Refolding of Lysozyme *In Vitro* Assisted by Colloidal Thermosensitive Poly(*N*-isopropylacrylamide) Brushes Grafted onto the Surface of Uniform Polystyrene Cores

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ABSTRACT: The renaturation of lysozyme assisted by a novel type of hairy particles was investigated. The particles used consisted of polystyrene (PS) cores onto which long chains of thermosensitive poly(*N*-isopropylacrylamide) (PNIPAM) were grafted as shell. Here, two kinds of particles were synthesized as sub-micron PS cores and 89 nm brush thickness (Particle A) and 122 nm brush thickness (Particle B), respectively. Thermosensitive characteristics of the above prepared hairy uniform PS particles with PNIPAM brushes were studied and the results indicated that the lower critical solution temperature (LCST) of PNIPAM brushes grafted on the surface of uniform PS cores was between 33 and 34°C. It was proved that the particles were quite efficient in assisting lysozyme renaturation at high initial protein concentration. When the protein concentration was increased to 500 μg/mL, the refolding yield of the recovery of lysozyme activity could achieve 58.0% (Particle A) and 71.5% (Particle B), compared with only 34.8% by simple dilution refolding. Furthermore, the kinetics of lysozyme refolding in the absence and the presence of the colloidal particles were studied accordingly. The results indicated that the time required for the refolding with colloidal particles was a little bit delayed than that by the simple dilution method owing to the hydrophobic interactions between lysozyme and the PNIPAM brushes. The mechanism of the enhancement for the hairy uniform PS particles with PNIPAM brushes assisted refolding was further discussed. All results above demonstrated that the spherical PNIPAM brushes presented an alternative way to assist protein renaturation *in vitro*. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 1270–1277, 2009

Key words: thermosensitive; poly(*N*-isopropylacrylamide); brushes; uniform particle; lysozyme; protein refolding

INTRODUCTION

Nowadays, in producing recombinant proteins, one of the most widely used expression system is *Escherichia coli* (*E. coli*) because it is a simple and relatively inexpensive means of obtaining large amounts of target proteins.[1] However, the overexpression of proteins in *E. coli* usually leads to the formation of misfolded and insoluble protein aggregates—inclusion bodies (IBs).[2] So protein refolding *in vitro*, which is used to convert the inactive and misfolded inclusion body proteins into soluble bioactive products, is a very important operation in the production of many recombinant proteins. Generally, protein refolding involves a two-step process: solubilization of IBs by a mixture composed of denaturant (such as 8 mol/L urea or 7 mol/L guanidine hydrochloride) and then refolding by removal of the denaturant. The simplest method to initiate refolding is based on dilution, and a final protein concentration of 10–100 μg/mL is usually applied in fast dilution procedures.[3] In recent years, many novel methods to improve the efficiency of refolding by reducing the formation of aggregation at high protein concentration have been reported.[4–12]

With the rapid development of interdisciplines, some polymer additives such as poly(ethylene glycol) (PEG) and poly(*N*-isopropylacrylamide) (PNIPAM) that can reduce the possibility of undesired protein–protein interactions and assist the protein refolding, have attracted increasing attention.[13] PNIPAM is one of the typical thermosensitive polymers and possesses a lower critical solution temperature (LCST) around 30–36°C.[14] PNIPAM as a nonionic polymer exhibits hydrophilic below the LCST and hydrophobic characteristics above the LCST. The phase transition properties of PNIPAM have been developed for application in many fields, such as controlled drug delivery, immobilization of

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enzymes,[15] biomedical materials,[16,17] biochemical separation engineering,[18] and reactivity control.[19] Recently researchers have explored the protein refolding *in vitro* assisted with PNIPAM and some interesting results were obtained. Lin et al.[20] applied linear PNIPAM to the renaturation of β-lactamase from inclusion bodies. It was observed that linear PNIPAM was effective in enhancing protein renaturation. At a concentration of 0.1%, linear PNIPAM improved the yield of β-lactamase activity by 41% from 46.5 to 65.4 IU/mL. With the sequential addition approach, the activity yield was increased by 60.5% from 46.5 to 74.6 IU/mL. Lu et al.[21] evaluated the effectiveness of linear PNIPAM with different molecular weights on the refolding of lysozyme (i.e., PNIPAM fractions with average molecular weights of 5.7, 22, and 53 kDa, respectively, termed as L-PNI, M-PNI, and H-PNI). For L-PNI and H-PNI, an optimal polymer concentration existed at which the refolding yield reached its maximum. In the case of M-PNI, the refolding yield continuously increased with the increase of the polymer concentration within the range of experimental conditions examined and the maximum refolding yield reached nearly 100% when the final lysozyme concentration was 1 mg/mL. Cui et al.[22,23] synthesized crosslinking PNIPAM particles by inverse suspension polymerization, and the particles were used for the refolding of lysozyme and bovine prethrombin-2 expressed as inclusion bodies. The results showed that 64 and 122% increase of enzyme activity recovery were obtained, respectively, compared with refolding by simple dilution under the optimized conditions.

