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A Temperature-sensitive Hydrogel Refolding System: Preparation of Poly(*N*-isopropyl acrylamide) and Its Application in Lysozyme Refolding*

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Abstract Temperature-sensitive hydrogel—poly(*N*-isopropyl acrylamide) (PNIPA) was prepared and applied to protein refolding. PNIPA gel disks and gel particles were synthesized by the solution polymerization and inverse suspension polymerization respectively. The swelling kinetics of the gels was also studied. With these prepared PNIPA gels, the model protein lysozyme was renatured. Within 24 h, PNIPA gel disks improved the yield of lysozyme activity by 49.3% from 3375.2 $U \cdot mg^{-1}$ to 5038.8 $U \cdot mg^{-1}$. With the addition of faster response PNIPA gel beads, the total lysozyme activity recovery was about 68.98% in 3h, as compared with 42.03% by simple batch dilution. The novel refolding system with PNIPA enables efficient refolding especially at high protein concentrations. Discussion about the mechanism revealed that when PNIPA gels were added into the refolding buffer, the hydrophobic interactions between denatured proteins and polymer gels could prevent the aggregation of refolding intermediates, thus enhanced the protein renaturation.

Keywords poly(*N*-isopropyl acrylamide) hydrogel, free radical polymerization, protein refolding, lysozyme

1 INTRODUCTION

It is known that heterologous proteins produced by recombinant DNA technology are often present in the form of inactive and insoluble aggregates called inclusion bodies[1]. These insoluble aggregates are the misfolded molecules. In order to obtain protein molecules with biological activity, these insoluble proteins must be dissolved by strong denaturants such as guanidine hydrochloride or urea under reducing condition[2]. With gradually decreasing concentrations of denaturants, the dissolved protein molecules fold spontaneously into its native conformation. Usually the refolding of the dissolved protein into its native conformation can be considered as a unimolecular reaction, whereas the intermolecular aggregation of dissolved protein molecules into insoluble aggregates proceeds as a multi-molecular reaction. At high protein concentrations, the formation of irreversible aggregates will predominate over the refolding of protein and lead to low renaturation yields. Therefore, in order to ensure high refolding efficiency, it is generally necessary to conduct the protein renaturation at concentrations as low as 10^{-6} $mol \cdot L^{-1}$[3].

In recent years, many attempts have been reported to improve the efficiency of refolding by minimizing the formation of aggregation at high protein concentrations. These approaches involve refolding in reversed micelles utilizing solid-liquid extraction[4], refolding with aqueous two-phase systems[5], and refolding with high performance liquid chromatography [size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEC) and affinity chromatography (AFC) *etc.*][6–9]. On the other hand, some additives such as L-arginine[10] and poly(ethylene glycol)[11] which can reduce the possibility of undesired protein-protein interactions are also employed. Although the use of additives can greatly improve the renaturation efficiency of proteins, the need to remove these additives before final product finishing presents another obstacle for the scale up of these processes. The membrane processes such as ultrafiltration are used to separate effectively these relatively low molecular weight compounds from proteins. Lin *et al.*[12] applied the non ionic, temperature-sensitive linear polymer-poly(*N*-isopropyl acrylamide)(PNIPA) in aqueous solution to the refolding of β-lactamase, thus improved the activity by 41%. The mechanism for this water-soluble polymer enhancing protein renaturation might be similar to that of PEG, which indicated that the non-ionic and temperature-sensitive linear polymer system may also be a practical method for protein refolding. Enlightened by them and from the point of easy to handling and recycling, *N*, *N'* -methylene-bisacrylamide (Bis) will be introduced as crosslinker to synthesize the solid-state PNIPA hydrogel and to form a new refolding method by PNIPA hydrogel in this work. On the other hand, as a kind of intelligent polymer which volume in solution is in response to temperature, PNIPA hydrogel has been broadly applied in the field of enzyme immobilization, d rug delivery, superabsorbent *etc.* Here the potential application of PINPA gel particles in the protein refolding will be explored.

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2 MATERIALS AND METHODS

2.1 Materials

N-isopropyl acrylamide(NIPA) was purchased from ACROS, *N*, *N'*-methylene-bisacrylamide (Bis) was from Fluka, Dithiothreitol(DTT), reduced and oxidized glutathione(GSH and GSSG), *N*, *N*, *N*, *N*-tetramethylethylenediamine (TEMED), lysozyme and *Micrococcus Lysodeikticus* were purchased from Sigma Co.. All other chemicals were of analytical grade.

