Effect of the SA Content of a Novel Thermo-Sensitive P(NIPAM-*co*-SA) Copolymer on Denatured Lysozyme Refolding *In Vitro*

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ABSTRACT: A novel hydrogel of P(NIPAM-*co*-SA) copolymer was synthesized by inverse suspension polymerization by adding sodium acrylate (SA) to improve the phase transition properties of poly(*N*-isopropylacrylamide) (PNIPAM). The morphologies, size distribution and thermosensitive characteristics of gel particles were studied and the maximal swelling ratio and LCST (Lower Critical Solution Temperature) of gel particles increased obviously with the addition of SA comonomer. When the protein concentration was 250 μg/mL, the optimized refolding conditions of denatured lysozyme with P(NIPAM-*co*-SA) hydrogel were that operating at the temperature of 35°C and a urea concentration of 2*M*, in which the mass ratio of P(NIPAM-*co*-SA) hydrogel with 4% SA copolymerized to lysozyme was 10 : 1. Under the optimized conditions, the activity recovery of lysozyme increased to 76.5% assisted by P(NIPAM-*co*-SA) gel particles compared with 55.6% by simple dilution. When refolding finished, the gel particles could be removed and recovered easily and the activity recovery of lysozyme was still as high as 61.5% after reused for 5 batches. With the addition of different amounts of SA comonomer, the hydrophobicity of the copolymer could be varied. Then the copolymerized hydrogel inhibits protein molecules aggregation more effectively through the moderate hydrophobic interactions between copolymers and protein molecules in the course of lysozyme refolding compared with the presence of PNIPAM polymer. All results above demonstrate that the P(NIPAM-*co*-SA) is a cost effective additive with tunable hydrophobicity for application in the refolding of recombinant proteins expressed as inclusion bodies *in vitro*. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 2597–2605, 2011

Key words: thermosensitive hydrogel; PNIPAM; sodium acrylate; copolymer; lysozyme; protein refolding

INTRODUCTION

The modern gene technology converts the dream of expression of recombinant proteins in prokaryote (i.e., *E. coli*) into reality. However, one main disadvantage of this method to produce target proteins in industry is the formation of inclusion bodies (IBs) in *E. coli* in the process. Then protein refolding *in vitro* is increasing the importance in production of recombinant proteins.[1,2] Till now many techniques have been developed in protein refolding, including direct dilution, chromatography refolding[3,4] and adding refolding aids.[5]

In the study for mechanisms of folding, the hydrophobic collapse model assumes that a protein buries its hydrophobic side chains from solvent water early during folding, forming a collapsed intermediate or molten globule species, from which the native state develops by searching within this conformationally restricted state. It is now clear, based on investigations of transient and equilibrium intermediates *in vitro*, that partially folded intermediates, as found with newly synthesized proteins in the cell, are particularly prone to aggregate, probably via specific intermolecular interactions between hydrophobic surfaces of structural subunits. Thus in the assisted assembling theory, the hydrophobic forces are the key factors to facilitate protein refolding thought to prevent misfolding and aggregation by binding to polypeptide chains that are not fully folded,[6] and polymers with rich hydrophobic groups are potential efficient refolding aids.

Poly(*N*-isopropylacrylamide) (PNIPAM) is one of typical thermosensitive polymers with a lower critical solution temperature (LCST) around 32°C.[7] The hydrogel exhibits hydrophilic characteristics below the LCST, but hydrophobic above the LCST. Hence, the PNIPAM polymer could be easily separated from solution at temperature above LCST. Because the LCST is close to the physiological temperature, PNIPAM is widely applied to the bioseparation,[8,9] drug delivery[10] and so on. Recently, researchers explored the application of hydrogel into protein

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refolding and studied the effect of PNIPAM on refolding process since it possesses a hydrophobic vinyl backbone and pendant isopropyl side groups. Lu et al.[11] investigated PNIPAM hydrogel for lysozyme refolding and discovered its inhibition of protein aggregation which led to higher refolding yield. Meanwhile they utilized the weak hydrophobic dextran-grafted-PNIPAM to assisted protein refolding and got better results than PNIPAM.[12] Our work on lysozyme refolding using disks and particles of PNIPAM hydrogel also demonstrated PNIPAM could improve refolding yield significantly.[13,14] Furthermore, the PNIPAM particles enhanced refolding of recombinant bovine prethrombin-2 from *E. coli* inclusion bodies with 122% increase of activity recovery compared with refolding by simple dilution under the optimized conditions.[15] The PNIPAM particles could be easily recovered from solution by centrifugation. After reutilization for eight batches, lysozyme refolding yield still increased from 44.9% to 55.0%.[13]