Although traditional linear PNIPAM and crosslinking PNIPAM polymers could enhance the refolding effect availably, some unresolved problems presented in the application of protein refolding simultaneously. First, the molecular weight and its distribution of linear PNIPAM were not easy to control, leading to the fluctuation in the activity recovery. Otherwise, the linear PNIPAM with low molecular weight could not be removed by centrifugation after protein refolding attributed to the comparative molecular weight of the polymers and the peptides in aqueous solution. Second, the swelling rate of crosslinking PNIPAM particles was relatively slower, leading to a longer phase to reach refolding equilibrium because of the complex network structure, especially in the interior. Meanwhile, the protein molecules penetrated into the polymer's network structure were difficult to be recovered by the deswelling of PNIPAM particles. To overcome the above problems, the technique of grafting linear PNIPAM brushes onto the support material surface was introduced and applied into protein refolding *in vitro*.[24,25] The hairy particles should possess both the advantages of linear PNIPAM and crosslinking PNIPAM polymers with rapid response and are easy to be separated and recycled.

In this work, we prepared a type of uniform polystyrene (PS) particles with PNIPAM brushes (i.e., hairy particles), which are made up of PS core and a large number of PNIPAM brushes as shell grafted on the surface. Then the hairy particles were used to assist the refolding of lysozyme *in vitro*. A novel protein refolding method that offers high refolding efficiency, minimum loss of protein aggregates, and ease of polymers recycling is proposed.

MATERIALS AND METHODS

Materials

Styrene (St) was purchased from Hangzhou Resin Manufactory (Hangzhou, China). Prior to use, it was first washed by 5% NaOH solution to remove the inhibitor; then deionized water was added to rinse the St and vacuum distillation was applied. Finally, the St monomers were sealed and stored at 4°C. The isopropyl benzophenone methylacrylate as photoinitiator was synthesized in our laboratory. Potassium persulfate ($K_2S_2O_8$) was purchased from Shanghai Aijian Reagent (Shanghai, China) and was further purified by recrystallization and then dried under vacuum at room temperature. *N*-isopropyl acrylamide (NIPAM) was purchased from ACROS (Morris Plain, NJ) and used without further purification. Dithiothreitol (DTT), reduced and oxidized glutathione (GSH and GSSG), lysozyme, and *Micrococcus lysodeikticus* were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical reagent grade and purchased commercially.

Preparation of the hairy particles

The hairy uniform PS particles with thermosensitive PNIPAM brushes were synthesized following the three key steps scheme shown in Figure 1.[26]

First, the submicron monodisperse PS particles were synthesized by the emulsifier-free emulsion polymerization by using $K_2S_2O_8$ as thermoinitiator at a given temperature for about 5 h. Second, the isopropyl benzophenone methylacrylate as photoinitiator with double bond was attached onto the surface of the above PS cores as a paper-thin shell by copolymerization by using "starve" addition with St based on the emulsifier-free emulsion polymerization. Finally, PNIPAM brushes were grafted on the surface of the PS particles by the "grafting from" approach with sequential addition of NIPAM using photoinitiation under the irradiation of 500 W ultraviolet. The product was collected with centrifugal

