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Synthesis and characterization of thermoresponsive hydrogels cross-linked with chitosan

Research article

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Abstract: Hydrogels composed of *N*-isopropylacrylamide (NIPAAm) and acrylic acid (AAc) were prepared by redox polymerization with degradable chitosan cross-linkers. Chitosan degradable cross-linkers were synthesized by the acrylation of the amine groups of glucosamine units within chitosan and characterized with ^{1}H NMR. With the chitosan cross-linkers, loosely cross-linked poly(*N*-isopropylacryamide-co-acrylic acid) [P(NIPAAm-*co*-AAc)] hydrogels were prepared, and their phase transition behavior, lower critical solution temperature (LCST), water content and degradation properties were investigated. The chitosan cross-linked P(NIPAAm-*co*-AAc) hydrogels were pliable and transparent at room temperature. The LCST could be adjusted at 32~39°C by alternating the feed ratio. Swelling was influenced by NIPAAm/AAc monomer ratio, cross-linking density, swelling media, and temperature. All hydrogels with different feeding ratios contained more than 95% water at 25°C in the ultra pure water and phosphate-buffered saline (PBS, pH = 7.4 ± 0.1), and had a prospective swelling in the simulated gastric fluids (SGF, pH = 1.2) > 72.54%. In degradation studies, breakdown of the chitosan cross-linked P(NIPAAm-*co*-AAc) hydrogels was dependent on the cross-linking density. The chitosan cross-linked P(NIPAAm-*co*-AAc) hydrogels which can be tailored to create environmentally-responsive artificial extracellular materials have great potential for future use.

Keywords: *Chitosan • Cross-linker • Thermoresponsive hydrogel • Synthesis*

1. Introduction

Many hydrogels have recently been developed as biomaterials for applications in the medical and pharmaceutical fields. Research studies have shown that the swelling behavior of hydrogels depends on the external environment [1,2]. Abrupt changes in the swelling behavior of the network structure, permeability, or mechanical strength may be exhibited in response to changes in pH, ionic strength, temperature, or electromagnetic radiation. The most commonly studied hydrogels having environmental sensitivity are responsive either to pH or temperature [3,4]. Poly(*N*-isopropylacryamide) (PNIPAAm) is a thermosensitive hydrogel that has received much attention for biomedical use because of its lower critical solution temperature (LCST) behavior at around 32 °C in an aqueous solution [5,6]. PNIPAAm chains hydrate to form expanded structures in water when the solution temperature is below its LCST but become a compact gel structure by dehydration when heated to a temperature above its LCST. Below its LCST, PNIPAAm is extremely soluble in water and appears transparent. However, as its temperature is increased above the LCST, it becomes hydrophobic from the increased interactions between the isopropyl groups and PNIPAAm precipitates out from the aqueous solution, appearing opaque. PNIPAAm hydrogels possess a three-dimensional network structure which is insoluble but has characteristics of reversible swelling [2]. The polymer chains undergo a coil (soluble)-globule (insoluble) transition when the external temperature cycles across its LCST at about 33°C [1,7]. Thus, at a temperature below the LCST, PNIPAAm hydrogels absorb water and exist in a swollen state but shrink and display an abrupt volume decrease when the environmental temperature is higher than

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the LCST. With this quick response to phase transition at body temperature, PNIPAAm may be an excellent candidate for drug delivery or injectable soft tissue replacement.

However, an important limitation of PNIPAAm hydrogel for biomedical applications is its lack of bioactivity and biodegradability. When degradable linkages are incorporated into the hydrogel, materials can be prepared which accomplish a number of interesting biomedical applications such as temporary implants [8]. Such degradable hydrogel comprises of cross-linking molecules with degradable segments. As degradation occurs, degradable linkages in each "arm" of the cross-linking molecules are cleaved systematically, lowering the average number of cross-links per kinetic chain with time and causing eventual mass loss [9]. One of the objectives of our work was to develop a new biodegradable cross-linker.

