

1 **Integrated system for temperature-controlled fast protein liquid chromatography. II.**
2 **Optimized adsorbents and ‘single column continuous operation’**

3

4 Ping Cao^{a,3} Tobias K.H. Müller^{b,1,3}, Benedikt Ketterer^b, Stephanie Ewert^{a,b}, Eirini
5 Theodosiou^{a,2}, Owen R.T. Thomas^{a,*}, Matthias Franzreb^{b,**}

6

7 ^aSchool of Chemical Engineering, College of Engineering and Physical Sciences, University
8 of Birmingham, Edgbaston, Birmingham B15 2TT, England, UK

9 ^bInstitute for Functional Interfaces, Karlsruhe Institute of Technology, Hermann-von-
10 Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

11

12 *Corresponding author. Tel. +44 121 414578; fax: +44 121 4145377.

13 **Corresponding author. Tel.: +49 721 608 23595; fax: +49 721 608 23478

14 *E-mail addresses:* o.r.t.thomas@bham.ac.uk (O.R.T. Thomas); matthias.franzreb@kit.edu
15 (M. Franzreb).

16

17 1 Present address: Evonik Industries AG, Rodenbacher Chausee 4, 63457 Hanau, Germany

18 2 Present address: Department of Chemical Engineering, School of Aeronautical,
19 Automotive, Chemical and Materials Engineering, Loughborough University, Loughborough
20 LE11 3TU, UK

21 3 These authors contributed equally to the experimental work in this study

22

23 **Abstract**

24 Continued advance of a new temperature-controlled chromatography system, comprising a
25 column filled with thermoresponsive stationary phase and a travelling cooling zone reactor
26 (TCZR), is described. Nine copolymer grafted thermoresponsive cation
27 exchangers(thermoCEX) with different balances of thermoresponsive (*N*-
28 isopropylacrylamide), hydrophobic (*N*-*tert*-butylacrylamide) and negatively charged (acrylic
29 acid) units were fashioned from three cross-linked agarose media differing in particle size and
30 pore dimensions. Marked differences in grafted copolymer composition on finished supports
31 were sourced to base matrix hydrophobicity. In batch binding tests with lactoferrin, maximum
32 binding capacity (q_{max}) increased strongly as a function of charge introduced, but became
33 increasingly independent of temperature, as the ability of the tethered copolymer networks to
34 switch between extended and collapsed states was lost. ThermoCEX formed from Sepharose
35 CL-6B (A2), Superose 6 Prep Grade (B2) and Superose 12 Prep Grade (C1) under identical
36 conditions displayed the best combination of thermoresponsiveness ($q_{max,50^{\circ}C} / q_{max,10^{\circ}C}$ ratios
37 of 3.3, 2.2 and 2.8 for supports ‘A2’, ‘B2’ and ‘C1’ respectively) and lactoferrin binding
38 capacity ($q_{max,50^{\circ}C} \sim 56, 29$ and 45 mg/g for supports ‘A2’, ‘B2’ and ‘C1’ respectively), and
39 were selected for TCZR chromatography. With the cooling zone in its parked position,
40 thermoCEX filled columns were saturated with lactoferrin at a binding temperature of $35^{\circ}C$,
41 washed with equilibration buffer, before initiating the first of 8 or 12 consecutive movements
42 of the cooling zone along the column at 0.1 mm/s. A reduction in particle diameter (A2 \rightarrow
43 B2) enhanced lactoferrin desorption, while one in pore diameter (B2 \rightarrow C1) had the opposite
44 effect. In subsequent TCZR experiments conducted with thermoCEX ‘B2’ columns
45 continuously fed with lactoferrin or ‘lactoferrin + bovine serum albumin’ whilst
46 simultaneously moving the cooling zone, lactoferrin was intermittently concentrated at
47 regular intervals within the exiting flow as sharp uniformly sized peaks. Halving the
48 lactoferrin feed concentration to 0.5 mg/mL, slowed acquisition of steady state, but increased

49 the average peak concentration factor from 7.9 to 9.2. Finally, continuous TCZR mediated
50 separation of lactoferrin from bovine serum albumin was successfully demonstrated. While
51 the latter's presence did not affect the time to reach steady state, the average lactoferrin mass
52 per peak and concentration factor both fell (respectively from 30.7 to 21.4 mg and 7.9 to 6.3),
53 and lactoferrin loss in the flowthrough between elution peaks increased (from 2.6 to 12.2 mg).
54 Fouling of the thermoCEX matrix by lipids conveyed into the feed by serum albumin is
55 tentatively proposed as responsible for the observed drops in lactoferrin binding and recovery.

56

57 **Keywords:** Copolymer modified agarose adsorbents; Ion exchange adsorption; Lower critical
58 solution temperature (LCST); *N*-isopropylacrylamide; Smart polymers; Travelling cooling
59 zone reactor

60

61 **1. Introduction**

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

83

84 Smart thermoresponsive polymers are ones that exhibit inverse temperature solubility
85 behaviour, i.e. they are water-soluble at low temperature and insoluble at high temperature,
86 above a critical temperature known as the lower critical solution temperature (LCST) [11].

87 The most studied smart thermoresponsive polymer by far is poly(*N*-isopropylacrylamide) or
88 pNIPAAm [12-14], and its successful and broad application within biomedicine and
89 biotechnology is extensively documented [15-18]. pNIPAAm undergoes a sharp reversible
90 ‘hydrophilic coil – hydrophobic globule’ phase transition in water at an LCST of 32–34 °C
91 [12,13]. A large body of work on endowing chromatographic packing materials with
92 temperature switchable behavior, through their modification with e.g. pNIPAAm or
93 pNIPAAm copolymers, has appeared since the 1990s [19-24]. Most of this has involved
94 modification of small pored inorganic or hydrophobic (polystyrene based) chromatography
95 supports for use in analytical separations of small biomolecules (especially steroids). In stark
96 contrast, very little has been done on the modification of softer macroporous media for
97 preparative separations of much larger macromolecules, such as proteins. [22-24]. Maharjan
98 et al. [22] and subsequently we [23] grafted lightly cross-linked networks of poly(*N*-
99 isopropylacrylamide-*co*-*N*-tert-butylacrylamide-*co*-acrylic acid) into the surfaces of cross-
100 linked agarose supports to produce thermoresponsive cation exchangers (hereafter abbreviated
101 to thermoCEX). In tests with the thermally robust protein lactoferrin (LF) and jacketed
102 columns of thermoCEX media, LF previously adsorbed at a higher temperature could be
103 desorbed by lowering the mobile phase and column temperature.

104

105 To exploit thermoresponsive chromatography media more effectively, we invented a bespoke
106 column arrangement [23], the so-called Travelling Cooling Zone Reactor (TCZR). TCZR
107 chromatography employs a vertically held stainless steel walled column filled with
108 thermoresponsive stationary phase and a computer-controlled motor-driven Peltier cooling
109 device (the travelling cooling zone, TCZ) surrounding a discrete zone of the column (Fig. 1).
110 In standard operation, a protein feed is administered to the column at an elevated temperature.
111 On completion of the loading phase, the column is irrigated with an equilibration buffer
112 whilst simultaneously moving the TCZ along the full length of the separation column

113 (multiple times) in the direction of the mobile phase, and at a velocity lower than that of the
114 interstitial fluid. With each TCZ arrival at the end of the column, a sharp concentrated protein
115 peak appears in the exiting flow, which can be collected by means of a fraction collector.

116

117 In this second follow up study, we push the boundaries of the TCZR chromatography concept
118 further. From 3 different agarose base matrices, we construct and fully characterize 9
119 thermoCEX media varying in particle size, pore diameter, and copolymer composition, and
120 subsequently identify, from batch adsorption and batch mode TCZR chromatography, the
121 thermoCEX variant best suited for operation in TCZR modified columns. We then
122 demonstrate, for the first time, how TCZR can be operated in continuous mode, to accumulate
123 and concentrate a model binding protein (LF), and then separate the same target molecule
124 from a simple protein mixture.

125

126 **2. Materials and methods**

127 **2.1 Materials**

128 The base matrices, Sepharose CL-6B (Cat. no. 170160-01, Lot no. 10040943), Superose 6
129 Prep Grade (Cat. no. 17-0489-01, Lot. no. 10037732) and Superose 12 Prep Grade (Cat.
130 no.17-0536-01, Lot. no. 10057699) were all supplied by GE Healthcare Life Sciences (Little
131 Chalfont, Bucks, UK). The chemicals, *N*-isopropylacrylamide (Cat. no. 415324, 97%;
132 NIPAAm), *N*-*tert*-butylacrylamide (Cat. no. 411779, 97%; *t*-BAAm), acrylic acid (Cat. no.
133 147230, anhydrous, 99%, AAc), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (Cat. no.
134 149837, $\geq 99\%$; EEDQ), 4,4'-azobis(4-cyanovaleric acid) (Cat. no. 11590, $\geq 98\%$; ACV), *N,N*-
135 dimethylformamide (Cat. no. 270547, $>99.9\%$; DMF), *N,N'*-methylenebisacrylamide (Cat.
136 No. 146072, 99%; MBAAm), epichlorohydrin (Cat. no. E1055, 99%; ECH), tetrahydrofuran
137 (Cat. no. 34865, $>99\%$; THF), diethyl ether (Cat. no. 309966, $>99.9\%$), sodium borohydride
138 (Cat. no. 71321, $>99\%$) and sodium hydroxide (Cat. no. S5881, anhydrous, $>98\%$) were

139 obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Absolute ethanol (Cat. no.
140 E/0650DF/17, 99.8+%) and ammonia solution (Cat. no. A/3280/PB15, AR grade, 0.88 S.G.,
141 35%) were acquired from Fisher Scientific UK Ltd (Loughborough, Leics, UK), and bottled
142 oxygen-free nitrogen gas was supplied by the British Oxygen Co Ltd (Windlesham, Surrey,
143 UK). Bovine whey lactoferrin (MLF-1, Lot. No. 12011506, ~96%) was a gift from Milei
144 GmbH (Leutkirch, Germany), and bovine serum albumin (BSA, Cat. No. A7906, lyophilized
145 powder, $\geq 98\%$ by agarose gel electrophoresis) and 'Blue Dextran MW 2,000,000' (Cat. No.
146 D-5751) were purchased from Sigma-Aldrich. Di-sodium hydrogen phosphate (Cat. no.
147 4984.1, dihydrate, $\geq 99.5\%$) was from Carl Roth GmbH + Co. KG (Karlsruhe, Germany).
148 Disodium hydrogen phosphate (dihydrate, $\geq 99.5\%$) and sodium chloride (ACS reagent,
149 $\geq 99.5\%$) were supplied by Carl Roth or Sigma-Aldrich, and citric acid monohydrate ($\geq 99\%$)
150 and Coomassie Brilliant Blue R250 (C.I. 42660) were from Merck Millipore (Darmstadt,
151 Germany). Pre-cast 15% mini-PROTEAN® TGX™ gels and Precision Plus Protein™ All
152 Blue Standards were supplied by Bio-Rad Laboratories Inc. (Hercules, CA, USA). All other
153 chemicals not stated above were from Sigma-Aldrich or Merck Millipore. The water used in
154 all experiments was deionized and purified using a Milli-Q Ultrapure system (Merck
155 Millipore, Darmstadt, Germany).

156

157 **2.2 Preparation of the thermoCEX media used in this work**

158 For detailed descriptions of the procedures involved in the four step conversion of
159 underivatized beaded agarose chromatography supports into thermoCEX media, the reader is
160 referred to our previous study [23]. Exactly the same methods were applied here to three
161 different beaded agarose starting materials (i.e. Sepharose CL-6B, Superose 6 Prep Grade and
162 Superose 12 Prep Grade; see Tables 1 and 2). The first three steps of the conversion, i.e.
163 epoxy activation, amine capping and immobilization of the ACV radical initiator were
164 identically performed, but in the fourth and final 'graft from' polymerization step the initial

165 AAc monomer concentration entering reactions with ACV anchored supports was
166 systematically varied between 25 and 150 mM (equivalent to 2.5 to 13.5% of the total
167 monomer concentration), whilst maintaining fixed concentrations of all other monomers, i.e.
168 900 mM NIPAAm, 50 mM tBAAm, and 10 mM of the cross-linking monomer, MBAAm. For
169 point of comparison, the AAc concentration used in our previous study [23] was 50 mM
170 (corresponding to ~5% of the total monomer composition).

171

172 **2.3 Batch adsorption experiments with LF**

173 In batch binding tests, portions of settled thermoCEX matrices (0.1 mL), previously
174 equilibrated with 10 mM sodium phosphate buffer, pH 6.5, were mixed with 0.5 mL aliquots
175 of varying initial LF concentration ($c_0 = 1 - 15$ mg/mL made up in the same buffer) and
176 incubated at 10, 20, 35 or 50 °C with shaking at 100 rpm in a Thermomixer Comfort shaker
177 (Eppendorf, Hamburg, Germany) for 1 h. After an additional 0.5 h at the selected temperature
178 without shaking, the supernatants were carefully removed and analyzed for residual protein
179 content (see 2.4 Analysis). The equilibrium loadings on supports (q^*) were computed from
180 the differences in initial (c_0) and equilibrium (c^*) bulk phase protein concentrations, and the
181 resulting q^* vs. c^* data were subsequently fitted to the simple Langmuir model (Eq. (1))

182

$$183 \quad q^* = q_{\max} \frac{c^*}{K_d + c^*} \quad (1)$$

184

185 where q_{\max} and K_d are respectively, the maximum protein binding capacity of the support and
186 the dissociation constant. Data fitting was performed by SigmaPlot® software version 11
187 (Systat Software Inc., San Jose, CA, USA) using the least squares method.