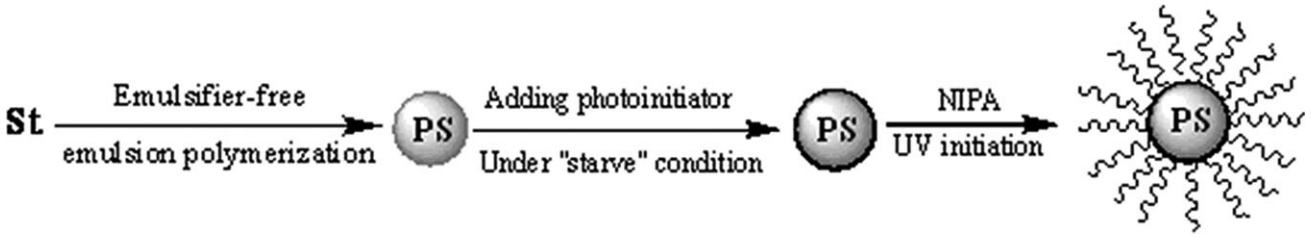

Figure 1 Schematic representation of the preparation approach for the uniform PS particles with PNIAM brushes.

separation and the hairy uniform PS particles with PNIPAM brushes were obtained. The reaction was performed in a 500 mL reactor fitted with a mechanical stirrer, a thermometer, a condenser, and the nitrogen inlet. A thermostatic water bath was used for constant isothermal control. The nitrogen was introduced to the reactor prior to reaction to remove air.

Morphology and thermosensitive characteristics of the hairy particles

The surface morphology and size characteristics of the microspheres were analyzed by using a scanning electron microscope (SEM; FEI, SIRON) and Bio-Scope SZ atomic force microscope (AFM; VEECO, USA). Layer thickness of the PNIPAM brushes grafted on the surface of uniform PS particles at different temperatures was observed through Malvern Zetasizer 3000 (Malvern, UK) with the given core-shell model, which needs refractive index of core (1.47 for PS) and Mark-Houwink parameters K and α of shell (0.00959 and 0.65 for PNIPAM, respectively), gaining the brush shell thickness based on the difference between diameter of the PS particles with PNIPAM brushes and diameter of PS cores. The results were analyzed to determine the LCST of the obtained PNIPAM brushes.

Refolding of the denatured lysozyme

A definite amount of lysozyme was 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 90–120 min. Then the denatured lysozyme was slowly diluted into 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, GSH : GSSG = 8 : 1). For the study of refolding with hairy particles, the denatured lysozyme was diluted into the refolding buffer containing a certain amount of hairy particles. The mixture was blended and shaken constantly in the incubator at 30°C at 120 rpm.

Assay of lysozyme activity

The activity of lysozyme was determined according to the method proposed by Stellmach and Qian[27] using *M. lysodeikticus* as the substrate. During an assay, the substrate cells were 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 at 450 nm. Then 10 μL of lysozyme solution at an appropriate concentration was added into 3 mL of the substrate solution and an absorbance measurement was obtained at 450 nm 2 min later. The activity of lysozyme was determined by eq. (1) as follows:

$$I(\mathrm{U/mg}) = \frac{E_1 - E_2}{E_w \times 2 \times 0.001} \quad (1)$$

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 into, and E_w is the quantity of lysozyme in the reaction system.

RESULTS AND DISCUSSION

Morphology and size characteristics of the hairy particles

Two kinds of hairy uniform PS particles with PNIPAM brushes were prepared (i.e., Particle A with 442 nm core and 89 nm brushes, Particle B with similar core and 122 nm brushes). The SEM images of the hairy PS particles A and B were shown in Figure 2. AFM images of the typical uniform PS cores and hairy PS particles with PNIPAM brushes (Particle B) were shown in Figure 3. We could identify that the particles were uniformly separated and had a relatively narrow size distribution for both cores and hairy microspheres. Furthermore, we could see clearly from the AFM images shown in Figure 3 that a large number of PNIPAM brushes had been grafted on the surface of the uniform PS cores. This result should be consistent with that by Malvern measurement.

