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Metal chelate affinity precipitation: Purification of BSA using poly(*N*-vinylcaprolactam-co-methacrylic acid) copolymers

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ABSTRACT

This investigation involves the metal chelate affinity precipitation of bovine serum albumin (BSA) using a copper ion loaded thermo-sensitive copolymer. The copolymer of *N*-vinylcaprolactam with methacrylic acid PNVCL-co-MAA was synthesized by free radical polymerization in aqueous solution, and Cu(II) ions were attached to provide affinity properties for BSA. A maximum loading of 48.1 mg Cu^{2+} per gram of polymer was attained. The influence of pH, temperature, BSA and NaCl concentrations on BSA precipitation and of pH, ethylenediaminetetraacetic acid (EDTA) and NaCl concentrations on elution were systematically probed. The optimum conditions for BSA precipitation occurred when pH, temperature and BSA concentration were 6.0, 10 °C and 1.0 mg/ml, respectively and the most favorable elution conditions were at pH 4.0, with 0.2 M NaCl and 0.06 M EDTA. The maximum amounts of BSA precipitation and elution were 37.5 and 33.7 mg BSA/g polymer, respectively. It proved possible to perform multiple precipitation/elution cycles with a minimal loss of polymer efficacy. The results show that PNVCL-co-MAA is a suitable matrix for the purification of target proteins from unfractionated materials.

1. Introduction

The selective isolation and purification of proteins by affinity chromatography using highly specific ligands has proven to be highly successful, but also complicated in terms of scale-up, and sample-treatment, suffering from low throughput of the products at high cost [1].

Immobilized metal affinity chromatography (IMAC) uses chelating compounds bound to polymeric supports to immobilize metal ions, and the latter act as affinity ligands for various proteins [2,3]. The technique has many advantages over other systems in terms of ligand stability, binding capacity, protein recovery and matrix regeneration [4]. Metal chelate affinity precipitation is a non-chromatographic process which combines specific affinity with precipitation [5,6].

The application of affinity precipitation often employs "intelligent" or "smart" polymers [7]. These materials can undergo reversible transitions between water soluble and insoluble phases in response to changes in environmental conditions (temperature, pH, light or solvent). Thermo-sensitive polymers have been extensively used in bioseparation and bioprocessing. They can very easily be separated from aqueous solutions by heating above the lower critical solution temperature (LCST) of the polymer [8–10]. Poly(*N*-vinylcaprolactam) (PNVCL), a water-soluble thermo-responsive polymer, has a LCST of about 35 °C, and so lies in the physiological temperature range [11]. Galaev and Mattiasson [12,13] have studied the use of PNVCL in affinity precipitation with promising results reported. PNVCL is stable to hydrolysis, non-toxic, and has potential applications in bioseparation, medicine and pharmacology [14].

To ensure that precipitation is both predictable and selective, affinity ligands should be attached to the polymer chain [5]. Zinc and copper ions are known to form stable complexes with histidine and cysteine amino acids in proteins [15]. Hence, they could be used to enhance the strength of interactions between a polymer and protein. PNVCL has no reactive groups that could be used to couple with affinity ligands, and hence reactive groups need to be introduced through copolymerization of *N*-vinylcaprolactam (NVCL) with monomers containing reactive groups (e.g. methacrylic acid) [16]. Galaev et al. [17] copolymerized NVCL with *N*-vinylimidazole for metal ion loading.

In the present study, we have synthesized a thermo-sensitive copolymer, poly(*N*-vinylcaprolactam-co-methacrylic acid) (PNVCL-co-MAA), comprising NVCL and methacrylic acid (MAA). MAA contains carboxylic groups, which were used to load copper ions onto the polymer. The cation-loaded copolymer was used as an affinity macroligand for the precipitation of bovine serum albumin (BSA). This affinity precipitation process with

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thermo-sensitive copolymers is found to be a system with the ability to significantly enhance bioseparation.

2. Material and methods

2.1. Chemicals and reagents

N-vinylcaprolactam (NVCL) and bovine serum albumin were purchased from the Sigma Chemical Corp., Shanghai, China. Methacrylic acid, ammonium persulfate (APS), and copper sulfate were obtained from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. All chemicals were analytic grade reagents, and were used without purification.

2.2. Synthesis of PNVCL-co-MAA

An aqueous solution of NVCL and MAA was used for copolymerization. A reaction mixture containing different amounts of the two monomers was degassed under vacuum for 30 min. APS (0.04 g) was then added as an initiator and the pH was adjusted to 7.0 with NaOH. The reaction was allowed to proceed under a nitrogen atmosphere for 3 h at 65 °C. The supernatant was removed and the precipitate dissolved in water. This process was repeated three times, with 0.2 M NaCl being added for the final precipitation phase (see Fig. 1a).

2.3. Polymer characterization

Fourier transform infrared spectroscopy (FT-IR) spectra of PNVCL-co-MAA, NVCL and MAA were obtained using a FT-IR spectrophotometer (NEXUS-670, Thermo Nicolet Corp., USA). The LCST of the copolymer for a 5.0 mg/ml aqueous solution was determined at 540 nm using a UV–vis spectrophotometer (Lambda 35, PerkinElmer Inc., USA) by heating from 0 to 50 °C at a heating rate of 1 °C/min. The molecular weight of the polymer was determined from viscosity measurements [18].

