

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

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1 Three beaded agarose media are grafted with networks of temperature
2 sensitive anionic copolymers.
3
4 Base matrix X-linking chemistry is shown to strongly influence the
5 grafted copolymer composition.
6
7 Travelling Cooling Zone Reactor chromatography uses a single column
8 operated isocratically.
9
10 Small diameter wide pored supports are best suited for protein
11 separations in TCZR chromatography.
12
13 TCZR chromatography can continuously accumulate, concentrate and
14 fractionate proteins.

15

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

17

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36

36 **Abstract**

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

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

69

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

73

73 **1. Introduction**

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

95

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

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

116

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

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

128

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

137

138 **2. Materials and methods**

139 **2.1 Materials**

140 The base matrices, Sepharose CL-6B (Cat. no. 170160-01, Lot no. 10040943), Superose 6
141 Prep Grade (Cat. no. 17-0489-01, Lot. no. 10037732) and Superose 12 Prep Grade (Cat.
142 no.17-0536-01, Lot. no. 10057699) were all supplied by GE Healthcare Life Sciences (Little
143 Chalfont, Bucks, UK). The chemicals, *N*-isopropylacrylamide (Cat. no. 415324, 97%;
144 NIPAAm), *N*-*tert*-butylacrylamide (Cat. no. 411779, 97%; *t*-BAAm), acrylic acid (Cat. no.
145 147230, anhydrous, 99%, AAc), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (Cat. no.
146 149837, $\geq 99\%$; EEDQ), 4,4'-azobis(4-cyanovaleric acid) (Cat. no. 11590, $\geq 98\%$; ACV), *N,N*-
147 dimethylformamide (Cat. no. 270547, $>99.9\%$; DMF), *N,N'*-methylenebisacrylamide (Cat.
148 No. 146072, 99%; MBAAm), epichlorohydrin (Cat. no. E1055, 99%; ECH), tetrahydrofuran
149 (Cat. no. 34865, $>99\%$; THF), diethyl ether (Cat. no. 309966, $>99.9\%$), sodium borohydride
150 (Cat. no. 71321, $>99\%$) and sodium hydroxide (Cat. no. S5881, anhydrous, $>98\%$) were

151 obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Absolute ethanol (Cat. no.
152 E/0650DF/17, 99.8+%) and ammonia solution (Cat. no. A/3280/PB15, AR grade, 0.88 S.G.,
153 35%) were acquired from Fisher Scientific UK Ltd (Loughborough, Leics, UK), and bottled
154 oxygen-free nitrogen gas was supplied by the British Oxygen Co Ltd (Windlesham, Surrey,
155 UK). Bovine whey lactoferrin (MLF-1, Lot. No. 12011506, ~96%) was a gift from Milei
156 GmbH (Leutkirch, Germany), and bovine serum albumin (BSA, Cat. No. A7906, lyophilized
157 powder, $\geq 98\%$ by agarose gel electrophoresis) and 'Blue Dextran MW 2,000,000' (Cat. No.
158 D-5751) were purchased from Sigma-Aldrich. Di-sodium hydrogen phosphate (Cat. no.
159 4984.1, dihydrate, $\geq 99.5\%$) was from Carl Roth GmbH + Co. KG (Karlsruhe, Germany).
160 Disodium hydrogen phosphate (dihydrate, $\geq 99.5\%$) and sodium chloride (ACS reagent,
161 $\geq 99.5\%$) were supplied by Carl Roth or Sigma-Aldrich, and citric acid monohydrate ($\geq 99\%$)
162 and Coomassie Brilliant Blue R250 (C.I. 42660) were from Merck Millipore (Darmstadt,
163 Germany). Pre-cast 15% mini-PROTEAN® TGX™ gels and Precision Plus Protein™ All
164 Blue Standards were supplied by Bio-Rad Laboratories Inc. (Hercules, CA, USA). All other
165 chemicals not stated above were from Sigma-Aldrich or Merck Millipore. The water used in
166 all experiments was deionized and purified using a Milli-Q Ultrapure system (Merck
167 Millipore, Darmstadt, Germany).

168

169 **2.2 Preparation of the thermoCEX media used in this work**

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

177 AAc monomer concentration entering reactions with ACV anchored supports was
178 systematically varied between 25 and 150 mM (equivalent to 2.5 to 13.5% of the total
179 monomer concentration), whilst maintaining fixed concentrations of all other monomers, i.e.
180 900 mM NIPAAm, 50 mM tBAAm, and 10 mM of the cross-linking monomer, MBAAm. For
181 point of comparison, the AAc concentration used in our previous study [23] was 50 mM
182 (corresponding to ~5% of the total monomer composition).

183

184 **2.3 Batch adsorption experiments with LF**

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

194

$$195 \quad q^* = q_{\max} \frac{c^*}{K_d + c^*} \quad (1)$$

196

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

200

201

202 2.3. TCZR chromatography experiments

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

218

219 2.3.2 Batch mode TCZR chromatography of LF

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

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

235

236 **2.3.3 Continuous TCZR chromatography**

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

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

256

$$257 \quad CF_{Peak,i} = \frac{c_{Peak,i}}{c_f} \quad (2)$$

258

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

262

$$263 \quad CF = \frac{\sum_j CF_{Peak,i}}{j} \quad (3)$$

264

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

268

269 **2.4 Analysis**

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

273

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

276

277 For qualitative FT-IR analysis of solid supports, oven dried samples (~3 mg) were mixed with
278 potassium bromide (300 mg), ground down to a fine powder and hydraulically pressed (15
279 tonne) into tablet form. Each tablet was subjected to 64 scans (averaged at a resolution of 2
280 cm^{-1}) in a Nicolet 380 FT-IR (Thermo Fisher Scientific, Waltham, MA, USA) in direct beam
281 mode. Quantitative estimation of 'NIPAAm + tBAAm' consumption by supports during
282 grafting reactions, by monitoring changes in area of the characteristic peak for N-H bending
283 ($1575 - 1500 \text{ cm}^{-1}$), was performed on liquid samples (150 μL) applied directly to the surface
284 of the Nicolet 30 FT-IR's Smart 53 Orbit diamond accessory. The samples were scanned 64
285 times at a resolution of 2 cm^{-1} in attenuated total reflectance mode (ATR FT-IR).

286

287 Gravimetric analyses was used to determine the immobilized ACV and copolymer contents
288 on solid supports and also of dried residues recovered from liquid samples, to allow
289 determination of free ungrafted copolymer content and unreacted monomers remaining in
290 solution post-grafting. Free copolymers were separated from unreacted monomers by rotary
291 evaporating to dryness, resuspending in tetrahydrofuran, precipitating with diethyl ether and
292 oven drying, and unreacted monomers remaining in the supernatants were recovered by rotary
293 evaporating to dryness.