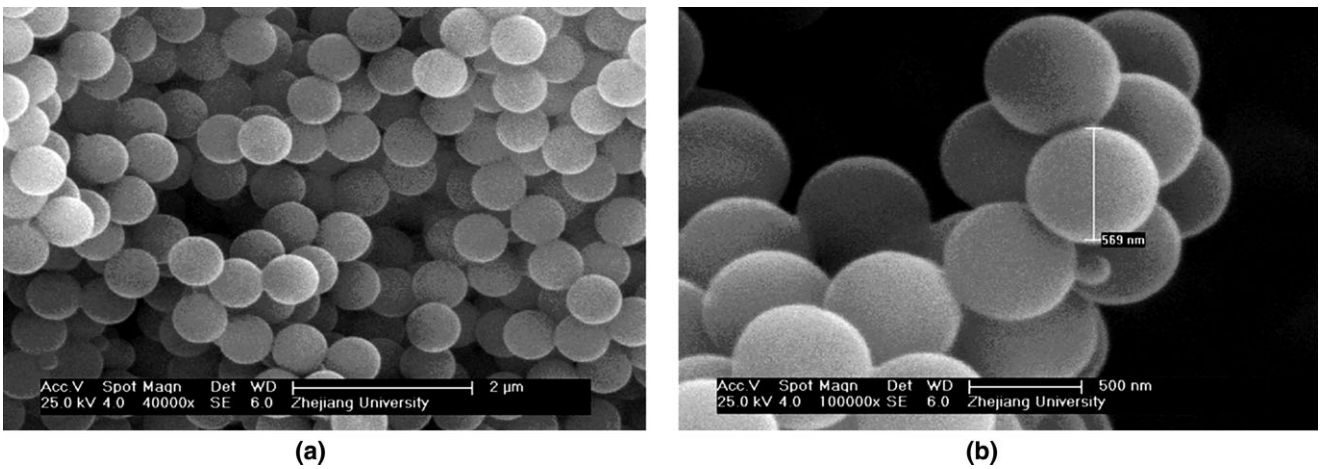

Figure 2 SEM images of the hairy particles. (a), Particle A; (b), Particle B.

Thermosensitive characteristics of the PNIPAM brushes

For the study of temperature sensitivity of PNIPAM brushes, the thickness of the brushes layer at different temperature should be measured; the results are shown in Figure 4. The layer thickness of the brushes appeared as a sharp decline between 30 and 37°C, with half collapse occurring at about 33–34°C. It was thus considered that the LCST of PNIPAM brush ranged between 33 and 34°C. The LCST of PNIPAM-grafted on the surface of PS particles was in accord with the results reported previously.

Refolding of lysozyme with hairy particles

Two kinds of hairy particles obtained above were subsequently used to study the effects on lysozyme refolding *in vitro*. In the refolding process, the denatured lysozyme was diluted into the refolding buffer with a certain amount of particles and shaken in the incubator at 30°C with 120 rpm for about 12 h. After refolding finished, the particles could be removed easily through heating above its LCST and centrifugal separation. The final refolding efficiency was expressed by the activity recovery, which was defined as the activity percent of lysozyme after refolding relative to the initial activity of the native lysozyme.

Influence of the particles concentration on lysozyme refolding

The influence of the hairy particles concentration on lysozyme refolding was investigated, where the

