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Studies on the application of temperature-responsive ion exchange polymers with whey proteins

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ABSTRACT

Several new types of temperature-responsive ion exchange resins of different polymer composition have been prepared by grafting the products from the co-polymerisation of *N*-phenylacrylamide, *N*-isopropylacrylamide and acrylic acid derivatives onto cross-linked agarose. Analysis of the binding isotherms for these different resins obtained under batch adsorption conditions indicated that the resin based on *N*-iso-propylacrylamide containing 5% (w/w) *N*-phenylacrylamide and 5% (w/w) acrylic acid resulted in the highest adsorption capacity, B_{max}, for the whey protein, bovine lactoferrin, e.g. 14 mg bovine lactoferrin/mL resin at 4 °C and 62 mg bovine lactoferrin/mL resin at 40 °C, respectively. Under dynamic loading conditions at 40 °C, 94% of the loaded bovine lactoferrin on a normalised mg protein per mL resin basis was adsorbed by this new temperature-responsive ion-exchanger, and 76% was eluted by a single cycle temperature shift to 4 °C without varying the composition of the 10 mM sodium dihydrogen phosphate buffer, pH 6.5, or the flow rate. The binding characteristics of these different ion exchange resins with bovine lactoferrin were also compared to results obtained using other resins based on *N*-isopropylacrylamide but contained *N*-*tert*-butylacrylamide rather than *N*-phenylacrylamide, where the corresponding dynamic capture and release properties for bovine lactoferrin required different temperature conditions of 20 °C and 50 °C, respectively for optimal desorption/adsorption. The cationic protein, bovine lactoperoxidase, was also adsorbed and desorbed with these temperature-responsive resins under similar conditions of changing temperature, whereas the anionic protein, bovine β-lactoglobulin, was not adsorbed under this regime of temperature conditions but instead eluted in the flow-through.

1. Introduction

Interest in stimuli-responsive polymeric materials, or so-called "smart polymers", has been heavily focused in recent years on their use in therapeutic and biomedical applications, including use in the solid phase sample extraction and analysis of steroids, adenosine nucleotides or various pharmaceuticals [1–9]. Many temperature-responsive polymeric materials are based on poly(*N*-isopropylacrylamide) (pNIPAM) together with other monomers, such as acrylic acid, *tert*-butylacrylamide or acrylamide to modulate the hydrophilic or hydrophobic properties of the resulting co-polymers. The properties of such copolymers enable "catch and release" mechanisms to be employed often as smart polymeric hydrogels with the target compounds resulting, for example, in their application in the controlled delivery of chemical and protein drugs such as insulin [2,9–13]. Moreover, similar stimuli-responsive polymers have found application in protein chromatography, affinity separations, immuno-assays and for cell attachment to surfaces [1,3]. To achieve additional selectivities, thermo-responsive materials of increased hydrophobicity have been prepared through the incorporation of more hydrophobic monomers, such as *N*-phenylacrylamide, into *N,N*-dimethylacrylamide- and *N,N*-diethylacrylamide-based polymers supported on a variety of substrata, ranging from silica to cellulose [14,15].

Temperature-responsive polymeric materials attached to silica particles or grafting onto gigaporous polystyrenes via surface-

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initiated atom transfer radical polymerization (ATRP) methods have been investigated at the analytical chromatographic scale for the separation of various low molecular weight analytes, small biomolecules such as peptides and some globular proteins, e.g. human serum albumin [16–19]. In addition, molecular dynamics simulations of poly(*N*-isopropylacrylamide) (PNIPAAm) grafted onto silica have provided useful insight into ways to reduce protein denaturation compared to the more traditionally used reversed phase LC procedures [20]. Typically, for ion exchange applications, the anionic poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-*N*-*tert*-butylacrylamide) (ItBA) has been used as a pH- and temperature-responsive copolymer. Recently, we reported that the separation of proteins can be achieved at larger process scales using the temperature-responsive copolymers, based on ItBA, attached to more process relevant and industrially compatible matrices, such as cross-linked agarose [21]. The use of this matrix avoids the instability of silica-based support materials under the high pH conditions required for clean-in-place (CIP) regeneration as part of an integrated approach to bioprocess chromatography [22–25]. In this paper, we explore the effect of varying the polymer composition on resin performance, in terms of impact on the lower critical solution temperature (LCST) of the free co-polymeric moieties, as well as their effectiveness when immobilised onto agarose materials as temperature-responsive ion exchange chromatographic resins for protein separation.

