results and Discussion

3.1. Modification Degree

It is challenging to accurately determine the modification degree of hydrogels, and so the molecular modification degree is often expressed by swelling degree. A HA hydrogel with higher modification degree is thought to have a more compact spatial network structure, which limits the ability of a solution to enter, thus causing a lower swelling degree. [8] The swelling degrees of Sample1-7 were determined and are shown in Fig.1. With the increase of BDDE concentration, the swelling degree showed a decreasing trend, indicating that the modification degree was increasing. However, this trend seemed irregular, as has been reported previously for the swelling properties of other hydrogels, indicating the lack of precision of this method to estimate the modification degree. [14] Since the swelling degree may be affected by many factors other than the modification degree, the swelling degree can only approximate the changing tendency of modification degree, but is not quantitative. Because of this, this method cannot be used to accurately predict the relationship between the degree of swelling and the BDDE concentration for unknown samples.

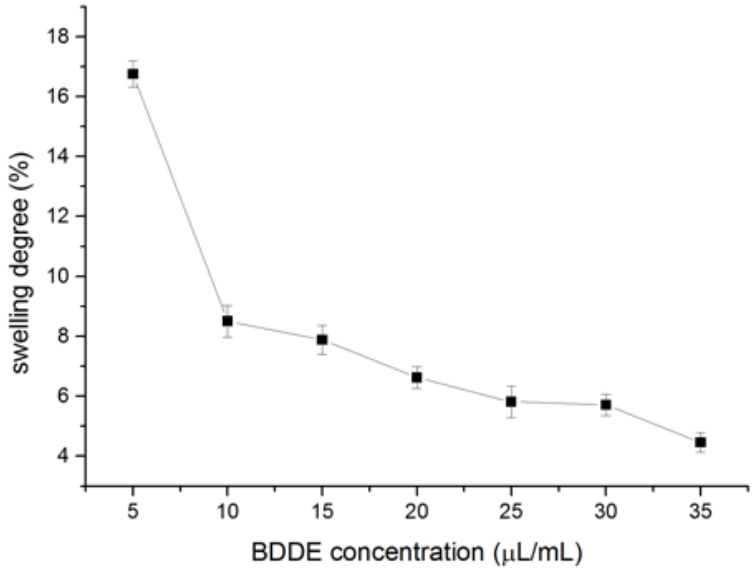

Figure 1. Swelling degrees of different hydrogel samples.

A new method has been developed to determine the modification degree by precise identification and quantification of the modified fragments in cross-linked HA hydrogel digestion. [7] The digestion products of Sample6 (BDDE concentration of 30μL/mL) included 4 main fractions (Fraction1, 2, 3, and 4). For the same chromatogram condition, HA solution digestion products included two main fractions (Fraction3' and 4'). Fraction 1, 2 had shorter retention times, and both Fraction3, 4 and Fraction3', 4' in each sample exhibited the same retention time (Fig. 2 (a) (b)). Fraction1-4 and Fraction3'-4' were collected by SEC and then were analysed by MS.