In the synthesis of PNIPAM hydrogel, we found that the strength of gel particles at the swollen state was not high enough and vulnerable to break up in subsequent refolding and recovery. Besides, the refolding temperature was quite limited due to the lower LCST of PNIPAM. Copolymerization is an alternative method to improve the mechanical properties[16] and change the LCST and swelling behavior of gel particles.[17] According to the study of Li et al.,[18] PNIPAM copolymerized with a small ratio of sodium acrylate (SA) would increase the LCST of the polymers. Meanwhile our previous work indicated that the copolymer of P(NIPAM-*co*-SA) had the capacity to enhance protein refolding *in vitro*[19] compared with simple dilution. But for hydrophilic proteins such as lysozyme, the hydrophobic environment was excess strong to inhibit the intramolecular interaction of protein intermediates, which is unfavorable to protein refolding. In addition, the hydrophilic environment was also needed to stabilize proteins after refolding. Hence the application of hydrogels would be extended if the hydrophobicity of PNIPAM could be regulated for different proteins. In this paper, we will synthesize P(NIPAM-*co*-SA) particles with different ratio of SA copolymerized and apply them to assist denatured lysozyme refolding *in vitro*. The hydrophobicity of hydrogels could be adjusted for the refolding of versatile proteins by changing the SA contents copolymerized.[20] The lysozyme refolding conditions mediated by P(NIPAM-*co*-SA) copolymers will be optimized and compared with PNIPAM assisted refolding. Then gel particles will be removed from solution and recovered for reutilization. Finally structure of lysozyme after refolding will be analyzed, which will help us to get further understanding about refolding assisted by the P(NIPAM-*co*-SA) hydrogel particles. A novel P(NIPAM-*co*-SA) hydrogel system will be proposed for application in the refolding of genetically engineered proteins expressed as inclusion bodies *in vitro*.

EXPERIMENTAL

Materials

N-isopropyl acrylamide (NIPAM) was purchased from ACROS. *N,N′*-methylene-bisacrylamide (Bis) was from Fluka. Dithiothreitol (DTT), reduced and oxidized glutathione (GSH and GSSG), and hen egg white lysozyme were from BBI. *Micrococcus Lysodeikticus* ATCC 4698 was purchased from Sigma. Sodium acrylate (SA) was prepared in our lab. All other chemicals were of analytical reagent grade and purchased commercially.

Preparation of P(NIPAM-*co*-SA) copolymers

Copolymers with different feed ratio of SA to NIPAM were synthesized by inverse suspension polymerization.[13,15,18] The contents of monomer and crosslinker were defined as follows:

$$T = \frac{W_{\mathrm{NIPAM}} + W_{\mathrm{Bis}}}{W_{\mathrm{NIPAM}} + W_{\mathrm{Bis}} + W_{\mathrm{H_2O}}} \times 100\% \quad (1)$$

$$C = \frac{W_{\mathrm{Bis}}}{W_{\mathrm{NIPAM}} + W_{\mathrm{Bis}}} \times 100\% \quad (2)$$

$$A = \frac{M_{\mathrm{SA}}(\mathrm{mol})}{M_{\mathrm{NIPAM}}\,(\mathrm{mol})} \times 100\% \quad (3)$$

where T is the mass percent of monomer NIPAM plus crosslinker Bis, C is the crosslinker Bis mass percent, while A is the molar ratio of SA to NIPAM. Here W_{NIPAM}, W_{Bis}, $W_{\mathrm{H_2O}}$ denote the mass of NIPAM, Bis, and H_2O respectively, and M_{SA}, M_{NIPAM} are the molar of SA and NIPAM.

The reaction was performed in a 500-mL cylindrical round-bottom glass flask fitted with a mechanical stirrer, nitrogen inlet, and oil-water separator. A thermostatic water bath was used for isothermal control. Paraffin oil (as a continuous phase) and Tween80 (as the dispersant) were first added into the flask and stirred. The monomer NIPAM, comonomer SA, crosslinker Bis and 1.7 wt % of initiator ammonium persulfate (APS) were dissolved in deionized water. When the solution was added into the flask for about 30 min, following the adding of activator *N,N,N′,N′*-tetramethylethylenediamine (TEMED), the polymerization took place and continued for 3 h. The whole process was carried out with purging of the nitrogen. The water was thus separated through a water-oil separator.

According to the previous work,[13] here the gels of $T = 14\%$, $C = 10\%$ were synthesized with different addition of SA i.e., $A = 0$, 2, 4, and 6% copolymerized respectively, since the gels with higher T and C could greatly facilitate the refolding of lysozyme.

Characterization of P(NIPAM-*co*-SA) particles

A microscope of Eclipse E200 (Nikon, Japan) equipped with a Canon camera for image analysis was used to observe morphologies of the hydrogel particles. A scanning electron microscope (SEM) of JSM-6390A (JEOL, Japan) was used to study the surface characteristics of the microspheres. The diameters of hydrogel particles in swelling state were measured by Mastersizer 2000 (Malvern, UK). The dried particles (with dry weight W_d) were ground into powders and packed into a graduated flask with deionized water. The volumes of wet gels were recorded as V_1. The density of the dried gels was about 1.25 g/mL, and the dried volumes were calculated (V_0). The swelling ratio (SR) was then obtained by dividing the swollen gel volume (V_1) by the dried gel volume (V_0).[15] SR was measured at different temperature, and LCST was ascertained as the temperature where the SR was half of the maximum SR.

Protein refolding

Native lysozyme was first dissolved in the denaturation solution (0.1 mol/L Tris-HCl, pH 8.5, 8 mol/L urea, and 30 mmol/L DTT) and shaken in the incubator at 37°C with 100 rpm for 90 min. Protein concentration was measured by absorbance at 280 nm as elsewhere.[12] The final protein concentration was 10 mg/mL. Then denatured lysozyme was mixed with the refolding buffer (0.1 mol/L Tris-HCl, pH 8.5, 3 mol/L urea, 1 mmol/L EDTA, 0.15 mol/L NaCl, 3 mmol/L GSH and 0.375 mmol/L GSSG, i.e., GSH : GSSG = 8 : 1), in the presence of P(NIPAM-*co*-SA) copolymers. The solution was shaken in the incubator at 30°C with 120 rpm overnight.

