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Integrated system for temperature-controlled fast protein liquid chromatography. II. Optimized adsorbents and 'single column continuous operation'

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ABSTRACT

Continued advance of a new temperature-controlled chromatography system, comprising a column filled with thermoresponsive stationary phase and a travelling cooling zone reactor (TCZR), is described. Nine copolymer grafted thermoresponsive cation exchangers (thermoCEX) with different balances of thermoresponsive (*N*-isopropylacrylamide), hydrophobic (*N*-*tert*-butylacrylamide) and negatively charged (acrylic acid) units were fashioned from three cross-linked agarose media differing in particle size and pore dimensions. Marked differences in grafted copolymer composition on finished supports were sourced to base matrix hydrophobicity. In batch binding tests with lactoferrin, maximum binding capacity (q_{max}) increased strongly as a function of charge introduced, but became increasingly independent of temperature, as the ability of the tethered copolymer networks to switch between extended and collapsed states was lost. ThermoCEX formed from Sepharose CL-6B (A2), Superose 6 Prep Grade (B2) and Superose 12 Prep Grade (C1) under identical conditions displayed the best combination of thermoresponsiveness ($q_{max,50\,^{\circ}C}/q_{max,10\,^{\circ}C}$ ratios of 3.3, 2.2 and 2.8 for supports 'A2', 'B2' and 'C1' respectively) and lactoferrin binding capacity ($q_{max,50\,^{\circ}C} \sim 56$, 29 and 45 mg/g for supports 'A2', 'B2' and 'C1' respectively), and were selected for TCZR chromatography. With the cooling zone in its parked position, thermoCEX filled columns were saturated with lactoferrin at a binding temperature of 35 °C, washed with equilibration buffer, before initiating the first of 8 or 12 consecutive movements of the cooling zone along the column at 0.1 mm/s. A reduction in particle diameter (A2 → B2) enhanced lactoferrin desorption, while one in pore diameter (B2 → C1) had the opposite effect. In subsequent TCZR experiments conducted with thermoCEX 'B2' columns continuously fed with lactoferrin or 'lactoferrin + bovine serum albumin' whilst simultaneously moving the cooling zone, lactoferrin was intermittently concentrated at regular intervals within the exiting flow as sharp uniformly sized peaks. Halving the lactoferrin feed concentration to 0.5 mg/mL, slowed acquisition of steady state, but increased the average peak concentration factor from 7.9 to 9.2. Finally, continuous TCZR mediated separation of lactoferrin from bovine serum albumin was successfully demonstrated. While the latter's presence did not affect the time to reach steady state, the average lactoferrin mass per peak and concentration factor both fell (respectively from 30.7 to 21.4 mg and 7.9 to 6.3), and lactoferrin loss in the flowthrough between elution peaks increased (from 2.6 to 12.2 mg). Fouling of the thermoCEX matrix by lipids conveyed into the feed by serum albumin is tentatively proposed as responsible for the observed drops in lactoferrin binding and recovery.

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1. Introduction

Today, liquid chromatography is universally recognized as a supremely effective and practical bioseparation tool [1,2]. There are a multitude of reasons for this, but perhaps the two most important are the technique's adaptability to analytical and preparative separation tasks [3] and the availability of a huge variety of differently functionalized chromatographic supports affording orthogonal separation mechanisms [4,5]. In typical adsorption chromatography, defined amounts of a given feed solution, containing a single target component and multiple contaminants, are loaded onto a fixed-bed of adsorbent contained in a chromatography column. While the target component adsorbs, to be recovered in a later dedicated elution step by changing the chemical composition of the mobile phase, contaminant species either flow through the column unhindered, or alternatively are washed out in a subsequent washing step and/or during elution procedures. In addition to modifying the mobile phase's chemical composition, physical parameters can also be manipulated to influence protein adsorption to and desorption from chromatographic supports; the most popular of these being temperature, especially in the case of Hydrophobic Interaction Chromatography, HIC [6–10]. According to the Gibbs–Helmholtz equation, an increase in temperature exerts an influence similar to that imposed by raising the cosmotropic salt content in the mobile phase during HIC, which leads to enhanced protein adsorption affinity [8]. However, the relatively small differences in working capacity, even across temperature differentials as high as 40 °C, makes HIC adsorbents unattractive materials for purely temperature mediated liquid chromatography. The anchoring of 'smart' temperature-sensitive polymers or 'smart' thermoresponsive polymers onto chromatography supports offers a potential means of overcoming this drawback.