2.2 Preparation of PNIPA hydrogel

Different ratio of NIPA (monomer) and Bis (crosslinker) based on total monomer contents (W_T, the mass percent of monomer plus crosslinker in the reaction solution) and crosslinking density (W_C, the crosslinker mass percent relative to the total monomer plus crosslinker) was dissolved in deionized water, then 1.7% (by mass) oxidant ammonium persulfate and 1.0% (by mass) activator TEMD with respect to total monomer were added. While aquoues solution polymerization carried out, the mixture was immediately injected into the space between two glass plates. Polymerization was carried out at room temperature for 24 h[13]. Then the gel membrane was cut into disks. As to inverse suspension polymerization[14], the reaction was performed in a 500 ml cylindrical round-bottom glass flask fitted with mechanical stirrer, nitrogen inlet and an oil-water separator. A thermostatic water bath was used for isothermal polymerization with *n*-hexane as continuous phase and Span 20 as the dispersant. The polymerization was carried out with nitrogen purge for 3h. Water in system was thus separated through a water-oil separator.

The gels obtained were immersed in deionized water and ethanol for about a week and the solution was refreshed every day to remove the residual monomer, then dried in a vacuum oven for two days at 60℃.

2.3 Measurement of swelling ratio(S_R)

The dried disk gels (dry mass W_d) were immersed in an excess amount of deionized water at different temperatures until swelling equilibrium was attained. The mass of wet sample (W_w) was determined after the surface water was removed by blotting with filter paper. The swelling ratio was then calculated, which is defined as

$$S_R = \frac{W_w - W_d}{W_d} \quad (1)$$

As to the gel particles, the dried particles were grinded into powder and packed into a measuring cylinder filled with deionized water. The volumes of the wet gels were recorded. The volume of dried particles could be calculated with W_d and the density 1.25 g·ml^{-1}, and the S_R was then obtained by dividing the swollen gel volume by the dried gel volume[15].

2.4 Dynamic swelling and deswelling

The dried gels (or the fully swollen gels at 4℃) were immersed in deionized water at different temperatures. The S_R was obtained by weighing the dried gels and samples at a definite time interval. The S_R was reported as a function of time.

2.5 Denaturation and refolding of lysozyme[16]

A definite amount of lysozyme was dissolved in the denaturation solution (0.1 mol·L^{-1} Tris-HCl, pH 8.5, 8 mol·L^{-1} urea and 30 mmol·L^{-1} DTT) and shaken in the 37℃ incubator, 120 r·min^{-1} for 90 min. The denatured lysozyme was slowly diluted into the refolding buffer [0.1 mol·L^{-1} Tris-HCl, pH 8.5, 1 mmol·L^{-1} EDTA, 0.15 mol·L^{-1} NaCl, 2 mol·L^{-1} urea, 3 mmol·L^{-1} GSH and 0.375 mmol·L^{-1} GSSG (GSH:GSSG = 8:1)]. For the study of refolding with PNIPA, the denatured lysozyme was diluted into the refolding buffer containing a certain amount of PNIPA gels. The mixture was vortexed and shaken in the incubator.

3 RESULTS AND DISCUSSION

3.1 Temperature sensitivity of PNIPA gel disks

When the gel composition was W_T = 5% and W_C = 2%, the gels looked transparent. With the increase of W_T and W_C, the gel disks prepared became more and more opaque due to the more compact structure and the heterogeneity of the gel network. The temperature sensitivity of the gel was shown in Fig. 1, which indicated their equilibria swelling ratio as a function of temperature. A sharp decline in the water absorption capacity of the gels appeared between 30℃ and 36℃, with half collapse occurring at 33℃. Then the low critical soluble temperature (LCST) of PNIPA gels was ascertained to be 33℃, which is in accord with the results reported by Lin *et al.*[17] Fig. 1 also showed that the gel composition had great influences on the swelling ratio below the collapse temperature. It was notable that only the gels with low monomer and crosslinker concentrations exhibited high swelling ratio. Above LCST, the water contents of all gels were quite low.