188

189

190 **2.3. TCZR chromatography experiments**

191 All chromatographic experiments were conducted using Travelling Cooling Zone Reactors
192 (TCZR_s) connected to ÄKTA Purifier UPC 10 or Explorer 100 Air chromatography
193 workstations (GE Healthcare, Uppsala, Sweden) For detailed descriptions of the TCZR
194 arrangement, the reader is referred to our recent study [23]. A brief, but necessary description
195 of the workings of the system is given here. The TCZR set-up features four components, i.e.
196 (i) a temperature controlled box housing, (ii) the thermoresponsive stationary phase contained
197 in (iii) a stainless steel walled (1 mm thick) fixed-bed column (length = 10 cm; internal
198 diameter = 6 mm; volume = 2.83 mL), and (iv) a movable assembly of copper blocks and
199 Peltier elements surrounding a small discrete zone of the column. The whole cooling unit can
200 be moved up or down the column's length via a ball bearing guided linear motorized axis, and
201 by adjusting the Peltier elements, the centre of the assembly can be cooled down by >20 °C.
202 In all of the work described here, the constant surrounding temperature was 35 °C, and the
203 velocity of the TCZ assembly (v_c) was the lowest attainable in the system (0.1 mm/s),
204 generating a maximum temperature difference of 22.6 °C corresponding to a minimum
205 temperature in the centre of the column of 12.4 °C extending across 2 cm of column length.

206

207 **2.3.2 Batch mode TCZR chromatography of LF**

208 LF ($c_f = 2$ mg/mL) in an equilibration buffer of 10 mM sodium phosphate, pH 6.5 was
209 continuously applied to beds of thermoCEX media (packing factor = 1.2) until almost
210 complete breakthrough had been achieved in each case (i.e. c/c_f approaching 1). At this point
211 the LF saturated columns were then washed with 5 CVs of equilibration buffer, before
212 moving the TCZ assembly multiple times (8 times in the case of thermoCEX-CL6B and
213 thermoCEX-S6pg, and 12 times for thermoCEX-S12pg) along the full separation column's
214 length at its minimum velocity of 0.1 mm/s, generating a minimum temperature in the centre
215 of the column of 12.4 °C [23]. On completion of TCZ's last movement residually bound LF

216 was dislodged from the columns using a 1 M NaCl step gradient. Constant mobile phase
217 velocities of 30 mL/h for the small particle sized Superose based thermoCEX media, or 60
218 mL/h for the larger Sepharose CL-6B derived thermoCEX adsorbent, were employed, giving
219 rise to interstitial velocities for the packed columns of thermoCEX-S6pg, thermoCEX-S12pg
220 and thermoCEX-CL6B of 0.70 mm/s, 0.84 mm/s and 1.89 mm/s respectively. The
221 percentages of LF in each of collected peaks were calculated by dividing the LF mass eluted
222 in each by the total mass of LF recovered in all of the elution peaks.

223

224 **2.3.3 Continuous TCZR chromatography**

225 In continuous chromatography experiments, feeds of LF ($c_f = 0.5$ or 1 mg/mL) or 'LF + BSA
226 (1 mg/mL of each) in 10 mM sodium phosphate equilibration buffer were continuously fed at
227 a temperature of 35 °C, and interstitial velocity of 0.74 mm/s on to a column filled with
228 thermoCEX-S6pg. During the first 2 h of operation the TCZ remained in its parking position
229 above the separation column, by which point the protein loading front had approached ~75%
230 of the column's length. At this stage slow constant movement of the TCZ along the column
231 was initiated resulting in the first elution peak. At the applied velocity ($v_c = 0.1$ mm/s) the
232 TCZ travelled down the column at less than a seventh the rate of the interstitial mobile phase
233 velocity. On reaching the base of the column, some ~20 minutes later, the elution peak left the
234 column, and the TCZ was once again moved, at high speed, to its parking position above the
235 column. Seven further movements of the TCZ along the column's length were conducted in
236 each experiment at regular 80 minute intervals, i.e. after 200, 280, 360 440, 520, 600 and 680
237 minutes had elapsed. During the continuous application of the TCZR system, desorbed
238 proteins were fractionated after every movement of the TCZ. The fractionation started when
239 the UV adsorption at 280 nm in the effluent showed values above 100 mAu. The protein
240 peaks were collected and the protein concentration was determined. Residual bound protein
241 finally was eluted by an increase to 1 M of sodium chloride in the mobile phase. The

242 concentration factor ($CF_{Peak,i}$) of every single protein peak i was determined by dividing the
243 protein peak concentration by the feed concentration (Eq. (2)):

244

$$245 \quad CF_{Peak,i} = \frac{c_{Peak,i}}{c_f} \quad (2)$$

246

247 where $c_{Peak,i}$ is the peak concentration of the respective fractionated protein peak. An averaged
248 concentration factor (CF) was determined by calculating the average of the peak
249 concentration factors when a steady-state was reached (Eq. (3)):

250

$$251 \quad CF = \frac{\sum CF_{Peak,i}}{j} \quad (3)$$

252

253 where j is the number of fractionated protein peaks in a steady-state. By multiplying the
254 protein concentration of the fractions and the fraction volume, the eluted protein mass could
255 be calculated.

256

257 **2.4 Analysis**

258 The battery of methods employed to characterize the various thermoCEX adsorbents prepared
259 in this work, including all intermediates in their manufacture, are summarized briefly below
260 and described in detail elsewhere [23].

261

262 Reactive epoxide contents introduced by activation with epichlorohydrin were determined as
263 described by Sundberg and Porath [25].

264

265 For qualitative FT-IR analysis of solid supports, oven dried samples (~3 mg) were mixed with
266 potassium bromide (300 mg), ground down to a fine powder and hydraulically pressed (15
267 tonne) into tablet form. Each tablet was subjected to 64 scans (averaged at a resolution of 2
268 cm^{-1}) in a Nicolet 380 FT-IR (Thermo Fisher Scientific, Waltham, MA, USA) in direct beam
269 mode. Quantitative estimation of 'NIPAAm + tBAAm' consumption by supports during
270 grafting reactions, by monitoring changes in area of the characteristic peak for N-H bending
271 ($1575 - 1500 \text{ cm}^{-1}$), was performed on liquid samples (150 μL) applied directly to the surface
272 of the Nicolet 30 FT-IR's Smart 53 Orbit diamond accessory. The samples were scanned 64
273 times at a resolution of 2 cm^{-1} in attenuated total reflectance mode (ATR FT-IR).

274

275 Gravimetric analyses was used to determine the immobilized ACV and copolymer contents
276 on solid supports and also of dried residues recovered from liquid samples, to allow
277 determination of free ungrafted copolymer content and unreacted monomers remaining in
278 solution post-grafting. Free copolymers were separated from unreacted monomers by rotary
279 evaporating to dryness, resuspending in tetrahydrofuran, precipitating with diethyl ether and
280 oven drying, and unreacted monomers remaining in the supernatants were recovered by rotary
281 evaporating to dryness.

282

283 The relative amounts of NIPAAm and tBAAm in ungrafted copolymers were obtained by
284 proton NMR spectroscopic analysis of CDCl_3 dissolved samples using a Bruker AV400 NMR
285 Spectrometer (Bruker-BioSpin Corporation, Billerica, MA, USA). 'NIPAAm:tBAAm' ratios
286 (Tables 1 & 2) were computed from characteristic chemical shifts in ^1H NMR spectra at 1.15
287 ppm and 1.34 ppm for two strong methyl proton peaks arising from the NIPAAm and tBAAm
288 side chains respectively [22,23].

289

290 Full temperature dependent ‘coil–globule’ transition profiles and lower critical solution
291 temperature (LCST) values for ungrafted copolymers were obtained by monitoring the optical
292 transmittance (at $\lambda = 500$ nm) of test solutions (0.5% w/v copolymer in 10 mM sodium
293 phosphate, pH 6.5) in a Cecil CE7500 UV/visible dual beam spectrometer equipped with a
294 water thermostatted cuvette holder.

295
296 H^+ exchange capacities of supports were determined by titration using GE Healthcare’s
297 method for determination of the ionic capacity of CM Sepharose media (No. 30407). The void
298 volumes of packed beds of thermoCEX media were determined by SEC of Blue Dextran (50
299 μ L, 1 mg/mL) under non binding conditions, using an equilibrating and mobile phase of 50
300 mM Tris-HCl, pH 7.5 supplemented with 100 mM KCl.

301
302 The protein content in samples was spectrophotometrically assayed at a wavelength of 280
303 nm either off-line during batch binding experiments using a NanoDrop® ND-1000
304 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) or quartz cuvettes in a
305 Lambda 20 UV-vis spectrophotometer (PerkinElmer Analytical Instruments, Shelton, CT,
306 USA), or on-line during chromatographic investigations using ÄKTA chromatography
307 workstations operated under Unicorn™ software (GE Healthcare, Uppsala, Sweden).

308
309 The composition of fractions generated during continuous TCZR chromatographic separation
310 of LF and BSA was examined by reducing SDS-PAGE [26] in 15% (w/v) pre-cast
311 polyacrylamide gels in a Mini-Protean® Tetracell electrophoresis system (Bio-Rad
312 Laboratories, Hercules, CA, USA). After electrophoresis, gels were stained with 0.1% (w/v)
313 Coomassie Brilliant Blue R250, dissolved in 40% (v/v) ethanol and 10% (v/v) acetic acid) for
314 1 h at room temperature, and were subsequently destained at the same temperature in a
315 solution composed of 7.5% (v/v) acetic acid and 10% (v/v) ethanol. The LF and BSA contents

316 were determined by densitometric analysis of scanned TIFF images of appropriately loaded
317 Coomassie Blue stained gels following electrophoresis. The images were captured with an HP
318 ScanJet C7716A flat bed scanner (Hewlett-Packard Company, Palo Alto, CA, USA) at a
319 resolution of 2400 dpi, and analyzed using ImageJ software [27].

320

321 **3. Results and discussion**

322 **3.1 Concept of single-column continuous chromatography**

323 Schematic concentration and loading profiles within the TCZR fitted column are illustrated in
324 Fig. 2 for the various operation phases. These are characterized by different positions of the
325 TCZ relative to the column. The profiles at three different positions of the TCZ are shown,
326 i.e.: (i) outside the column (Fig. 2a); and after travelling (ii) a quarter (Fig. 2b) and (iii) three-
327 quarters (Fig. 2c) of the separation column's length. When the TCZ is parked 'outside' (Fig
328 2a), the whole column is operated at an increased temperature (T_B). Protein is continuously
329 loaded and binds to the adsorbent. In keeping with the binding strength of the protein to the
330 support at T_B , sharp concentration and loading fronts of the protein propagate through the
331 system at a constant velocity, v_{pf} (Eq. (4)):

332

$$333 \quad v_{pf} \cong \frac{u_i}{1 + \frac{1 - \varepsilon_b}{\varepsilon_b} \frac{\partial q}{\partial c}} \quad (4)$$

334

335 Here, v_{pf} is a function of the slope of the isotherm, u_i , the interstitial fluid velocity, u_i , and the
336 phase ratio between the solid and liquid phases expressed by the bed voidage, ε_b , of the
337 column packing.

338

339 When the TCZ starts to move along the column, previously bound protein desorbs at the front
340 of the zone, resulting in a strong increase in mobile phase protein concentration (Fig. 2b,
341 lower trace). Because the TCZ's velocity (v_c) is slower than that of the mobile phase (i.e. $v_c <$
342 u_i), the flow transports the desorbed protein further along the column into the adjacent region,
343 which is still at the elevated temperature, T_B . At T_B , this increased protein concentration leads
344 to a corresponding rise in the local protein loading at this point within the column. When,
345 sometime later, the constantly moving TCZ reaches this 'protein-laden' part of the column, its
346 action desorbs the bound protein, resulting in an even larger surge in mobile phase protein
347 concentration. In essence, what results within the column, provided that mass transfer
348 limitations are small, are sharp concentration and loading waves formed in front of the
349 moving TCZ. The concentration of adsorbing species will be much higher than that in the
350 feed, and protein loading closely approaches the maximum capacity of the adsorbent. With
351 progressive movement of the TCZ along the column more and more protein is desorbed at its
352 front, to continuously supply the immediately flanking high temperature (T_B) region ('over-
353 travelled' column section) with an increasing protein challenge (Fig. 2c. lower trace). Given
354 that the adsorbent's protein binding capacity cannot be exceeded (Fig. 2c, upper trace) the
355 protein concentration wave broadens (Fig. 2c, lower trace).

356

357 Closer scrutiny of the plots in Fig. 2 reveals further noteworthy features of the TCZR
358 principle highlighted as follows:

359 1. When the rate of progress of the feed concentration front along the column is slower than
360 that of the TCZ, an increasingly wide region of low protein loading and concentration forms
361 in the TCZ's wake (compare Figs 2b & 2c). The extent of reduction in protein loading and
362 concentration, and therefore in effect the operational working capacity of the TCZR, are
363 determined by the TCZ's efficiency in eluting the adsorbed protein.

364 2. When the protein wave cresting the TCZ reaches the column outlet, protein elutes in high
365 concentration. In the meantime, the protein feed is continuously applied at the other end of the
366 column (inlet), protein loading of the front section of the column attains equilibrium with the
367 feed, and once this loaded section reaches ~70% of the column length, another movement of
368 the TCZ is initiated, giving rise to a second elution peak, and so on and so forth.