2. Experimental

2.1. Chemicals

Crosslinked agarose (Sepharose 6 Fast Flow™) with particle sizes in the range of 45–165 μm, was obtained from GE Healthcare (Sydney, Australia). Bovine lactoferrin, (Mw 87 kDa, pI 8.0, ~95% purity), bovine β-lactoglobulin (Mw 18 kDa, pI 5.2, ~95% purity) and bovine lactoperoxidase (Mw 77.5 kDa, pI 8.1, ~90% purity) were provided by Food Science Australia/MG Nutritionals (Melbourne, Australia). *N*-isopropylacrylamide (NIPAAm), *tert*-butylacrylamide (tBAAm), acrylic acid (AAc), methacrylic acid (MAAc) and *N*-phenylacrylamide (PhAAm) 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), 4,4′-azobis(4-cyanovaleric acid) (ACV), anhydrous *N*,*N*-dimethylformamide (DMF), *N*,*N*′-methylenebisacrylamide (MBAAm) and epichlorohydrin were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide, sodium borohydride, 28% (v/v) aqueous ammonia solution, absolute and 96% ethanol, anhydrous tetrahydrofuran and deuterated chloroform ($CDCl_3$) were obtained from Merck–Millipore (Darmstadt, Germany). Acetonitrile was obtained from Unichrom (Seven Hills, Australia). The buffers employed in these investigations were (a) buffer/mobile phase A, 10 mM sodium dihydrogen phosphate, pH 6.5; and (b) buffer/mobile phase B, 1 M NaCl in 10 mM sodium dihydrogen phosphate, pH 6.5.

2.2. Preparation of free, ungrafted copolymers

(Poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-*N*-*tert*-butylacrylamide (ItBA)), poly-(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-*N*-phenylacrylamide (IPhA)) and poly(*N*-isopropylacrylamide-*co*-methacrylic acid-*co*-*N*-phenylacrylamide (IPhMA)) were individually prepared in tetrahydrofuran with a total monomer concentration of 1 mmol/mL and 4,4′-azobis(4-cyanovaleric acid) (ACV; initiator) content of 0.15% of total monomers based on procedures described previously [21]. The solution mixture was degassed by applying a vacuum (20 torr) for 5 min and the reaction was allowed to proceed under an argon atmosphere at 65 °C for 3 h. The polymers were precipitated in diethyl ether, collected on Buchner funnel, washed with diethyl ether and then suction dried under vacuum. The monomers used with *N*-isopropyl-acrylamide (NIPAAm) were either (i) *N*-phenylacrylamide (PhAm), (ii) *N*-*tert*-butylacryl-amide (tBAAm) with acrylic acid (AAc) or (iii) methacrylic acid (MAAc). The various compositions of the monomer feeds used with 1% (w/w) *N*,*N*′-methylenebisacrylamide (MBAAm) as crosslinker were as follows:

(A) For the synthesis of the I-5tB material, the polymer was prepared from the monomers NIPAAm, 95% w/w, tBAAm, 5% w/w, no AAc. For the synthesis of the I-5tB-5AA polymer (coded as ItBA in our previous work [21]) the monomers used were NIPAAm, 90% w/w, tBAAm, 5% w/w and AAc, 5% w/w. For the synthesis of the I-5tB-10AA polymer the monomers used were NIPAAm, 85% w/w, tBAAm, 5% w/w and AAc, 10% w/w.
(B) For the synthesis of the I-5Ph-5AA material, the polymer was prepared from the monomers NIPAAm, 90%w/w, PhAAm, 5% w/w and AAc, 5% w/w. For the synthesis of the I-5Ph-10AA polymer, the monomers used were NIPAAm, 85%w/w, PhAAm 5% w/w and AAc, 10% w/w. For the synthesis of the I-5Ph-20AA polymer, the monomers used were NIPAAm, 75%w/w, PhAAm, 5% w/w and AAc, 20% w/w.
(C) For the synthesis of the I-5Ph-5MAA material, the polymer was prepared from the monomers NIPAAm, 95% w/w, PhAAm, 5% w/w and MAAc, 5% w/w.

2.3. Preparation of polymer grafted cross-linked agarose

Different variations of the resin materials above were synthesised by immobilisation of the initiator onto the agarose support material, followed by addition of the appropriate monomers and crosslinker and polymerisation carried out using our protocol described for the synthesis of ItBA-based resins [21], but with variation of the compositions of the monomers.

2.4. Characterisation of the LCST of the bulk pre-formed co-polymers

The lower critical solution temperature (LCST) profiles of the ungrafted copolymers were determined by measuring the optical transmittance of a 0.5% (w/w) aqueous solution of the copolymer in 10 mM sodium dihydrogen phosphate buffer, pH 6.5, at 500 nm at temperatures ranging from 16 °C to 40 °C using a UV/visible spectrometer (SpectraMax Plus^{384}, Molecular Devices, Sunnyvale, CA, USA) with a temperature controlled cuvette chamber, with the data analysed based on procedures described in our previous studies [21].

2.5. Batch adsorption procedures

The batch adsorption data of the selected resin and proteins were obtained based on methods described previously [21]. In brief, bovine lactoferrin solutions of different concentrations were prepared in 10 mM sodium dihydrogen phosphate buffer, pH 6.5, and mixed with the resin samples (0.1 g) for 1 h using a reciprocating mixing system. The mixtures were then centrifuged at 15,000 × *g* for 5 min using Eppendorf Centrifuge 5417R. The bovine lactoferrin concentration in the supernatants was determined by measuring the absorbance at 280 nm. The amount of bovine lactoferrin adsorbed to the resin was determined from the mass balance equation. Similar methods were employed with the other proteins.

2.5.1. Adsorption profiles at different temperatures

To obtain the adsorption characteristics of the selected resin and protein at various temperatures, a solution of the protein (1 mL of

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