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Integrated system for temperature-controlled fast protein liquid chromatography comprising improved copolymer modified beaded agarose adsorbents and a travelling cooling zone reactor arrangement

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ABSTRACT

An integrated approach to temperature-controlled chromatography, involving copolymer modified agarose adsorbents and a novel travelling cooling zone reactor (TCZR) arrangement, is described. Sepharose CL6B was transformed into a thermoresponsive cation exchange adsorbent (thermoCEX) in four synthetic steps: (i) epichlorohydrin activation; (ii) amine capping; (iii) 4,4′-azobis(4-cyanovaleric acid) immobilization; and 'graft from' polymerization of poly(*N*-isopropylacrylamide-*co*-*N*-*tert*-butylacrylamide-*co*-acrylic acid-co-*N*,*N*′-methylenebisacrylamide). FT-IR, ^{1}H NMR, gravimetry and chemical assays allowed precise determination of the adsorbent's copolymer composition and loading, and identified the initial epoxy activation step as a critical determinant of 'on-support' copolymer loading, and in turn, protein binding performance. In batch binding studies with lactoferrin, thermoCEX's binding affinity and maximum adsorption capacity rose smoothly with temperature increase from 20 to 50 °C. In temperature shifting chromatography experiments employing thermoCEX in thermally jacketed columns, 44–51% of the lactoferrin adsorbed at 42 °C could be desorbed under binding conditions by cooling the column to 22 °C, but the elution peaks exhibited strong tailing. To more fully exploit the potential of thermoresponsive chromatography adsorbents, a new column arrangement, the TCZR, was developed. In TCZR chromatography, a narrow discrete cooling zone (special assembly of copper blocks and Peltier elements) is moved along a bespoke fixed-bed separation columnfilled with stationary phase. In tests with thermoCEX, it was possible to recover 65% of the lactoferrin bound at 35 °C using 8 successive movements of the cooling zone at a velocity of 0.1 mm/s; over half of the recovered protein was eluted in the first peak in more concentrated form than in the feed. Intra-particle diffusion of desorbed protein out of the support pores, and the ratio between the velocities of the cooling zone and mobile phase were identified as the main parameters affecting TCZR performance. In contrast to conventional systems, which rely on cooling the whole column to effect elution and permit only batch-wise operation, TCZR chromatography generates sharp concentrated elution peaks without tailing effects and appears ideally suited for continuous operation.

1. Introduction

Bioprocess chromatography suffers from several problems that compromise its sustainability. Current 'adsorption–desorption' processes are expensive, suffer from low productivity (given that most involve batch-wise operation in fixed beds), and employ large volumes of buffer for equilibrating, washing, eluting and cleaning of columns, generating as a consequence excessive quantities of waste [1–3]. Effective solutions to these significant problems have not yet been forthcoming, but would clearly make for much leaner, greener

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and more sustainable manufacturing of valuable bio-commodities [3].

In this context, we introduce an integrated bioseparation concept, which makes use of discrete 'local' changes in temperature to control adsorption–desorption equilibria, and relies on the combined use of chromatographic supports modified with a methylene bisacrylamide (MBAAm) cross-linked 'smart' temperature-sensitive anionic copolymer, i.e. poly(*N*-isopropylacrylamide-*co*-*N*-*tert*-butylacrylamide-*co*-acrylic acid) (abbreviated to pNIPAAm-*co*-tBAAm-*co*-AAc), and a novel device that permits continuous thermally mediated bioseparation.

Smart temperature-sensitive or thermoresponsive polymers are polymers that exhibit inverse temperature solubility behaviour, i.e. they are water-soluble at low temperature, and insoluble at high temperature above a critical temperature known as the lower critical solution temperature (LCST) [4]. They have been shown as potentially useful in diverse biomedical and biotechnological applications [3,5–7]. By far the most studied species is pNIPAAm [8–10]. pNIPAAm undergoes an abrupt reversible 'hydrophilic coil–hydrophobic globule' phase transition in water at an LCST of 32–34 °C [8,9], and importantly its LCST is relatively insensitive to variation in pH, concentration or chemical environment [9,10].

