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Synthesis and Characterization of P(NIPAAm-co-AAc) Hydrogels with L-Lysine-cross-linker

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In order to improve the biocompatibility of the hydrogels, hydrogels composed of N-isopropylacrylamide (NIPAAm) and acrylic acid (AAc) were prepared by redox polymerization with L-lysine-cross-linker. L-lysine-cross-linker was synthesized by the acrylation of the amino groups of the L-lysine and characterized by ^{1}H NMR. With L-lysine-cross-linker, loosely cross-linked poly(N-isopropylacrylamide-co-acrylic acid) [P(NIPAAm-co-AAc)] hydrogels were synthesized in phosphate-buffered saline (PBS, pH = 7.4 ± 0.1) and their phase transition behaviour, low critical solution temperature (LCST), water content were investigated. The LCST could be adjusted at 35-38 ºC by changing the hydrogel's composition. The hydrogels contained more than 90 % water content at 37 ºC in the ultra pure water and have a prospective swelling in the PBS (> 84.72 %), which was controlled by the molar ratio of NIPAAm/AAc, swelling media and cross-linking density. This L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels which can be tailored to create environmentally-responsive artificial extracellular materials will have a potential use in the future.

Key Words: Thermoresponsive hydrogels, Lysine, Cross-link, Low critical solution temperature, Synthesis.

INTRODUCTION

Hydrogels are hydrophilic polymers that are crosslinked to form insoluble, but water-swellable networks. Hydrogels have been widely used in many biomedical applications including contact lenses, wound dressings, artificial organs due to their high degree of compatibility[1,2], especially the application in delivery carriers for the bioactive reagents. The applications of hydrogels are flexibility in tailoring physiochemical properties, such as permeability and swelling and the ability to load drugs without the loss of their bioactivity. More hydrogels have been extensively studied for diffusion-

controlled and swelling-controlled delivery devices[3-5]. In the design of drug delivery devices used as implants, however, it is highly desirable to utilize biocompatible hydrogels. Their biocompatibility is likely related to their high water content and low interfacial tension with the surrounding biological environment. Various bioresponsive hydrogels have been developed for drug delivery based on thermoresponsive polymer poly(N-isopropylacrylamide) (PNIPAAm) due to its unique volume phase transition at a lower critical solution temperature (LCST) in water around 32 °C[6,7], which is close to body temperature. They swell and collapse significantly in an aqueous environment at temperatures below and above the LCST, respectively. But the PNIPAAm homopolymeric hydrogel is not a favoured matrix for biomedical applications because of its transition temperature and rigid network structure. A desirable phase transition temperature of the three dimensional matrix should be at or near the physiologic temperature (37 °C). In addition, the gel matrix should possess high water content but still exhibit temperature sensitive properties at 37 °C. Thus, incorporating a hydrophilic monomer, acrylic acid (AAc), into the PNIPAAm backbone is a good approach to modulating the properties of PNIPAAm hydrogel.

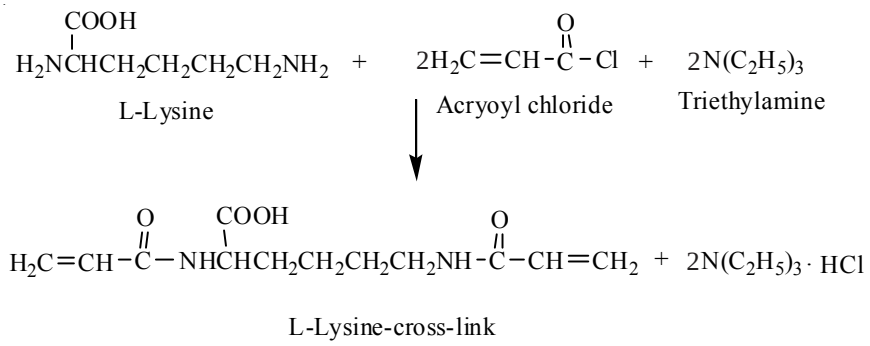
However, an important limitation of PNIPAAm hydrogel for biomedical application is their lack of biocompatibility and biodegradability. By incorporating natural amino acid or peptide into hydrogel, the material can accomplish a number of interesting biomedical applications[8]. Kim and Healy[9] synthesized poly(N-isopropylacryamide-co-acrylic acid) [P(NIPAAm-co-AAc)] hydrogels with degradable peptides cross-linkers. Several investigations had shown that the phase behaviour and swelling properties of PNIPAAm hydrogels could be modified by using a novel cross-linker for desired drug delivery[10-12].