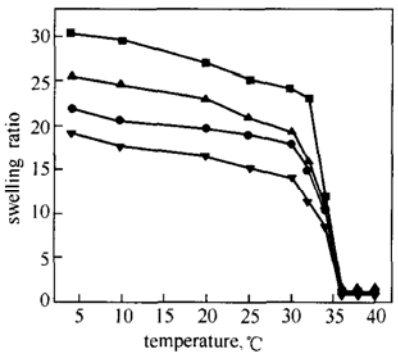

Figure 1 Swelling ratio of PNIPA gel at different temperatures
(W_T, W_C), %: ■ 5, 2; ▲ 10, 2; ● 5, 5; ▼ 10, 5

3.2 Swelling and deswelling kinetics of the PNIPA gel disks

Figures 2 and 3 showed that the time needed for the swelling and deswelling equilibrium of gel disks was about 24h. The lower the temperature, the longer the time required for the gel to reach equilibrium. This could be explained by the acceleration of the thermal motion of molecules.

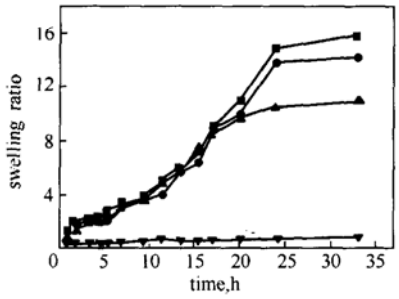

Figure 2 Swelling rate of PNIPA gel at different temperatures (W_T = 10%, W_C = 5%)
T, ℃: ■ 25; ● 30; ▲ 34; ▼ 36

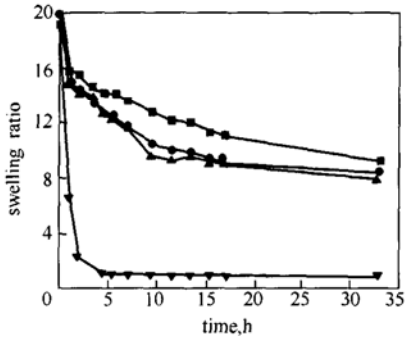

Figure 3 Deswelling rate of PNIPA gel at different temperatures (W_T = 10%, W_C = 5%)
T, ℃: ■ 25; ● 30; ▲ 34; ▼ 36

3.3 Refolding of lysozyme by PNIPA gel disks

Figure 4 indicated the influence of the crosslinking contents and the monomer concentrations on the refolding of lysozyme. It could be seen that the addition of the PNIPA did enhance the lysozyme renaturation. With the increase of crosslinking contents, the effects became more obvious. It is well known that the hydrophobic effects between the molecules of protein during refolding must be controlled to suppress the aggregation of the denatured protein and its intermediates. With the presence of PNIPA, the hydrophobic groups of the polymer chain can interact with the protein, thus would suppress the hydrophobic effects of the protein which could cause the protein aggregation[12]. The higher the crosslinking contents, the more hydrophobic the gel became, which was more favourable to the lysozyme refolding. Similarly, as the monomer concentrations increased, the hydrophobic effects between the polymer chains and the protein became strong enough to prevent the congregation of the protein, as shown in Fig. 4. The highest lysozyme activity (with PNIPA gel: W_T = 10%, W_C = 10%) was 5038.8 U·mg^{-1}. While using simple dilution in the lysozyme refolding, only 3375.2 U·mg^{-1} can be got. In a word, PNIPA gel disks improved the yield of lysozyme activity by 49.3%, thus the positive effect of PNIPA in the protein refolding was primarily proved by above experiments.

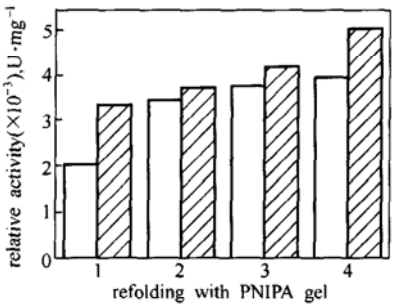

Figure 4 Effect of PNIPA gel disks on the refolding of lysozyme
(protein concentration 10 mg·ml^{-1}, dilution factor 20)
W_T, %: □ 5; ▨ 10

But due to the slow swelling rate of the gel disks, the refolding process was gel-swelling limited which had to take 24h, hence the fast responsive PNIPA gel particles were further synthesized by the reversed phase suspension as described.

3.4 Swelling and deswelling kinetics of PNIPA gel particles

The temperature sensitivity of PNIPA particles is quite similar to that of the PNIPA gel disks. Results of the swelling and deswelling rate of PNIPA gel particles showed that they were faster responsive compared with gel disks, and the equilibrium could be reached within only about 3 min as shown in Fig. 5 (4℃) and Fig. 6 (fully swollen under 4℃ and then deswelled at 25℃).