Chitosan is a linear polysaccharide. Structurally, it is considered a copolymer of glucosamine and *N*-acetylglucosamine units linked by *β*-1, 4 glycosidic bonds. It is usually obtained by *N*-deacetylation of chitin, which is the principal component of crustacean shells and is the most abundant natural polysaccharide on the earth after cellulose. Because of the presence of reactive amino groups, chitosan can easily be modified to create derivatives. Because of its particular structure and properties, chitosan has attracted significant interest in a broad range of scientific areas including the biomedical, agricultural, and environmental fields. In particular, chitosan and its derivatives have been considered as biomaterials because of their biocompatibility, biodegradability, low immunogenicity, and biological activities [10-12]. Water soluble chitosan can be prepared by hydrogen peroxide induced random degradation of the chitosan [13].

Therefore, the objective of this study was to develop biocompatible and biodegradable P(NIPAAm-co-AAc) hydrogels with chitosan derivatives (*N*-acryl chitosan, NAC) as cross-linkers. NAC was synthesized by the acrylation of the amine groups of glucosamine units within chitosan and characterized with ^{1}H NMR. A series of thermoresponsive and degradable P(NIPAAm-co-AAc) hydrogels were obtained by redox polymerization with degradable chitosan cross-linkers. To evaluate the thermoresponsive property, the LCST and water content of hydrogels were characterized. The degradation properties of hydrogels were also investigated by weight loss in PBS.

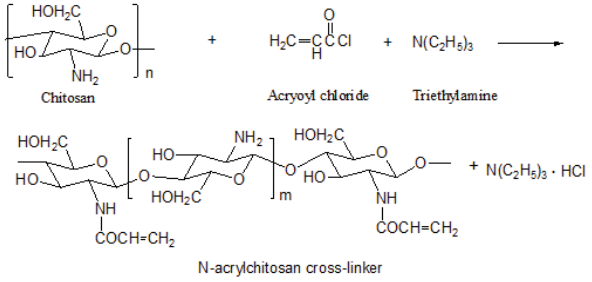
2. Experimental

2.1. Materials

Soluble chitosan (Mn < 2000, prepared by hydrogen peroxide induced random degradation of chitosan according to the literature[13]), *N*-isopropylacrylamide (NIPAAm, Tokyo Kaset Kogyo Co. Ltd.), acrylic acid (AAc), *N,N,N',N'*-tetramethylethylenediamine (TEMED, analytic ultrapure grade), ammonium peroxodisulfate (APS, analytic ultrapure grade), Acryloyl chloride (analytic ultrapure grade), triethylamine, dimethylacetamide (DMAc), phosphate-buffered saline (PBS, pH = 7.4 ± 0.1), similar gastric fluid (SGF, pH = 1.2). All materials excepted NIPAAm were purchased from Shanghai Fine Chemical Co. Ltd., China, and used as received without further purification.

2.2. Synthesis of N-acrylchitosan cross-linker

As shown in Scheme 1, *N*-acrylchitosan (NAC) cross-linker was synthesized through the reaction between the amine groups of glucosamine units within the chitosan backbone and acryloyl chloride. Specifically, 3.0 g of soluble chitosan was added in 80 mL of DMAc with triethylamine, and the appropriate amount of acryloyl chloride was added dropwise to the solution with stirring. Different acryloyl chloride/glucosamine residue of chitosan molar ratios were shown in Table 1. The reaction temperature was kept at 0-5°C. After all of the acryloyl chloride was added, the reaction continued with stirring for 4 h at 0-5°C and 20 h at room temperature. Then triethylamine hydrochloride salts were removed by filtration. The resulting solution was dialyzed against ultrapure water (UPW, 18 MΩ cm) for 48 h with periodic bath changes (every 8 h) to eliminate unreacted compounds. The final dialysis product was lyophilized for 24 h using a freeze dryer (LGJ-10, Beijing Song Yuan Hua Xing technology company, China) connected to a vacuum pump (2XZ-2, Zhejiang qiujing vacuum company, China).



Scheme 1. Synthesis of the N-acrylchitosan cross-linker.

Table 1. Description of synthesis of the Cross-Linker.