294

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

301

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

307

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

313

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

320

321 The composition of fractions generated during continuous TCZR chromatographic separation
322 of LF and BSA was examined by reducing SDS-PAGE [26] in 15% (w/v) pre-cast
323 polyacrylamide gels in a Mini-Protean® Tetracell electrophoresis system (Bio-Rad
324 Laboratories, Hercules, CA, USA). After electrophoresis, gels were stained with 0.1% (w/v)
325 Coomassie Brilliant Blue R250, dissolved in 40% (v/v) ethanol and 10% (v/v) acetic acid) for
326 1 h at room temperature, and were subsequently destained at the same temperature in a
327 solution composed of 7.5% (v/v) acetic acid and 10% (v/v) ethanol. The LF and BSA contents

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

332

333 **3. Results and discussion**

334 **3.1 Concept of single-column continuous chromatography**

335 Schematic concentration and loading profiles within the TCZR fitted column are illustrated in
 336 Fig. 2 for the various operation phases. These are characterized by different positions of the
 337 TCZ relative to the column. The profiles at three different positions of the TCZ are shown,
 338 i.e.: (i) outside the column (Fig. 2a); and after travelling (ii) a quarter (Fig. 2b) and (iii) three-
 339 quarters (Fig. 2c) of the separation column's length. When the TCZ is parked 'outside' (Fig
 340 2a), the whole column is operated at an increased temperature (T_B). Protein is continuously
 341 loaded and binds to the adsorbent. In keeping with the binding strength of the protein to the
 342 support at T_B , sharp concentration and loading fronts of the protein propagate through the
 343 system at a constant velocity, v_{pf} (Eq. (4)):

344

$$345 \quad v_{pf} \cong \frac{u_i}{1 + \frac{1 - \varepsilon_b}{\varepsilon_b} \frac{\partial q}{\partial c}} \quad (4)$$

346

347 Here, v_{pf} is a function of the slope of the isotherm, $\frac{\partial q}{\partial c}$, the interstitial fluid velocity, u_i , and
 348 the phase ratio between the solid and liquid phases expressed by the bed voidage, ε_b , of the
 349 column packing.

350

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

368

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

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

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

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

388

389 **3.2 Manufacture and characterization of thermoresponsive CEX adsorbents**

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

401

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

421

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

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

447

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

454 functional cross-linkers conducted in aqueous solvent [30,32]. Superose media are thus less
455 hydrophilic than Sepharose CL supports, and it is this fundamental difference that likely: (i)
456 contributes to the 30 – 50% greater number of immobilized oxiranes introduced into Superose
457 matrices (Table 2, 878 $\mu\text{mol/g}$ for S6pg, 1018 $\mu\text{mol/g}$ for S12pg) by epichlorohydrin
458 activation (step 1) under identical conditions *cf.* Sepharose CL-6B (Table 1, 662 $\mu\text{mol/g}$);
459 leads in turn (ii) both to higher immobilized ACV contents (568 – 611 $\mu\text{mol/g}$ for S6pg, 878
460 $\mu\text{mol/g}$ for S12pg *cf.* 380 $\mu\text{mol/g}$ for Sepharose CL-6B) and elevated grafted polymer yields
461 (6005 ± 68 $\mu\text{mol/g}$ for ThermoCEX-Superose adsorbents *cf.* 4675 ± 97 $\mu\text{mol/g}$ for
462 ThermoCEX-CL6B); and (iii) significantly higher incorporation of the charged AAc
463 monomer into the grafted copolymers on ThermoCEX-CL6B *cf.* ThermoCEX adsorbents
464 fashioned from Superose media across all AAc input concentrations during grafting (see
465 Tables 1 and 2).

466

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

472

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

479 and tBAAm into pNIPAAm's backbone ('A1', 'B1' & 'C1') at the expense of 12.1 to 16.9%
480 of its NIPAAm content (Tables 1 & 2), had the effect of lowering the LCST (by 1.4 °C for
481 'A1', 2.3 °C for 'B1', 1.5 °C for 'B2' and 0.9 °C for 'C1') and broadening the transition
482 temperature range in both directions (i.e. 28.3 to 36.7 °C for 'A1', 26.5 to 34.8 °C for 'B1',
483 26.5 to 37.7 °C for 'B2', and 28.5 to 36.8 °C for 'C1'). With mounting NIPAAm replacement
484 ('A3', 'A4', 'B3', 'B4'), smart thermoresponsive behaviour became increasingly
485 compromised. For example, the LCST for the '67.3:15.8:16.9' copolymer (Fig. 4d, 'A4')
486 reached 36.6 °C and temperature range over which full 'coil – globule' transition range
487 extended >25 °C, i.e. from 20.5 to 46 °C.

488

489 **3.3 Temperature dependent adsorption of LF on thermoCEX adsorbents**

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

499

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

505 (Fig. 1, top right), as the distance between neighboring charged AAc units within the
 506 collapsed copolymer decreased and previously shielded/buried negative charges became
 507 exposed [22]. However, the degree of thermoresponsiveness exhibited varied significantly
 508 between and within each thermoCEX family, and a clear trend emerged. For the least
 509 substituted supports in the Sepharose CL-6B and Superose 6pg thermoCEX families, i.e. 'A1'
 510 (Fig. 5a, Table 1) and 'B1' (Fig. 5b, Table 2), LF binding capacity was strongly temperature
 511 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
 512 of <22 mg/mL for 'B1' and <27 mg/mL for 'A1'). With increasing NIPAAm replacement by
 513 hydrophobic tBAAm and negatively charged AAc monomers (\rightarrow 'A2' \rightarrow 'A3' \rightarrow 'A4', Fig. 5a;
 514 \rightarrow 'B2' \rightarrow 'B3' \rightarrow 'B4'; Fig. 5b), LF binding increased dramatically ($q_{max, 50^{\circ}\text{C}}$ rising to ~52
 515 mg/mL for 'B4' and ~83 mg/mL for 'A4'), but became increasingly temperature independent
 516 ($q_{max, 50^{\circ}\text{C}} / q_{max, 10^{\circ}\text{C}}$ ratios of 1.06 for A4 & 1.1 for 'B4'), as the ability of the tethered
 517 copolymers to transition between extended and collapsed states was lost. ThermoCEX
 518 supports with moderate levels of NIPAAm replacement, i.e. 'A2', 'B2' & 'C1', displayed the
 519 best combination of thermoresponsiveness and LF binding capacity (i.e. reasonable $q_{max, 50^{\circ}\text{C}}$
 520 and low $q_{max, 10^{\circ}\text{C}}$ values), and were therefore selected for further study.