369 3. Thus, by careful selection of both the velocity of the TCZ and the timing between
370 successive movements of the device, a quasi-stationary state operation should be attained,
371 where similar concentration and protein loading profiles are generated before every
372 movement of the TCZ. This confers unique capabilities on the TCZR system, namely the
373 possibility of continuously loading protein at one end of column whilst simultaneously
374 desorbing previously bound protein from the other, in a single-column installation without
375 need of additional steps of regeneration and/or equilibration.

376

377 **3.2 Manufacture and characterization of thermoresponsive CEX adsorbents**

378 Three types of beaded cross-linked agarose matrices (Sepharose CL-6B, Superose 6 Prep
379 Grade and Superose 12 Prep Grade) differing in particle diameter, agarose content and pore
380 size (see Tables 1 and 2) were fashioned into thermoCEX adsorbents in four successive steps,
381 i.e. epoxide activation, amine capping, ACV initiator immobilization and graft-from
382 polymerization using improved protocols detailed previously [23]. The initial composition of
383 monomers entering the final copolymer grafting step was systematically varied to create
384 families of thermoCEX materials with different balances of thermoresponsive, hydrophobic
385 and charged building blocks in the copolymers anchored to their exteriors and lining their
386 pores. The resulting thermoCEX adsorbents and intermediates in their manufacture (both
387 supports and reaction liquors) underwent rigorous qualitative and quantitative
388 physicochemical analysis prior to use.

389

390 FTIR spectra obtained during the stepwise conversion of Superose 6 Prep Grade into the
391 thermoCEX-S6pg adsorbent family are shown in Fig. 3. Identical sets of spectra were
392 obtained with Superose 12 Prep Grade and Sepharose CL-6B subjected to the same
393 procedures. During the various steps, the expected peaks previously assigned in converting
394 Sepharose CL-6B into a thermoresponsive cation exchanger [23], were also observed during
395 the manufacture of the Superose based thermoCEX media in this work. Of special note are: (i)
396 the growth in peak heights between 1474 and 1378 cm^{-1} in the spectrum of epoxy-activated
397 S6pg due to increased alkyl group content, consistent with incorporation of glycidyl moieties
398 into S6pg; (ii) sharpening and growth of the signal at 1378 cm^{-1} following amination of
399 epoxy-activated S6pg, likely arising from diminished flexibility, and therefore reduced
400 variance in the vibrational frequency of the CH_2 groups in the backbone; (iii) the appearance
401 of two new peaks in the FTIR spectrum of ACV immobilized S6pg (1736 cm^{-1} for carboxylic
402 acid C=O stretching, and 1552 cm^{-1} for azo N=N stretching and/or amide N-H bending); the
403 growth of (iv) amide N-H bending (1570 cm^{-1}) and amide C=O stretching (1670 cm^{-1})
404 contributions from incorporated NIPAAm, tBAAm and MBAAm units, and (v) of carboxylic
405 acid C=O stretching (1736 cm^{-1}), arising from the presence of AAc in the grafted copolymer
406 on the thermoCEX supports; and finally that (vi) despite marked differences in the
407 compositions of grafted copolymers on thermoCEX supports B1-B4 (see Table 2), their FTIR
408 spectra appear identical.

409

410 Analysis of the immobilized copolymer compositions of Sepharose CL-6B and Superose
411 based thermoCEX (Tables 1 and 2 respectively) illustrates marked differences. Under
412 identical reaction conditions, unique monomer consumption preferences appear to be
413 displayed by the different ACV-coupled supports. Compare supports 'A2', 'B2' and 'C1' for
414 example. When normalized against the initial monomer composition entering polymerization
415 reactions with ACV immobilized supports, ACV-immobilized Sepharose CL-6B consumed

416 tBAAm and AAc roughly equally (32.5% *cf.* 30.4%), and ~2.4 times more readily than
417 NIPAAm (13.2%); by contrast, ACV-coupled S6pg consumed tBAAm (33.5%) in >1.8 fold
418 preference to both AAc (19.0%) and NIPAAm (18.7%), whereas ACV-linked S12pg
419 consumed tBAAm (39.2%) ~1.5 and ~2.2 fold more readily than AAc (25.4%) and NIPAAm
420 (17.6%) respectively. Thus, despite the higher initial epoxide density (878 $\mu\text{mol/g}$) driving
421 increased ACV immobilization (568 $\mu\text{mol/g}$) and ~1.4 fold higher mass of grafted copolymer,
422 the ionic capacity of the thermoCEX-S6pg was 38% lower than that on thermoCEX-CL6B
423 (i.e. 293 *cf.* 469 $\mu\text{mol/g}$ dried support), whereas its NIPAAm and tBAAm contents had both
424 increased (by 42% and 3% respectively). We observed this phenomenon previously during
425 fabrication of thermoCEX adsorbents fashioned from ostensibly very similar cross-linked 6%
426 agarose media, and suggested that differences were likely linked to the epoxide densities
427 introduced in the first synthetic step, rather than to subtle chemical disparities between the
428 two base matrices [23]. Based on findings in this study with 15 support materials, i.e. 9
429 finished thermoCEX and 6 intermediates in their manufacture (Tables 1 & 2), we no longer
430 believe this to be the case. Though sharing agarose backbones and being subjected to identical
431 polymer modification reactions, it appears here that differences in proprietary modification
432 (principally cross linking) of the base matrix starting materials [29-33] influence the initial
433 epoxy activation level, subsequent immobilized initiator density, loading and composition of
434 grafted copolymer of the finished thermoCEX adsorbents.

435

436 Sepharose CL-6B is prepared by first reacting Sepharose 6B with 2,3-dibromopropanol under
437 strongly alkaline conditions and then desulphating post cross-linking, by reducing alkaline
438 hydrolysis, to give a cross-linked matrix with high hydrophilicity and very low content of
439 ionizable groups [30,33]. In the manufacture of Superose media, cross-linking to confer
440 rigidity occurs in two stages, i.e. initial priming reaction with a cocktail of long-chain bi- and
441 poly- functional epoxides in organic solvent, followed by cross-linking via short-chain bi-

442 functional cross-linkers conducted in aqueous solvent [30,32]. Superose media are thus less
443 hydrophilic than Sepharose CL supports, and it is this fundamental difference that likely: (i)
444 contributes to the 30 – 50% greater number of immobilized oxiranes introduced into Superose
445 matrices (Table 2, 878 $\mu\text{mol/g}$ for S6pg, 1018 $\mu\text{mol/g}$ for S12pg) by epichlorohydrin
446 activation (step 1) under identical conditions *cf.* Sepharose CL-6B (Table 1, 662 $\mu\text{mol/g}$);
447 leads in turn (ii) both to higher immobilized ACV contents (568 – 611 $\mu\text{mol/g}$ for S6pg, 878
448 $\mu\text{mol/g}$ for S12pg *cf.* 380 $\mu\text{mol/g}$ for Sepharose CL-6B) and elevated grafted polymer yields
449 ($6005 \pm 68 \mu\text{mol/g}$ for ThermoCEX-Superose adsorbents *cf.* $4675 \pm 97 \mu\text{mol/g}$ for
450 ThermoCEX-CL6B); and (iii) significantly higher incorporation of the charged AAc
451 monomer into the grafted copolymers on ThermoCEX-CL6B *cf.* ThermoCEX adsorbents
452 fashioned from Superose media across all AAc input concentrations during grafting (see
453 Tables 1 and 2).

454
455 The temperature dependent phase transition behaviour of ungrafted free copolymer solutions
456 emanating from various grafting reactions with ACV-immobilized Sepharose CL-6B and
457 Superose Prep Grade are compared in Fig. 4. Figures 4a and 4b display the raw transmittance
458 vs. temperature profiles, and Fig. 4c examines the wider impact of NIPAAm replacement on
459 the LCST and full transition temperature ranges of the copolymers.

460
461 In accord with literature reports [12-14], the LCST at 50% optical transmittance ($T_{50\%}$) for the
462 ‘smart’ homopolymer pNIPAAm was 32.3 °C and sharp transition from fully extended
463 ‘hydrophilic coil’ ($T_{90\%}$) to fully collapsed ‘hydrophobic globule’ ($T_{0.4\%}$) occurred between 31
464 and 35 °C (dashed line traces in Figs 4 a-c). Copolymerizing NIPAAm with more
465 hydrophobic monomers leads to a reduction in the LCST [34,35], whereas incorporation of
466 more hydrophilic species increases it [34]. Here, simultaneous low level substitution of AAc

467 and tBAAm into pNIPAAm's backbone ('A1', 'B1' & 'C1') at the expense of 12.1 to 16.9%
468 of its NIPAAm content (Tables 1 & 2), had the effect of lowering the LCST (by 1.4 °C for
469 'A1', 2.3 °C for 'B1', 1.5 °C for 'B2' and 0.9 °C for 'C1') and broadening the transition
470 temperature range in both directions (i.e. 28.3 to 36.7 °C for 'A1', 26.5 to 34.8 °C for 'B1',
471 26.5 to 37.7 °C for 'B2', and 28.5 to 36.8 °C for 'C1'). With mounting NIPAAm replacement
472 ('A3', 'A4', 'B3', 'B4'), smart thermoresponsive behaviour became increasingly
473 compromised. For example, the LCST for the '67.3:15.8:16.9' copolymer (Fig. 4d, 'A4')
474 reached 36.6 °C and temperature range over which full 'coil – globule' transition range
475 extended >25 °C, i.e. from 20.5 to 46 °C.

476

477 **3.3 Temperature dependent adsorption of LF on thermoCEX adsorbents**

478 The optimum condition for an effective thermoresponsive adsorbent is one where the binding
479 of a given target is strongly temperature dependent; in the ideal case being effectively
480 switched 'on' (powerful adsorption) and 'off' (no adsorption) by a small change in bulk phase
481 temperature across the LCST of the thermoresponsive copolymer. In practice this has not
482 been achieved, i.e. the sharp 'coil – globule' transitions observed in free solution are not
483 mirrored by protein binding vs. temperature plots [22,23]. The collapse and extension of
484 surface-anchored thermoresponsive copolymer chains is considerably more constrained [36]
485 and complex [23,36,37] than that of the free untethered species. As a consequence, changes to
486 the binding interface in response to a thermal trigger are gradual in nature.

487

488 The effect of temperature on the maximum LF adsorption capacity (q_{max}) for all nine
489 thermoCEX supports is illustrated in Fig. 5. In all cases, q_{max} rose linearly with increase in
490 temperature over the examined range (10 – 50 °C), as the tethered copolymer networks
491 gradually transitioned from predominantly hydrophilic and fully extended low charge density
492 states (Fig. 1, bottom right) to increasingly flattened, hydrophobic and highly charged ones

493 (Fig. 1, top right), as the distance between neighboring charged AAc units within the
494 collapsed copolymer decreased and previously shielded/buried negative charges became
495 exposed [22]. However, the degree of thermoresponsiveness exhibited varied significantly
496 between and within each thermoCEX family, and a clear trend emerged. For the least
497 substituted supports in the Sepharose CL-6B and Superose 6pg thermoCEX families, i.e. ‘A1’
498 (Fig. 5a, Table 1) and ‘B1’ (Fig. 5b, Table 2), LF binding capacity was strongly temperature
499 dependent ($q_{max, 50^{\circ}\text{C}} / q_{max, 10^{\circ}\text{C}}$ ratios of 2.2 for ‘A1’ & 3.3 for ‘B1’), but low ($q_{max, 50^{\circ}\text{C}}$ values
500 of <22 mg/mL for ‘B1’ and <27 mg/mL for ‘A1’). With increasing NIPAAm replacement by
501 hydrophobic tBAAm and negatively charged AAc monomers (\rightarrow ‘A2’ \rightarrow ‘A3’ \rightarrow ‘A4’, Fig. 5a;
502 \rightarrow ‘B2’ \rightarrow ‘B3’ \rightarrow ‘B4’; Fig. 5b), LF binding increased dramatically ($q_{max, 50^{\circ}\text{C}}$ rising to ~52
503 mg/mL for ‘B4’ and ~83 mg/mL for ‘A4’), but became increasingly temperature independent
504 ($q_{max, 50^{\circ}\text{C}} / q_{max, 10^{\circ}\text{C}}$ ratios of 1.06 for A4 & 1.1 for ‘B4’), as the ability of the tethered
505 copolymers to transition between extended and collapsed states was lost. ThermoCEX
506 supports with moderate levels of NIPAAm replacement, i.e. ‘A2’, ‘B2’ & ‘C1’, displayed the
507 best combination of thermoresponsiveness and LF binding capacity (i.e. reasonable $q_{max, 50^{\circ}\text{C}}$
508 and low $q_{max, 10^{\circ}\text{C}}$ values), and were therefore selected for further study.

509

510 Fig. 6 shows adsorption isotherms obtained for the binding of LF to these at different
511 temperatures, and Table 3 presents the fitted Langmuir parameters. At the lowest temperature
512 of 10 °C, the binding of LF to all three thermoCEX adsorbents is rather weak (K_d values
513 between 0.82 and 4.8 mg/mL) and of low capacity (q_{max} values <17 mg/mL), but as the
514 temperature is gradually stepped up, the tightness and capacity of LF sorption rise strongly.
515 At the highest temperature of 50 °C, the paired values for q_{max} and tightness of binding (initial
516 slope, q_{max}/K_d) are 55.9 mg and 631 for support ‘A2’, 28.6 mg/mL and 168 for ‘B2’, and 44.7
517 mg/mL and 213 for support ‘C1’ (Table 3). An identical pattern of behaviour in response to
518 temperature (between 20 and 50 °C) was noted previously by Maharjan et al. [22] for a

519 thermoCEX support fashioned out of Sepharose 6 FF. The ranking of static LF binding
520 performance of this family of thermoCEX supports of ‘Sepharose CL-6B > Superose 12 Prep
521 Grade > Superose 6 Prep Grade > Sepharose 6 FF’ (initial slope values at 50 °C of 610, 213,
522 168 and 120 respectively) is not correlated with mass of copolymer attached (respectively
523 4177, 5495, 5733, 2060 $\mu\text{mol/g}$ dried support), nor the tBAAm content (respectively 501,
524 604, 516, 165 $\mu\text{mol/g}$ dried support), but rather with the support’s intrinsic ionic capacity (i.e.
525 469 μmol > 391 μmol > 293 μmol > 154 $\mu\text{mol H}^+/\text{g}$ dried support).