Samples	Acryloyl chlorides / Glucosamine residue (mol/mol)	Water-Solubility	Cross-Linked ability
1	1.0/10.0	excellent	absence
2	1.0/5.0	excellent	absence
3	1.0/3.8	excellent	excellent
4	1.0/3.0	good	excellent
5	1.0/2.5	commonly	good
6	1.0/1.9	commonly	good
7	1.0/1.5	undissolved	commonly

2.3. Characterization of NAC cross-linker

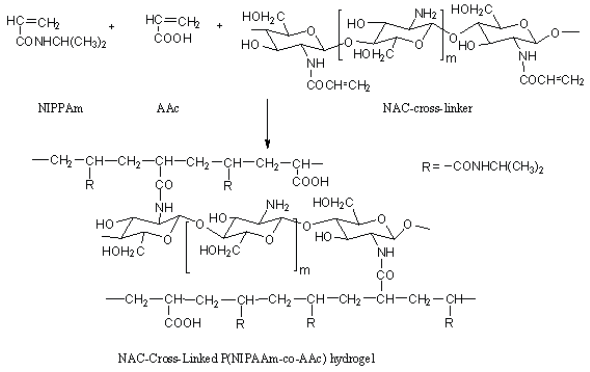
^{1}H Nuclear magnetic resonance (NMR) spectroscopy was used to confirm the synthesis of the NAC-cross-linker. ^{1}H NMR spectra were obtained on a Bruker AM 500 MHz instrument. The samples were dissolved in D_2O. Chemical shifts are quoted in ppm.

2.4. Synthesis of NAC-cross-linked P(NIPAAm-co-AAc) hydrogels

The P(NIPAAm-co-AAc) hydrogels were prepared with NAC as cross-linker by redox polymerization in the presence of APS as initiator and TEMED as accelerator in PBS. The hydrogels were prepared by varying the molar ratio of NIPAAm/AAc and the amount of NAC cross-linker in the feed (shown in Table 2). The total monomer amount of NIPAAm and AAc in the feed was always 5% w/v of PBS. Nitrogen gas was bubbled through a mixture of NIPAAm and AAc and NAC in 50 mL of PBS in a glass beaker covered with a plastic film for 15 min to remove dissolved oxygen. Following the nitrogen gas purge, 0.8 wt% (based on total monomer) of APS and 8% v/w (based on total monomer) of TEMED was added as the initiator and accelerator, respectively. The mixture was stirred vigorously for 20 s and allowed to polymerize at room temperature for 24 h. Following the polymerization, the hydrogel was washed three times for 20 min each in excess ultrapure water to remove unreacted compounds, and freeze-dried. A schematic representation of the NAC-cross-linked P(NIPAAm-co-AAc) hydrogel synthesis is shown in Scheme 2.

2.5. LCST measurements

The LCST of the hydrogel samples was determined as the abscissa of the inflection point of the transmittance vs. temperature curves [14]. All samples were immersed in deionized water at room temperature and allowed to swell for at least 24 h to reach the equilibrium state. The thermal analyses were performed from 25 to 45°C at rates of 0.05~0.1°C /min. Temperature was controlled by a high-constant temperature bath. The changes in transmittance of the hydrogel with temperature were captured using an UV-vis spectrophotometer (Spectrum 723p, Shanghai Spectrum Instruments CO., LTD., China). The data of the hydrogels were then analyzed by graph-processing software to determine their LCST.



Scheme 2. Preparation of the NAC-cross-linked P(NIPAAm-co-AAc) hydrogel.

2.6. Water content studies

The freeze-dried hydrogel samples were weighed upon removal from the freeze-dryer and were immersed in excess UPW, PBS or SGF for 24 h at 25°C and 37°C. The swelling ratios of the hydrogels were measured gravimetrically after wiping off the excess water on the surface with filter paper. The water content was calculated using the following formula,

$$Water\ Content = \frac{W_s - W_d}{W_s} \times 100\% \qquad (1)$$

where W_s is the weight of the swollen gel and W_d is the weight of the dry gel.