Assay of lysozyme activity

Lysozyme activity was determined according to the method proposed by Stellmach et al.[21] The substrate of *Micrococcus lysodeikticus* was first ground and dissolved in a buffer of pH 6.2, 1/15 mol/L Na_2HPO_4-KH_2PO_4 with an initial absorbance between 0.6 and 0.7 at 450 nm. Then 10 μL of lysozyme solution at an appropriate concentration was added into 3 mL of substrate solution and an absorbance measurement was obtained 2 min later. The activity of lysozyme was determined by eq. (4) as following.

$$I(\mathrm{U/mg}) = (E_1 - E_2)/(E_w \times 2 \times 0.001) \quad (4)$$

where I is the enzymatic activity of the lysozyme sample, E_1 is the absorbance of the substrate solution at 450 nm, E_2 is the absorbance of the substrate solution 2 min after the addition of lysozyme solution, and E_w is the quantity of lysozyme in the reaction system.

Fluorescence measurement

Intrinsic protein fluorescence spectra were measured with fluorescence spectrophotometer of F-4500 (Hitachi, Japan) by exciting at 280 nm. Then the emission spectra within 300–500 nm were measured.

Circular dichroism spectra

Circular dichroism (CD) was performed with spectropolarimeter of J-815 (Jasco, Japan) to determine the secondary structure of lysozyme after refolding. The spectra within 200–250 nm were measured.

Nonreductive SDS-PAGE

The nonreductive sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done similarly to SDS-PAGE but without DTT in the sample buffer. The resolving gel was 15%, and the stacking gel was 5%.

RESULTS AND DISCUSSION

Characterization of P(NIPAM-*co*-SA) hydrogels

In this experiment, four kinds of copolymers with different ratio of SA to NIPAM were synthesized. The micrographs of P(NIPAM-*co*-SA) gel particles in dried and swelling state are shown in Figure 1, indicating that hydrogel particles were spherical and well dispersed in aqueous solution. From the SEM images shown in Figure 2, we can identify that numbers of pores decreased with the increase of SA content which resulted in a decrease in the swelling rate. The average diameters of swelling gel particles are shown in Table I. The diameters were between 200 and 400 μm with a relatively narrow distribution.

For the study of temperature sensitivity of P(NIPAM-*co*-SA) gel particles, the swelling ratios of the particles at different temperature were investigated and the results are shown in Figure 3. The LCST of the P(NIPAM-*co*-SA) hydrogels are also summarized in Table I. From Figure 3 and Table I, we know that the LCST increased from 32°C to around 40°C with the addition of SA. Furthermore, the maximum swelling ratio also obviously increased from 6.5 to 13.75. This will facilitate