521

522 Fig. 6 shows adsorption isotherms obtained for the binding of LF to these at different
 523 temperatures, and Table 3 presents the fitted Langmuir parameters. At the lowest temperature
 524 of 10 °C, the binding of LF to all three thermoCEX adsorbents is rather weak (K_d values
 525 between 0.82 and 4.8 mg/mL) and of low capacity (q_{max} values <17 mg/mL), but as the
 526 temperature is gradually stepped up, the tightness and capacity of LF sorption rise strongly.
 527 At the highest temperature of 50 °C, the paired values for q_{max} and tightness of binding (initial
 528 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
 529 mg/mL and 213 for support 'C1' (Table 3). An identical pattern of behaviour in response to
 530 temperature (between 20 and 50 °C) was noted previously by Maharjan et al. [22] for a

531 thermoCEX support fashioned out of Sepharose 6 FF. The ranking of static LF binding
532 performance of this family of thermoCEX supports of ‘Sepharose CL-6B > Superose 12 Prep
533 Grade > Superose 6 Prep Grade > Sepharose 6 FF’ (initial slope values at 50 °C of 610, 213,
534 168 and 120 respectively) is not correlated with mass of copolymer attached (respectively
535 4177, 5495, 5733, 2060 $\mu\text{mol/g}$ dried support), nor the tBAAm content (respectively 501,
536 604, 516, 165 $\mu\text{mol/g}$ dried support), but rather with the support’s intrinsic ionic capacity (i.e.
537 469 μmol > 391 μmol > 293 μmol > 154 $\mu\text{mol H}^+/\text{g}$ dried support).

538

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

556

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

575

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

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

588

589 **3.4 Continuous protein accumulation experiments**

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

597

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

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

613

614 Another important parameter influenced by the protein concentration in feed is averaged
615 concentration factor, CF , (Eq. (3)) attainable during steady state operation. The CF reached
616 9.2 at $c_f = 0.5$ mg/mL (Fig. 8a), but dropped to 7.9 on increasing the LF concentration in the
617 feed twofold (Fig. 8b). An explanation for the slight reduction in CF with increasing LF
618 concentration can be found in the isotherm describing LF adsorption to thermoCEX-S6pg
619 ‘B2’ at 35 °C (Fig. 6b); i.e. isotherm starts to become non-linear between 0.5 and 1 mg LF per
620 mL.

621

622 **3.5 Continuous separation of a binary protein mixture**

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

635 returned to roughly constant threshold of ~500 mAU. Following the 8th TCZ motion,
636 residually bound protein was desorbed from the column by a step change in ionic strength (S).
637

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

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

663

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

675

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

687 extraction with organic phases at elevated temperatures or sorption of free fatty acids (FFAs)
688 onto activated charcoal at elevated temperature under acidic–neutral conditions [41,42].
689 Under the operating conditions employed here, i.e. mobile phase of pH 6.5, temperature of 35
690 °C, FFA binding to BSA is weak [42] and FFA solubility is comparatively high [40]; it is
691 conceivable that FFAs bind to and foul the copolymer binding surface, thereby reducing both
692 the adsorbent's binding affinity and occupancy for LF.

693

694 **4. Conclusions**

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

706

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

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

731

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

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

743

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746 project ‘Advanced Magnetic nano-particles Deliver Smart Processes and Products for Life’
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748

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- 883

883 **Figure legends**

884

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

896

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

906

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

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

912

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

923

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

929

930 **Fig. 6.** Equilibrium isotherms for the adsorption of LF to thermoCEX supports initially
 931 selected for TCZR chromatography (Fig. 7) 'A2' (a), 'B2' (b) and 'C1' (c) at 10, 20, 35 and
 932 50 °C. The solid lines through the data points represent fitted Langmuir curves with parameter
 933 values presented in Table 3.

934

935 **Fig. 7.** Chromatograms arising from TCZR tests conducted with thermoCEX supports ‘A2’
936 (a), ‘B2’ (b) and ‘C1’ (c), employing multiple movements of the TCZ at a velocity, v_c , of 0.1
937 mm/s. Columns were saturated with LF ($c_f = 2$ mg/mL) and washed at the binding
938 temperature (35°C) prior to initiating the first of eight (a & b) or twelve (c) sequential
939 movements of the TCZ. Unshaded regions indicate column operation at a temperature of 35
940 °C with the TCZ in its ‘parked’ position. Gray shaded zones indicate the periods when the
941 TCZ moves along the column. The solid and dashed lines represent the absorbance and
942 conductivity signals respectively.

943

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

950

951 **Fig. 9.** Chromatogram arising from TCZR test conducted with thermoCEX support ‘B2’
952 during continuous feeding of a binary protein mixture (1 mg/mL LF + 1 mg/mL BSA) and
953 movement of the TCZ at a velocity, v_c , of 0.1 mm/s. Unshaded regions indicate column
954 operation at a temperature of 35 °C with the TCZ ‘parked’. Gray shaded zones indicate the
955 periods when the TCZ moves along the column. The solid and dashed lines represent the
956 absorbance and conductivity signals respectively. SDS-PAGE analysis of pooled flowthrough
957 (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
958 in Fig. 10.

959

960 **Fig. 10.** Reducing SDS 15% (w/v) polyacrylamide gel electrophoretogram corresponding to
961 the chromatogram shown in Fig. 9. Key: molecular weight markers (M); feed (F);
962 flowthrough fractions (*a, c, e, g, i, k, m, o & q*); peak fractions (*b, d, f, h, j, l, n & p*); and salt-
963 stripped fraction (S).
964