Therefore, the objective of this study was to develop biocompatible and injectable P(NIPAAm-co-AAc) hydrogels. To synthesize biocompatible cross-linker, we used L-lysine, a natural amino acid, which has active amino groups and is useful in promoting cell adhesion. L-lysine cross-linker has several advantages, such as good biocompatibility, plentiful active amino groups and relatively good solubility in water. L-lysine cross-linker could act as a relative 'friendly' and 'soft' linker between the hydrogel and bioactive molecules. We synthesized the L-lysine cross-linker with reactive acrylate groups and prepared biocompatible P(NIPAAm-co-AAc) hydrogel networks with L-lysine cross-linker *via* radical addition polymerization. To evaluate the feasibility as injectable thermoresponsive hydrogels, the LCST and water content of the L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels were characterized.

EXPERIMENTAL

N-isopropylacrylamide (NIPAAm, Tokyo Kaset Kogyo Co. Ltd.), acrylic acid (AAc), N,N,N',N'-tetramethylethylenediamine (TEMED, analytic ultrapure grade), ammonium peroxodisulfate (AP; analytic ultrapure grade), acryloyl chloride (analytic ultrapure grade), triethylamine, dimethylacetamide (DMAc), L-lysine (chemzymes ultrapure grade), phosphate-buffered saline (PBS, pH = 7.4 ± 0.1). All materials except N-isopropylacrylamide were purchased from Shanghai Fine Chemical Co. Ltd., China, used as received without further purification.

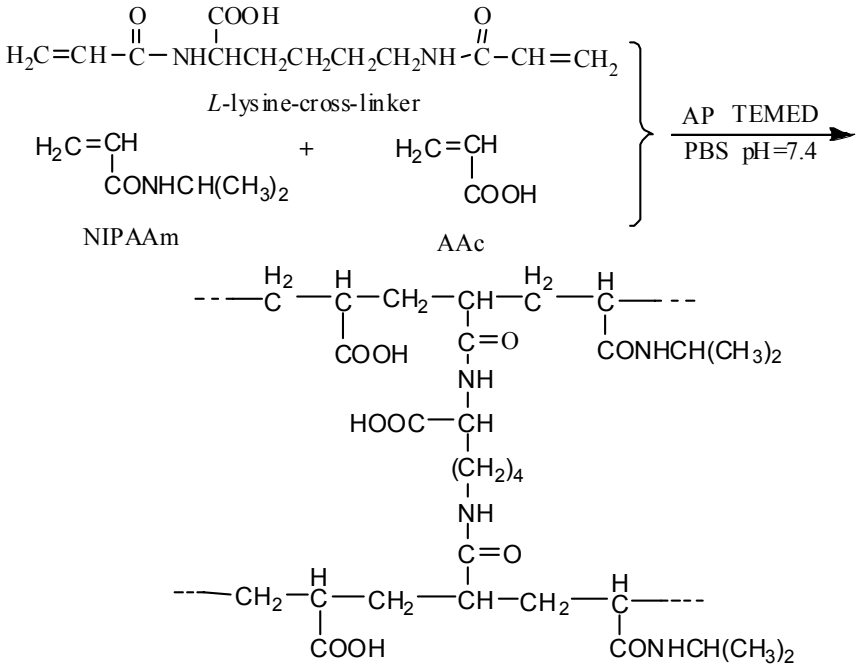
Synthesis of lysine-cross-linker: As shown in **Scheme-I**, the L-lysine-cross-linker with bifunctional acryl groups was synthesized through the reaction between the amine groups of lysine and acryloyl chloride. 2 g of lysine was added in 60 mL of dimethylacetamide (DMAc) with triethylamine and acryloyl chloride solution (5 mL in 15 mL DMAc) was added dropwise to the solution with stirring. The reaction temperature was kept at 0-5 °C. After all of the acryloyl chloride, the reaction continued with stirring for 4 h at 0-5 °C and 20 h at room temperature. Subsequently, 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 to remove unreacted compounds. The final dialysis product was lyophilized for 24 h using a freeze-dryer (LGJ-10) attached to a vacuum pump.