A significant body of work on the modification of chromatographic packing materials with thermoresponsive polymers (mostly pNIPAAm or pNIPAAm based copolymers) has appeared since the first reports in the early-mid 1990s [11–13]. Most of this has concentrated on exploiting the principle of thermally induced extension and collapse of polymer chains for modulating the fractionation range in size exclusion chromatography [11,12,14], hydrophobicity in HPLC [13,15–18], and balancing electrostatic and hydrophobic interactions in ion exchange chromatography, through the use of copolymers containing both thermoresponsive and ion exchange components [19–25], rather than mitigating ligand masking and 'forced elution' in pseudoaffinity chromatography [3,26]. To this day, the vast body of work on thermoresponsive chromatography pertains to the modification and subsequent use of small pored inorganic (glass, silica) or hydrophobic (polystyrene based) chromatography supports in analytical HPLC separations of small biomolecules (especially steroids). By contrast, applications involving soft macroporous supports, appropriate for bioprocess scale separations of large macromolecular targets (globular proteins, nucleic acids, viruses) have received very little attention thus far. In some respects this is surprising, given that the use of temperature responsive chromatography materials offers cost-effective environmentally friendly solutions to the isolation of commercially valuable biocomponents from biopharma, bioindustry, agricultural, food and other complex process streams [3,24]. Though not discussed in the literature, one of the main reasons for this is likely to be difficulties in scaling up thermo-responsive chromatography with such media and column formats, and further, that macromolecules tend to be much more thermally labile than their smaller counterparts. On the latter point, Maharjan and coworkers [24] highlighted the attraction of this type of chromatography for the separation of thermally robust targets, such as lactoferrin (LF) from ultra-high volumes of bovine whey feedstocks. Using methods originally applied to silica matrices, these authors were the first to prepare thermoresponsive cation exchange adsorbents (modified with lightly cross-linked pNIPAAm-*co*-tBAAm-*co*-AAc) from cross-linked beaded agarose supports. In the same report the authors subsequently demonstrated both temperature dependent adsorption of LF and thermally mediated elution of the adsorbed protein in batch and dynamic column experiments; the latter being conducted by tempering the whole column in a water bath.

In this paper, we extend upon Maharjan and coworkers [3,24] work by integrating the use of thermoresponsive cation exchangers (hereafter referred to as thermoCEX) with a new column arrangement specifically tailored for thermoresponsive chromatography. Our solution is, rather than subject the whole column to temperature changes in a water bath or via a surrounding jacket, we subject only a small part of it via a computer controlled motor-driven travelling Peltier block arrangement. In this study involving inverse temperature responsive copolymer modified adsorbents, where elution is observed on cooling, we term the set up a travelling cooling zone reactor (TCZR). Note, in the opposite case, i.e. where the modifying polymer exhibits normal temperature solubility behaviour and/or elution occurs on heating, a travelling heating zone reactor (THZR) would be appropriate.

We first describe the preparation, characterization and comparison of thermoCEX adsorbents prepared for this study with those reported by Maharjan et al. [24]. Subsequently, we compare their chromatographic behaviour in the TCZR (under batch-wise operation) with that in a jacketed column, and further, identify the main parameters affecting the TCZR's performance, as intra-particle diffusion of desorbed protein out of the pores of the support, and the ratio between the velocities of the cooling zone and mobile phase. Finally, we address the steps that will be required to make preparative TCZR chromatography, and temperature-controlled adsorption–desorption chromatography in general, attractive propositions for the future.

2. Experimental

2.1. Materials

Bovine whey lactoferrin (MLF-1, 97%) was received as a gift from Milei GmbH (Leutkirch, Germany). Sepharose CL-6B was obtained from GE Healthcare Life Sciences (Little Chalfont, Bucks, UK). The chemicals, *N*-isopropylacrylamide (97%; NIPAAm), *N*-*tert*-butylacrylamide (97%; *t*-BAAm), acrylic acid (anhydrous, 99%, AAc), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (≥99%; EEDQ), 4,4′-azobis(4-cyanovaleric acid) (≥98%; ACV), *N*,*N*-dimethylformamide (>99.9%; DMF), *N*,*N*′-methylenebisacrylamide (99%; MBAAm), epichlorohydrin (99%; ECH), tetrahydrofuran (>99%; THF), diethyl ether (>99.9%), sodium borohydride (>99%) and sodium hydroxide (anhydrous, >98%) were purchased from the Sigma–Aldrich Company Ltd (Poole, Dorset, UK), whereas absolute ethanol (99.8+%) and ammonia solution (AR grade, 0.88 S.G., 35%) were acquired from Fisher Scientific UK Ltd (Loughborough, Leics, UK), di-sodium hydrogen phosphate (dihydrate, ≥99.5%) was from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), and bottled oxygen-free nitrogen gas was supplied by the British Oxygen Co Ltd (Windlesham, Surrey, UK).

2.2. Preparation of thermoresponsive cation exchange (thermoCEX) chromatography adsorbents

The methods used to convert underivatized Sepharose CL-6B supports into thermoresponsive cation exchange matrices (thermoCEX) involve four successive steps – epoxide activation, amine capping, initiator immobilization and 'graft from' polymerization. These are detailed below and summarized schematically in Fig. 1.

2.2.1. Epoxy activation [27]

Washed suction-drained supports (50 g, 71 ml) were mixed with 85 ml of water in 250 ml Pyrex® conical flasks and 40 ml of 2 M NaOH before placing in a 40 °C shaking water bath (Julabo SW22, Labortechnik GmbH, Seelbach, Germany) reciprocating at 150 rpm for 0.5 h. Ten millilitres of ECH (99%) was then added to a final concentration of ~5% (v/v), and reaction was allowed to proceed at 40 °C with shaking for an additional 2 h. The resulting