526

527 Figure 7 compares chromatographic profiles arising from batch TCZR chromatography of LF
528 on fixed beds of three thermoCEX manufactured under identical conditions from cross-linked
529 agarose base matrices differing in particle diameter and pore size (see Tables 1 & 2), i.e.
530 thermoCEX-CL6B ‘A2’ (Fig. 7a), thermoCEX-S6pg ‘B2’ (Fig. 7b) and thermoCEX-S12pg
531 ‘C1’ (Fig. 7c). A striking similarity is instantly evident, namely that every movement of the
532 TCZ results in a sharp LF elution peak. Of greater importance, however, are the differences.
533 Following 8 movements of the TCZ along beds of the thermoCEX-CL6B ‘A2’ (Fig. 7a) and
534 thermoCEX-S6pg ‘B2’ (Fig. 7b), the percentages of eluted LF recovered in peaks ‘a’ to ‘h’
535 combined were practically the same, i.e. 64.6% of that initially bound for thermoCEX-CL6B
536 ‘A2’ *cf.* 67.1% for thermoCEX-S6pg ‘B2’. But whereas just over half (54.4%) of the
537 thermally eluted LF from all 8 peaks (‘a – h’) was recovered by the first movement of the
538 TCZ (peak ‘a’) along the thermoCEX-CL6B ‘A2’ column (Fig. 7a), peak ‘a’ accounted for
539 more three-quarters (76.6%) of the combined thermally eluted LF (a – h inclusive) from
540 thermoCEX-S6pg ‘B2’ (Fig. 7b). Twelve movements of the TCZ were employed during
541 TCZR chromatography on thermoCEX-S12pg ‘C1’. In this case, eluted LF recovered in peaks
542 ‘a – l’ was just 58.3%, and the first movement of the TCZ accounted for only 17.5% of
543 combined thermally eluted LF (‘a – l’).

544

545 Achieving significantly higher LF desorption with a single movement of the TCZ, i.e.
546 approaching the 100% level, would require the absence of both mass transport limitations and
547 LF binding at the minimum column temperature of 12.4 °C. Clearly, neither condition
548 applies. Inspection of Fig. 6 and Table 3 confirm that LF is still able to bind to the
549 thermoCEX media at 10 °C albeit weakly, thus at 12.4 °C in the TCZR system some LF will
550 inevitably remain bound. Further, evidence of significant limitations on mass transport during
551 TCZR mediated elution (though noticeably less marked for thermoCEX-S6pg ‘B2’) is
552 provided by the observation that LF elution continued up to and including the last TCZ
553 movement for all three matrices (Fig. 7). Because the total amounts of LF eluted on
554 approaching equilibrium (after 8 movements of the TCZ) were essentially the same for
555 thermoCEX-CL6B ‘A2’ and thermoCEX-S6pg ‘B2’, the lower LF binding strength of
556 thermoCEX-S6pg ‘B2’ cannot adequately explain its improved LF recovery following the
557 first TCZ movement (peak ‘a’ in Figs 7 a & b). Instead, differences in particle size and pore
558 diameter (highlighted in Tables 1 & 2) manifested in form of pore diffusion limitation, likely
559 account for the significant disparity in % LF desorption observed following the first TCZ
560 movement along packed beds of the three different thermoCEX media, i.e. 76.6% for
561 thermoCEX-S6pg ‘B2’ *cf.* 55.4% for thermoCEX-CL6B ‘A2’ *cf.* 17.5% for thermoCEX-
562 S12pg ‘C1’.

563

564 Consider the case of a target protein adsorbed close to the centre of a thermoCEX support
565 particle. If such a species is to be desorbed by the temperature change effected by the TCZ, it
566 must diffuse out of the support particle’s pores and into the mobile phase to be eluted from
567 the column. However, should the time required for this diffusion process be greater than the
568 TCZ’s contact time with the region of the column where the support resides, the target protein
569 will re-adsorb en route and hence will not contribute to the elution peak. The advantage of
570 smaller adsorbent particle diameters and adequately large pores for TCZR application with

571 protein adsorbates is therefore clear. Smaller particles dictate shorter diffusion paths, while
572 large pores provide less of an impediment to mass transfer of large macromolecules [38]. This
573 combination leads to reduced times for the diffusion process, culminating practically in fewer
574 numbers of movements of the TCZ to achieve a desired target desorption yield, and is
575 displayed best in this work by the Superose 6 Prep Grade based thermoCEX matrix.

576

577 **3.4 Continuous protein accumulation experiments**

578 In our first series of continuous TCZR chromatography experiments, the influence of the
579 target protein concentration (c_f) on total system performance during continuous feeding and 8
580 movements of the TCZ along the column was examined using LF as the model binding
581 component and thermoCEX-S6pg 'B2' as the column packing material. Chromatograms
582 corresponding to continuous feeding of LF at 0.5 mg/mL and 1 mg/mL are shown in Figs 8a
583 and 8b respectively. In both cases, residual bound LF not desorbed by the TCZ was eluted
584 after the last movement by raising the mobile phase's ionic strength.

585

586 Visual comparison of the 8 individual eluted peaks ($a - g$) within each chromatogram
587 suggests a certain time is required before the profiles and peak areas become uniform (i.e. 4
588 movements at $c_f = 0.5$ mg/mL and 3 movements at $c_f = 1$ mg/mL), and the quantitative
589 analysis in Table 4 confirms this. Quasi-stationary states are effectively reached from peaks
590 ' d ' ($c_f = 0.5$ mg/mL) and ' c ' ($c_f = 1$ mg/mL) onwards, where the mass of LF eluted in each
591 peak remains essentially constant (i.e. 14.2 ± 2.1 mg and 30.7 ± 1.3 mg for the low and high
592 LF feed concentrations respectively) and small traces of LF are lost in the flowthrough
593 between successive individual elution peaks (i.e. averages of 1.5 and 2.6 mg for low and high
594 LF feed conditions respectively). The observation that steady state was reached later, when
595 feeding the lower strength LF feed, merits explanation. Attaining a quasi-stationary condition
596 is only possible after the TCZ has completed its transit and the protein loading across the full

597 column length approaches equilibrium. Under such circumstances, the amount of protein
598 temporarily loaded at the elevated temperature T_B , (with the TCZ parked outside, Fig. 2a) will
599 be constant. From this it follows that raising the protein concentration in the feed should
600 speed the acquisition of a steady state.

601
602 Another important parameter influenced by the protein concentration in feed is averaged
603 concentration factor, CF , (Eq. (3)) attainable during steady state operation. The CF reached
604 9.2 at $c_f = 0.5$ mg/mL (Fig. 8a), but dropped to 7.9 on increasing the LF concentration in the
605 feed twofold (Fig. 8b). An explanation for the slight reduction in CF with increasing LF
606 concentration can be found in the isotherm describing LF adsorption to thermoCEX-S6pg
607 'B2' at 35 °C (Fig. 6b); i.e. isotherm starts to become non-linear between 0.5 and 1 mg LF per
608 mL.

609

610 **3.5 Continuous separation of a binary protein mixture**

611 The ability of TCZR to function continuously having been established (Fig. 8, Table 4), the
612 next step was to test TCZR's feasibility to not only accumulate and concentrate a single target
613 protein, but to continuously separate it from a protein mixture. For this we employed a simple
614 binary protein mixture consisting 1 mg/mL LF and 1 mg/mL BSA in a 10 mM sodium
615 phosphate pH 6.5 buffer. At this pH the binding species LF carries an overall cationic charge,
616 whereas the BSA is negatively charged. The experiment was conducted in a similar manner
617 to the LF accumulation study reported above (Section 3.4, Fig. 8), and Fig. 9 shows the
618 chromatogram obtained. Shortly after applying the protein mix to the column, the UV signal
619 stepped steeply to ~350 mAU where it remained (first flowthrough pooled 'a') until directly
620 after the first of 8 individual movements of the TCZ (highlighted by shaded gray bars). Each
621 TCZ movement led immediately to a sharp (>2750 mAU) symmetrical elution peak, and the
622 UV signal of the flowthrough between each temperature mediated elution peak rapidly

623 returned to roughly constant threshold of ~500 mAU. Following the 8th TCZ motion,
624 residually bound protein was desorbed from the column by a step change in ionic strength (S).
625
626 SDS-PAGE analysis of the feed (F), pooled flowthrough (*c, e, g, i, k, m, o & q*), peak (*b, d, f,*
627 *h, j, l, n & p*) and salt-stripped (S) fractions corresponding to the chromatogram in Fig. 9 is
628 presented in Fig. 10. Commercial BSA and LF were employed in this work, and neither
629 protein was subjected to further purification prior to use. Thus, the binary 'LF+BSA' mixture
630 used actually contained many additional species present in trace quantities. Cation exchange
631 chromatography had been employed as the main purification step for the ~96% pure LF, and
632 all but one contaminant species observable in Fig. 10 (esp. noticeable in pool 'S') emanates
633 from this preparation. Only two species within the BSA preparation are observed in
634 Coomassie Blue stained electrophoretograms following SDS-PAGE, i.e. the 66.4 kDa
635 monomer accounting for >98% of the BSA content, and a much lower intensity 130.5 kDa
636 dimer contaminant. The intensities of the lower migrating BSA monomer and upper dimer
637 species remain constant across all flowthrough and peak fractions indicating their continued
638 presence in the mobile phase throughout the run. The early UV signal surge to 350 mAU in
639 Fig. 9 (flowthrough pool 'a') is primarily due to breakthrough of BSA (see Fig. 10, only a
640 small percentage of the feed's LF is noted in pool 'a'); the sharp strong peaks, on the other
641 hand, arise from the accumulation on and subsequent temperature mediated elution of LF
642 along with small traces of numerous contaminants of the LF preparation (Fig. 10) from the
643 adsorbent bed. The increase in UV signal from 350 mAU for the flowthrough pool 'a' to 500
644 mAU for all inter peak flowthroughs (pools *c, e, g, i, k, m, o, & q*) is due to LF leakage. The
645 addition of 1 mg/mL BSA to the 1 mg/mL LF feed did not disturb the time taken to attain
646 quasi-stationary state; in both cases this was reached from the third peak on (compare Figs 8b
647 and 9). However, the average eluted LF mass per peak and concentration factors were
648 significantly lower (i.e. 21.4 mg *cf.* 30.7 mg & *CF* = 6.3 *cf.* 7.9), and the loss of LF in the

649 flowthrough between successive elution peaks was much higher (12.2 *cf.* 2.6 mg).
650 Nevertheless, the mean purity of LF in eluted peaks was ~86%.

651

652 Reasons for impaired LF recovery in the presence of BSA are presently unclear. The presence
653 of small amounts of BSA in the NaCl stripped pool 'S' (Fig. 10) illustrates that a tiny fraction
654 of applied BSA had been adsorbed sufficiently strongly to resist desorption by 8 movements
655 of the TCZ. The occurrence of BSA in the strip fraction 'S' and unbound fractions (pools *a, c,*
656 *e, g, i, k, m, o, & q*) raises the possibility of two or more distinct BSA species, i.e. those that
657 electrostatically repelled, and others that bind strongly and possibly unfold and spread on the
658 thermoCEX matrix at 35 °C. Such a scenario could occur were the distribution of exposed
659 hydrophobic and charged monomers within the collapsed copolymer non-uniform, such that
660 highly hydrophobic clusters or islands are created occupying a few percent of the overall
661 binding surface. In this instance a slight drop in the adsorbent's LF binding capacity, but not
662 in its LF binding affinity, would be anticipated.

663

664 A more satisfactory explanation for the reduction in both LF binding strength and capacity in
665 the presence of BSA is fouling of binding interface by the lipids it carries. Long-chain free
666 fatty acids (FFAs) are found in many bioprocess liquors (e.g. fermentation broths), but their
667 influence on fouling of chromatography media and membrane units has gone largely ignored
668 owing to their low concentrations and poor solubility [39,40]. Serum albumin's principal role
669 *in vivo* is to bind otherwise insoluble long-chain fatty acids released into the blood from
670 adipose cells and transport them within circulating plasma, and the effectiveness with which it
671 binds FFAs is highlighted by the fact that the solution concentration of a given long-chain
672 FFA can be increased as much 500 fold in its presence [41]. To date, most chromatographic
673 studies with BSA have employed commercial preparations substantially pure with respect to
674 protein, but not free of lipids. Procedures for delipidation of serum albumin usually involve

675 extraction with organic phases at elevated temperatures or sorption of free fatty acids (FFAs)
676 onto activated charcoal at elevated temperature under acidic–neutral conditions [41,42].
677 Under the operating conditions employed here, i.e. mobile phase of pH 6.5, temperature of 35
678 °C, FFA binding to BSA is weak [42] and FFA solubility is comparatively high [40]; it is
679 conceivable that FFAs bind to and foul the copolymer binding surface, thereby reducing both
680 the adsorbent's binding affinity and occupancy for LF.