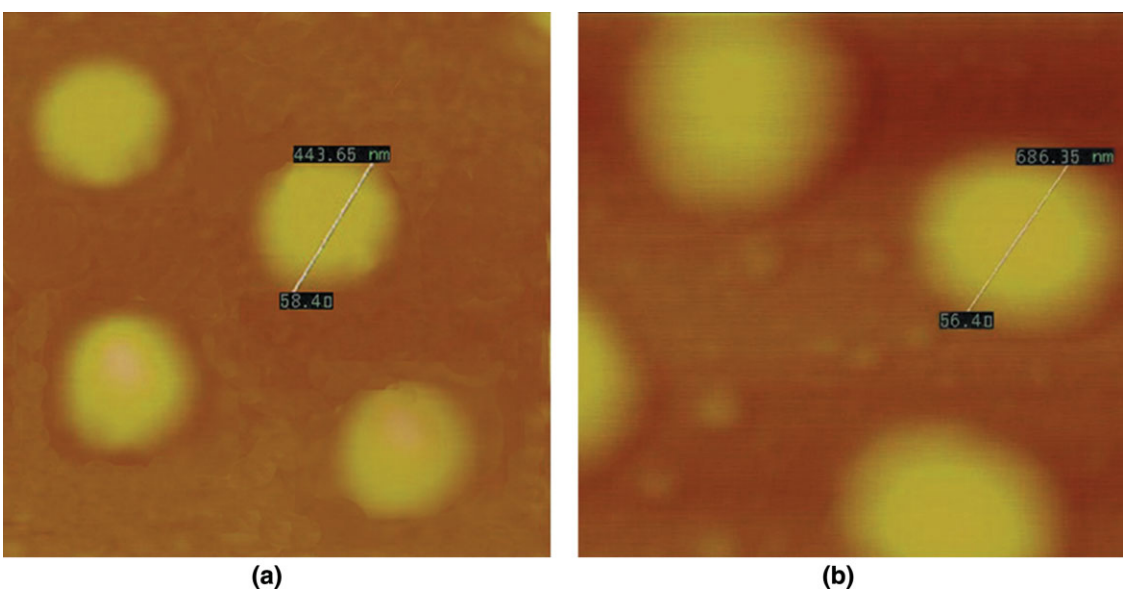

Figure 3 AFM images of the typical uniform PS cores and hairy PS particles with PNIPAM brushes (Particles B). (a), PS cores; (b), hairy PS particles with PNIPAM brushes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

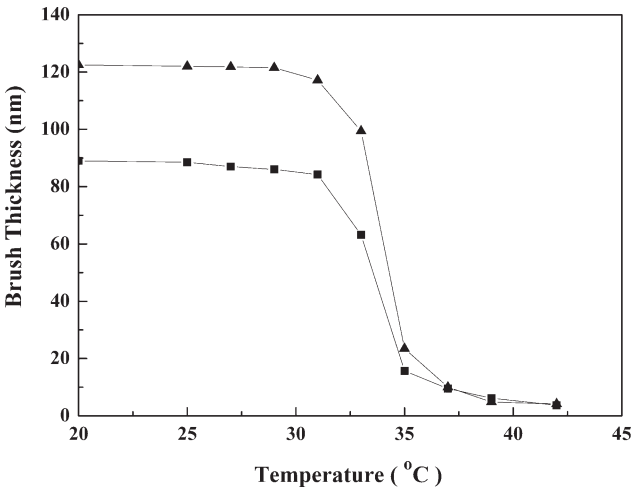

Figure 4 Layer thickness change of PNIPAM brushes grafted on the surface of uniform PS particles at different temperatures ■, Particle A; ▲, Particle B.

initial denatured protein concentration was 10 mg/mL, and dilution factors were 20 and 40 (namely, the final protein concentration were 500 μg/mL and 250 μg/mL), respectively. In this experiment, the different amounts of particles were added and the results were shown in Figure 5, Figure 6 (Particle A), and Figure 7 (Particle B). From Figures 5 and 6, it could be seen that the activity recovery of lysozyme reached the maximum 58.0 and 71.1% when the concentration ratio of Particle A to protein was 1 : 1. Compared with only 34.8 and 57.2% by simple dilution refolding, 23.2 and 13.9% increase were obtained, respectively, which showed that colloidal particles could facilitate the protein refolding evidently at higher protein concentration. Similarly, Figure 7 indicated that the concentration ratio of

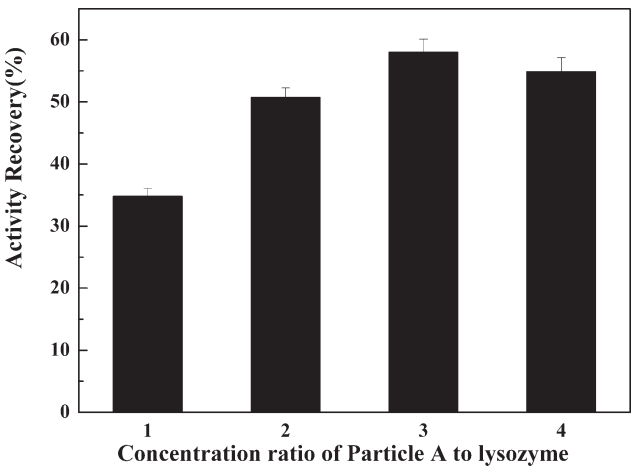

Figure 5 Effect of the concentration ratio of Particle A to lysozyme on activity recovery of lysozyme (the final protein concentration was 500 μg/mL). 1: Refolding by simple dilution; 2: the concentration ratio was 2 : 3; 3: the concentration ratio was 1 : 1; 4: the concentration ratio was 3 : 2.