Scheme-I: Synthesis of the L-lysine-cross-linker

Synthesis of L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels: The P(NIPAAm-co-AAc) hydrogels were prepared with L-lysine-cross-linker by free radical polymerization in aqueous media. The hydrogels were prepared by varying the molar ratio of NIPAAm/AAc and the amount of L-lysine-cross-linker in the feed. The total monomer amount of NIPAAm

and AAc in the feed was always 5% w/v, NIPAAm/AAc molar ratios of 97/3, 96/4 and 95/5, feed molar ratio of cross-linker (mol %) of 0.90, 1.26 and 1.62 were used. The polymerization formulations of the L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels are described in Table-1. Dry nitrogen gas was bubbled through a mixture of NIPAAm and AAc and L-lysine-cross-linker in 50 mL of phosphate-buffered saline (PBS) in a glass beaker covered with a plastic film for 15 min to remove dissolved oxygen. Following the nitrogen gas purge, 0.02 g (0.0876 mmol) of ammonium peroxysulfate (AP) and 0.20 mL (0.0013 mol) of TEMED were added as the initiator and accelerator, respectively. The mixture was stirred vigorously for 20 s and allowed to polymerize at 20 °C or 0 °C for 24 h in the glass beaker. Following the polymerization, the L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogel was washed three times for 20 min each in excess ultrapure water to remove unreacted compounds. A schematic representation of the P(NIPAAm-co-AAc) hydrogel synthesis is shown in **Scheme-II**.



Scheme-II: Preparation of the *L*-lysine-cross-linked P(NIPAAm-co-AAc) hydrogel

TABLE-1
DESCRIPTION OF P(NIPAAm-co-AAc) HYDROGEL SAMPLES[a]

Hydrogel samples	Polymerization temperature (ºC)	NIPAAm/AAc monomer ratio in feed	Feed molar ratio of cross-linker (mol %)
1	20	97/3	0.90
2	20	96/4	0.90
3	20	95/5	0.90
4	20	97/3	1.26
5	20	97/3	1.62
6	0	97/3	0.90
7	0	97/3	0.90
8	0	97/3	0.90

[a]All hydrogels were synthesized in PBS as a reaction solvent. The total amount of NIPAAm and AAc in feed was always 5 % w/v of reaction media (PBS).

Characterization of L-lysine-cross-linker: ^{1}H NMR spectroscopy was used to identify the synthesis of the L-lysine-cross-linker. The acrylolylation of amine groups in the lysine was analyzed with a 500 MHz ^{1}H NMR spectrometer (Bruker AMX-500); dried L-lysine-cross-linker samples were dissolved in DMSO with dropwise few D_2O at room temperature.