681

682 **4. Conclusions**

683 The fabrication and detailed characterisation of porous beaded thermoCEX adsorbents
684 varying in particle size, pore dimensions and grafted poly(*N*-isopropylacrylamide-*co*-*N*-tert-
685 butylacrylamide-*co*-acrylic acid) composition has been done for two main reasons, i.e. to (i)
686 challenge an earlier hypothesis [23] that intra-particle diffusion of desorbed protein out of the
687 support pores is the main parameter affecting TCZR performance; and (ii) identify an
688 effective adsorbent customized for TCZR chromatography. ThermoCEX matrices with
689 moderate levels of NIPAAm replacement (by *N*-tert-butylacrylamide and acrylic acid)
690 displayed the best combination of thermoresponsiveness and LF binding capacity. Head-to-
691 head batch TCZR chromatography tests with LF and three such materials confirmed the
692 advantage of small particles with adequately sized pores, namely faster diffusion leading to
693 fewer numbers of TCZ movement to attain a set desorption yield.

694

695 Chromatographic separations of proteins are typically performed in batch mode requiring
696 sequential steps of equilibration, loading, washing elution and regeneration. Unless fully
697 optimized, a common feature is inefficient use of the separation medium. The switch from
698 batch to continuous operation promises several advantages, key of which is more efficient
699 utilization of the bed [43]. Several continuous chromatography formats have been developed
700 and applied for the separation of biomacromolecules thus far, including Continuous Annular

701 Chromatography [44], Continuous Radial Flow Chromatography [43], Simulated Moving Bed
702 in various guises [45-49], and Periodic Counter-current Chromatography [50]. The new
703 addition described here, Continuous Travelling Cooling Zone Reactor Chromatography,
704 employs a single column operated isocratically. The simplicity of its configuration
705 notwithstanding, the main benefits over batchwise operation include reduced solvent and
706 buffer component usage, time savings and increased productivity. The continuous steady state
707 accumulation on and regular cyclic elution of the thermostable basic protein, LF, from a fixed
708 bed of a thermoresponsive cation exchange adsorbent in the form of sharp uniformly sized
709 peaks has been demonstrated in this work. The time required to reach quasi steady state
710 operation and the degree of concentration attained on TCZ mediated elution appear inversely
711 related to the concentration of LF being continuously supplied to the bed. The addition of the
712 non-binding species, BSA, to the LF feed had unexpectedly deleterious effects on lactoferrin
713 accumulation and recovery; the latter being tentatively attributed to fouling of the thermoCEX
714 matrix by lipids carried into the feed by serum albumin. No evidence of temperature induced
715 protein unfolding during TCZR chromatography was observed in the current study, but it
716 remains a potential problem, especially for more thermolabile proteins. Non-NIPAAm based
717 thermoresponsive polymers tuned to transition at lower temperatures should mitigate this
718 concern [23,51].

719

720 Currently, the primary limiters on TCZR system throughput come from the necessary use of
721 low flow rates – a direct consequence of the prevailing mass transfer limitations. In the
722 present example the sorbate, LF, must diffuse into and back out of individual thermoCEX
723 adsorbent beads within a period of ~3 minutes. A small particle diameter combined with
724 sufficiently large pores is necessary in this instance (hence Superose 6 Prep Grade's
725 superiority over Sepharose CL-6B and Superose 12 Prep Grade), and the magnitude of the
726 interstitial fluid velocity is practically constrained to ~1 mm/s (i.e. ~10 times the TCZ's

727 minimum speed of 0.1 mm/s). It should be possible to overcome the above issues by using
728 thermoresponsive adsorbents fashioned from either more pressure tolerant smaller uniformly
729 sized support particles with similar pore dimensions, or monolithic materials [51-53]. Future
730 work on TZCR will explore this tenet.

731

732 **Acknowledgements**

733 This work was funded by the European Framework 7 large scale integrating collaborative
734 project ‘Advanced Magnetic nano-particles Deliver Smart Processes and Products for Life’
735 (MagPro²Life, CP-IP 229335-2).

736

737 **References**

738 [1] S.C. Goheen, B.M. Gibbins, Protein losses in ion-exchange and hydrophobic interaction
739 high-performance liquid chromatography, *J. Chromatogr. A* 890 (2000) 73 – 80.

740 [2] J.A. Asenjo, B.A. Andrews, Protein purification using chromatography: selection of type,
741 modelling and optimization of operating conditions, *J. Molec. Recognit.* 22 (2009) 65 – 76.

742 [3] G. Guiochon, Preparative liquid chromatography, *J. Chromatogr. A* 965 (2002) 129 – 161.

743 [4] Y. Shi, R. Xiang, C. Horvath, J.A. Wilkins, The role of liquid chromatography in
744 proteomics, *J. Chromatogr. A* 1053 (2004) 27 – 36.

745 [5] C.J. Venkatramani, Y. Zelechok, An automated orthogonal two-dimensional liquid
746 chromatograph, *Anal. Chem.* 75 (2003) 3484 – 3494.

747 [6] D. Haidacher, A. Vailaya, C. Horváth, Temperature effects in hydrophobic interaction
748 chromatography, *Proc. Nat. Acad. Sci.* 93 (1996) 2290 – 2295.

749 [7] S. Hjertén, Some general aspects of hydrophobic interaction chromatography, *J.*
750 *Chromatogr.* 87 (1973) 325 – 331.

751 [8] J.A. Queiroz, C.T. Tomaz, J.M.S. Cabral, Hydrophobic interaction chromatography of
752 proteins, *J. Biotechnol.* 87 (2001) 143 – 159.

- 753 [9] R. Muca, W. Piatkowski, D. Antos, Altering efficiency of hydrophobic interaction
754 chromatography by combined salt and temperature effects, *J. Chromatogr. A* 1216 (2009)
755 6716-6727.
- 756 [10] R. Muca, W. Piatkowski, D. Antos, Effects of thermal heterogeneity in hydrophobic
757 interaction chromatography, *J. Chromatogr. A* 1216 (2009) 8712 – 8721.
- 758 [11] L. Taylor, D. Cerankowski, Preparation of films exhibiting a balanced temperature
759 dependence to permeation by aqueous solutions – a study of lower consolute behavior, *J.*
760 *Polym. Sci., Part A: Polym. Chem.* 13 (1975) 2551 – 2570.
- 761 [12] M. Heskins, J.E. Guillet, Solution properties of poly(N-isopropylacrylamide), *J.*
762 *Macromolec. Sci. A* 2 (1968) 1441 – 1455.
- 763 [13] K. Kubota, S. Fujishige, I. Ando, Single-chain transition of poly(N-isopropylacrylamide)
764 in water, *J. Phys. Chem.*, 94 (1990) 5154 – 5158.
- 765 [14] H.G. Schild, Poly(N-isopropylacrylamide): Experiment, theory and application, *Prog.*
766 *Polym. Sci.* 17 (1992) 163 – 249.
- 767 [15] P. Maharjan, B.W. Woonton, L.E. Bennett, G.W. Smithers, K. DeSilva, M.T.W. Hearn,
768 Novel chromatographic separation – The potential of smart polymers, *Innov. Food Sci.*
769 *Emerg. Technol.* 9 (2008) 232 – 242.
- 770 [16] I.Y. Galaev, B. Matthiasson, 'Smart' polymers and what they could do in biotechnology
771 and medicine, *Trends Biotechnol.* 17 (1999) 335 – 340.
- 772 [17] A.S. Hoffman, P. Stayton, Bioconjugates of smart polymers and proteins: synthesis and
773 applications, *Macromol. Symp.* 207 (2004) 139 – 151.
- 774 [18] N. Matsuda, T. Shimizu, M. Yamato, T. Okano, Tissue Engineering based on cell sheet
775 technology, *Adv. Mater.* 19 (2007) 3089 – 3099.
- 776 [19] K. Hoshino, M. Taniguchi, T. Kitao, S. Morohashi, T. Sasakura, Preparation of a new
777 thermo-responsive adsorbent with maltose as a ligand and its application to affinity
778 precipitation, *Biotechnol. Bioeng.* 60 (1998) 568 – 579.

779 [20] H. Kanazawa, K. Yamamoto, Y. Matsushima, N. Takai, A. Kikuchi, Y. Sakurai, T.
780 Okano, Temperature-responsive chromatography using poly(N-isopropylacrylamide)-
781 modified silica, *Anal. Chem.* 68 (1996) 100 – 105.

782 [21] K. Yamamoto, H. Kanazawa, Y. Matsushima, T. Nobuhara, A. Kikuchi, T. Okano,
783 *Chromatogr.* 21 (2000) 209 – 215.

784 [22] P. Maharjan, M.T. Hearn, W.R. Jackson, K. De Silva, B.W. Woonton, Development of a
785 temperature-responsive agarose-based ion-exchange chromatographic resin, *J. Chromatogr. A*
786 1216 (2009) 8722 – 8729.

787 [23] T.K.H. Müller, P. Cao, S. Ewert, J. Wohlgemuth, H. Liu, T.C. Willett, E. Theodosiou,
788 O.R.T. Thomas, M. Franzreb, Integrated system for temperature-controlled fast protein liquid
789 chromatography comprising improved copolymer modified beaded agarose adsorbents and a
790 travelling cooling zone reactor arrangement, *J. Chromatogr. A* 1285 (2013) 97 – 109.

791 [24] N.S. Terefe, O. Glagovskaia, K. De Silva, R. Stockmann, Application of stimuli
792 responsive polymers for sustainable ion exchange chromatography, *Food Bioprod. Process.*
793 92 (2014) 208 – 225.

794 [25] L. Sundberg, J. Porath, Preparation of adsorbents for biospecific affinity
795 chromatography: I. Attachment of group-containing ligands to insoluble polymers by means
796 of bifunctional oxiranes, *J. Chromatogr.* 90 (1974) 87 – 98.

797 [26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of
798 Bacteriophage T4, *Nature (London)* 227 (1970) 680 – 685.

799 [27] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image
800 analysis, *Nat. Methods* 9 (2012) 671 – 675.

801 [28] M.D. Oza, R. Meena, K. Prasad, P. Paul, A.K. Siddhanta, Functional modification of
802 agarose: A facile synthesis of a fluorescent agarose–guanine derivative, *Carbohydr. Polym.* 81
803 (2010) 878 – 884.

804 [29] J. Porath, J.-C. Janson, T Låås, Agar derivatives for chromatography, electrophoresis and
805 gel-bound enzymes, *J. Chromatogr.* 60 (1971) 167 – 177.

806 [30] T. Andersson, M. Carlsson, L. Hagel, P.-A. Pernemalm, J.-C. Janson, Agarose-based
807 media for high-resolution gel filtration of biopolymers, *J. Chromatogr.* 326 (1985) 33 – 44.

808 [31] G.E.S. Lindgren, Method of cross-linking a porous polysaccharide gel, US Patent
809 4,973,683, Publication date: 11/27/1990.

810 [32] P.A. Pernemalm, M. Carlsson, G. Lindgren, Separation material and its preparation,
811 European Patent EP 0132244, Publication date: 12/17/1986.

812 [33] M. Andersson, M. Ramberg, B.-L. Johansson, The influence of the degree of cross-
813 linking, type of ligand and support on the chemical stability of chromatography media
814 intended for protein purification, *Process Biochem.* 33 (1998) 47 – 55.

815 [34] A.S. Hoffman, P. Stayton, V. Bulmus, G. Chen, J. Chen, C. Cheung, A. Chilkoti, Z.
816 Ding, L. Dong, R. Fong, C.A. Lackey, C. J. Long, M. Miura, J.E. Morris, N. Murthy, Y.
817 Nabeshima, T.G. Park, O.W. Press, T. Shimoboji, S. Shoemaker, H.J. Yang, N. Monji, R.C.
818 Nowinski, C.A. Cole, J.H. Priest, J.M. Harris, K. Nakamae, T. Nishino, T. Miyata, Really
819 smart bioconjugates of smart polymers and receptor proteins, *J. Biomed. Mater. Res.* 52
820 (2000) 577 – 586.

821 [35] Y. Yoshimatsu, B.K. Lesel, Y. Yonamine, J.M. Beierle, Y. Hoshino, K.J. Shea,
822 Temperature-responsive “catch and release” of proteins by using multifunctional polymer-
823 based nanoparticles, *Angew. Chem. Int. Ed.* 51 (2012) 2405 – 2408.

824 [36] M. Andersson, S. Hietala, H. Tenhu, S.L. Maunu, Polystyrene latex particles coated with
825 crosslinked poly(N-isopropylacrylamide), *Colloid Polym. Sci.* 284 (2006) 1255 – 1263

826 [37] K.N. Plunkett, Z., Xi, J.S. Moore, D.E. Leckband, PNIPAM chain collapse depends on
827 the molecular weight and grafting density *Langmuir* 22 (2006) 4259 – 4266.

828 [38] J.F. Langford, M.R. Schure, Y. Yao, S.F. Maloney, A.M. Lenhoff, Effects of pore
829 structure and molecular size on diffusion in chromatographic adsorbents, *J. Chromatogr. A*
830 1126 (2006) 95 – 106.

831 [39] J. Jin, S. Chhatre, N.J. Titchener-Hooker, D.G. Bracewell, Evaluation of the impact of
832 lipid fouling during the chromatographic purification of virus-like particles from
833 *Saccharomyces cerevisiae*, *J. Chem. Technol. Biotechnol.* 85 (2010) 209 – 215.

834 [39] J. Brinck, A.-S. Jönsson, B. Jönsson, J. Lindau, Influence of pH on the adsorptive fouling
835 of ultrafiltration membranes by fatty acid, *J. Membr. Sci.* 164 (2000) 187 – 194.