Smart thermoresponsive polymers are ones 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) [11]. The most studied smart thermoresponsive polymer by far is poly(*N*-isopropylacrylamide) or pNIPAAm [12–14], and its successful and broad application within biomedicine and biotechnology is extensively documented [15–18]. pNIPAAm undergoes a sharp reversible 'hydrophilic coil – hydrophobic globule' phase transition in water at an LCST of 32–34 °C [12,13]. A large body of work on endowing chromatographic packing materials with temperature switchable behavior, through their modification with e.g. pNIPAAm or pNIPAAm copolymers, has appeared since the 1990s [19–24]. Most of this has involved modification of small pored inorganic or hydrophobic (polystyrene based) chromatography supports for use in analytical separations of small biomolecules (especially steroids). In stark contrast, very little has been done on the modification of softer macroporous media for preparative separations of much larger macromolecules, such as proteins [22–24]. Maharjan et al. [22] and subsequently we [23] grafted lightly cross-linked networks of poly(*N*-isopropylacrylamide-*co*-*N*-tert-butylacrylamide-*co*-acrylic acid) onto the surfaces of cross-linked agarose supports to produce thermoresponsive cation exchangers (hereafter abbreviated to thermoCEX). In tests with the thermally robust protein lactoferrin (LF) and jacketed columns of thermoCEX media, LF previously adsorbed at a higher temperature could be desorbed by lowering the mobile phase and column temperature.

To exploit thermoresponsive chromatography media more effectively, we invented a bespoke column arrangement [23], the so-called Travelling Cooling Zone Reactor (TCZR). TCZR chromatography employs a vertically held stainless steel walled column filled with thermoresponsive stationary phase and a computer-controlled motor-driven Peltier cooling device (the travelling cooling zone, TCZ) surrounding a discrete zone of the column (Fig. 1). In standard operation, a protein feed is administered to the column at an elevated temperature. On completion of the loading phase, the column is irrigated with an equilibration buffer whilst simultaneously moving the TCZ along the full length of the separation column (multiple times) in the direction of the mobile phase, and at a velocity lower than that of the interstitial fluid. With each TCZ arrival at the end of the column, a sharp concentrated protein peak appears in the exiting flow, which can be collected by means of a fraction collector.

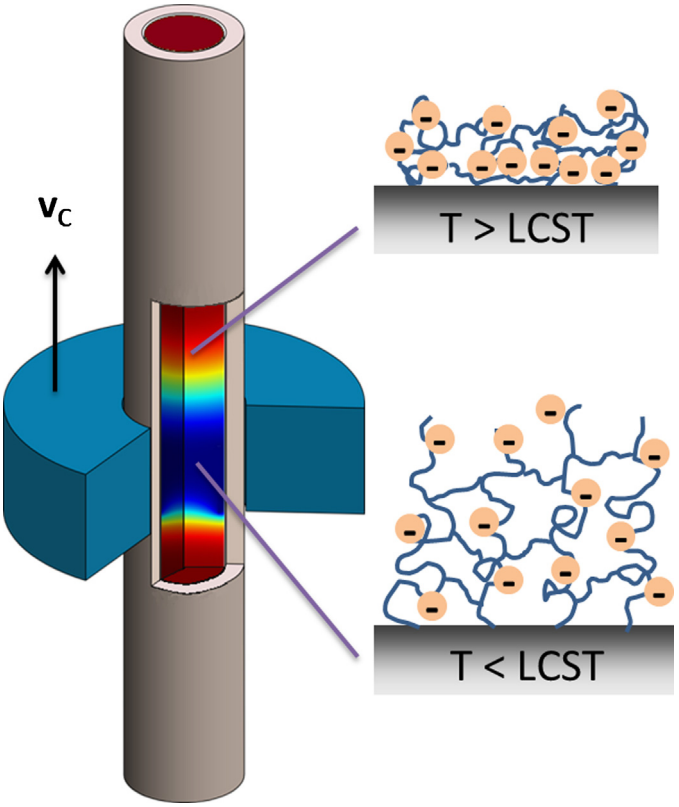

Fig. 1. Schematic illustration of the TCZR principle. A stainless steel column filled with thermoresponsive copolymer modified chromatographic media is contained in a temperature-controlled environment at a value above the copolymer's LCST. At this temperature (indicated by red) the grafted thermoresponsive copolymer network exists in a collapsed and highly charged state (top right) that affords high protein binding affinity. For elution a motor-driven Peltier cooling device, the travelling cooling zone or TCZ (shown as a turquoise ring), is moved along the column's full length at a velocity (v_c) lower than that of the mobile phase. Within the cooled zone (shown in blue) generated by the TCZ as it travels along the column, the tethered thermoresponsive copolymer expands, the charge density drops (bottom right) and bound protein detaches from the support surfaces and is carried away in the exiting mobile phase. For more details the reader is referred to Sections 2.3, 3.1 and 3.3 of the text (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) .

In this second follow up study, we push the boundaries of the TCZR chromatography concept further. From three different agarose base matrices, we construct and fully characterize nine thermoCEX media varying in particle size, pore diameter, and copolymer composition, and subsequently identify, from batch adsorption and batch mode TCZR chromatography, the thermoCEX variant best suited for operation in TCZR modified columns. We then demonstrate, for the first time, how TCZR can be operated in continuous mode, to accumulate and concentrate a model binding protein (LF), and then separate the same target molecule from a simple protein mixture.