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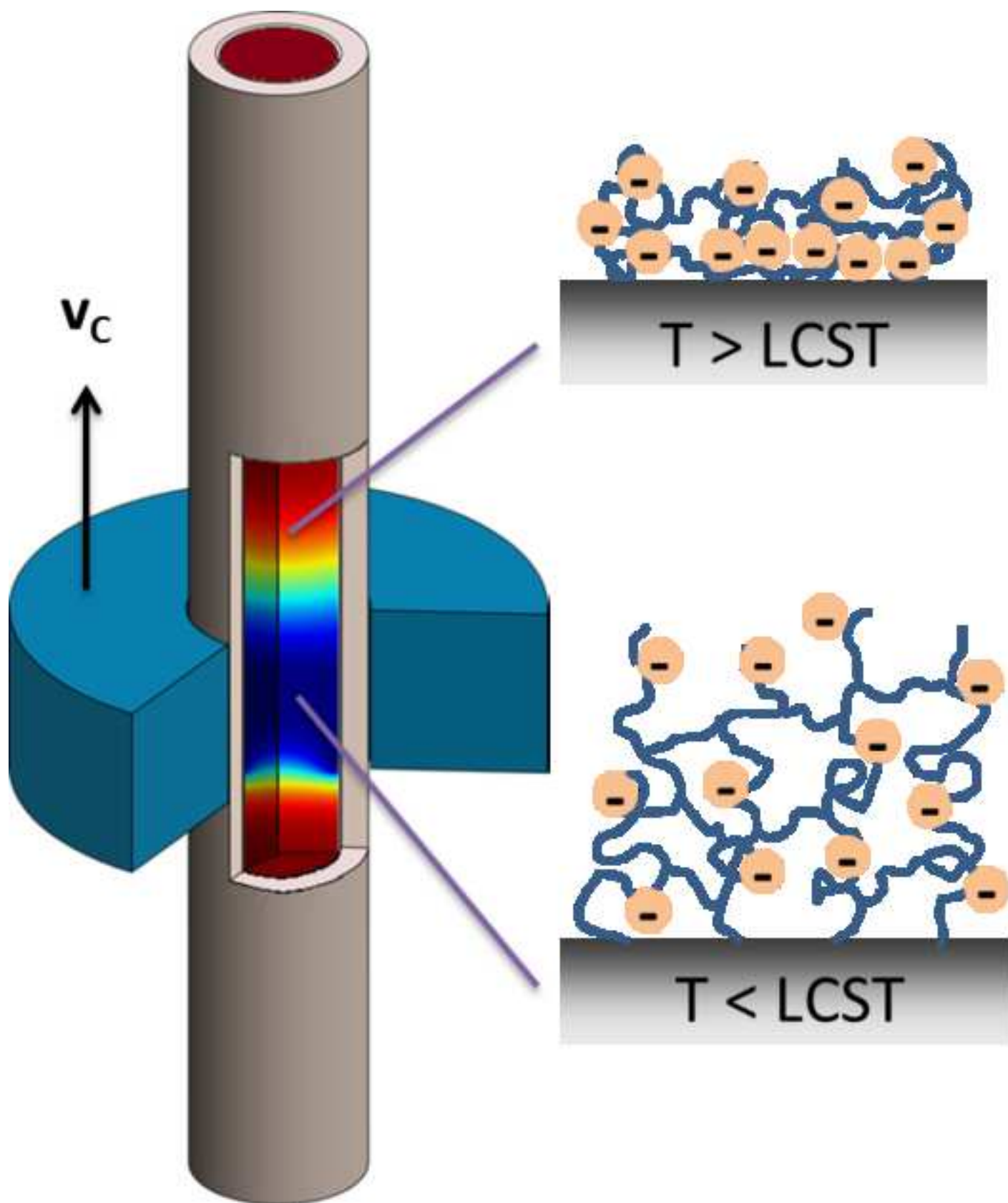
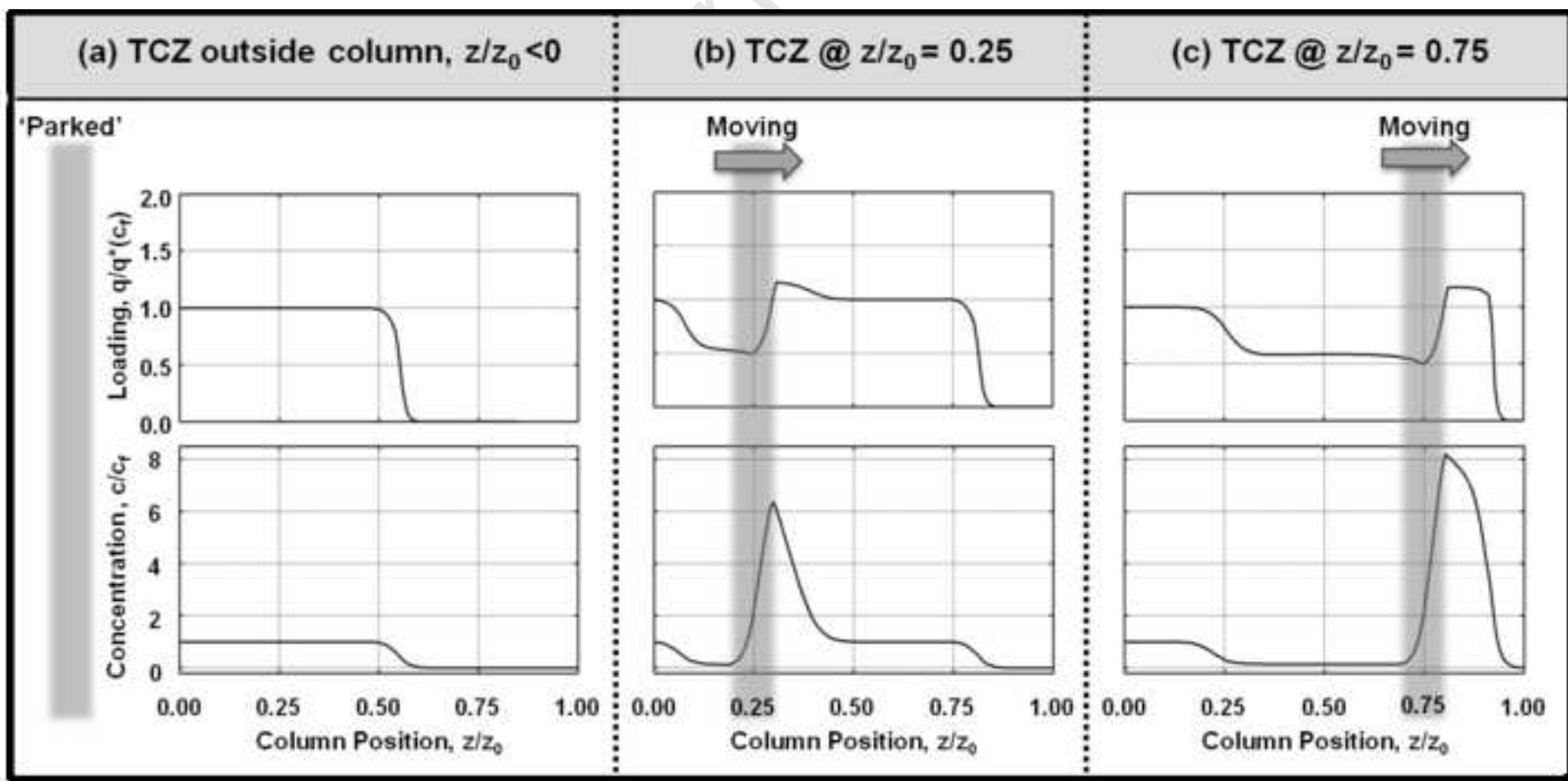
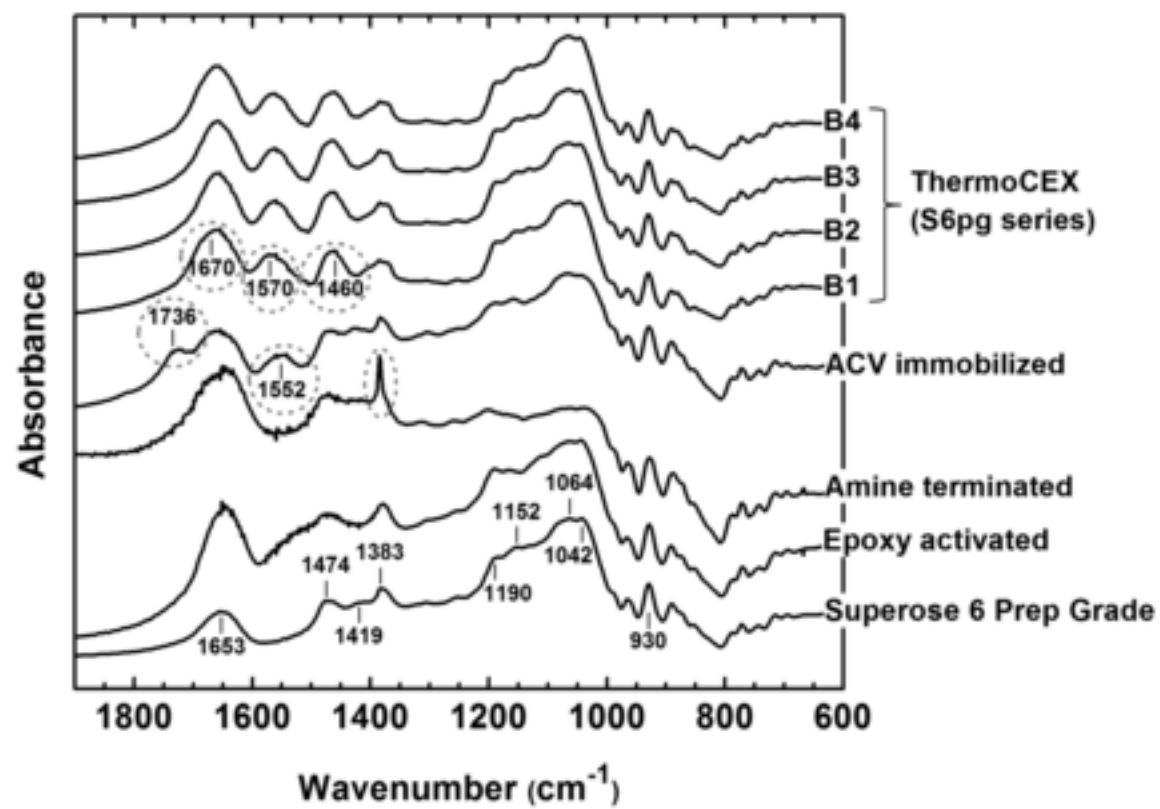