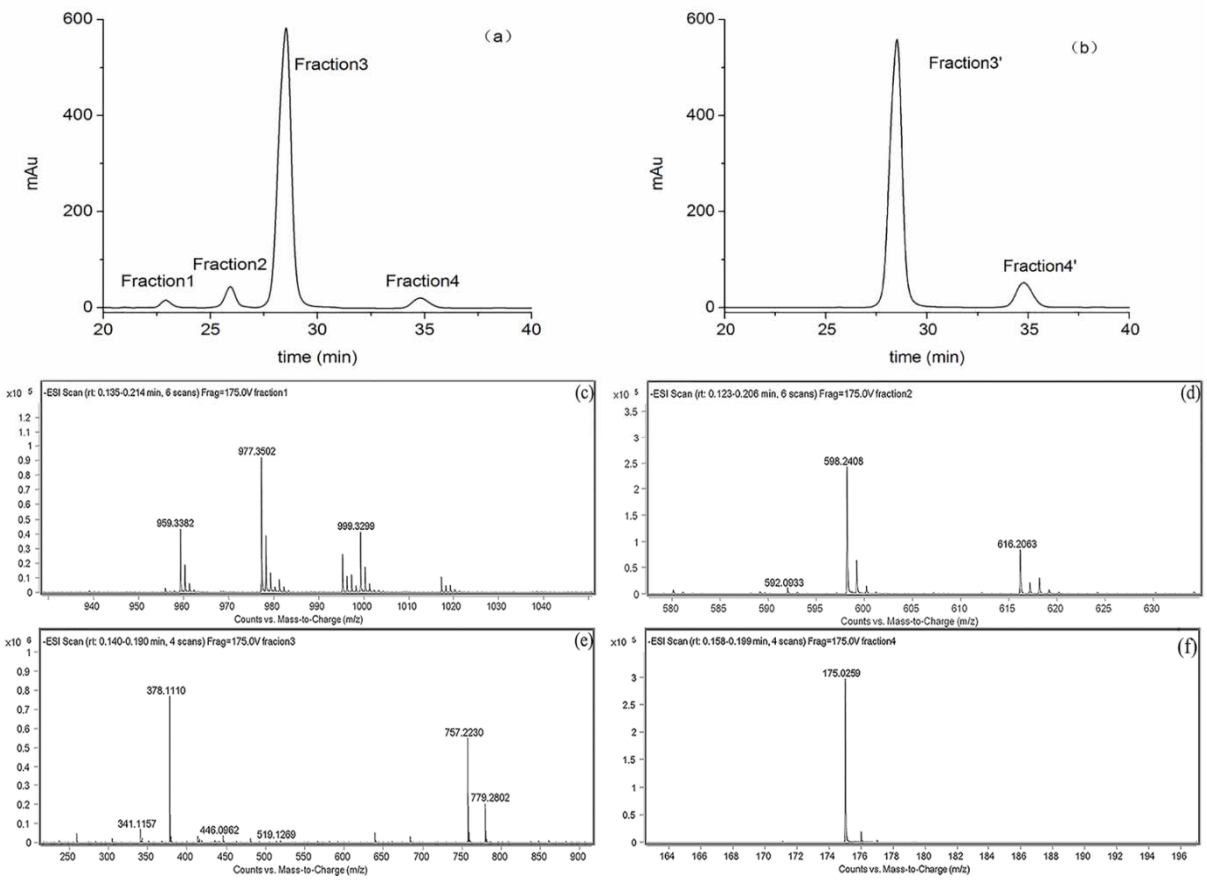

Figure 2. Separation and exploration of components in enzymatic hydrolysates: (a) SEC- HA hydrogel; (b) SEC- HA solution; (c) MS- Fraction 1; (d) MS- Fraction 2; (e) MS- Fraction 3, 3'; (f) MS- Fraction 4, 4'.

The accurate molecular weight (MW) information of each fraction was determined by MS analysis (Fig. 2 (c) (d) (e) (f)). The composition of F3 and F3', and F4 and F4' were highly similar. The fragments in each fraction are listed in Table.1, together with the cross-linking and enzymatic reaction conditions and the MW information. The spatial steric conformation of each fragment is presented in Fig.3.