836 [41] A.A. Spector, K. John, J.E. Fletcher, Binding of long-chain fatty acids to bovine serum
837 albumin, *J. Lipid Res.* 10 (1969) 56 – 67.

838 [42] R. Chen, Removal of fatty acids from serum albumin by charcoal treatment removal of
839 fatty acids by charcoal treatment from serum albumin, *J. Biol. Chem.* 242 (1967) 173 – 181.

840 [43] M.C. Lay, C.J. Fee, J.E. Swan, Continuous radial flow chromatography of proteins, *Food*
841 *Bioprod. Process.* 84 (2006) 78 – 83.

842 [44] R. Giovanni, R. Freitag, Continuous isolation of plasmid DNA by annular
843 chromatography, *Biotechnol. Bioeng.* 77 (2002) 445 – 454.

844 [45] J. Andersson, B. Mattiasson, Simulated moving bed technology with a simplified
845 approach for protein purification: Separation of lactoperoxidase and lactoferrin from whey
846 protein concentrate, *J. Chromatogr. A* 1107 (2006) 88 – 95.

847 [46] B.J. Park, C.H. Lee, S. Mun, Y.M. Koo, Novel application of simulated moving bed
848 chromatography to protein refolding, *Process Biochem.* 41 (2006) 1072 – 1082.

849 [47] S. Palani, L. Gueorguieva, U. Rinas, A. Seidel-Morgenstern, G. Jayaraman, Recombinant
850 protein purification using gradient-assisted simulated moving bed hydrophobic interaction
851 chromatography. Part I: Selection of chromatographic system and estimation of adsorption
852 isotherms, *J. Chromatogr. A* 1218 (2011) 6396 – 6401.

853 [48] M. Bisschops, BioSMB™ Technology: Continuous Countercurrent Chromatography
854 Enabling a Fully Disposable Process, in: G. Subramanian (Ed.), Biopharmaceutical
855 Production Technology, Volume 1 & Volume 2 Wiley-VCH Verlag GmbH & Co. KGaA,
856 Weinheim, Germany, 2012, pp. 769 – 791.

857 [49] M. Angarita, T. Mueller-Spaeth, D. Baur, R. Lievrouw, G. Lissens, M. Morbidelli, Twin-
858 column CaptureSMB: A novel cyclic process for protein A affinity chromatography, J.
859 Chromatogr. A 1389 (2015) 85 – 95.

860 [50] R. Godawat, K. Brower, S. Jain, K. Konstantinov, F. Riske, V. Warikoo, Periodic
861 counter-current chromatography – design and operational considerations for integrated and
862 continuous purification of proteins, Biotechnol. J. 7 (2012) 1496 – 1508.

863 [51] N. Li, L. Qi, Y. Shen, Y. Li, Y. Chen, Thermoresponsive oligo(ethylene glycol)-based
864 polymer brushes on polymer monoliths for all-aqueous chromatography, ACS Appl. Mater.
865 Interfaces, 5 (2013) 12441 – 12448.

866 [52] E.C. Peters, F. Svec, J.M.J. Frechet, Thermally responsive rigid polymer monoliths, Adv.
867 Mater. 9 (1997) 630 – 632.

868 [53] K. Nagase, J. Kobayashi, A. Kikuchi, Y. Akiyama, H. Kanazawa, T. Okano, Thermally
869 modulated cationic copolymer brush on monolithic silica rods for high-speed separation of
870 acidic biomolecules, ACS Appl. Mater. Interfaces 5 (2013) 1442 – 1452.

871

872 **Figure legends**

873

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

885

886 **Fig.2.** Schematic illustrations of single protein loading (top) and concentration (bottom)
887 profiles at different stages during TCZR operation. The profiles correspond to three discrete
888 z/z_0 positions ($<0, 0.25, 0.75$) of the TCZ illustrated by the gray shaded vertical bars, i.e.: (i)
889 parked outside the separation column resulting in conventional operation with slowly
890 progressing concentration and loading profiles; (ii) shortly after initiation of TCZ movement,
891 where a sharp concentration peak evolves just ahead of the TCZ; and (iii) as the TCZ nears
892 the end of its journey along the column. At this point, in addition to further protein
893 accumulation within the elution peak, new loading and concentration profiles arising from
894 constant feed flow at the column inlet become clearly visible.

895

896 **Fig. 3.** FT-IR spectra during the fabrication of thermoCEX-S6pg supports (B1-B4)
897 characterized in Table 2. All spectra are normalized for peak height at the 'fingerprint'

898 wavenumber for agarose of 930 cm^{-1} characteristic of 3,6 anhydro moiety [28], and functional
899 groups expected of cross-linked agarose [23,28] are identified on the spectrum for Superose 6
900 Prep Grade.

901

902 **Fig. 4.** Optical transmittance (500 nm) vs. temperature profiles for 0.5% (w/v) solutions of
903 ungrafted free poly(NIPAAm-co-tBAAm-co-AAc-co-MBAAm) arising during fabrication of
904 (a) Sepharose CL-6B and (b) Superose based thermoCEX supports (detailed in Tables 1 and
905 2), and (c) the influence of NIPAAm content on temperature transition behaviour of the
906 copolymers. The symbols in ‘c’ indicate the determined LCST values at 50% transmittance
907 ($T_{50\%}$), capped bars define the temperature range over which phase transition occurred, and
908 the lower dotted and upper dashed lines respectively delineate the temperatures at which full
909 collapse ($T_{0.4\%}$) and extension ($T_{90\%}$) of the copolymer chains occurred. Key: pNIPAAm (★);
910 Sepharose CL6B series (A1 – □, A2 – ○, A3 – △, A4 – ▽); Superose series (B1 – ■, B2 –
911 ●, B3 – ▲, B4 – ▼, C1 – ●).

912

913 **Fig. 5.** Effect of temperature on the maximum adsorption capacity (q_{max}) of LF on (a)
914 thermoCEX-CL-6B supports (open circles) and (b) thermoCEX-S6pg (filled black circles)
915 and thermoCEX-S12pg (filled gray circles). The asterisks next to supports ‘A2’, ‘B2’ and
916 ‘C1’ indicate those initially selected for TCZR chromatography (Fig. 7). The solid lines
917 represent linear fits to the data.

918

919 **Fig. 6.** Equilibrium isotherms for the adsorption of LF to thermoCEX supports initially
920 selected for TCZR chromatography (Fig. 7) ‘A2’ (a), ‘B2’ (b) and ‘C1’ (c) at 10, 20, 35 and
921 50 °C. The solid lines through the data points represent fitted Langmuir curves with parameter
922 values presented in Table 3.

923

924 **Fig. 7.** Chromatograms arising from TCZR tests conducted with thermoCEX supports ‘A2’
925 (a), ‘B2’ (b) and ‘C1’ (c), employing multiple movements of the TCZ at a velocity, v_c , of 0.1
926 mm/s. Columns were saturated with LF ($c_f = 2$ mg/mL) and washed at the binding
927 temperature (35°C) prior to initiating the first of eight (a & b) or twelve (c) sequential
928 movements of the TCZ. Unshaded regions indicate column operation at a temperature of 35
929 °C with the TCZ in its ‘parked’ position. Gray shaded zones indicate the periods when the
930 TCZ moves along the column. The solid and dashed lines represent the absorbance and
931 conductivity signals respectively.

932

933 **Fig. 8.** Chromatograms arising from TCZR tests conducted with thermoCEX support ‘B2’
934 employing eight movements of the TCZ at a velocity, v_c , of 0.1 mm/s. The columns were
935 continuously supplied with LF at concentrations c_f of (a) 0.5 mg/mL and (b) 1.0 mg/mL.
936 Unshaded regions indicate column operation at a temperature of 35 °C with the TCZ ‘parked’.
937 Gray shaded zones indicate the periods when the TCZ moves along the column. The solid and
938 dashed lines represent the absorbance and conductivity signals respectively.

939

940 **Fig. 9.** Chromatogram arising from TCZR test conducted with thermoCEX support ‘B2’
941 during continuous feeding of a binary protein mixture (1 mg/mL LF + 1 mg/mL BSA) and
942 movement of the TCZ at a velocity, v_c , of 0.1 mm/s. Unshaded regions indicate column
943 operation at a temperature of 35 °C with the TCZ ‘parked’. Gray shaded zones indicate the
944 periods when the TCZ moves along the column. The solid and dashed lines represent the
945 absorbance and conductivity signals respectively. SDS-PAGE analysis of pooled flowthrough
946 (*a, c, e, g, i, k, m, o & q*); peak (*b, d, f, h, j, l, n & p*) and salt-stripped (S) fractions are shown
947 in Fig. 10.

948

949 **Fig. 10.** Reducing SDS 15% (w/v) polyacrylamide gel electrophoretogram corresponding to
950 the chromatogram shown in Fig. 9. Key: molecular weight markers (M); feed (F);
951 flowthrough fractions (*a, c, e, g, i, k, m, o & q*); peak fractions (*b, d, f, h, j, l, n & p*); and salt-
952 stripped fraction (S).
953

(a) TCZ outside column, $z/z_0 < 0$

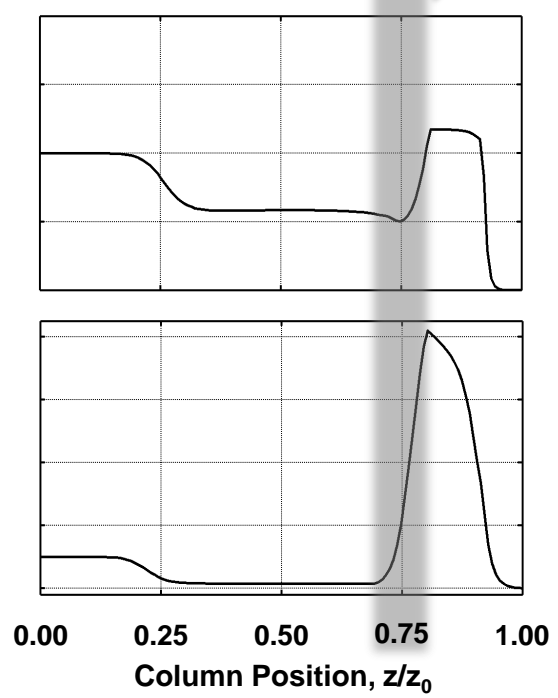
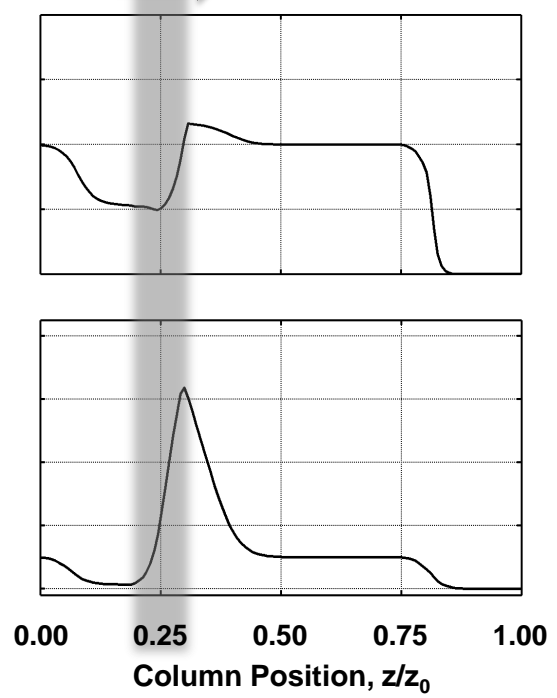
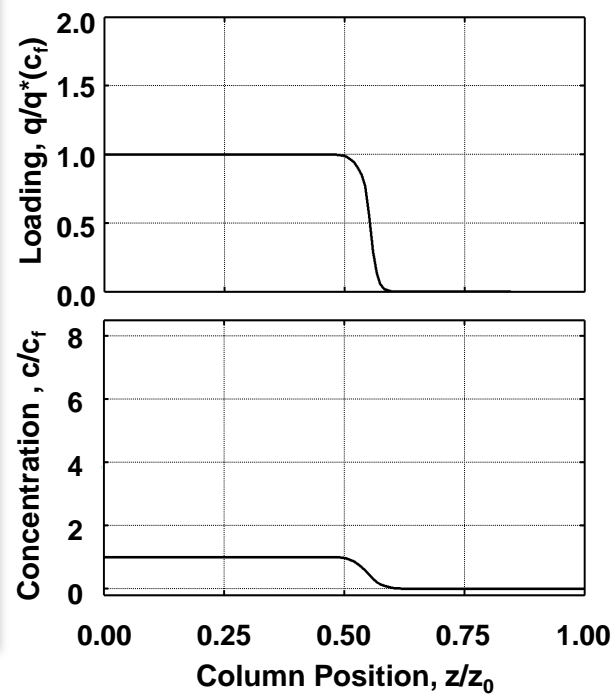
(b) TCZ @ $z/z_0 = 0.25$