Figure 2





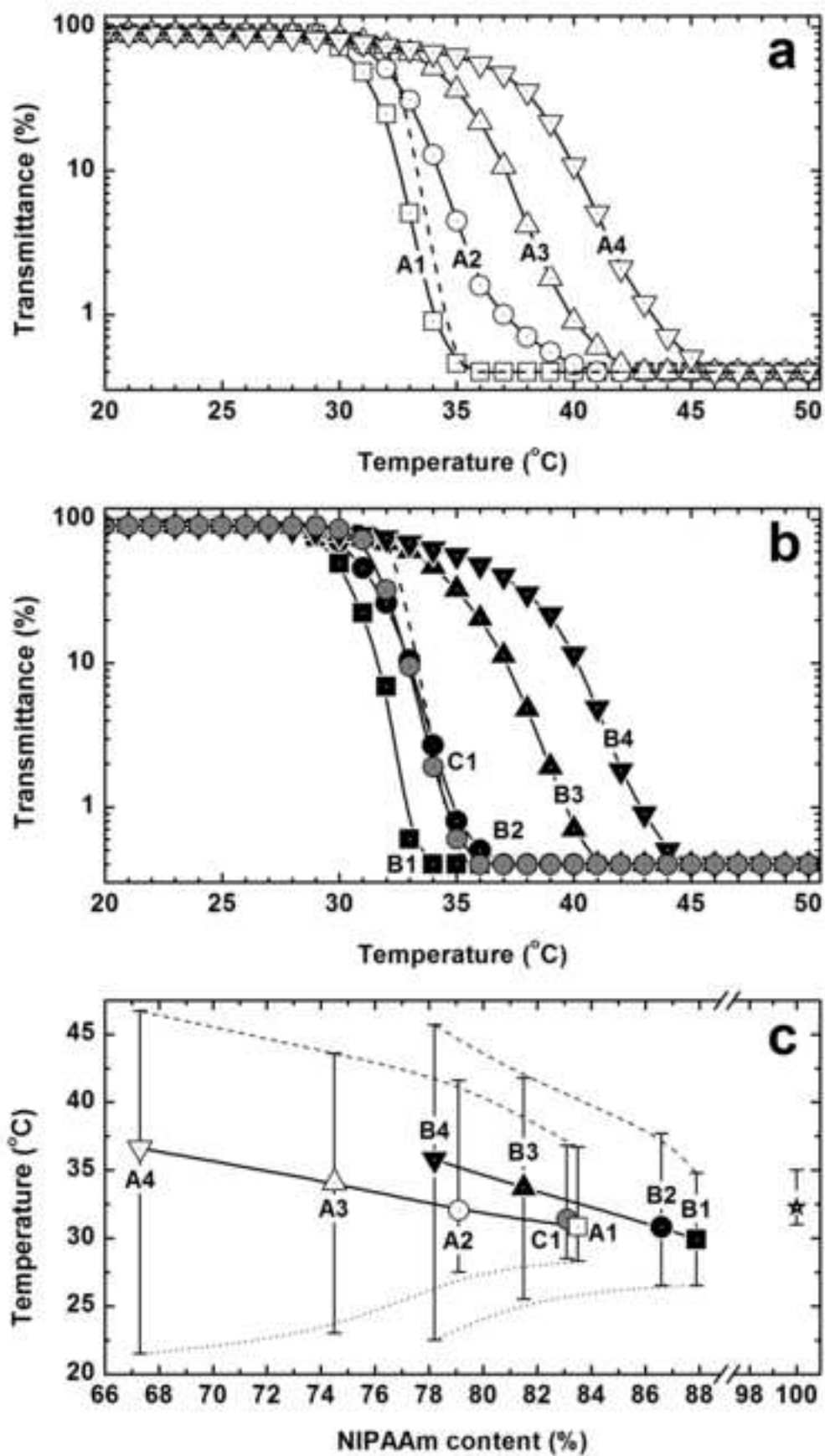


Figure 5

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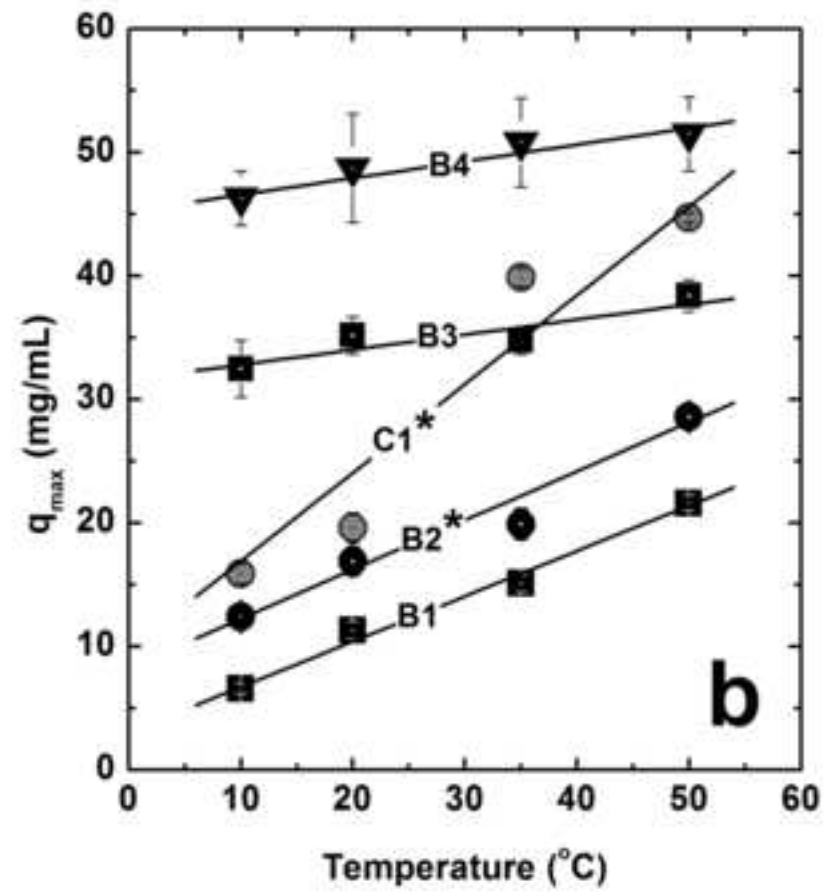
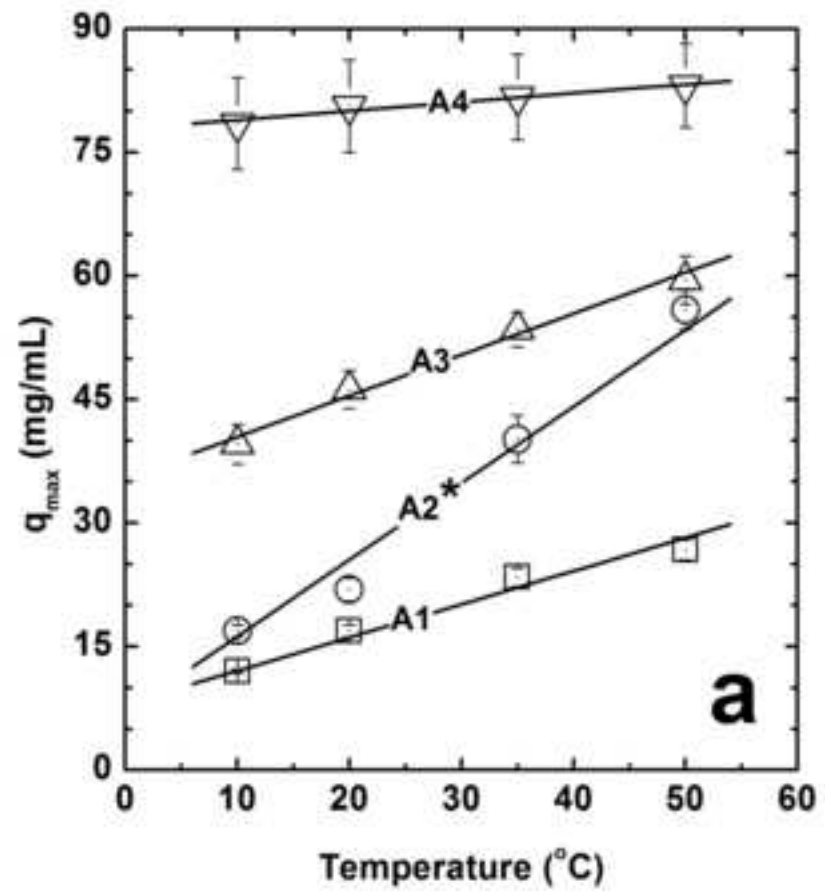