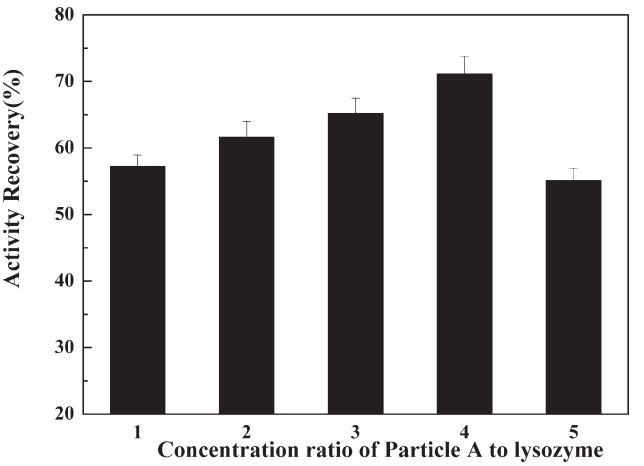

Figure 6 Effect of the concentration ratio of Particle A to lysozyme on activity recovery of lysozyme (the final protein concentration was 250 μg/mL). 1: Refolding by simple dilution; 2: the concentration ratio was 2 : 5; 3: the concentration ratio was 4 : 5; 4: the concentration ratio was 1 : 1; 5: the concentration ratio was 1.7 : 1.

Particle B to protein controlled at 0.5 was optimal for the refolding of lysozyme. In this test, the activity recovery could reach to 71.5 and 88.9%, so 36.7 and 31.7% increase were obtained compared with refolding by simple dilution. All results above demonstrated that the activity recovery first increased with the concentration of the particles and achieved the maximum at an optimal concentration; then a further increase of particles concentration led to a decreasing activity recovery on the contrary. During the refolding process, it is well known that the hydrophobic effects between the molecules of protein must be avoided to suppress the aggregation of the denatured proteins and its folding intermediates. Thus, with the presence of appropriate concentration

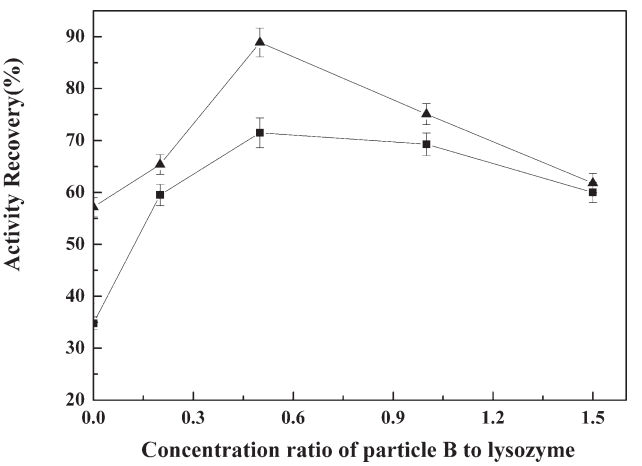

Figure 7 Effect of the concentration ratio of Particle B to lysozyme on activity recovery of lysozyme: ■, the final protein concentration was 500 μg/mL; ▲, the final protein concentration was 250 μg/mL.

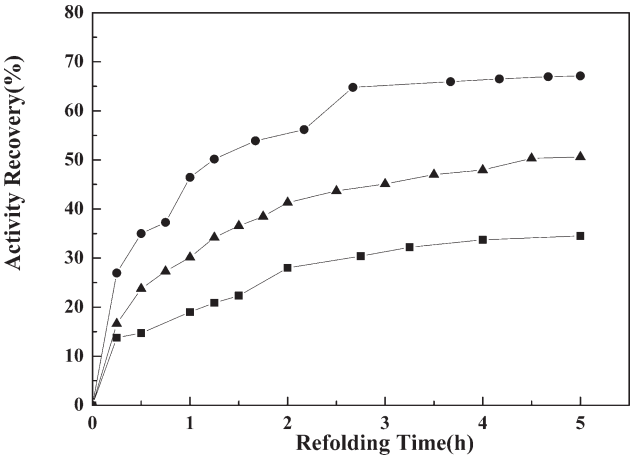

Figure 8 Time course of the lysozyme refolding with or without hairy particles (the final protein concentration was 500 μg/mL, the concentration ratios of Particle A and Particle B to protein were 1 and 0.5): ■, refolding by simple dilution; ▲, refolding with Particle A; ●, refolding with Particle B.