Low critical solution temperature measurements: The phase transition of the hydrogel samples was measured using an UV-Vis spectrophotometer (Spectrum 723p, Shanghai Spectrum Instruments Co., Ltd., China). The transmittance of visible light (λ = 600 nm; path length = 3 cm) through the hydrogel was recorded as a function of temperature[13]. At the start of each experiment, hydrogel samples were swollen in ultra pure water to reach their equilibrium state, the spectrophotometer was calibrated with ultra pure water. The temperature of the cuvette chamber was regulated through the high-constant temperature bath. Temperature was manually ramped at rates of *ca*. 0.05-0.10 °C /min for all runs. The LCST of hydrogel samples was determined as the abscissa of the inflection point of the transmittance *vs.* temperature curves.

Water content studies: The L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogel samples were freeze-dried for 24 h. The freeze-dried hydrogel samples were weighed upon removal from the freeze-dryer and were immersed in excess phosphate-buffered saline or ultrapure water for 24 h at different temperature (25 and 37 °C) to determine the effects of swelling water content. The water content was calculated on the basis of the weight difference of the hydrogel samples before and after swelling (water content = $(W_s-W_d)/W_s \times 100$, where W_s is the weight of the swollen gel and W_d is the weight of the dry gel).

RESULTS AND DISCUSSION

The ^{1}H NMR spectra of the lysine (D_2O) and the L-lysine-cross-linker in DMSO were shown as follows, the lysine (D_2O) δ 1.3 (m, 2H, C**H**$_2$), δ 1.6 (m, 2H, C**H**$_2$), δ 1.8 (m, 2H, C**H**$_2$), δ 2.9 (t, 2H, C**H**$_2$), δ 3.6 (t, 1H, C**H**) and the L-lysine-cross-linker in DMSO δ 1.2 (m, 2H, -C**H**$_2$), δ 1.5 (m, 2H, -C**H**$_2$), δ 1.9 (q, 2H, -C**H**$_2$-CH-), δ 3.5 (t, 2H, -C**H**$_2$-N), δ 3.9 (t, 1H, -C**H**), δ 5.8-6.1 (m, 4H, C**H**$_2$=), δ 6.4 (t, 2H, C**H**=). The ^{1}H NMR spectrum of L-lysine-cross-linker showed the proton peaks of acryl group (5.8, 6.1 and 6.4 ppm) and the newly formed amide bond (3.5 and 3.9 ppm). Acryloyl chlorides reacted with the amine groups to give amide groups 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.

Synthesis of the P(NIPAAm-co-AAc) hydrogels: The loosely L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels were synthesized as shown in **Scheme-II**. At room temperature, the hydrogels were light yellow, transparent and extremely pliable. When heated above the 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. The L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels became less pliable than at room temperature and turned cloudy.

Low critical solution temperature characterization: The LCST observations of the L-lysine-cross-linked hydrogels are similar to the body temperature. Furthermore all hydrogels have very well fluidity which can be injectable through a 2 mm aperture without demonstrating appreciable macroscopic fracture at room temperature. Fig. 1 shows the transmittance (per cent) of visible light (λ = 600 nm; path length = 3 cm) through L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels as a function of temperature. Each line represents a single experiment with one hydrogel sample. The L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogel showed phase transition at about 36 °C. Increasing the amount of AAc in P(NIPAAm-co-AAc) hydrogel increased the LCST. The L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogel (feed molar ratio of the cross-link = 0.90 %) with different NIPAAm/AAc molar ratio of 97/3, 96/4 and 95/5 had the LCST at 35.1, 36.3 and 37.6 °C, respectively. The hydrophilic monomer AAc strongly influenced changes in the hydrophilic/hydrophobic nature of the polymer, where the incorporation of more hydrophilic monomer to hydrogels increases the LCST value because the hydrophilic monomer hinders the dehydration of the polymer chains and acts to expand the collapsed structure. However, with different amount of the L-lysine-cross-links in the range of 0.90-1.62 mol %, the L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels with the same NIPAAm/AAc molar ratio of 97/3 had the same LCST at *ca*.

35.1 °C as shown in Fig. 1. So the LCST of the L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels were insensitive to the amount of the L-lysine-cross-linker in the range of investigated (0.90-1.62 mol %).