(c) TCZ @ $z/z_0 = 0.75$

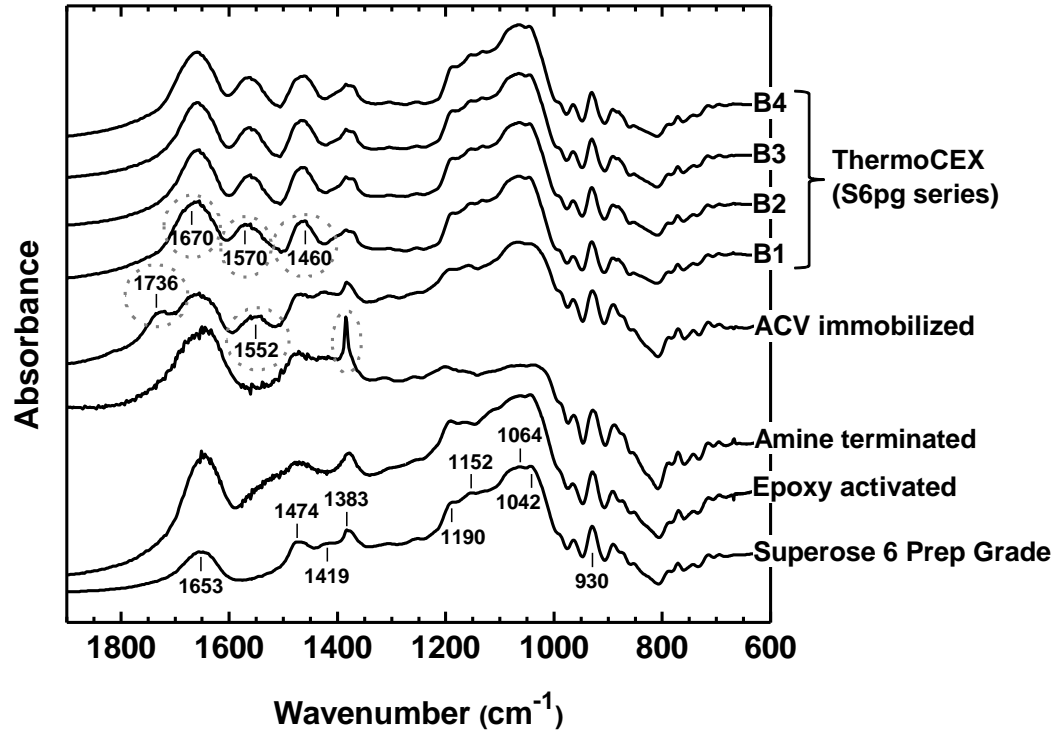
'Parked'

Moving

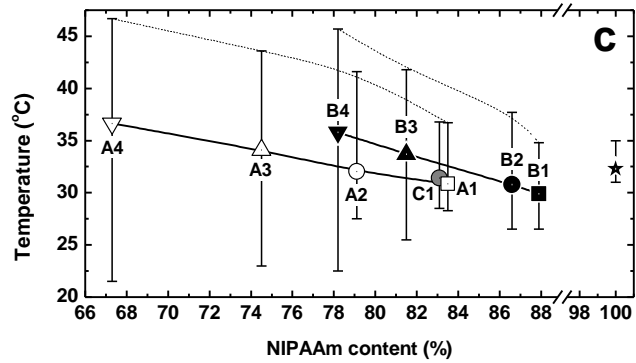
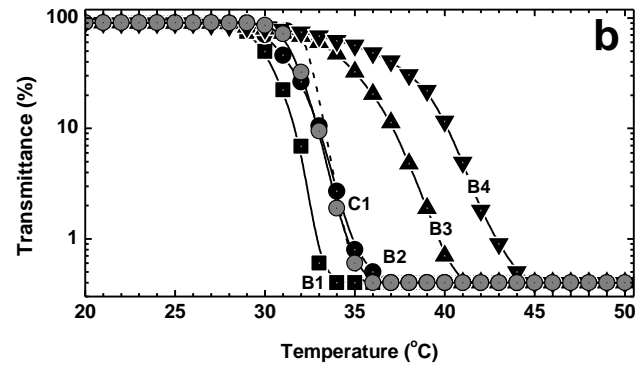
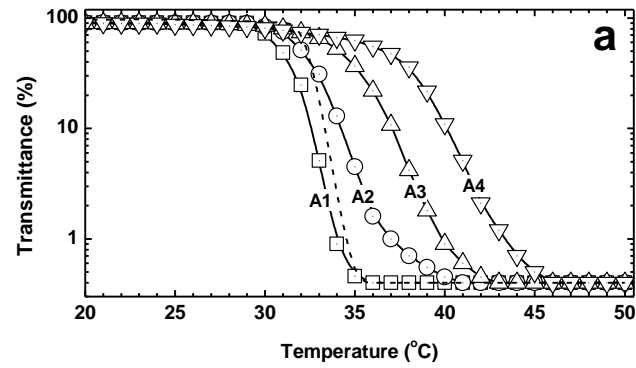
Moving



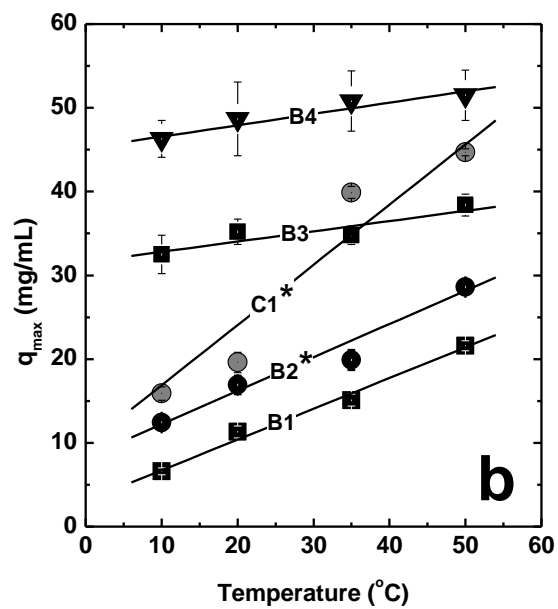
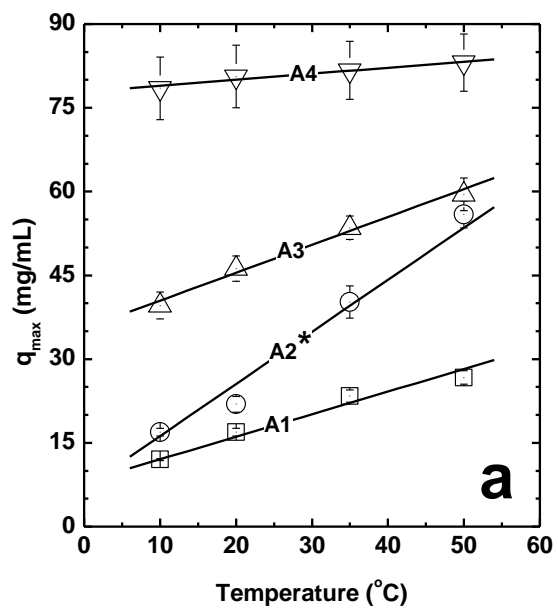
FIG_1



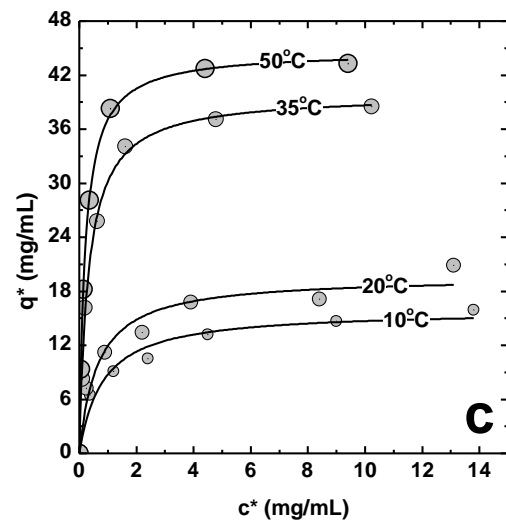
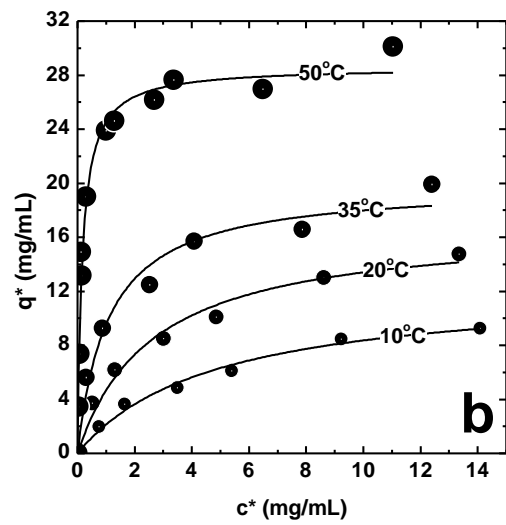
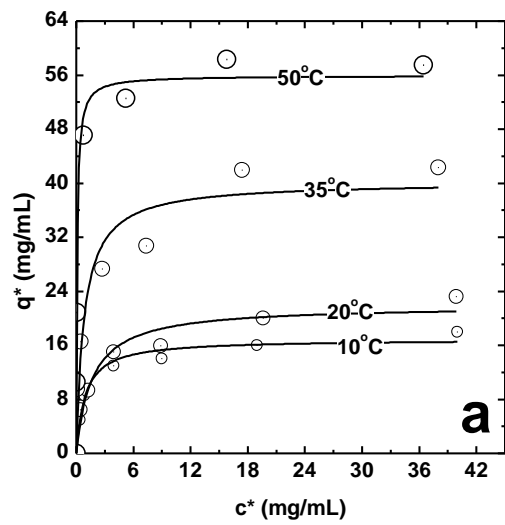
FIG_2



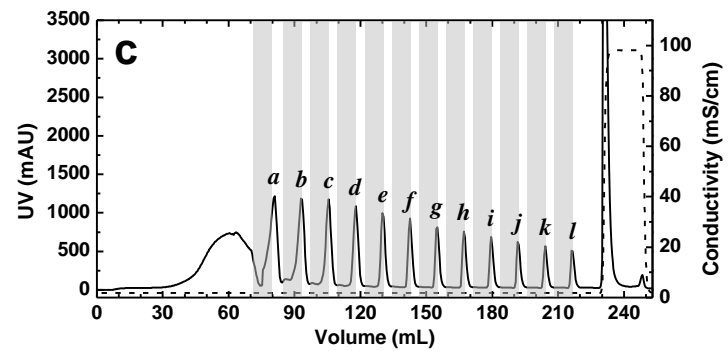
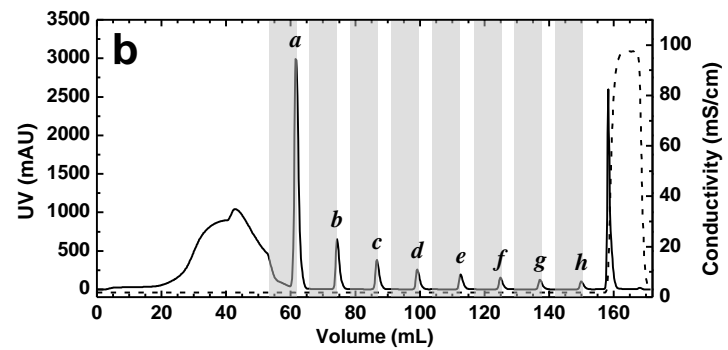
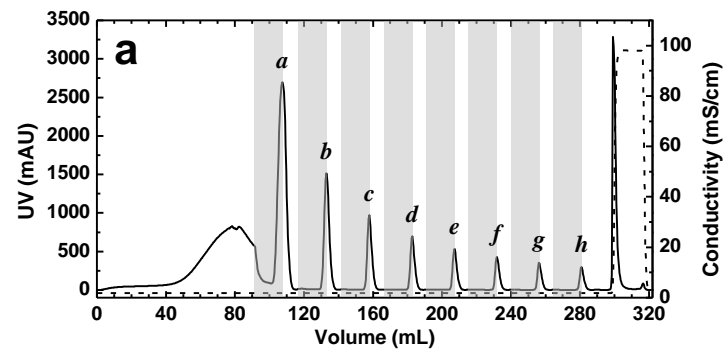
FIG_3



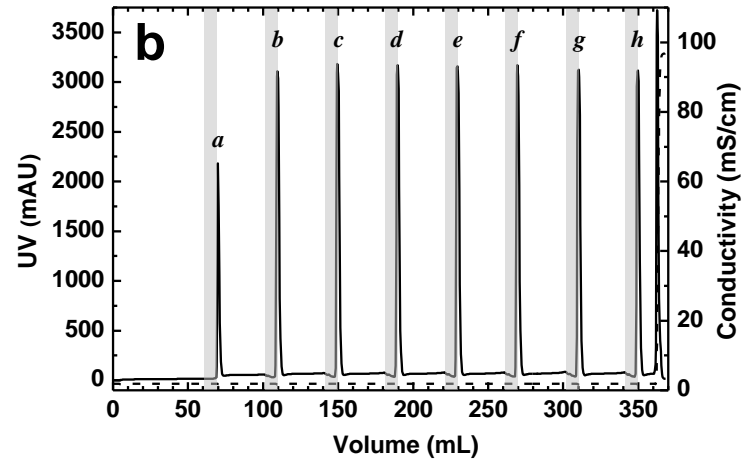
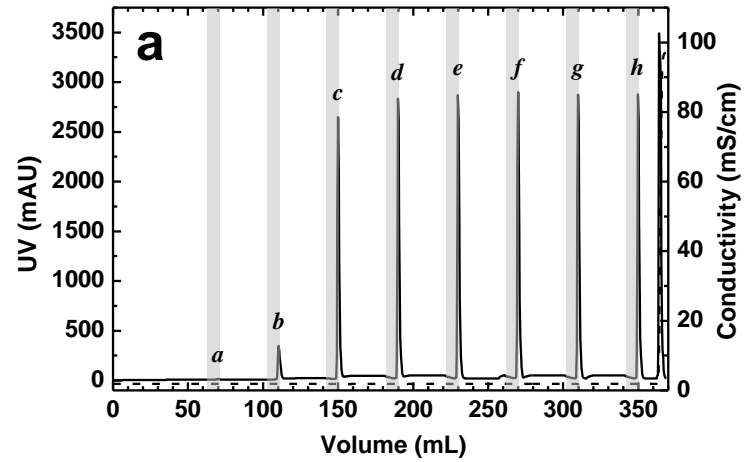
FIG_4