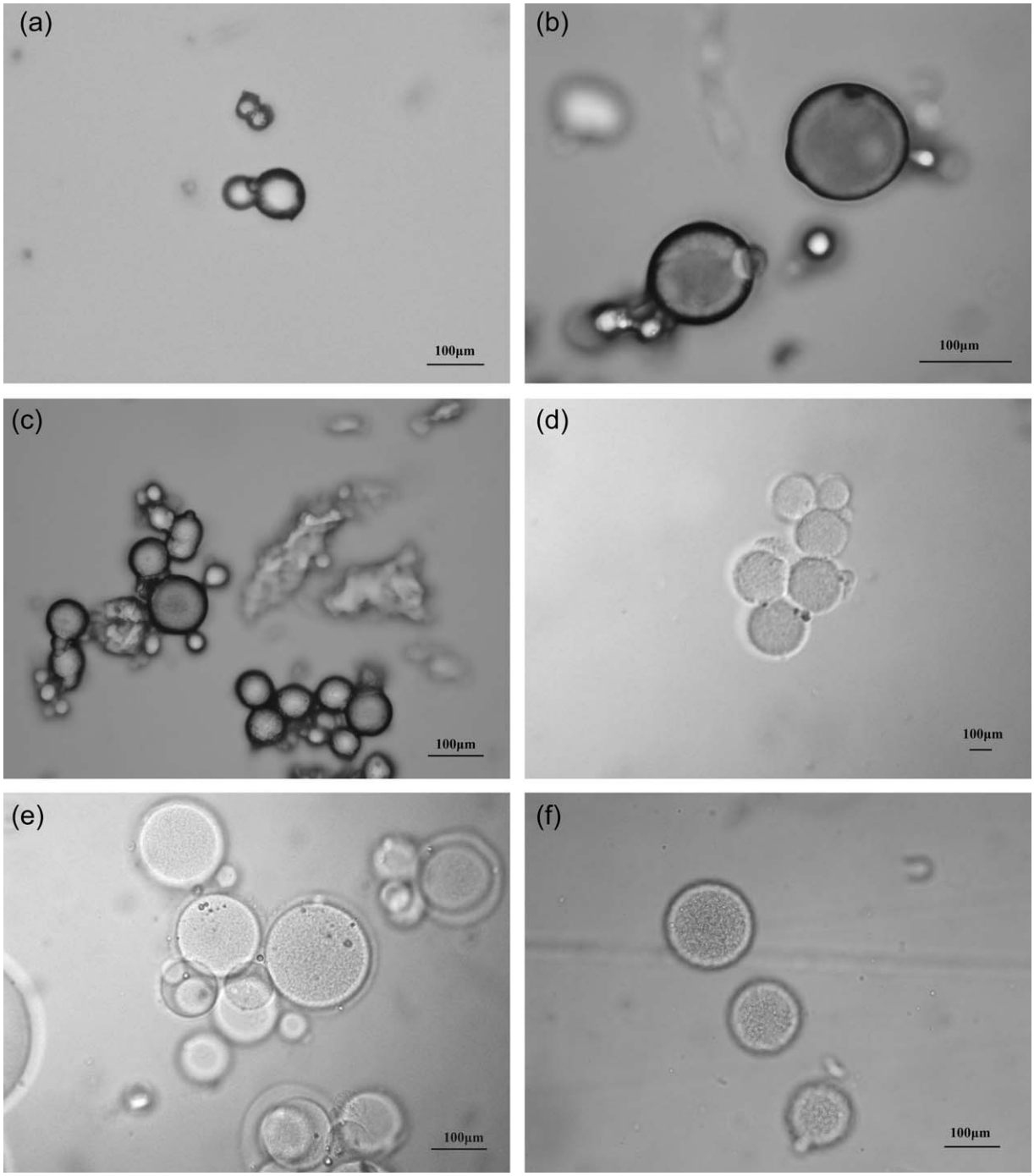

Figure 1 Micrographs of P(NIPAM-*co*-SA) Gel Particles. (a) $T = 14\%$, $C = 10\%$, $A = 2\%$ at dry state; (b) $T = 14\%$, $C = 10\%$, $A = 4\%$ at dry state; (c) $T = 14\%$, $C = 10\%$, $A = 6\%$ at dry state; (d) $T = 14\%$, $C = 10\%$, $A = 2\%$ at swelling state; (e) $T = 14\%$, $C = 10\%$, $A = 4\%$ at swelling state; (f) $T = 14\%$, $C = 10\%$, $A = 6\%$ at swelling state.

protein refolding because the water absorption capacity of hydrogels was greatly improved. When the SA was copolymerized, the thermosensitivity decreased which slowed down the rate of the phase transition. These phenomena are consistent with the research of other groups.[17,22]

In general, phase transition is driven by the interaction among hydrophobic groups. When the hydrophilic SA is added, the introduction of ionic groups of hydrogels leads to increase of water content hydrogen bonded to polymers. Therefore the interaction among hydrophobic groups causing breakage of hydrogen bonds needs more energy, and LCST is higher than the nonionic PNIPAM. Because the breakage of hydrogen bonds is likely to occur at various temperatures, the phase transition crosses a broader temperature range, i.e., thermosensitivity decreased. That means the hydrophobicity of

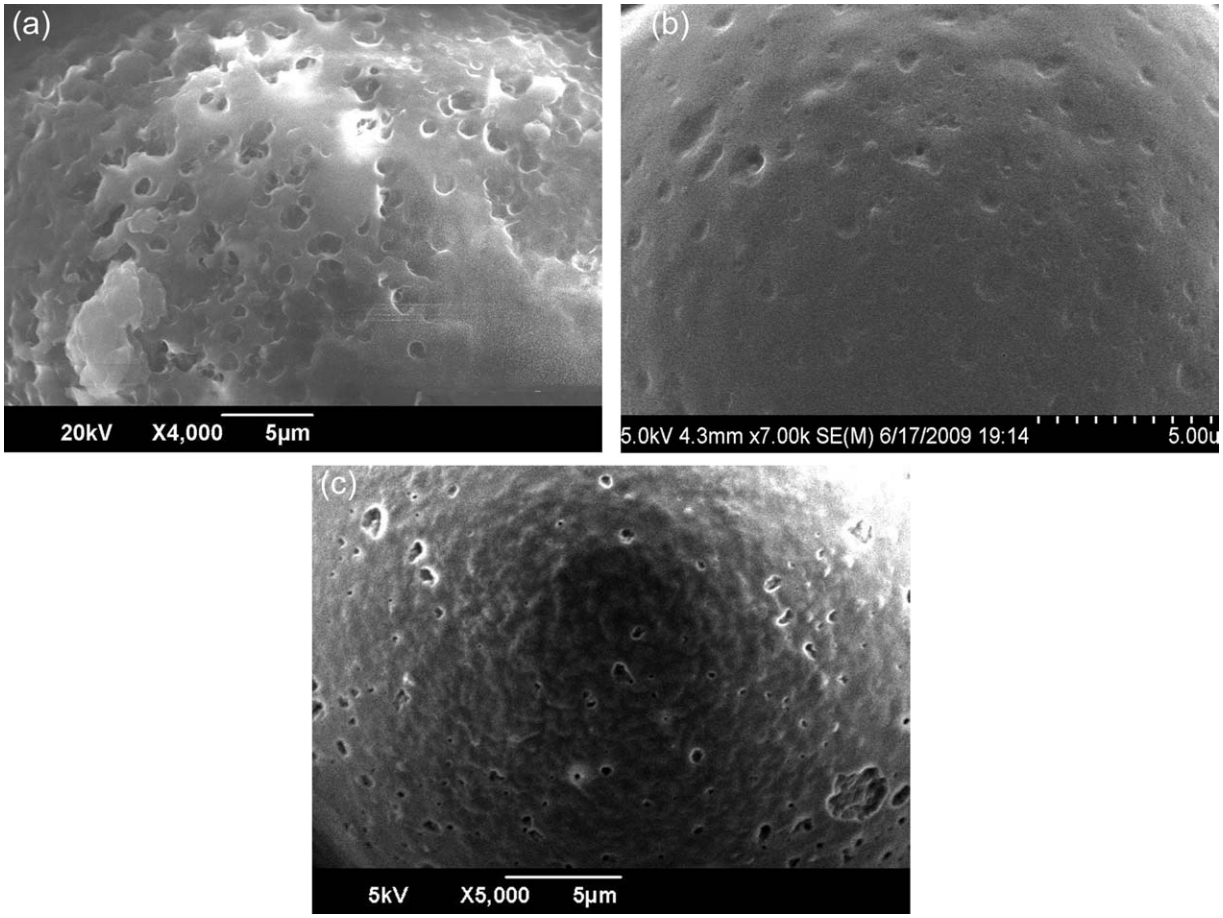

Figure 2 SEM Photographs of P(NIPAM-*co*-SA) Gel Particles. (a), $T = 14\%$, $C = 10\%$, $A = 2\%$; (b) $T = 14\%$, $C = 10\%$, $A = 4\%$; (c) $T = 14\%$, $C = 10\%$, $A = 6\%$.