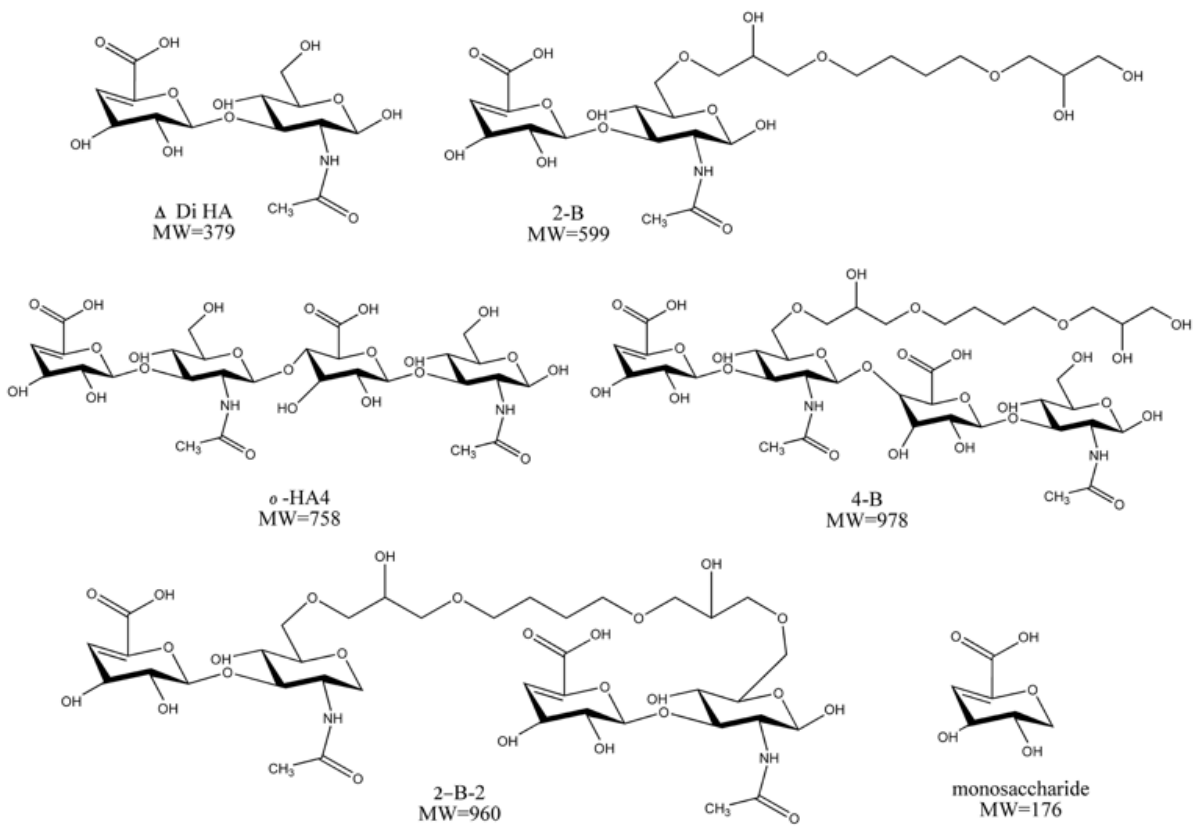

Figure 3. Chemical structures of pendent and cross-link modified fragments.

Table 1. Fragments of the digested hydrogel in Fraction 1-4, confirmed by MS

Fraction	Fragments	ESI-MS observations(m/z)
Fraction1	4-B	977.3(-1),999.3(-1)[a]
	2-B-2	959.3(-1)
Fraction2	2-B	598.2(-1),616.2(-1)[b]
Fraction3,3’	o-HA4	757.2(-1),779.2(-1)[a]
	Δ-DiHA	378.1(-1)
Fraction4,4’	Monosaccharide	175.0(-1)

[a][M-2H+Na]$^-$ in negative mode
[b][M+OH]$^-$ in negative mode

Fraction4 contained monosaccharide ([M-H]$^-$=175.0), which was converted from GlcA. HAase, fermented by *Arthrobacter globiformis* A152, is a hyaluronate lyase and can hydrolyze β-1, 4 bonds between GlcNAc and GlcA. The detection of monosaccharides might indicate that the ends of HA chains may have single GlcA units that did not connect with GlcNAc. Fraction3 and Fraction3' contained DiHA ([M-H]$^-$=378.1) and o-HA4 ([M-H]$^-$=757.2) ([M-2H+Na]$^-$=779.2). However, 2-B fragments ([M-H]$^-$=598.2) ([M-2H+Na]$^-$=616.2) of lower molecular weight were found in Fraction2. This suggests that the separation of fractions by SEC using a Superdex 30 Increase 10/300 GL column might not correspond exactly according to molecular weight, but mobility may also be affected by different structures of modified and unmodified molecules. Fraction2 and Fraction1 had less retention time, and were two unique fractions of cross-linked molecular hydrolysates. Fraction1 was a mixture of 4-B and 2-B-2, with sizes too close to be separated sufficiently. Thus the t-MOD was equal to the total area

percentage of Fraction1 and 2 in the SEC chromatogram (Table 2). As the concentration of BDDE increased, the variation trend of t- MOD was obtained (Fig. 4).