2.7. Degradation of NAC-cross-linked P(NIPAAm-co-AAc) hydrogels

Monitoring the weight-loss of hydrogels is a suitable and reliable method and it has been widely employed for the investigation of degradation behaviors of hydrogels. NAC-cross-linked P(NIPAAm-co-AAc) hydrogels samples were cut with a scissor to approximately 0.2 g. Samples were weighed individually and immerged in excess PBS without any enzyme at 37°C. At various times during the course of the experiment, the samples were removed from PBS. Each sample was freeze-dried and reweighed. The weight loss was calculated using the following formula,

$$Weight\ Loss = \frac{W_0 - W_t}{W_0} \times 100\% \qquad (2)$$

where W_0 is the initial weight of the dried sample before degradation and W_t is the weight of the redried sample

at the end of that time point. The degradation behavior of hydrogel samples was determined as the coordinate of the inflection point of the weight loss vs. time curves [15,16].

3. Results and discussion

3.1. Synthesis of NAC cross-linker

NAC-cross-linkers were synthesized by the acrylation of the amine groups of glucosamine units within chitosan backbone as shown in Scheme 1. Fig. 1 shows the ^{1}H NMR spectra of the original chitosan, and the NAC-cross-linker in D_2O. As seen in Fig. 1, the ^{1}H NMR spectrum of NAC-cross-linker showed the proton peaks of acryl group (5.5, 5.9, and 6.0 ppm). Acryloyl chlorides reacted with amines of chitosan to give amides by the addition-elimination mechanism beginning with attack of the nucleophilic amine nitrogen at the carbonyl carbon. Subsequently, the liberated acid reacted to form a salt with an additional equivalent of amine such as triethylamine. Because the reaction was performed in homogeneous conditions, the *N*-acryloyl groups should be distributed randomly along the chitosan backbone. Seven samples with different acryloyl chloride/glucosamine residue molar ratio ranging from 1.0/10.0 to 1.0/1.5 were obtained, and their water-solubility and cross-linked ability were shown in Table 1. As seen in Table 1, the water-solubility and cross-linked ability were significantly influenced by the feed molar ratio of acryloyl chloride. As the amounts of acryloyl chloride increased, the degree of substitution of acryloyl group increased, and the cross-linked ability of the NAC-cross-linker was enhanced but the water-solubility decreased at the same time. So we chose (acryloyl chloride)/(glucosamine residue) molar ratio as 1.0/3.8 for the NAC-cross-linker synthesis, giving excellent cross-linked ability and water-solubility (Sample 3 in Table 1).

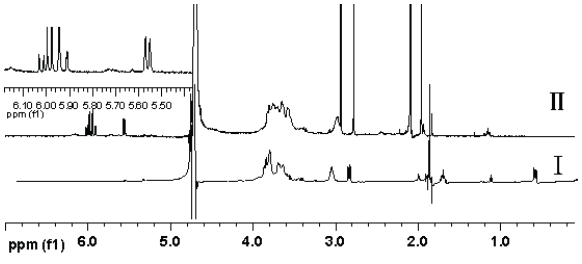


Figure 1. ^{1}H NMR spectra of chitosan (I) and NAC-cross-linker II).

3.2. Synthesis of the NAC-cross-linked P(NIPAAm-co-AAc) hydrogels

To improve the biocompatibility and biodegradability of the hydrogels, a series of P(NIPAAm-co-AAc) hydrogels were prepared in PBS by redox polymerization with NAC as cross-linker. Fig. 2 shows the thermoresponsive phase transformation of NAC-cross-linked P(NIPAAm-co-AAc) hydrogel (Sample 2 in Table 2) when the external temperature cycles across its LCST (from 25 to 40°C, and then to 25°C). As seen in Fig. 2, the NAC-cross-linked P(NIPAAm-co-AAc) hydrogel was light yellow, transparent, and extremely pliable at room temperature. When heated above its LCST, the hydrogel exhibited a considerable amount of collapse, released a large fraction of water contained in the pores of hydrogel, and became stiff and opaque.