NIPAM hydrogels could be regulated accurately around LCST.

Effect of P(NIPAM-*co*-SA) gel particles on lysozyme refolding

The mass ratio of hydrogel particles to lysozyme

The influence of hydrogel particles concentration on lysozyme refolding was investigated firstly, where the initial denatured protein concentration was 10 mg/mL, and the final protein concentration was 250 μg/mL. The hydrogel particles with 4% SA copolymerized were used here. From Figure 4, it could be seen that the activity recovery of lysozyme reached the maximum 71.1% when the concentration ratio of gel particles to protein was 10 : 1. Compared with refolding by simple dilution, addition of P(NIPAM-*co*-SA) could increase refolding yield by

TABLE I
Average Diameter and LCST of P(NIPAM-*co*-SA) Gel Particles ($T = 14\%$, $C = 10\%$)

A (%)	Diameter (μm)	LCST (°C)
0	313.7	32
2	260.8	38
4	216.2	40
6	246.1	41

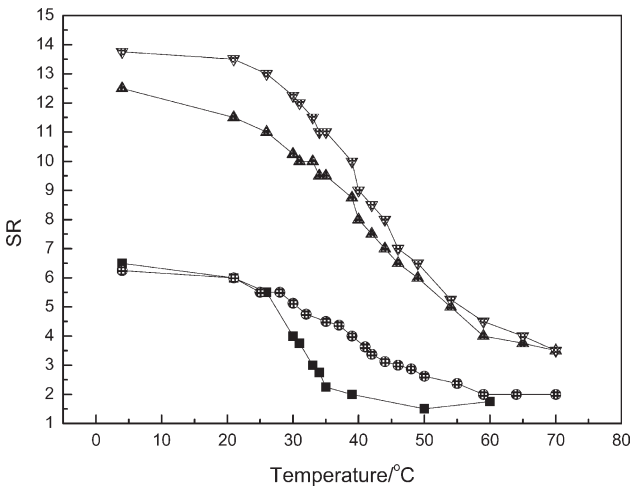

Figure 3 Swelling Ratio of PNIPAM and P(NIPAM-*co*-SA) Hydrogels at Different Temperature (■, A = 0%; ●, $A = 2\%$; ▲, $A = 4\%$; ▼, $A = 6\%$).

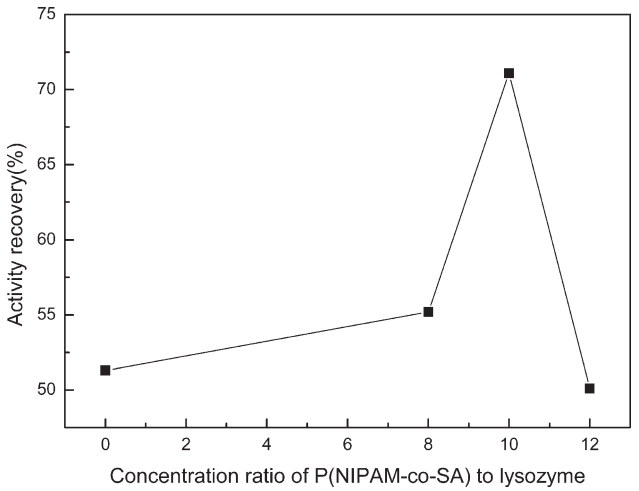

Figure 4 Effect of Hydrogels Concentration on Lysozyme Refolding. (Refolding was initiated by diluting lysozyme in Tris-HCl buffer containing 3M urea and different ratios of P(NIPAM-*co*-SA) (A = 4%) at 30°C. The final protein concentration was 250 μg/mL).

38.6%. Similarly to PNIPAM, further increase in P(NIPAM-*co*-SA) concentration resulted in a reduction in lysozyme refolding yield.

Effect of the temperature on lysozyme refolding

As temperature has a strong influence on the hydrophobicity of the copolymers, thus obviously affect protein refolding yield. Refolding was conducted at different temperature in this experiment. The dilution refolding yield decreased as expected with the increase of temperature. In the presence of hydrogel particles, we discovered from Figure 5 that when the temperature was below the LCST, the refolding yield increased with the temperature. But the refolding yield decreased sharply if the temperature was beyond the LCST. This is because that hydrogels collapse and release most of its swelling solution at temperature above LCST. The surface area for interaction between hydrogels and protein molecules gets smaller, which is unfavorable to protein refolding. The maximum refolding yield was obtained at 35°C with the presence of copolymers, while 30°C was the optimal temperature for refolding with PNIPAM. The optimal refolding temperature also increased for the addition of SA as LCST did. Meanwhile the addition of SA can achieve the accurate temperature control around the LCST to get the suitable hydrophobicity in the refolding of lysozyme.