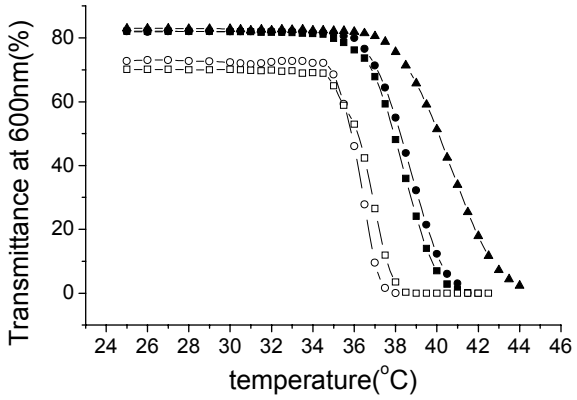


Fig. 1. Transmittance as a function of temperature for L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogel, NIPAAm/AAc molar ratio of (filled squares) 97/3 and (filled circles) 96/4 and (filled triangles) 95/5(cross-linker molar ratio = 0.90 %) and cross-linker molar ratio of (open circles) 1.26 % (97/3) and (open squares) 1.62 % (97/3)

In addition, hydrogels sample were synthesized at 0 and 20 °C, respectively. The transmittance *vs.* temperature plots of samples are presented in Fig. 2. When using the same feed molar ratio, the LCST of the hydrogels (35.1, 36.3 and 37.6 °C) synthesized at 20 °C is higher than the LCST of the hydrogels (34.2, 34.5 and 36.2 °C) polymerized at 0 ºC obviously. At the low temperature the higher molecules were synthesized by free radical polymerization that made the collapsed structure regular than others. It needs less kilocalories for transfer from a hydrophilic to hydrophobic phase. Thus, the LCST of the samples which were synthesized at 0 ºC would be lower than others.

Water content of P(NIPAAm-co-AAc) hydrogel depending on temperature: Table-2 shows the water content of L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels in different swelling media at 25 and 37 °C. At 25 °C, all of the hydrogel samples exhibited water contents of > 90 % in ultra pure water and PBS. The water contents of L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels were lower in the PBS than in the ultra pure water. This tendency was greater above the LCST as delineated in Table-2. The effect of the media on the swelling behaviour can be attributed to the shielding of COO^- repulsion, which prevents collapse of the gel, by the interactions between COO^- groups in AAc and the ions present in the PBS.

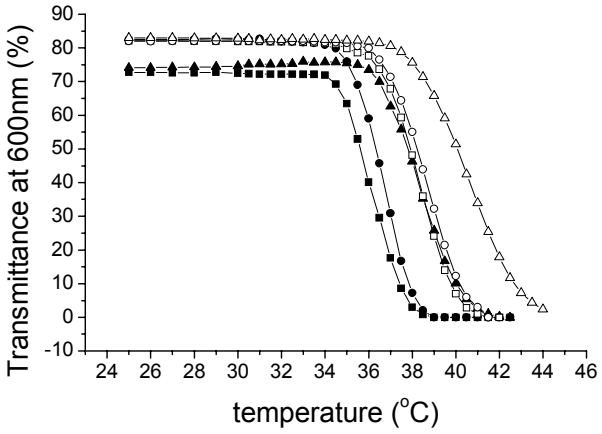


Fig. 2. Transmittance as a function of temperature for L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogel, NIPAAm/AAc molar ratio of (filled squares) 97/3 (0 ºC) and (filled circles) 96/4 (0 ºC) and (filled triangles) 95/5 (0 ºC) and (open squares) 97/3 (20 ºC) and (open circles) 96/4 (20 ºC) and(open triangles) 95/5 (20 ºC), feed molar ratio of the cross-linker = 0.90 %