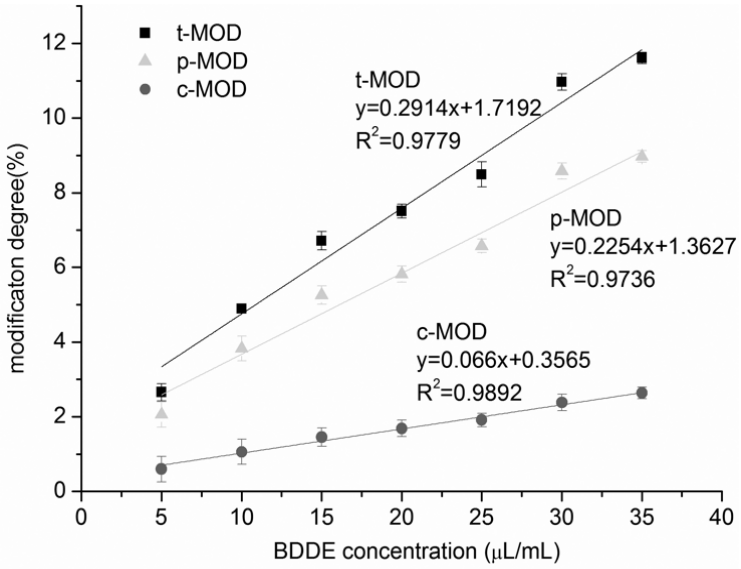

Figure 4. Modification degree of t-MOD, c-MOD, and p-MOD.

Table 2. Modification degree of different samples

	Area percentage (%)		T-MOD (%)	C%	P%	C-MOD (%)	P-MOD (%)
	Fraction1	Fraction2					
Sample1	0.79	1.87	2.66	22.41	77.59	0.60	2.06
Sample2	1.34	3.55	4.89	21.72	78.28	1.06	3.83
Sample3	1.75	4.96	6.71	21.64	78.36	1.45	5.26
Sample4	1.91	5.60	7.51	22.43	77.57	1.68	5.83
Sample5	2.22	6.27	8.49	22.53	77.47	1.91	6.58
Sample6	2.87	8.09	10.96	21.75	78.25	2.38	8.58
Sample7	3.25	8.37	11.62	22.72	77.28	2.64	8.98

For a known t-MOD value, TIC by SEC-MS can be used to quantify the relative amounts of the modified fragments. [15,16] The content of pendent modified fragments (p%) was given by the sum of the area percentage of ions from 2-B and 4-B, and the content of cross-link modified fragments (c%) was given by the area percentage of ions from 2-B-2. In this way, the c-MOD and p-MOD values were calculated (Table. 2), according to Eq. (3).

$$\text{c-MOD} = \text{t-MOD} \times \text{c\%},\ \text{p-MOD} = \text{t-MOD} \times \text{p\%} \quad (3)$$

As the BDDE concentration increased (from 5μL/mL to 35μL/mL), the t-MOD (y=0.2914x+1.7192, R^2=0.9779) increased linearly. This indicates that the amount of BDDE actually involved in the reaction accounted is directly related to the total amount of BDDE that was added to the reaction. For a reaction, the amount of BDDE that participates in the reaction can be controlled by adjustment of the BDDE concentration. Studies have shown that different BDDE cross-linked HA hydrogels had different ratios of c-MOD to t-MOD due to differences in reaction conditions such as temperature and time. However, if the other reaction conditions were kept constant and only the BDDE concentration changed, the c-MOD ratio did not change. [7] This was also observed here. Although the BDDE concentration changed, the c-MOD ratio was always approximately 21%. As the amount of BDDE concentration increased

(from 5μL/mL to 35μL/mL), c-MOD also showed a linear trend (y=0.066x+0.3565, R^2=0.9892). Therefore, adjusting the BDDE concentration can not only effectively control the amount of BDDE that actually participates in the reaction, but also can be used to control the amount of BDDE that exerts an actual effective cross-linking effect. This finding will facilitate the design of custom HA hydrogels with different modification degrees.