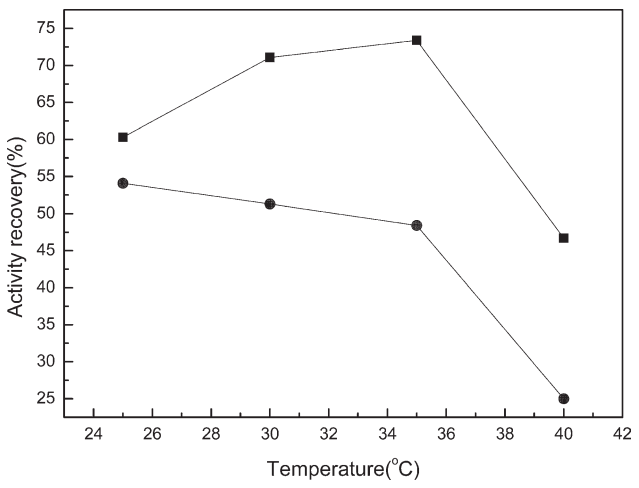

Figure 5 Effect of Temperature on Lysozyme Refolding. (Refolding was initiated by diluting lysozyme in Tris-HCl buffer containing 3M urea at different temperature. The mass ratio of P(NIPAM-*co*-SA) particles (A = 4%) to lysozyme was 10 : 1. The final protein concentration was 250 μg/mL). (■, Refolding with P(NIPAM-*co*-SA) (A = 4%); ●, Refolding by simple dilution).

Effect of urea concentration on lysozyme refolding

As urea could affect the hydrophobic environments, it definitely has great influence on protein refolding yield. Usually urea at low concentrations could effectively inhibit protein intermolecules hydrophobic interactions, which is advantageous to improve refolding yield. Otherwise, urea at high concentrations would inhibit the protein intramolecular hydrophobic interactions, which is against to protein refolding. Urea even causes protein denaturation, if the concentration is above 4 mol/L. So there must be an optima urea concentration in protein refolding process. For the P(NIPAM-*co*-SA) copolymer (A = 4%) assisted refolding, the optima urea concentration is 2 mol/L, as shown in Figure 6.

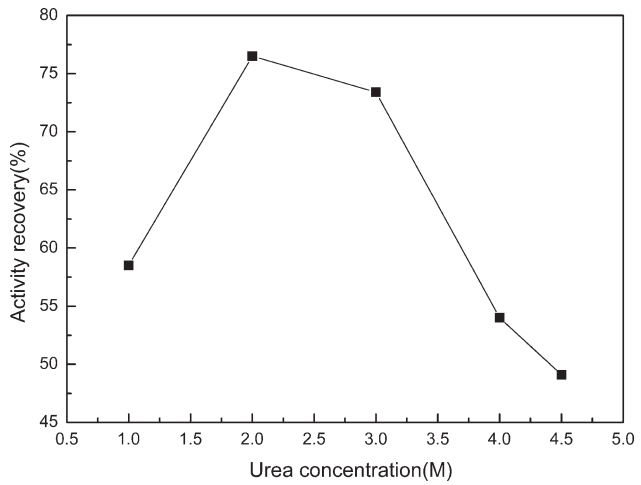

Figure 6 Effect of Urea Concentration on Lysozyme Refolding. (Refolding was initiated by diluting lysozyme in Tris-HCl buffer containing different concentration of urea at 35°C. The mass ratio of hydrogel particles (A = 4%) to lysozyme was 10 : 1. The final protein concentration was 250 μg/mL).

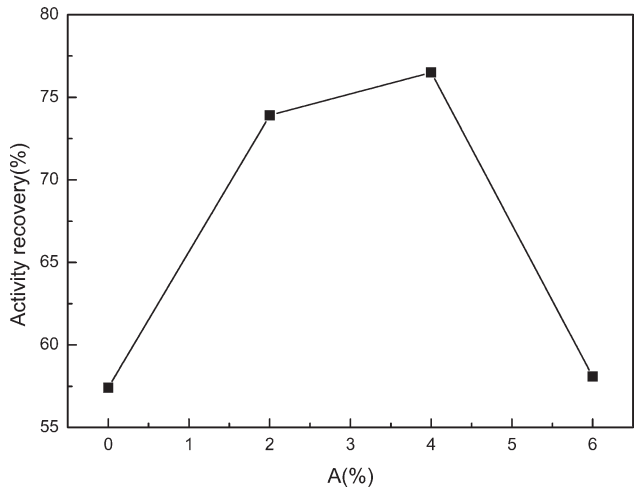

Figure 7 Effect of Sodium Acrylate on Lysozyme Refolding. (Refolding was initiated by diluting lysozyme in Tris-HCl buffer containing 2*M* urea at 35°C. The mass ratio of hydrogel particles to lysozyme was 10 : 1. The final protein concentration was 250 μg/mL).

SA amount on protein refolding

From above results, the SA amounts showed strong indirect effect on refolding yield. Refolding was done with the four different P(NIPAM-*co*-SA) gel particles under the optimized conditions. Figure 7 shows that the refolding yield was 76.5% at protein concentration of 250 μg/mL when A is 4%, about 33% higher than refolding using PNIPAM. This result demonstrated that the SA addition had positive effect on refolding kinetics.