PNIPAM brushes, the hydrophobic groups of the polymer chain could interact with the protein molecules and consequently would suppress the aggregation of protein[20] and enhance the activity recovery. However, when the PNIPAM brushes concentration was excessive, a stronger hydrophobic effect and the size exclusion effect would occur and some lysozyme molecules would be adsorbed on PNIPAM brushes or locked in a narrow space, which resulted in the decrease of activity recovery. It could also be used to explain why Particle B with a larger thickness of PNIPAM brush layer had a more evident effect of assisting lysozyme refolding at a relatively lower optimal concentration.

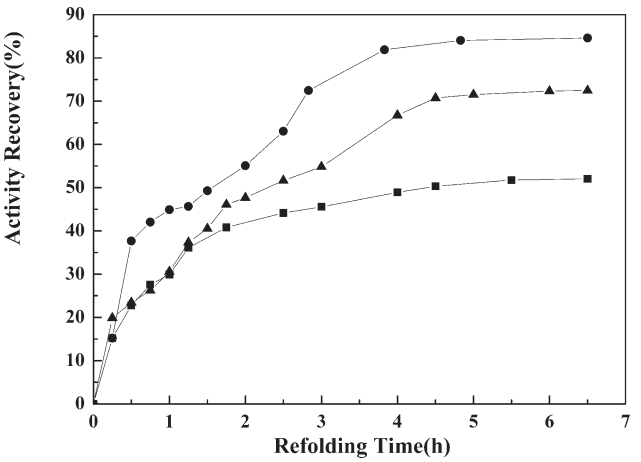

Figure 9 Time course of the lysozyme refolding with or without hairy particles (the final protein concentration was 250 μg/mL, the concentration ratios of Particle A and Particle B to protein were 1 and 0.5): ■, refolding by simple dilution; ▲, refolding with Particle A; ●, refolding with Particle B.

Time course of the lysozyme refolding assisted by hairy particles

Figures 8 and 9 showed the refolding process of lysozyme in the presence or absence of Particle A and Particle B, where the initial denatured lysozyme concentration was 10 mg/mL and dilution factor was 20 and 40, respectively. In the processes above, the concentration ratios of Particle A and Particle B to protein were 1 and 0.5, respectively.

From the results above, it could be seen that the process of lysozyme refolding by simple dilution could reach equilibrium within 4.5 h. As to the refolding in the presence of the Particle A and Particle B, the time to achieve equilibrium was extended to 5 to 6 h, which was a bit longer than the dilution refolding because of the hydrophobic interactions between the proteins and PNIPAM brushes. However, with the assisting of particles in the lysozyme refolding, the activity recovery could be greatly increased, that is, 16.1% (Particle A, dilution factor 20); 20.4% (Particle A, dilution factor 40); 32.2% (Particle B, dilution factor 20); and 33.0% (Particle B, dilution factor 40) activity increase could be obtained with the presence of PNIPAM brushes.

Influence of different dilution factors on lysozyme refolding

The influence of different dilution factors on lysozyme refolding with or without hairy particles was studied too. The results are shown in Figure 10, where the initial denatured protein concentration was 10 mg/mL, and the concentration ratios of

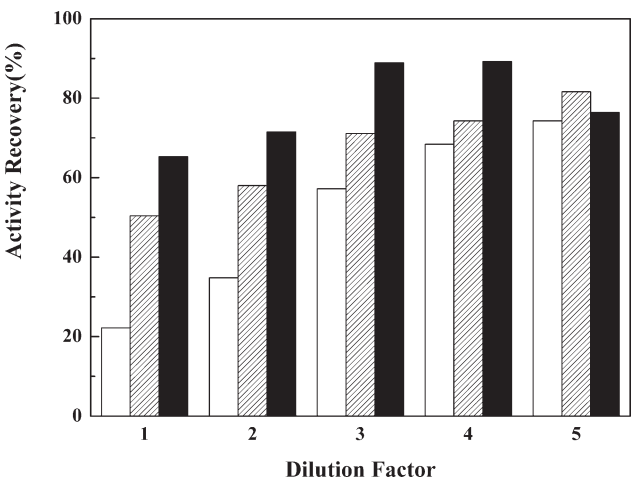

Figure 10 Influence of different dilution factors on lysozyme refolding process with or without particles (the initial denatured protein concentration was 10 mg/mL, concentration ratio of Particle A and Particle B were 1 and 0.5). 1: Dilution factor was 10; 2: dilution factor was 20; 3: dilution factor was 40; 4: dilution factor was 50; 5: dilution factor was 100. □, refolding by simple dilution; ▨, refolding with Particle A; ■, refolding with Particle B.