3.2. Micro morphology

As shown in Fig.5, SEM was used to characterize the interior morphology of the prepared HA hydrogels. Both the lyophilized original HA solution and the lyophilized HA hydrogel samples showed three-dimensional network structures. As the BDDE concentration increased, more HA molecules were involved in the construction of the hydrogel network structure, resulting in the formation of a more robust hydrogel backbone structure. From HA solution to HA hydrogel, the skeletons varied from fine to coarse, from loose to dense, and from dendritic to leafy. The difference in the surface morphology of the hydrogel indicated that a higher cross-linked degree meant more cross-linked points, making the network structure more compact.[17,18] It was noteworthy that in addition to the framework structure, a filamentous cross-linked structure was also observed that was not observed in the lyophilized HA samples. Fig.5(c, d, and e) shows the morphologies of lyophilized hydrogel samples of Sample2 (BDDE concentration of 10μL/mL), Sample4 (BDDE concentration of 20μL/mL), and Sample6 (BDDE concentration of 30μL/mL), respectively. The results showed that with increased modification, the filamentous cross-linked structure became more complex and denser. The filamentous structure may contribute to gel strength and hardness.

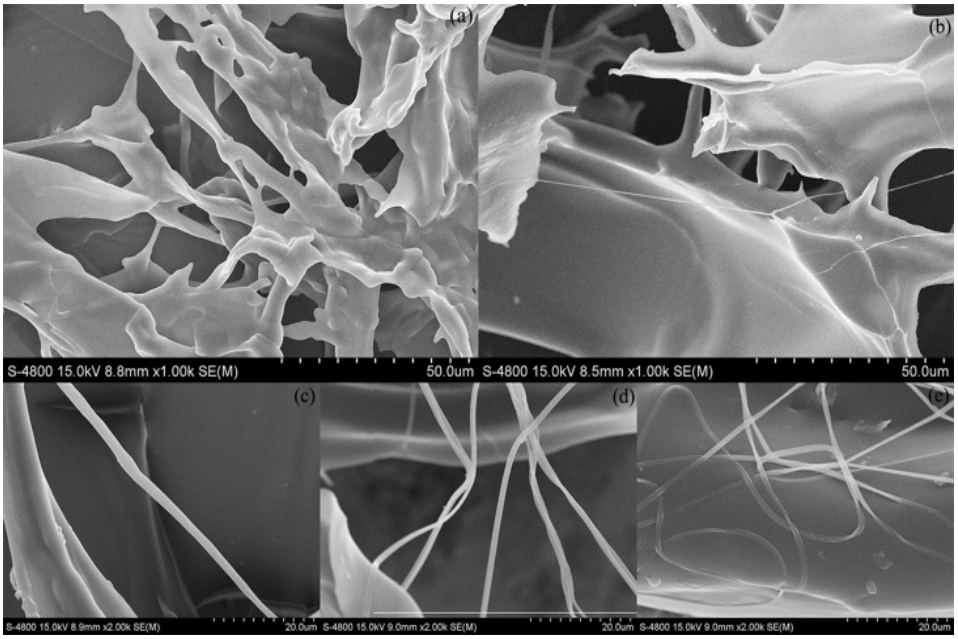

Figure 5. SEM images of HA hydrogel samples and HA solution: (a) HA solution; (b) Sample4; (c) Sample2; (d) Sample 4; (e) Sample6.

3.3. Anti-enzymatic analysis

The anti-enzyme property of cross-linked HA hydrogel is a strongly desired characteristic. HA hydrogels with higher modification degree showed stronger and better resistance to enzymes, resulting in increased retention time in vivo. In contrast, HA hydrogel with lower modification degree was softer and more rapidly degraded. [19] HAase has been used to resolve an arterial embolism caused by HA injection. [20] For drug delivery applications, the anti-enzyme abilities of the hydrogel dramatically

affected the release rate of the entrapped drug and a stiff hydrogel released proteins slower than a softer hydrogel. [4].

The HA content in the hydrogels measured by the carbazole method was measured as shown in Fig.6 (a). With increased BDDE concentration (from 5μL/mL to 35μL/mL), the content of HA in the hydrogel samples increased linearly (y=0.0854x+1.0727, R^2=0.99703). Based on the more accurate determination of the modification degree, from a molecular modification point of view, increased BDDE in the reaction resulted in denser cross-linking. Overall, the number of HA molecules in the hydrogel changed with increased BDDE concentration.