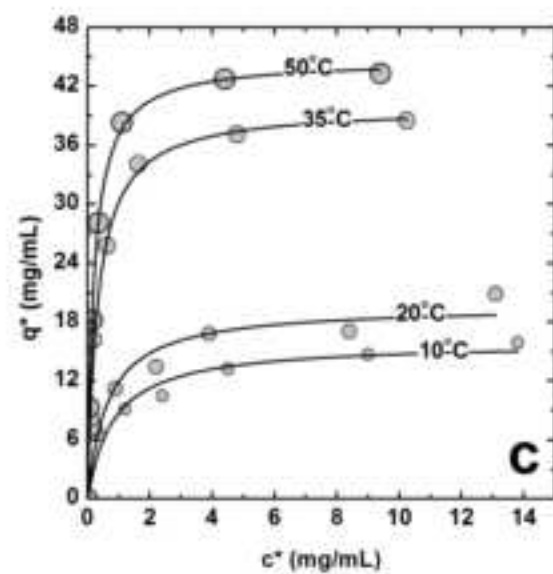
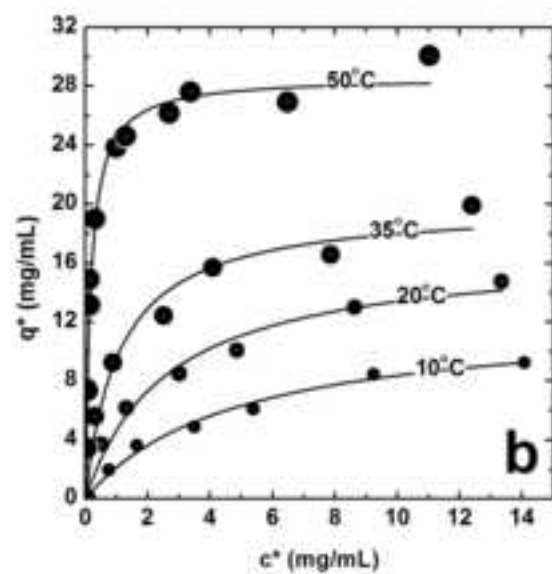
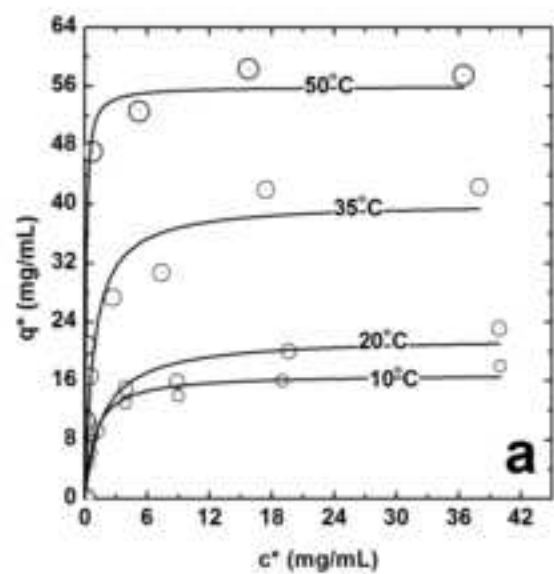
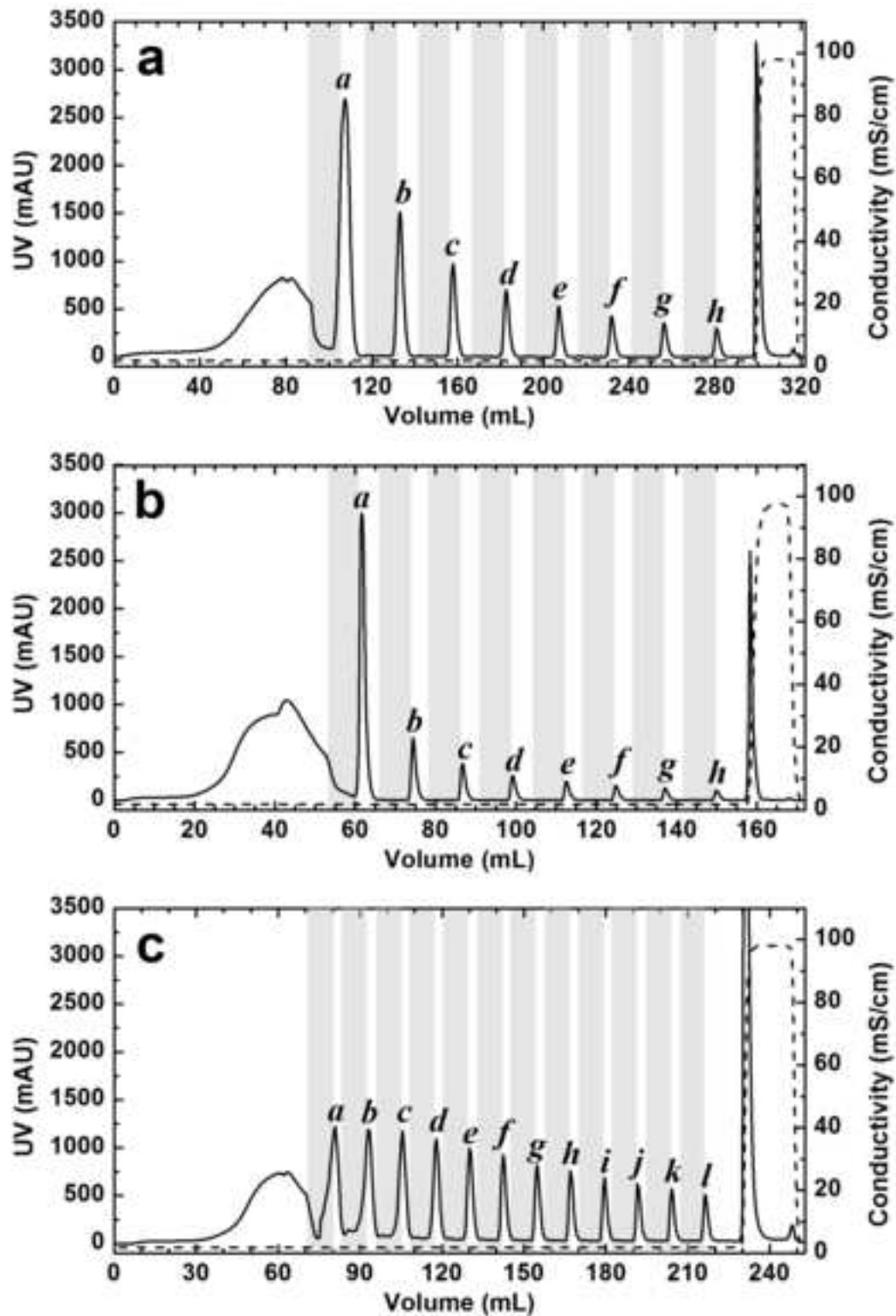