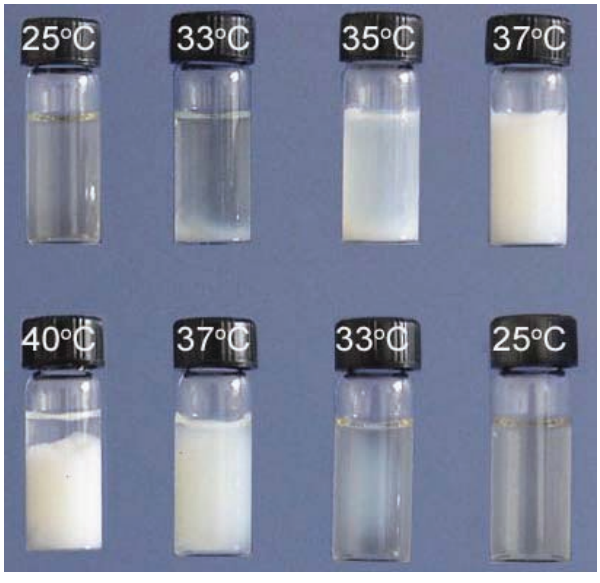


Figure 2. Thermoresponsive phase transformation of the hydrogel (sample 2 in table 2) when the external temperature cycles across its LCST (from 25°C to 40°C, and then to 25°C).

3.3. LCST characterization

Fig. 3 shows the transmittance (percent) of visible light (λ = 600 nm) through the NAC-cross-linked P(NIPAAm-co-AAc) hydrogels as a function of temperature. Each line represents a single experiment with one hydrogel sample. As seen in Fig. 3, the hydrogels showed phase transitions, and the LCST of the hydrogels was found to vary in the range from 32-39°C with different NIPAAm/AAc monomer molar ratios. Increasing the amount of AAc in NAC-cross-linked P(NIPAAm-co-AAc) hydrogel gradually increased the LCST, and a much broader phase transition was observed. The hydrophilic monomer AAc strongly influenced changes in the hydrophilic/hydrophobic nature of the polymer,

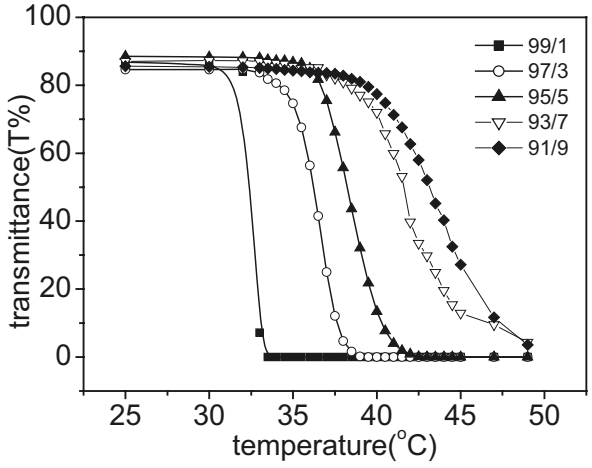


Figure 3. Transmittance as a function of temperature for NAC-cross-linked P(NIPAAm-co-AAc) hydrogel with different NIPAAm/AAc molar ratios.

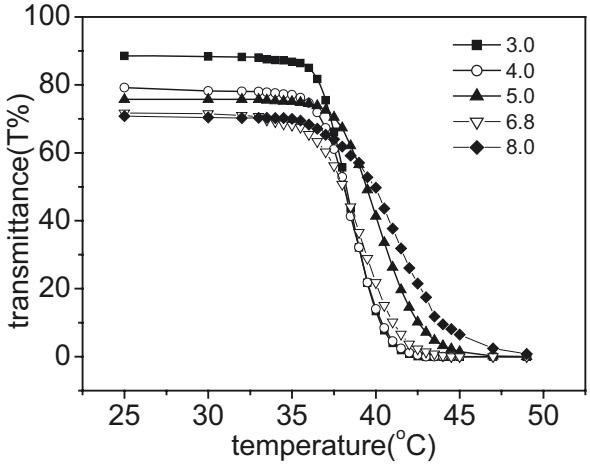


Figure 4. Transmittance as a function of temperature for NAC-cross-linked P(NIPAAm-co-AAc) hydrogel with different amount of the cross-linker (wt %).

where the incorporation of more hydrophilic monomer to P(NIPAAm) hydrogels increases the LCST value because the ionized -COO^- groups are sufficiently soluble to counteract the aggregation of the hydrophobic temperature sensitive units. Also, the repulsion of the -COO^- groups or the formation of hydrogen bonds between the amide groups in NIPAAm and the -COO^- groups in AAc may impede the collapse induced by the NIPAAm components, increasing the LCST. It is important to note that the LCST of the P(NIPAAm-co-AAc) hydrogel can change with the amount of AAc included in the polymerization formulation.