The swelling ratio of the particles in water and in the refolding buffer was also investigated. Fig. 7 showed the capacity of gel absorbing water was depressed in the refolding buffer since the hydrogen bond was partly weakened by the component of the buffer such as NaCl, which is in accord with reported work[18].

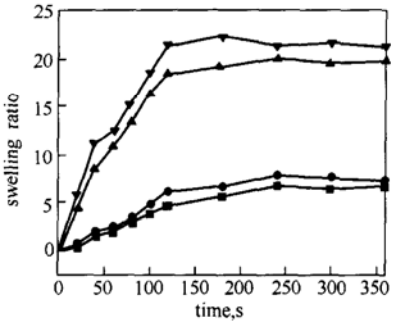

Figure 5 The swelling rate of PNIPA gel particles
(W_T, W_C), %: ▼ 5, 5; ▲ 5, 10; ● 14, 5; ■ 14, 10

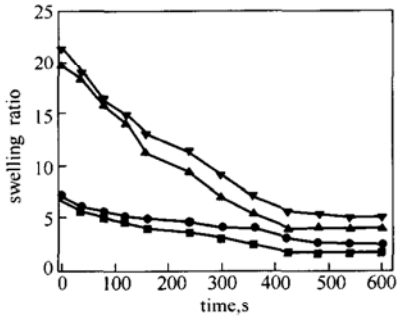

Figure 6 The deswelling rate of PNIPA gel particles
(W_T, W_C), %: ▼ 5, 5; ▲ 5, 10; ● 14, 5; ■ 14, 10

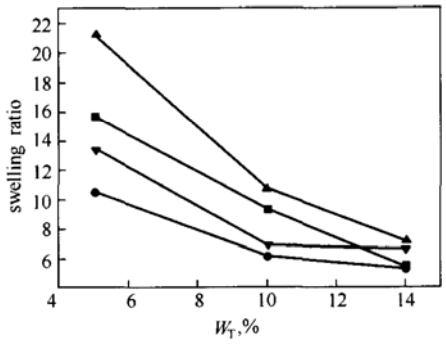

Figure 7 The swelling ratio of gel particles in water and refolding buffer
▲ deionized water, W_C = 5%; ▼ deionized water, W_C = 10%; ■ refolding buffer; W_C = 5%; ● refolding buffer; W_C = 10%

3.5 Refolding of lysozyme with PNIPA gel particles

The effects of the rotating speed and temperature on the refolding of lysozyme were taken into account first. Based upon the above results of refolding with PNIPA gel disks, the gel with higher W_T and W_C component (W_T = 14%, W_C = 10%) was used in the refolding of lysozyme at 25℃. The experimental results were shown in Fig. 8. It can be seen that 120 r·min^{-1} is optimal for both systems. So the following refolding experiment was carried out at 120 r·min^{-1}.

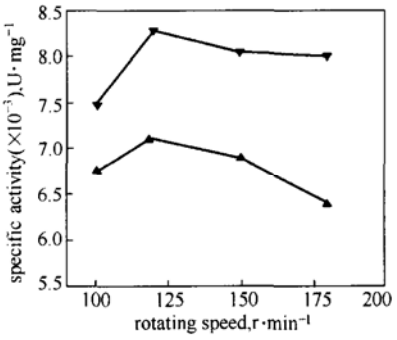

Figure 8 Effect of rotating speed on lysozyme refolding
(protein concentration 10 mg·ml^{-1}, dilution factor 40)
▲ refolding by dilution; ▼ refolding with PNIPA gel

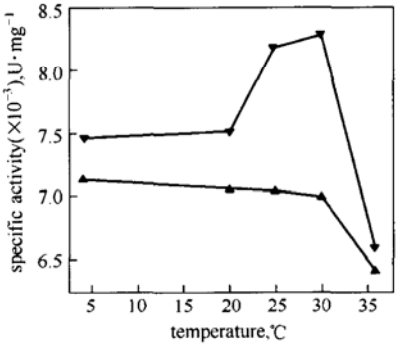

Figure 9 Effect of temperature on lysozyme refolding
(protein concentration 10 mg·ml^{-1}, dilution factor 40)
▲ refolding by dilution; ▼ refolding with PNIPA gel