Figure 6

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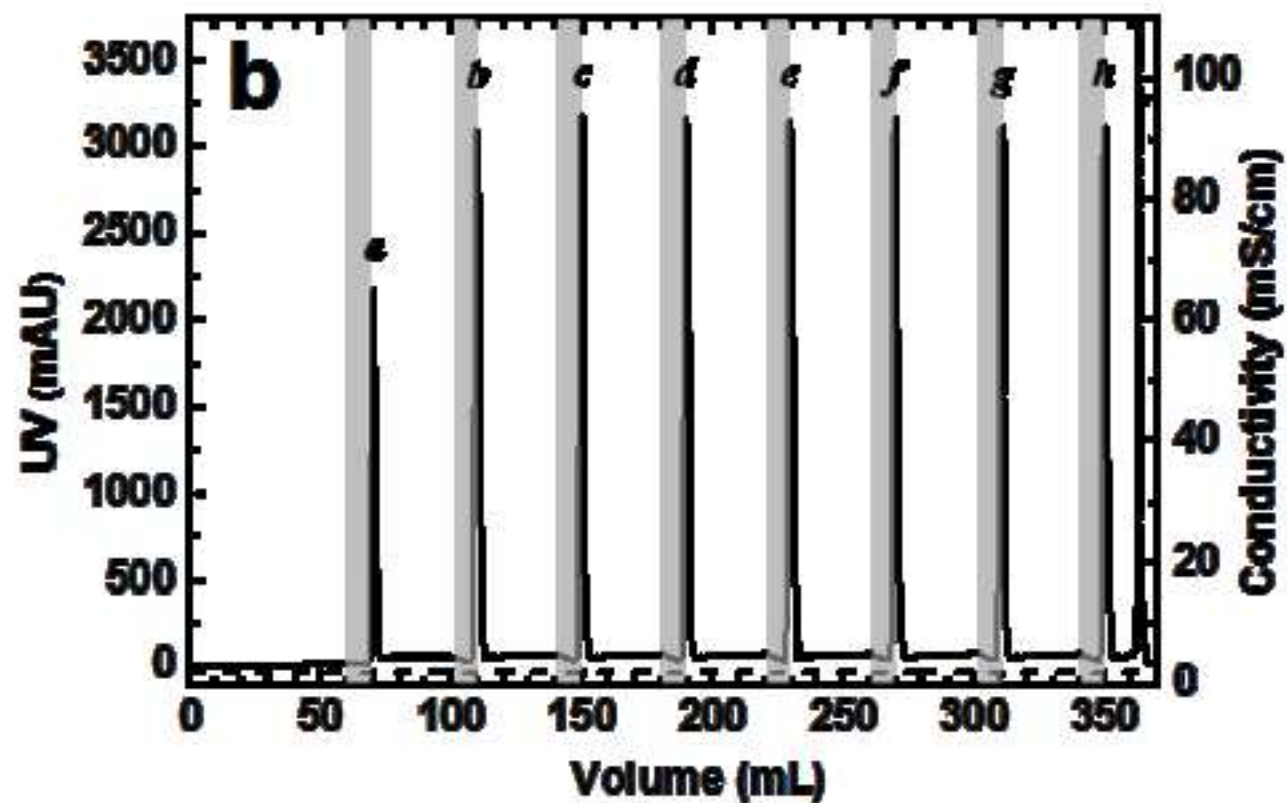
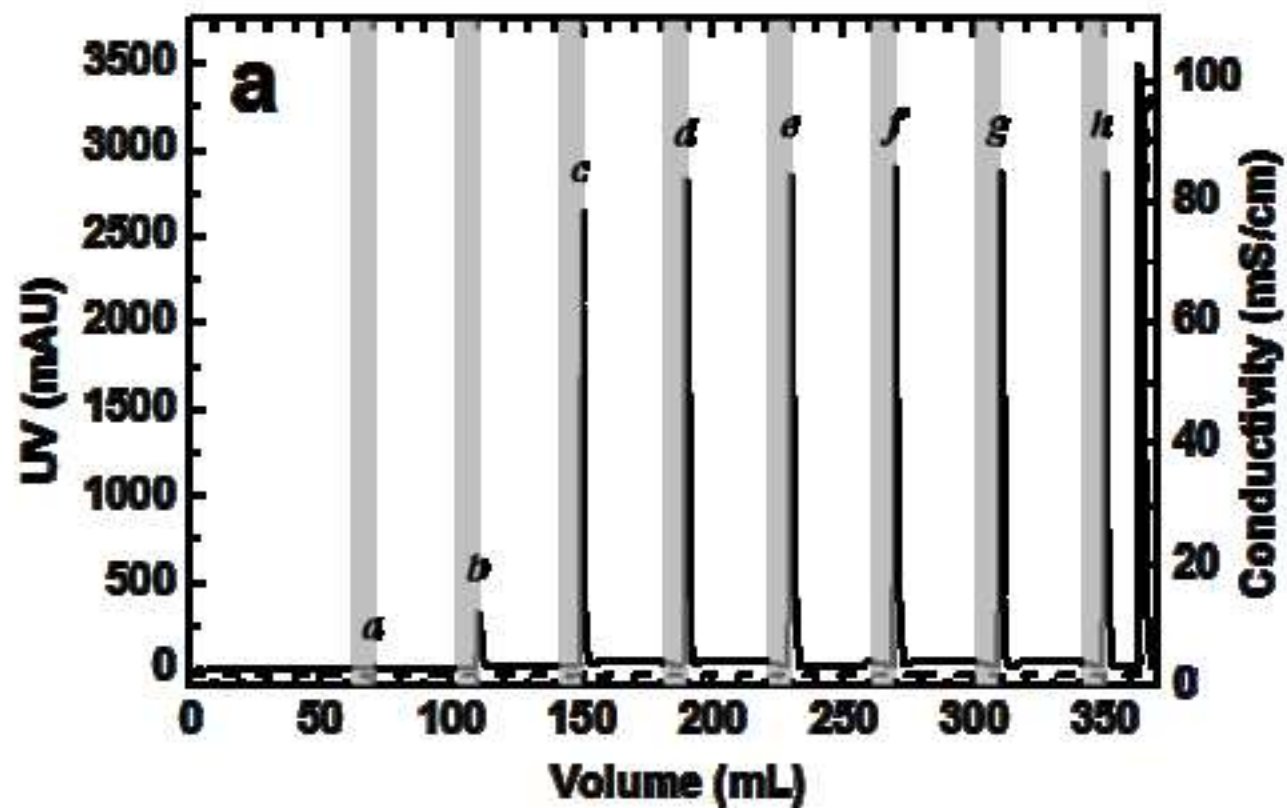