2.4. Loading of copper

Cu^{2+} loading onto PNVCL-co-MAA was carried out by adding copper sulfate solution to 5 ml of a 10% (w/v) PNVCL-co-MAA copolymer aqueous solution [4,7]. $CuSO_4$ (10 ml) was added slowly under stirring at room temperatures. The cation-loaded copolymers were stirred for 1 h and then precipitated by adding 2 ml 0.2 M NaCl and heating at 50 °C for 15 min. The supernatant was decanted, and the precipitates dissolved in water. This procedure was repeated 3 times, and finally the copolymers were dissolved in water to give a 2% (w/v) solution. A summary of the process is provided in Fig. 1b. The metal ion loading was calculated from inductively coupled plasma optical emission spectroscopy data (Prodigy, Leeman, USA).

2.5. Affinity precipitation

Protein affinity precipitation experiments were carried out by adding 0.5 g PNVCL-co-MAA-Cu to aqueous BSA solutions (0.25, 0.5, 1.0, 1.5, 2.0 or 4.0 mg/ml) to give a final copolymer content of 2.5% (w/v). Precipitation of BSA was studied at various ionic strengths (in 0, 0.1, 0.2, 0.3, 0.4, 0.5 or 0.6 M NaCl solution) and pH values (4.0, 5.0, 6.0, 7.0, or 8.0) in a 0.05 M Tris–HCl buffer. Affinity precipitation experiments were conducted for 1 h at different temperatures (0, 10, 20 or 30 °C), and were followed by a heat treatment at 40 °C for 10 min (to raise the polymer above its LCST and render it insoluble). The amount of precipitated BSA was determined by measuring the initial and final concentrations of BSA in the adsorption medium using UV spectroscopy at 280 nm (UV-2102PC spectrophotometer, Unico, China)[19].

The amount of precipitated BSA was calculated using Eq. (1):

$$q_p = \frac{(C_i - C_t)V_s}{m} \quad (1)$$

where q_p is the amount of BSA precipitated per unit mass of copolymer (mg/g), C_i and C_t are the concentrations of BSA in the solutions before and after precipitation (mg/ml), V_s is the volume of BSA solution (ml), and m is the mass of the copolymer (g).

2.6. Elution profiling

Elution was carried out after precipitation of BSA at pH 6.0, with 1.0 mg/ml BSA and at 10 °C. The copolymer/protein complex was added to a 0.05 M Tris–HCl buffer to extract the precipitated protein. Experiments were performed at different pH values at 4 °C. Thereafter, various concentrations of NaCl and ethylenediaminetetraacetic acid (EDTA) were added. The solution was heated at 40 °C for 10 min and the supernatant withdrawn and analyzed by UV spectroscopy. The amount of eluted BSA was calculated using Eq. (2):

$$q_e = \frac{(C_i - C_w)V_s}{m} \quad (2)$$

where q_e is the amount of BSA eluted per unit mass of copolymer (mg/g), C_i and C_w are the concentrations of BSA in the solutions before and after elution (mg/ml), V_s is the volume of BSA solution (ml), and m is the mass of the copolymer (g).

2.7. Recycling of PNVCL-co-MAA-Cu

To determine the reusability of the copolymer, the precipitation and elution cycle was repeated five times using the optimal conditions for each process. The precipitation of BSA was at pH 6.0, 10 °C, with 1.0 mg/ml. Elution was carried out after precipitation at pH 4.0, 0.2 M NaCl and 0.06 M EDTA. Since the elution buffer contained the chelating agent EDTA, which would have caused some copper ions to be removed from the copolymer during recycling, Cu^{2+} was supplemented by adding copper sulfate solution (pH 5.0, containing 2.0 mg/ml Cu^{2+}) before each precipitation process. The summary of metal-chelate affinity precipitation including the process of precipitation, elution and recycling is provided in Fig. 2.

3. Results and discussion

3.1. Synthesis and characterization of PNVCL-co-MAA

PNVCL-co-MAA was synthesized using free radical polymerization of NVCL and MAA, with APS acting as an initiator. Fig. 1a presents the reaction scheme for the introduction of carboxylic groups using MAA and Table 1 shows the various conditions investigated for the synthesis of PNVCL-co-MAA. To confirm the successful preparation of PNVCL-co-MAA, FT-IR and viscosity measurements were carried out.

Fig. 3 shows the FT-IR spectra of NVCL (Fig. 3a), MAA (Fig. 3b), and PNVCL-co-MAA (Fig. 3c). The C=C stretching band at 1654 cm^{-1} in the spectrum of the NVCL monomer and at 1641 cm^{-1} in the spectrum of MAA monomer disappeared in PNVCL-co-MAA. The amide I and II band of NVCL were observed in the spectrum of PNVCL-co-MAA at 1626 and 1481 cm^{-1}, respectively. In addition to the NVCL bands, an absorption band is visible at 1709 cm^{-1} in the PNVCL-co-MAA spectrum. This corresponds to the stretching of C=O in carboxylic acids.

By changing the MAA: NVCL molar ratio as listed in Table 1, various PNVCL-co-MAA products with different molecular weights