TABLE-2
WATER CONTENT OF *L*-LYSINE-CROSS-LINKED P(NIPAAm-co-AAc) HYDROGELS DEPENDING ON TEMPERATURE AND SWELLING MEDIA

Hydrogel samples	NIPAAm/AAc monomer ratio	Polymerization temperature (ºC)	Feed molar ratio of cross-linker (mol %)	25 ºC		37 ºC	
				UPW	PBS	UPW	PBS
1	97/3	20	0.90	97.18	94.48	95.87	89.92
2	96/4	20	0.90	97.30	95.19	96.68	91.04
3	95/5	20	0.90	98.65	96.60	97.11	91.98
4	97/3	20	1.26	96.75	93.27	95.49	86.88
5	97/3	20	1.62	95.76	92.85	95.09	85.43
6	97/3	0	0.90	97.65	95.92	96.36	84.72
7	96/4	0	0.90	97.88	96.36	96.59	88.94
8	95/5	0	0.90	98.44	97.42	97.65	91.58

Water content of hydrogel = $(W_s - W_d)/W_s \times 100$, where W_s is the weight of swollen gel and W_d is the weight of dry gel. Each water content value represents the average of three samples.

In addition, the water content of the hydrogels (feed molar ratio of the cross-linker = 0.90 %) with different NIPAAm/AAc monomer molar ratios (NIPAAm/AAc) 97/3, 96/4 and 95/5 were different. The water content of

the hydrogels in the ultra pure water and PBS at 25 and 37°C increased with the AAc content in the hydrogel (Table-2). The effect suggested that COO^- groups strongly influenced changes in the hydrophilic/hydrophobic nature of the polymer that prevent collapsing of the hydrogel.

The effect of cross-linking density on the water content for hydrogels with the same NIPAAm/AAc monomer molar ratio of 97/3 was investigated (sample 1, 4 and 5). As the cross-linking density within the hydrogel increased, the water content decreased in ultra pure water and in PBS. The high cross-linking density made the collapsed structure tightly than others. And the porosity of the sample is smaller than others. Thus the water content of the samples with higher cross-linking density would be lower. The difference in water content between in ultra pure water and in PBS due to the shielding of COO^- repulsion increased with increasing cross-linking density. For the hydrogels with higher cross-linking density (feed molar ratio of cross-linker = 1.62 mol %), a lower water content was observed, compared with hydrogels with lower cross-linking density (cross-linker feed molar ratio = 0.90 and 1.26 mol %) in PBS. This result indicated that the effect of interactions between COO^- groups in AAc in the hydrogel and the ions in the PBS was reduced by increase of the cross-linking density.

The water content of the L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels (sample 1, 2 and 3 compare with sample 6, 7 and 8) synthesized at different temperature (0 and 20 °C) did not show a regular difference in the PBS and in the ultra pure water at 25 °C, whereas the water content of the L-lysine-cross-linked P(NIPAAm-co-AAc) hydrogels exhibited media-dependent behaviour at 37 °C (near the LCST) in the PBS. The water contents of the hydrogels in the PBS at 37 °C were higher in higher polymerization temperature (20 °C) than in lower polymerization temperature (0 °C). At the low polymerization temperature the samples have more tightly structure and smaller pores, thus the water content was lower than others.

Conclusion

A L-lysine-cross-linker was synthesized and characterized using L-lysine as material. The loosely cross-linked P(NIPAAm-co-AAc) hydrogels were synthesized by free-radical polymerization in aqueous media with the L-lysine-cross-linker. The lysine-cross-linked P(NIPAAm-co-AAc) hydrogels had some of the attractive properties such as, transparently and extremely pliable at room temperature. Furthermore, the low critical solution temperature of hydrogels was significantly influenced by monomer molar ratio of NIPAAm/AAc, whereas cross-linking density did not affect the LCST. The hydrogels contained more than 90 % water content at 37 ºC in the ultra pure water and have a prospective swelling in the PBS (> 84.72 %), which was controlled by the molar ratio of NIPAAm/AAc, swelling media and cross-linking density.

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