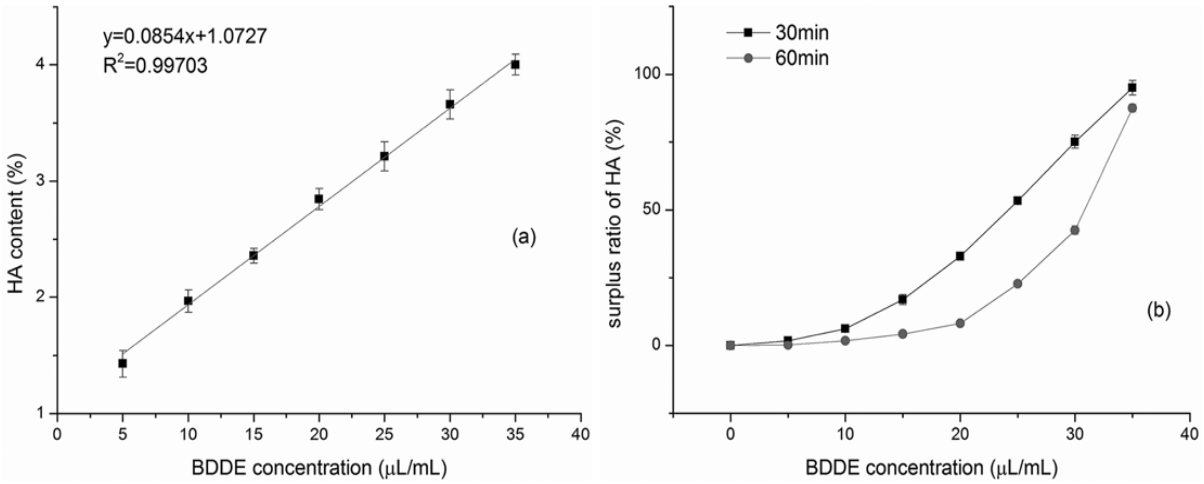

Figure 6. HA content (a) and the enzymatic residual ratio (b).

To assess the suitability of these materials for in vivo implantation, the likely persistence abilities of HA hydrogel in vivo were predicted from the measured anti-enzymatic abilities of HA hydrogel in vitro. [11] After the enzymatic reaction was performed for 30 and 60 min, the HA contents in the remaining solid were compared with the HA content in the original hydrogel. The anti-enzymatic abilities of the hydrogel samples prepared with different concentrations of BDDE were determined and are shown in Fig.6 (b). The amount of residual hydrogel was compared to the amount present after 30 or 60 min reaction. The relationship between anti-enzymatic ability and BDDE concentration was not linear. After 30 min, the concentration of BDDE increased from 5μL/mL to 15μL/mL, and the surplus ratio of hydrogel increased by approximately 19%, and with an increase in BDDE from 20μL/mL to 30μL/mL, the surplus ratio of hydrogel was increased by approximately 45%. This phenomenon was more pronounced after 60 minutes of reaction time. Obviously, with changing BDDE concentration, the change trend of the enzymatic residual rate was not approximately linear like the HA content. This might be because the chemical bonds between BDDE and HA stabilized the spatial structure of the entangled polymer macromolecule. [21] According to the SEM experiments described above, as the degree of cross-linking increased, the content of HA changed, but the spatial structure also changed. The coarser framework structure limited binding between the enzyme and the substrate, decreased the contact area of the hydrogel and enzymes, and the denser filamentous cross-linked structure limited enzyme mobility in the hydrogel. The anti-enzyme ability of the highly cross-linked hydrogel was thus further enhanced. Overall, the in vitro anti-enzymatic ability of the HA hydrogel can be influenced by many factors. This discovery may provide guidance for the custom design of HA hydrogels with the desired properties for varied applications.

4. Conclusion

We explored the effect of BDDE concentration on the properties of the prepared hydrogels, using a marine hyaluronidase. The properties of cross-linked HA hydrogels are greatly affected by different BDDE concentrations. HA hydrogels with different modification degrees and HA contents can be

customized by adjusting the BDDE concentration. However, some properties of hydrogels may not change linearly due to factors such as the spatial network structure. These properties need to be considered comprehensively when preparing HA hydrogels.

Acknowledgements
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