During protein refolding, too strong hydrophobic environment may hinder refolding of intermediates,[11] since the folded and partially folded proteins are usually hydrophilic. When the hydrophilic SA segments are randomly introduced, the hydrophobic NIPAM segments are divided into short ones, and hydrophobic forces decrease. Therefore hydrogels with SA addition could provide the hydrophilic environment and stabilize the native-like structure of proteins. On the other hand, the hydrophobic index of lysozyme is only −14.9 calculated from Janin's research,[23] compared to carbonic anhydrase B (about −45) and other recombinant proteins.[6] For the hydrophilic proteins such as lysozyme, PNIPAM was too strong to reach higher refolding yield. After addition of SA, the hydrophobicity could be slightly adjusted to facilitate the refolding of proteins with comparatively less hydrophobic residues. When adding an excess of SA ($A = 6\%$), the hydrophobicity of copolymer particles was not enough to hinder protein-protein interactions. As a result, refolding yield decreased, which was consistent with our previous work.[19] For protein refolding, suitable hydrophobic environment could be provided by the addition of moderate SA.

Refolding yields at different protein concentrations

We investigated the enhanced effect of copolymers at different lysozyme concentration, and the results are shown in Figure 8. Obviously refolding yield assisted by copolymers increased compared with simple dilution. When the protein concentration was 250 μg/mL, the addition of P(NIPAM-*co*-SA) increased refolding yield from 55.6% to 76.5%; while the protein concentration was 1000 μg/mL, P(NIPAM-*co*-SA) increased refolding yield from 12.9% to 42.1%. Obviously the copolymers greatly improved refolding yield at high lysozyme concentration. As a result, we could refold proteins with less refolding buffer to cut cost attributing to the P(NIPAM-*co*-SA) hydrogels function.

Reutilization of copolymers

The hydrogel particles were easily recovered by centrifugation above LCST. The reutilization experiment results are presented in Figure 9. The activity recovery of lysozyme was still as high as 61.5% after reused for five batches, compared with 55.6% by dilution refolding. This indicated that the gel particles could be reused and recycled for batches, which is advantageous to further industrial application.

Lysozyme structure analysis after refolding

To get further understanding about refolding assisted by the P(NIPAM-*co*-SA) hydrogel particles,

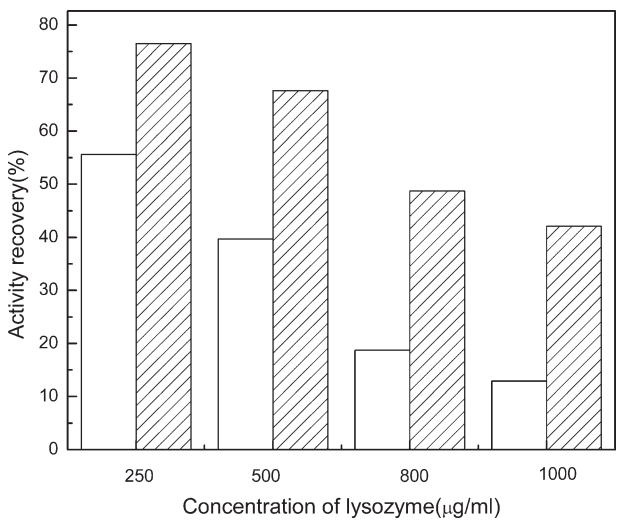

Figure 8 Lysozyme Refolding Assisted by Hydrogel Particles at Different Concentration. (Refolding was initiated by diluting lysozyme in Tris-HCl buffer containing 2*M* urea at 35°C. The mass ratio of P(NIPAM-*co*-SA) particles ($A = 4\%$) to lysozyme was 10 : 1. blank, Refolding by simple dilution; shadow, Refolding with copolymers).

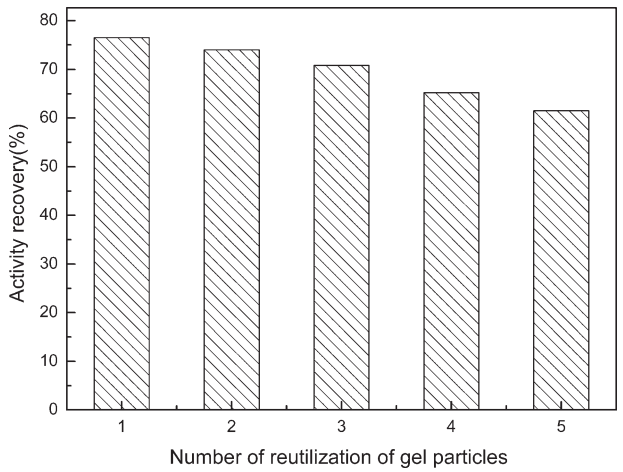

Figure 9 Reutilization of P(NIPAM-*co*-SA) Hydrogel Particles on Lysozyme Refolding. (Refolding was initiated by diluting lysozyme in Tris-HCl buffer containing 2*M* urea at 35°C. The mass ratio of P(NIPAM-*co*-SA) particles (A = 4%) to lysozyme was 10 : 1. The final protein concentration was 250 μg/mL).

we assayed lysozyme molecules after refolding by nonreductive SDS-PAGE. The result is shown in Figure 10, in which lysozyme was refolded in Tris-HCl buffer containing 2*M* urea at 35°C. In dilution refolding (Lanes 1–4), weak bands appeared at the position of the dimmer and trimer. When P(NIPAM-*co*-SA) particles were present in the process, the dimmer and other oligomer disappeared, indicating that the hydyogel particles obviously inhibited the aggregation of protein molecules. This also demonstrated the refolding mechanism proposed by Lu et al.[11] The hydrophobic interactions between the protein and the P(NIPAM-*co*-SA) particles could inhibit forces between protein molecules which led to the formation of aggregates.

