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Refolding of recombinant human interferon gamma inclusion bodies *in vitro* assisted by colloidal thermo-sensitive poly(N-isopropylacrylamide) brushes grafted onto the surface of uniform polystyrene cores

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ABSTRACT

Recombinant human interferon gamma (rhIFN-γ) is a protein with great potential for clinical therapy, but rhIFN-γ expressed in *Escherichia coli* is usually in the form of insoluble inclusion bodies which should be refolded *in vitro*. A novel type of hairy particles (PNIPAM-grafted-PS) consisted of submicron polystyrene cores and brushes of thermo-sensitive poly(N-isopropylacrylamide) grafted onto the cores was prepared and then applied to assist the refolding of rhIFN-γ *in vitro*. Two kinds of PNIPAM-grafted-PS particles with different thickness of brush layer (55 nm and 110 nm) were synthesized, which were spherical shape with good dispersion properties and the LCST was about 33 °C. The effect of thickness of brush layer, particle concentration and temperature on the refolding process was investigated, it was shown that particles with larger thickness of brush layer were more effective and the final rhIFN-γ activity could be up to more than 21 times of that in dilution refolding when initial rhIFN-γ concentration was 50 μg/mL. The optimal refolding condition was the concentration ratio of particle to rhIFN-γ 1:1 and refolding temperature of 15 °C. All results above demonstrated that PNIPAM-grafted-PS particles could assist rhIFN-γ refolding which presented an alternative way to facilitate recombinant protein refolding *in vitro*.

1. Introduction

Human interferons (hIFNs) are a family of secretory proteins mainly consisting of hIFN-α, hIFN-β and hIFN-γ *etc.* [1]. As a cytokine with a myriad of effects in both host defense and immune regulation such as antiviral, antimicrobial, and antitumor activity, hIFN-γ is more potent than the other two and becomes a protein drug which plays an important role in clinical medicine [2]. Natural hIFN-γ is secreted by lymphocytes stimulated by mitogen but the sources are quite limited [3]. Production of recombinant human interferon gamma (rhIFN-γ) was achieved about three decades ago and researchers have made great efforts to improve the expression level of rhIFN-γ in *E. coli* [1,3–5]. Although rhIFN-γ is not glycosylated at two sites as native hIFN-γ, it is still physiologically active [5]. Thus rhIFN-γ is a good solution to meet the requirements of clinical therapy in large amounts.

Nevertheless, one main disadvantage of producing rhIFN-γ in *E. coli* host is that the target protein will overexpress and accumulate to form inactive and insoluble aggregates called inclusion bodies (IBs) in the process [6], which should be refolded to its native conformation. Many methods have been studied to assist rhIFN-γ refolding *in vitro*, for example using size exclusion chromatography [7], expanded bed adsorption [8], an immobilized sht GroEL 191-345 column [9,10] and reversed phase column [11] *etc.* But the efficient methods to refold rhIFN-γ at high concentration with high yield are still being pursued.

For protein refolding *in vitro*, the use of some additives to inhibit the intermolecular interactions which lead to aggregation is a convenient strategy to enhance protein refolding [6,12]. In assisted assembling theory, the hydrophobic forces offered are the key factors to suppress misfolding and aggregation by binding additives to polypeptide chains that are not fully folded, hence polymers with rich hydrophobic groups are potential efficient refolding aids [13]. As a novel polymer additive, poly(N-isopropylacrylamide) (PNIPAM) has attracted increasing attentions [14–20], since it contains a hydrophobic vinyl backbone and pendant isopropyl side groups which can reduce the possibility of undesired protein-protein interactions. PNIPAM is one of the typical thermo-sensitive polymers and possesses a lower critical solution temperature (LCST) around 30–36 °C [21]. As a nonionic polymer, PNIPAM exhibits hydrophilic below the LCST and hydrophobic characteristics above the LCST

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[20]. The phase transition properties of PNIPAM make the polymer easily to be separated from solution at the temperature above LCST.

PNIPAM polymers are presented in three common physical forms [13], *i.e.*, linear free chains, covalently cross-linked reversible gels and chains adsorbed or surface-grafted form. Traditional linear PNIPAM chains could enhance the refolding process availably, but the linear PNIPAM hydrogel with low molecular weight might not be quickly removed by centrifugation after protein refolding [14]. On the contrary, crosslinking PNIPAM particles could be easily separated, but they were not as effective as linear PNIPAM chains when used to assist protein refolding because the protein molecules penetrated into the polymer network structure were difficult to be recovered [19,20]. To overcome the above problems, hairy particles (PNIPAM-grafted-PS) which grafted linear PNIPAM brushes onto the support material of polystyrene (PS) core were introduced with both the advantages of linear PNIPAM chains and crosslinking PNIPAM polymers. Ge et al. [22] in our lab had attempted to apply PNIPAM-grafted-PS microspheres to the renaturation of denatured lysozyme *in vitro*, where it was observed that PNIPAM-grafted-PS microspheres were quite effective in facilitating protein folding pathway. For PNIPAM-grafted-PS particles, the isopropyl groups on PNIPAM chains were loose and could interact with protein molecules randomly as the linear PNIPAM chains, while they also could be separated easily after refolding as crosslinking PNIPAM polymer because of their PS cores.