In Fig. 4, the effect of temperature on the transmittance of visible light (λ = 600 nm) through the NAC-cross-linked P(NIPAAm-co-AAc) hydrogels with the same NIPAAm/AAc molar ratio of 95/5 and different amount of the NAC-cross-linker is shown. The hydrogels had the same LCST at ~36.5°C with different amounts of the NAC-cross-linker (3.0~8.0 wt %). So the amount of the NAC-cross-linker did not influence the LCST and the phase transition behavior of hydrogels evidently in the range of 3.0-8.0 wt %.

3.4. Water content of NAC-cross-linked P(NIPAAm-co-AAc) hydrogel

After swelling in different media for 24 h at 25°C and 37°C, the water contents of the NAC-cross-linked P(NIPAAm-co-AAc) hydrogels were determined. Results are shown in Table 2. At 25°C, all of the hydrogel samples had water contents > 95% in UPW and PBS. There was no significant difference in water content between samples swelled in UPW and PBS at 25°C. However the water contents of hydrogels in SGF at 25°C were significantly lower at about 72.54%. This tendency was greater at 37°C, above the LCST, as shown in Table 2. The effect of the media on the swelling behavior could be attributed to the shielding of COO^- repulsions, which prevent collapse of the gel, by the interactions between COO^- groups in AAc and the ions present in the PBS. The swelling behavior of hydrogels was also dependent on the pH of the medium because of the carboxyl groups. In the presence of SGF (pH = 1.2), the interactions between NH_3^+ of the glucosamine repeat units within chitosan and the -COOH groups presumably disrupted more hydrogen bonds, and interfered with the swelling of the hydrogel at 25°C and 37°C.

As seen in Table 2 (Samples 1, 2, 3, 4, and 5), the water content of NAC-cross-linked P(NIPAAm-*co*-AAc) hydrogels with different NIPAAm/AAc monomer molar ratios (amount of cross-linker = 3.0 wt %) did not change extensively at 25°C regardless of the swelling medium. The water content of the hydrogels was not significantly influenced by the introduction of hydrophilic monomer AAc at the temperatures below the LCST where PNIPAAm has a hydrophilic and expanded structure. At 37°C, the water contents of the hydrogels in the UPW, PBS and SGF increased with increase of the AAc content in the hydrogel. The effect suggested that COO^- groups strongly influenced changes in the hydrophilic/hydrophobic nature of the polymer that prevented collapsing of the hydrogels above the LCST.

The effect of cross-linking density on the water content for hydrogels with the same NIPAAm/AAc monomer molar ratio of 95/5 was also investigated (Table 2, sample 3, 6, 7, 8, and 9). As the cross-linking density within the hydrogel increased, the water content difference was not statistically significant at 25°C. At 37°C, however, for the hydrogels with higher cross-linking density, lower water content was observed,

Table 2. Water Content of NAC-Cross-Linked P(NIPAAm-co-AAc) Hydrogels Depending on Temperature and Swelling Media.

Hydrogel samples	NIPAAm/AAc molar ratio	Amount of NAC cross-linker (wt %)	25°C			37°C		
			UPW	PBS	SGF	UPW	PBS	SGF
1	99/1	3.0	97.14	95.64	79.23	54.25	45.71	23.04
2	97/3	3.0	98.20	97.33	78.03	80.30	55.68	26.56
3	95/5	3.0	98.01	97.13	80.46	87.23	65.16	26.89
4	93/7	3.0	97.99	96.98	82.99	89.81	80.93	29.43
5	91/9	3.0	98.98	97.22	83.21	90.33	81.73	31.53
6	95/5	4.0	98.30	97.40	82.99	86.67	61.72	31.85
7	95/5	5.2	98.47	97.27	79.18	85.22	61.06	30.06
8	95/5	6.8	97.93	97.68	73.43	83.85	54.24	28.17
9	95/5	8.0	96.11	96.63	72.54	83.51	57.64	26.72