To explore the effect of temperature on protein refolding in the presence of PNIPA, the renaturation of denatured lysozyme was conducted at temperature from 4℃ to 36℃ and results were shown in Fig. 9. As expected, in the simple dilution refolding, the lower the incubation temperature, the higher the renaturation yield. While in the presence of PNIPA, when the temperature was below the LCST of the polymer, the renaturation efficiency increased with temperature. For example, when adding 100 mg·ml^{-1} PNIPA the relative activity obtained was increased by 11.1% from 7456.7 U·mg^{-1} at 4℃ to 8285.2 U·mg^{-1} at 30℃. The enhancement of protein refolding was a result of the hydrophobic interactions between folding intermediates and polymers, which greatly depressed the inclination of protein aggregation. But when at 36℃, which was above the LCST of PNIPA, the refolding efficiency was declined due to the hydrophobic interaction between PNIPA polymers. So in the following experiment 30℃ was chosen.

Figure 10 showed the refolding of lysozyme with PNIPA gel particles of different compositions. To improve further the activity recovery, the supernatant was collected after refolding. Then by heating the swelling gel particles till 37℃ for 10 min, the lysozyme entrapped in the gel network could be retrieved. The solution expelled from the gel also retained most of the enzyme activity. So the final refolding efficiency could be expressed by the total activity recovery (the total activity percent of lysozyme after refolding relative to the initial activity of the native lysozyme). The result of Fig. 10 is in consistent with that in Fig. 4, that is, the polymer with higher W_T and W_C is optimal for the refolding of protein. Since the solubility of NIPA and Bis in aqueous solution is not very high, the highest W_T and W_C available in the experiment is 14% and 10% respectively. With the addition of faster response PNIPA gel (W_T = 14% and W_C = 10%), the total lysozyme activity recovery was about 68.98% in 3h, as compared with 42.03% by simple batch dilution. Finally the refolding of lysozyme with different dilution factors was investigated, as represented in Fig. 11. Compared with refolding by dilution, the

effect of PNIPA was more notable especially at low dilution, which indicated that PNIPA could facilitate the protein refolding especially at high protein concentration.

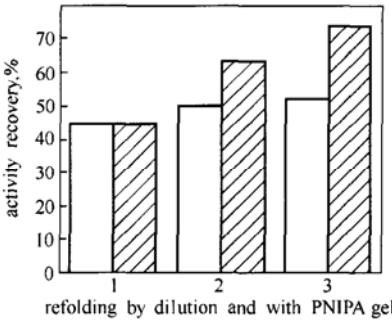

Figure 10 Refolding of lysozyme with PNIPA gel particles
(initial protein concentration 10 mg·ml^{-1}, PNIPA concentration 100 mg·ml^{-1})
W_T, %: □ 10; ▨ 14

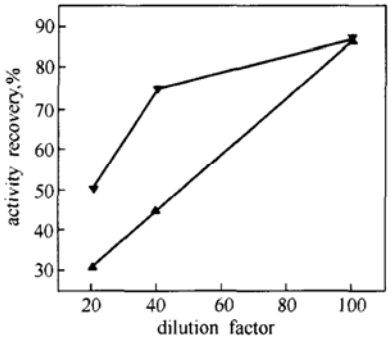

Figure 11 Effect of dilution factor on the refolding of lysozyme
(initial protein concentration 10 mg·ml^{-1}, PNIPA concentration 100 mg·ml^{-1})
▲ refolding by dilution; ▼ refolding with PNIPA gel

4 CONCLUSIONS

In order to obtain high protein refolding efficiency, the possibility in the refolding with high protein concentration was investigated using a temperature-sensitive polymer—poly (N-isopropyl acrylamide) hydrogel, which can significantly improve the refolding yield of denatured lysozyme *via* the hydrophobic interaction between the dissolved protein or the refolding intermediates and the polymer. By heating above its LCST the polymer could be easily recycled. The microcosmic structure of PNIPA hydrogel and relationship between gel structure and protein during refolding will be further discussed in another work and the refolding mechanism about the enhancement of PNIPA to protein renaturation will be described in detail.

NOMENCLATURE

LCST	low critical soluble temperature, ℃
S_R	swelling ratio of PNIPA hydrogel
W_C	the crosslinker mass percent relative to the total monomer plus crosslinker, %
W_d	mass of PNIPA hydrogel at dry state, mg
W_T	the total mass percent of monomer plus crosslinker in the reaction solution, %
W_w	mass of PNIPA hydrogel after swelling, mg

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