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Sequential tentacle grafting and charge modification for enhancing charge density of mono-sized beads for facilitated protein refolding and purification from inclusion bodies

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ABSTRACT

We have previously found that addition of like-charged media in a refolding solution can greatly enhance the refolding of pure proteins by suppressing protein aggregation. Herein, negatively charged mono-sized microspheres with sulfonic groups were fabricated to explore the facilitating effect of like-charged media on the refolding of enhanced green fluorescent protein (EGFP) expressed as inclusion bodies (IBs). A sequential polymer-tentacle grafting and sulfonate modification strategy was developed to increase the charge density of mono-sized poly(glycidyl methacrylate) (pGMA) beads (2.4 μm). Namely, GMA was first grafted onto the beads by grafting polymerization to form poly(GMA) tentacles on the pGMA beads, and then the epoxy groups on the tentacles were converted into sulfonic groups by modification with sodium sulfite. By this fabrication strategy, the charge density of the beads reached 793 μmol/g, about 2.8 times higher than that modified without prior grafting of the pGMA beads (285 μmol/g). The negatively charged beads of different charge densities were used for facilitating the refolding of like-charged EGFP from IBs. The refolding yield as well as refolding rate increased with increasing charge density. The anti-aggregation effects of urea and like-charged microspheres were synergetic. In addition, partial purification of EGFP was achieved because the ion-exchange adsorption led to 52% removal of positively charged contaminant proteins in the refolded solution. Finally, reusability of the tentacle beads was demonstrated by repetitive EGFP refolding and recovery cycles.

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1. Introduction

Bacterial expression systems have been widely used for the production of recombinant proteins in recent years [1]. However, heterologous proteins expressed by the systems often form inactive aggregates known as inclusion bodies (IBs) [2]. Thus, IBs refolding operation for active protein recovery is a crucial step in the production of the recombinant proteins [3]. During a refolding process, hydrophobic interaction-induced aggregation between folding intermediates compete with the on-path way folding, leading to a low refolding yield [4]. Therefore, inhibition of the aggregation is the key strategy for enhancing protein refolding [5,6].

To inhibit protein aggregation, various folding aids have been developed [7–9]. However, most of the folding aids effective in suppressing protein aggregation also compose the intramolecular hydrophobic interactions, leading to a low refolding rate [10,11]. Protein refolding on chromatographic columns has also been widely investigated in recent years [12,13]. Using the on-column techniques, refolding and partial purification of the target protein can be achieved simultaneously [14–16]. However, some drawbacks of on-column refolding remain, such as the decrease of folding rate caused by the adsorption [17] and the dilution of the recovered product [18].

Recently, our group has found that addition of like-charged resin particles in a refolding solution can greatly enhance the refolding yield at high protein concentrations [18]. A series of porous agarose gels [19] and nonporous mono-sized microspheres [20,21] with different charge group densities, ligand chemistries and particle sizes

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were subsequently fabricated to study the effect of solid phase properties on the refolding of like-charged lysozyme. Moreover, soluble polyelectrolytes were used to build homogeneous refolding systems to explore the mechanism of protein refolding facilitated by like-charged polymers/particles [22]. It is considered that the electrostatic repulsion between like-charged polymers/particles and the protein (folding intermediates) induced an oriented alignment of the folding protein molecules, leading to the suppression of protein aggregation. However, the earlier studies mainly focused on the facilitating effect of like-charged media on the refolding of pure proteins, and the behavior of protein refolding from IBs that contain many contaminant proteins has not been studied. Therefore, we herein report the refolding of enhanced green fluorescent protein (EGFP) expressed in *Escherichia coli* as IBs. To enhance the refolding performance of the negatively charged protein (at pH 8.5), we developed a sequential polymer-tentacle grafting and sulfonate modification strategy to fabricate mono-sized microspheres of high charge densities. The effect of charge density on EGFP refolding from IBs, EGFP purification in the refolding process, and the reusability of the high-charged beads were investigated. This research is expected to deepen our understanding of the facilitating effect of like-charged media on protein refolding from a practical source.

2. Materials and methods

2.1. Materials

Glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EDMA), isopropyl β-D-thiogalactopyranoside (IPTG) and kanamycin were obtained from Sigma–Aldrich (St. Louis, MO, USA). Urea, tris(hydroxymethyl)aminomethane (Tris) and dithiothreitol (DTT) were from Dingguo Biotech (Beijing, China). Ethylenediaminetetraacetic acid disodium (EDTA), polyvinylpyrrolidone (PVP K-30) and azodiisobutyronitrile (AIBN) were purchased from Guangfu Fine Chemical Research Institute (Tianjin, China). Other chemicals [such as ceric ammonium nitrate (CAN)] were all commercially available reagents of analytical grade and used without further purification. All solutions were prepared using deionized water.