In this paper, two kinds of PNIPAM-grafted-PS particles with same submicron PS core but different thickness of brush layer will be synthesized and characterized, then applied to assist the refolding of rhIFN-γ inclusion bodies which were produced by cultivation of the genetic engineering *E. coli*. The effect of thickness of brush layer, particle concentration and temperature on the refolding will be investigated and the structure of rhIFN-γ after refolding will also be analyzed. A novel protein refolding method with high refolding efficiency, minimum loss of protein aggregates and ease of polymers recycling will be proposed for application in the refolding of rhIFN-γ inclusion bodies *in vitro*.

2. Materials and methods

2.1. Materials

Genetically engineered *E. coli* (pBV220/IFN-γDH5α) was stored by Institute of Biological Engineering, Zhejiang University. Human IFN-gamma Platinum ELISA kit was purchased from Bender Medsystems (Austria). Guanidinium hydrochloride (GdmHCl), ethylene diamine tetraacetic acid (EDTA), Triton X-100, tryptone, yeast extract, ampicillin, and Tris (hydroxymethyl aminomethane) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). All other chemicals were of analytical reagent grade and obtained commercially.

2.2. Preparation and characterization of PNIPAM-grafted-PS particles

Two kinds of particles with same submicron PS cores but different brush thickness were obtained *via* three key steps [22,23]. PS cores were first synthesized using emulsifier-free emulsion polymerization, and then the photoinitiator (isopropyl benzophenone methylacrylate) was attached onto the surface of PS cores as a paper-thin shell by copolymerization. Finally, PNIPAM brushes were grafted on the surface of the PS particles by the "grafting from" approach with sequential addition of N-isopropylacrylamide (NIPAM) using photoinitiation. The amount of PNIPAM grafting onto PS cores (the mass ratio of PNIPAM to PS cores) was 50.6% (Particle A) and 36.8% (Particle B) respectively. A BioScope SZ atomic force microscope (AFM) (Veeco, USA) was used to analyze the surface morphology and size characteristics of the microspheres. The diameter of PNIPAM-grafted-PS particles at different temperature and the diameter of PS cores were determined by dynamic light scattering (DLS) using Malvern Zetasizer 3000 (Malvern, UK), gaining the layer thickness of the PNIPAM brushes based on the difference between diameter of the PNIPAM-grafted-PS particles and PS cores. The results of brush shell thickness at different temperature were calculated to determine the LCST of the obtained PNIPAM brushes.

2.3. Fermentation and purification of rhIFN-γ inclusion bodies

A single bacterial colony of genetically engineered *E. coli* pBV220/IFN-γDH5α which encoded hIFN-γ-cDNA and inserted thermal sensitive P_RP_L promoter was inoculated into 10 mL of LB medium containing 100 μg/mL of ampicillin and shaken at 200 rpm and 30 °C for 12 h. Then 1.5 mL of seed culture was inoculated into 150 mL of fermentation culture medium [9] and incubated at 30 °C with a rotating rate of 220 rpm until the value of OD_{600} was about 1.0. Gene expression was immediately induced by heating to 42 °C and the culture was allowed to continue for further 6 h. At the end of fermentation, cells were collected by centrifugation (6000 rpm at 4 °C for 10 min) and washed with buffer A (10 mM EDTA, 100 mM NaCl, 100 mM Tris–HCl, pH 8.0). After resuspension in the same buffer (10 mL buffer per g cells), cells were disrupted by sonication in an ice bath (3 s on, 5 s off, 200 rounds under 500 W of power) and crude inclusion bodies were collected by centrifugation (8000 rpm at 4 °C for 15 min). The crude inclusion bodies were then suspended in buffer B (0.5% Triton X-100, 2 M urea, 1 mM EDTA, 0.15 M NaCl, 100 mM Tris-HCl, pH 8.0). After stirring for 2 h, centrifugation was carried out to obtain purified rhIFN-γ inclusion bodies.

2.4. Refolding of the rhIFN-γ inclusion bodies

Firstly, 1 g of purified inclusion bodies were solubilized in 10 mL of denaturation buffer (6 M GdmCl, 1 mM EDTA, 100 mM Tris-HCl, pH 8.0) and stirred for 5 h, then centrifuged (10,000 rpm at 4 °C for 30 min) to remove insoluble materials. Then denatured rhIFN-γ with a concentration of 10 mg/mL was diluted by 200-fold into refolding buffer (1 mM EDTA, 100 mM Tris-HCl, pH 8.0, containing a certain amount of PNIPAM-grafted-PS microspheres), and the solution was shaken in the incubator at 100 rpm for 48 h. Finally, the refolded protein solution was centrifuged (12,000 rpm at 4 °C for 20 min) to collect supernatant for the activity assay and fluorescence analysis.

2.5. Assay of refolded rhIFN-γ

Protein concentration was measured by Coomassie brilliant blue method. The activity of refolded rhIFN-γ was indirectly determined by Avidin Biotin System-ELISA (ABS-ELISA) with human IFN-gamma Platinum ELISA kit [4]. Intrinsic protein fluorescence spectra were recorded with spectrophotometer of F-4500 (Hitachi, Japan) by exciting at 295 nm, and the emission spectra within 200–500 nm were measured. Reducing SDS-PAGE was used for the analysis of inclusion bodies and denatured protein, and non-reducing SDS-PAGE and native PAGE with samples not treated by high temperature were used for the analysis of refolded protein.