Particle A and Particle B to lysozyme were 1 and 0.5, respectively. Compared with simple dilution refolding, the effect of particles on the refolding of lysozyme was more remarkable at lower dilution factors. In this experiment, both particles could enhance the activity recovery of lysozyme compared with simple dilution, and Particle B worked more efficiently than Particle A. For example, when the dilution factor was 20, the activity recovery could achieve 58 and 71.5% in the presence of Particle A and Particle B. Compared with only 34.8% by simple dilution, 23.2 and 36.7% increment were obtained. Whereas when the dilution factor was 50, only an increase of only 5.9 and 20.8% was observed, respectively. It was proved that the adding of hairy particles could assist the lysozyme refolding efficiently at high initial protein concentration. This is vital because proteins are prone to aggregate at high concentration in the protein refolding process and lower protein concentration usually leads to larger reactor volume, more reagents, and higher capital cost.

The comparison of hairy uniform PS particles with PNIPAM brushes and PNIPAM particles in assisting lysozyme refolding

Compared with the activity recovery of lysozyme refolding assisted by crosslinking PNIPAM particles as reported,[22] the effect of hairy particles on lysozyme refolding, as shown in Figure 10, was even more evident. For example, when the initial lysozyme concentration was 250 μg/mL, the activity recovery could achieve 88.9% assisted by hairy particles (Particle B) compared with 74.4% by PNIPAM particles. Furthermore when the initial lysozyme concentration was increased to 500 μg/mL, the activity recovery could achieve 71.5% assisted by hairy particles (Particle B), 21.5% higher than that by PNIPAM particles.

Egg white lysozyme has 129 amino acid residues and four pairs of disulfide bonds, which form four rings with different sizes. The three-dimensional structure indicated that the amino acid residues embedded at the interior of lysozyme were mostly nonpolar, and the hydrophobic interaction played an important role in the conformation. At the initial stage of the refolding process, denatured lysozyme is rapidly transformed into a molten-globule with some disulfide bonds and a higher surface hydrophobicity, which will easily lead aggregation. With the addition of additive with abundant hydrophobic pendants, the hydrophobic interactions between protein molecules will be blocked and the aggregation will be inhibited. For the hairy particles, the hydrophobic isopropyl groups of the particles were located at PNIPAM chains. Therefore, the isopropyl groups were loose and could interact with protein molecules randomly to suppress the aggregation of the denatured protein and its folding intermediates. On the contrary, the isopropyl groups of PNIPAM particles were embedded in the network structure, so the hydrophobic interaction between isopropyl groups and protein molecules was limited to some extent.

After lysozyme refolding, by heating up the solution until 37°C for 15 min, the PNIPAM brushes collapsed completely. Then a majority of hairy particles could be removed by centrifugal separation at 10,000 rpm for about 10 min and recycled.

CONCLUSIONS

Two kinds of thermosensitive hairy particles (Particle A and Particle B) were synthesized; the morphologies, surface characteristics, and LCST of the particles were studied. The application of hairy particles in the lysozyme refolding showed that the particles were effective in assisting protein refolding especially at high initial protein concentration, and Particle B, which has thicker PNIPAM brushes layer, was more efficient than Particle A. Because of the hydrophobic interactions between the protein and PNIPAM brushes, the time needed for the refolding process with hairy particles was a bit longer than the simple dilution refolding. The study of refolding mechanism indicated that the plentiful hydrophobic pendants–isopropyl groups of the PNIPAM chains played an important role in the protein refolding. These hydrophobic groups could interact with protein molecules and then could prevent the intermolecular hydrophobic interactions and suppress the aggregation of the denatured protein and its folding intermediates. After refolding, the particles could be removed easily through heating above its LCST and centrifugal separation. Refolding with hairy particles is a novel and promising protein renaturation method with the advantages of high efficiency and easy handling.

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