2.2. Fabrication of mono-sized microspheres

Mono-sized poly(glycidyl methacrylate) (pGMA) microspheres were prepared by dispersion polymerization following the method described earlier [23] with minor modifications in the concentrations of the monomer and stabilizer. Briefly, PVP K-30 dispersed in 90% ethanol solution was used as the continuous phase. Predetermined amounts of GMA and AIBN were dissolved in the solution under nitrogen atmosphere. The polymerization system was kept under stirring (200 rpm) and reflux condensation at 70 °C for 3 h. Then, the crosslinker (EDMA) was fed into the mixture, and the polymerization was kept going for another 21 h. After cooling, pGMA microspheres were collected by washing with ethanol and distilled water to remove any unreacted monomers or other organic matter.

2.3. Modification of pGMA microspheres

The reaction scheme for the modification of pGMA beads is illustrated in Fig. 1. The pGMA beads were directly modified by reaction with sodium sulfite to prepare pGMA-SO_3^- (Fig. 1a). In brief [24], 2 g of pGMA beads was added into 100 mL of Na_2SO_3 solution (100 g/L). Then, two droplets of isopropanol were added into the suspension. The system was kept in a shaking incubator at 170 rpm and 40 °C for 8 h. The modified product, pGMA-SO_3^- beads, was collected by centrifugation and washed with deionized water.

In another reaction route, the pGMA beads were sequentially grafted with poly(GMA) tentacles and modified with sulfonic groups (Fig. 1b). In the procedure, the epoxy groups on the pGMA bead surface were first hydrolyzed by immersing the beads in 0.5 mol/L sulfuric acid solution at 60 °C for 5 h. The beads with hydroxyl groups were then activated with 0.1 mol/L CAN in 1 mol/L HNO_3 solution at 60 °C for 4 h. In the third step, the solution for the activation in the previous step was displaced with GMA solution and the graft polymerization proceeded at 40 °C for 20 min to prepare pGMA beads with poly(GMA) tentacles. The polymerization reaction was terminated by rapid cooling the system to 4 °C followed by extensive washing with pure methanol and deionized water. Finally, the beads with poly(GMA) tentacles were modified with sulfonic groups to prepare pGMA-[GMA-SO_3^-]$_n$ beads with the reaction condition similar to that in the preparation of the pGMA-SO_3^- beads.

2.4. Characterization of microspheres

The morphology of microspheres was characterized by a S4800 scanning electron microscopy (SEM) (Hitachi, Tokyo, Japan). Particle size distribution was assayed by Mastersizer 2000U particle size analyzer (Malvern Instruments, Worcestershire, UK). The mean particle size was represented by volume–weighted average diameter. The structural characterization of the microspheres was performed by a Nexus Fourier transform infrared spectroscopy (FTIR) (Thermo Nicolet, Madison, WI, USA) [20]. The epoxy group content of the pGMA beads was determined following the method reported earlier [25]. The charge density of the beads was assayed by the acid–base titration [26].

2.5. Preparation of enhanced green fluorescent protein (EGFP) IBs

The *E. coli* strain harboring the pET28a-EGFP vector for EGFP expression was described previously [21]. Native EGFP and EGFP IBs were obtained following the same procedures described in the literature [27]. In brief, the recombinant cells were cultured and harvested by centrifugation. After cell disruption by sonication, the homogenate was separated by centrifugation, and the supernatant was used for the purification of native EGFP by immobilized metal (nickel) affinity chromatography (IMAC), while the precipitate was washed to purify the IBs [28]. The purified IBs pellet was stored at 4 °C for further use. The purified native EGFP was used as a standard of EGFP for the determination of refolding efficiency [27].

2.6. Denaturation and refolding of EGFP IBs

The EGFP IBs were solubilized by dissolving in the denaturation/reduction buffer (10 mol/L urea, 20 mmol/L DTT, 20 mmol/L Tris–HCl and 1 mmol/L EDTA, pH 8.5) to a final concentration of 20 mg/mL. The protein solution was incubated at 25 °C for 12 h. EGFP IBs were completely reduced and denatured under this condition.

The denatured EGFP IBs solution was diluted by refolding buffer (20 mmol/L DTT, 20 mmol/L Tris–HCl, 1 mmol/L EDTA, and predetermined concentrations of urea and microspheres, pH 8.5) to a final solution containing 0.2 mg/mL EGFP, 2 mol/L urea, 20 mmol/L DTT, 0–250 mg/mL beads (dry weight), 20 mmol/L Tris–HCl and 1 mmol/L EDTA (pH 8.5). The refolding was performed in 1.5 mL microcentrifuge tubes kept in a shaking incubator at 170 rpm and 25 °C for 12 h. The experiments were performed in triplicate and the average value was adopted.

Kinetics of the refolding were also measured 1.5 mL microcentrifuge tubes kept in the shaking incubator at 170 rpm and 25 °C.

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