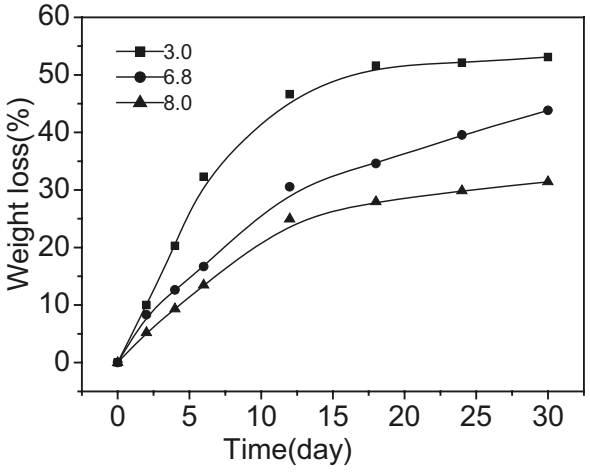


Figure 5. Weight loss as a function of time for NAC-cross-linked P(NIPAAm-co-AAc) hydrogel with different amount of the cross-linker (wt %).

compared to hydrogels with lower cross-linking density. This result indicated that high cross-linking density made the structure collapse more tightly than in the other samples. It slows the diffusion of chains and thus delays the dilatation of three dimensional net structures of hydrogels. And the porosity of the sample was smaller than others. Thus the effect of interactions between COO^- groups in hydrogel and the ions in the PBS and SGF was increased with increasing cross-linking density.

3.5. Degradation behavior of NAC-cross-linked P(NIPAAm-co-AAc) hydrogels

Weight loss of the hydrogels as a function of time in the PBS, at 37°C, in the absence of enzyme, is shown in Fig. 5. The NAC-cross-linked P(NIPAAm-co-AAc) hydrogels showed significant weight loss after immersing in the PBS without any enzyme at 37°C for 30 days. There are two important factors that can contribute to the weight loss due to degradation of these hydrogels. The hydrogels were designed to have a significant portion of NAC cross-linker, which was a copolymer of glucosamine and *N*-acrylglucosamine units linked by *β*-1,4 glycosidic bonds. With hydrolytic degradation of the glycosidic bonds and the amide bonds, NAC cross-linker could be released from the network, lowering the average number of cross-links per kinetic chain with time and causing eventual weight loss. In addition, the degradation products of the NIPAAm and AAc repeat unit could be released from the network, contributing to the weight loss.

In general, the degradation of hydrogels in solution (weight loss from the networks) is linked to several network parameters such as number of cross-links per backbone chain, number of vinyl groups on the cross-linking molecule, molecular weight of backbone, and proportion of degradable groups in the main and side chain. The degradation behavior of NAC-cross-linked hydrogels depended on the cross-linking density. Fig. 5 shows the degradation profiles of NAC-cross-linked hydrogels prepared by using different amounts of the NAC-cross-linker in the PBS without any enzyme. The degradation rate gradually decreased with increasing cross-linking density. As the cross linking density increased, additional degradable units must be broken to degrade the hydrogel. This effect results in longer inhibition time for the weight loss with increasing cross-linking density.

4. Conclusions

N-acrylchitosan (NAC) was synthesized by the acrylation of the amine groups of glucosamine units within water-soluble chitosan containing multiple hydrolytically degradable units, and characterized with ^1H NMR. With NAC as cross-linkers, a series of biodegradable P(NIPAAm-co-AAc) hydrogels was prepared in PBS (pH = 7.4 ± 0.1) through redox polymerization of NIPAAm and AAc. Their phase transition behavior, lower critical solution temperature (LCST), and water content were

investigated. The LCST could be adjusted in the range 32-39°C by alternating the feed ratio. Swelling was influenced by NIPAAm/AAc monomer ratio, cross-linking density, swelling media, and temperature. All hydrogels with different feeding ratios contained more than 95% water at 25°C in UPW and PBS, and have a significant swelling in SGF (> 72.54%). The degradation of hydrogels was also investigated by weight loss in PBS at 37°C. The degradation rate gradually decreased with increasing cross-linking density.

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