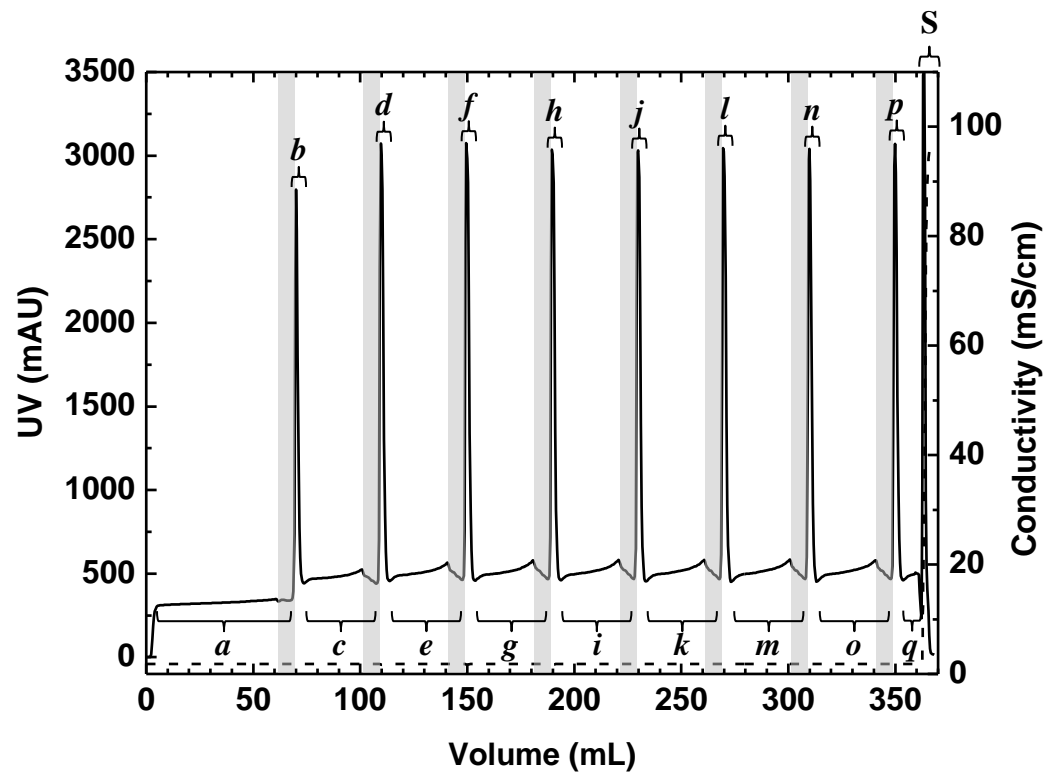
FIG_5



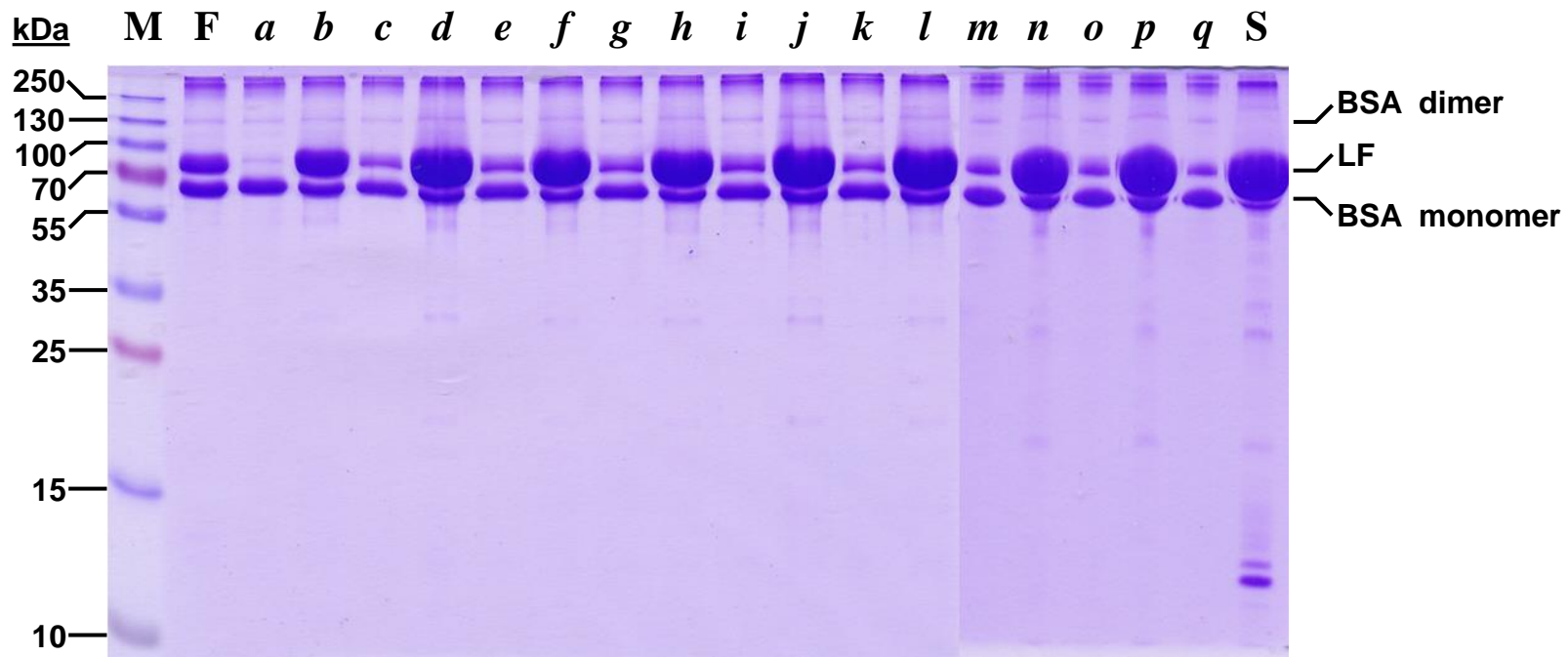
FIG_6



FIG_7



FIG_8



FIG_9

Table 1. Characterization of the Sepharose CL6B based thermoCEX supports prepared and used in this work. See text for details.

Parameter	ThermoCEX-CL-6B			
<i>Support ID</i>	A1	A2	A3	A4
<i>Base matrix</i>	Sepharose CL-6B (lot 10061497)			
Particle size distribution	45 – 165 μm (98%)			
Globular protein fractionation range, M_r	$1 \times 10^4 - 4 \times 10^6$ Da			
<i>Step 1. Immobilized oxirane content ($\mu\text{mol/g}$ dried support)^a</i>	662			
<i>Step 3. Immobilised ACV content ($\mu\text{mol/g}$ dried support)^b</i>	380			
<i>Step 4. Free monomers entering reaction:</i>				
‘NIPAAm:tBAAm:AAc’ (mM)	900:50:25	900:50:50	900:50:100	900:50:150
‘NIPAAm:tBAAm:AAc’ ratio	90:5:2.5	90:5:5	90:5:10	90:5:15
<i>Immobilized copolymer composition of thermoCEX support:</i>				
‘NIPAAm + tBAAm’ content ($\mu\text{mol/g}$ dried support) ^{b,c}	4227	4177	4131	3974
‘NIPAAm:tBAAm’ ratio ^d	90:10	88:12	85:15	81:19
NIPAAm content ($\mu\text{mol/g}$ dried support)	3804	3676	3511	3219
tBAAm content ($\mu\text{mol/g}$ dried support)	423	501	620	755
Ion exchange capacity ($\mu\text{mol H}^+/\text{g}$ dried support) ^e	330	469	582	811
‘pNIPAAm + tBAAm + AAc’ content ($\mu\text{mol/g}$ dried support)	4557	4646	4713	4785
‘NIPAAm:tBAAm:AAc’ ratio	83.5:9.3:7.2	79.1:10.8:10.1	74.5:13.2:12.3	67.3:15.8:16.9
<i>% monomer consumed by support:</i>				
NIPAAm + tBAAm + AAc	15.1	15.0	14.5	14.1
NIPAAm	13.7	13.2	12.6	11.6
tBAAm	27.4	32.5	40.2	49.0
AAc	42.8	30.4	18.9	17.5

Key: Determined by: ^aoxirane ring opening reaction followed by titration [25]; ^bgravimetric measurement; ^cATR-FTIR spectrometry of supernatant samples before and after polymerization reactions, ^dProton NMR of the ungrafted free copolymer in CDCl_3 ; ^etitration.

Table 2. Characterization of the Superose 6 and 12 based thermoCEX supports prepared and used in this work. See text for details.

Parameter	ThermoCEX-S6pg				ThermoCEX-S12pg
<i>Support ID</i>	B1	B2	B3	B4	C1
<i>Base matrix</i>	Superose 6 Prep Grade (lot 10037732)				Superose 12 Prep Grade (lot 10057699)
Particle size distribution	20 – 40 μm (83%)				20 – 40 μm (88%)
Globular protein fractionation range, M_r	$5 \times 10^3 - 5 \times 10^6$ Da				$1 \times 10^3 - 3 \times 10^5$ Da
<i>Step 1</i> Immobilized oxirane content ($\mu\text{mol/g}$ dried support) ^a	878				1018
<i>Step 3.</i> Immobilised ACV content ($\mu\text{mol/g}$ dried support) ^b	568				611
<i>Step 4. Free monomers entering reaction:</i>					
‘NIPAAm:tBAAm:AAc’ (mM)	900:50:25	900:50:50	900:50:100	900:50:150	900:50:50
‘NIPAAm:tBAAm:AAc’ ratio	90:5:2.5	90:5:5	90:5:10	90:5:15	90:5:5
<i>Immobilized copolymer composition of thermoCEX support:</i>					
‘NIPAAm + tBAAm’ content ($\mu\text{mol/g}$ dried support) ^{b,c}	5745	5733	5659	5641	5495
‘NIPAAm:tBAAm’ ratio ^d	92:8	91:9	87:13	84:16	89:11
NIPAAm content ($\mu\text{mol/g}$ dried support)	5285	5217	4923	4738	4891
tBAAm content ($\mu\text{mol/g}$ dried support)	460	516	736	903	604
Ion exchange capacity ($\mu\text{mol H}^+/\text{g}$ dried support) ^e	268	293	382	416	391
‘NIPAAm + tBAAm + AAc’ content ($\mu\text{mol/g}$ dried support)	6013	6026	6041	6057	5886
‘NIPAAm:tBAAm:AAc’ ratio	87.9:7.6:4.5	86.6:8.5:4.9	81.5:12.2:6.3	78.2:14.9:6.9	83.1:10.3:6.6
<i>% monomer consumed by support:</i>					
NIPAAm + tBAAm + AAc	19.9	19.5	18.6	17.8	19.1
NIPAAm	19.0	18.7	17.6	17.0	17.6
tBAAm	29.8	33.5	47.7	58.5	39.2
AAc	34.8	19.0	12.4	9.0	25.4

Key: Determined by: ^aoxirane ring opening reaction followed by titration [25]; ^bgravimetric measurement; ^cATR-FTIR spectrometry of supernatant samples before and after polymerization reactions, ^dProton NMR of the ungrafted free copolymer in CDCl_3 ; ^etitration.

Table 3. Langmuir parameters^a describing the adsorption of LF at 10, 20, 35 and 50 °C to three different thermoCEX media prepared under identical conditions using a common initial ‘NIPAAm:tBAAM:AAc’ ratio of ‘90:5:5’ (Tables 1 & 2).

Support (ionic capacity)	Temperature (°C)	q_{max} (mg/mL)	K_d (mg/mL)	Initial slope, q_{max}/K_d
thermoCEX-CL6B ‘A2’ (40.7 $\mu\text{mol H}^+$ /mL)	10	16.9 \pm 0.7	0.93 \pm 0.19	18.2
	20	21.9 \pm 1.4	1.66 \pm 0.48	13.2
	35	40.2 \pm 2.9	0.83 \pm 0.34	48.4
	50	55.9 \pm 2.4	0.09 \pm 0.03	621.1
thermoCEX-S6pg ‘B2’ (25.4 $\mu\text{mol H}^+$ /mL)	10	12.4 \pm 0.9	4.79 \pm 0.83	2.6
	20	16.9 \pm 1.1	2.63 \pm 0.52	6.4
	35	19.9 \pm 1.2	1.09 \pm 0.26	18.3
	50	28.6 \pm 0.7	0.17 \pm 0.02	168.2
thermoCEX-S12pg ‘C1’ (34.8 $\mu\text{mol H}^+$ /mL)	10	15.9 \pm 0.8	0.82 \pm 0.20	19.39
	20	19.6 \pm 1.2	0.65 \pm 0.20	30.2
	35	39.9 \pm 0.7	0.32 \pm 0.03	124.7
	50	44.7 \pm 0.4	0.21 \pm 0.01	212.9

^aFigure 5 adsorption data were fitted to the Langmuir model (Eq. (1)).

Table 4. LF contents in peaks *a* – *h* obtained following eight sequential movements of the cooling zone during continuous feeding of LF ($c_f = 0.5$ and 1 mg/mL) to a column filled with the thermoCEX-S6pg ‘B2’ matrix (see Table 2).

LF concentration in feed (mg/mL)	LF content (mg) in peak:							
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>h</i>
0.5	0.0	1.46	10.5	12.3	13.4	14.5	17.8	13.2
1.0	7.54	28.9	31.3	31.2	31.4	30.6	31.7	28.1