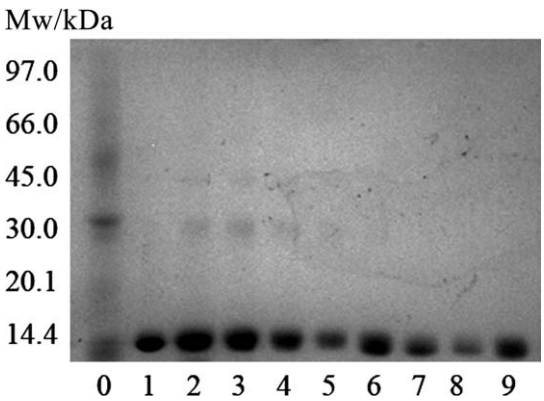

Figure 10 Nonreductive SDS-PAGE of Lysozyme after Refolding at Different Protein Concentration. (Lane 0 was protein molecular weight marker, Lanes 1–4 were samples with concentration of 1000 μg/mL, 800 μg/mL, 500 μg/mL, and 250 μg/mL refolded by dilution, Lanes 5–8 were samples with concentration of 1000 μg/mL, 800 μg/mL, 500 μg/mL, and 250 μg/mL refolded by copolymers, Lane 9 was native lysozyme molecules).

The conformation of correct lysozyme structure after refolding was carried out both by fluorescence and the far-UV circular dichroism. According to fluorescence spectra [see Fig. 11(a)], lysozyme refolding by dilution showed lower fluorescence intensity than lysozyme in the presence of hydrogel particles. Also the fluorescence emission maximum was a little larger for lysozyme refolding by dilution compared with lysozyme refolding in the presence of P(NIPAM-*co*-SA). Lysozyme molecules after refolding with P(NIPAM-*co*-SA) copolymers have similar tertiary structure as the native lysozyme. The far-UV

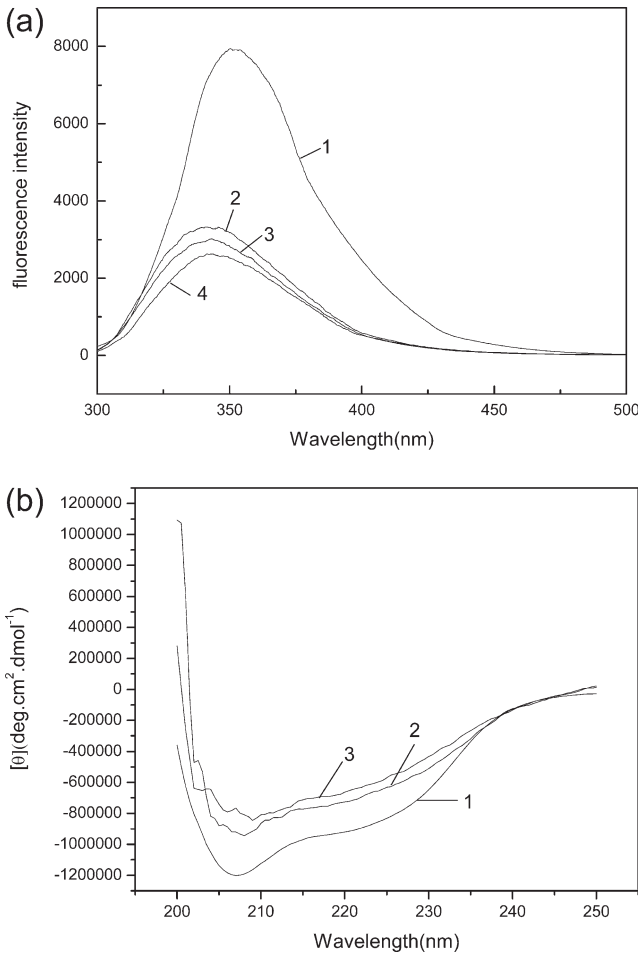

Figure 11 (a) Fluorescence spectra of native lysozyme and samples after refolding. (The lysozyme concentration was 250 μg/mL. 1, denatured lysozyme; 2, native lysozyme; 3, lysozyme refolding with P(NIPAM-*co*-SA); 4, lysozyme refolding by dilution.) (b) Far –UV circular dichroism spectra of native lysozyme and samples after refolding. (The lysozyme concentration was 250 μg/mL. 1, native lysozyme; 2, lysozyme refolding with P(NIPAM-*co*-SA); 3, lysozyme refolding by dilution).

circular dichroism spectra in Figure 11(b) also demonstrated that lysozyme molecules after refolding with hydrogel particles had native-like secondary structure, which was close to the native lysozyme structure. The intrinsic fluorescence spectra and the far-UV circular dichroism spectra in Figure 11 both validated that P(NIPAM-*co*-SA) copolymers could facilitate lysozyme refolding *in vitro*.

CONCLUSIONS

Four kinds of thermosensitive P(NIPAM-*co*-SA) gel particles with different amounts of SA copolymerized were synthesized by inverse suspension polymerization and the characterization including morphologies, surface characteristics and LCST of the P(NIPAM-*co*-SA) gels was studied. The LCST increased obviously with the addition of comonomer SA. The addition of SA with different ratio affects refolding yields by adjusting hydrophobic interactions between hydrogels and proteins molecules, and the hydrogel with 4% SA copolymerized is suitable for lysozyme refolding compared with PNIPAM. The application of hydrogel particles in the lysozyme refolding indicates that the particles are effective in assisting protein refolding especially at high initial protein concentration. After refolding the particles could be removed easily by centrifugation above LCST for reutilization. P(NIPAM-*co*-SA) exhibits tunable hydrophobicity both by changing SA content and temperature with a broad range and presents potential application in the refolding of genetically engineered proteins expressed as inclusion bodies *in vitro*.

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