Figure 9

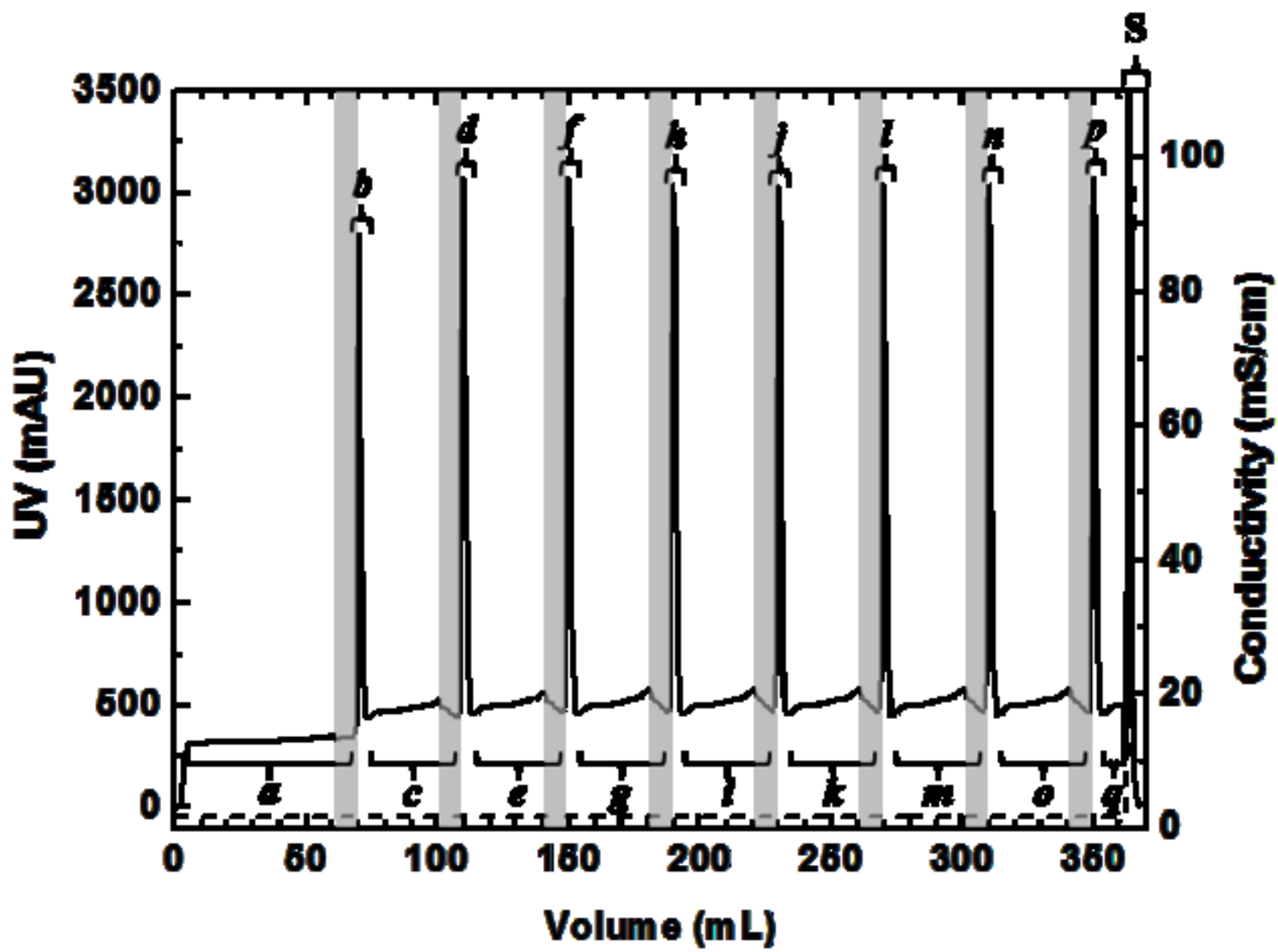


Figure 10

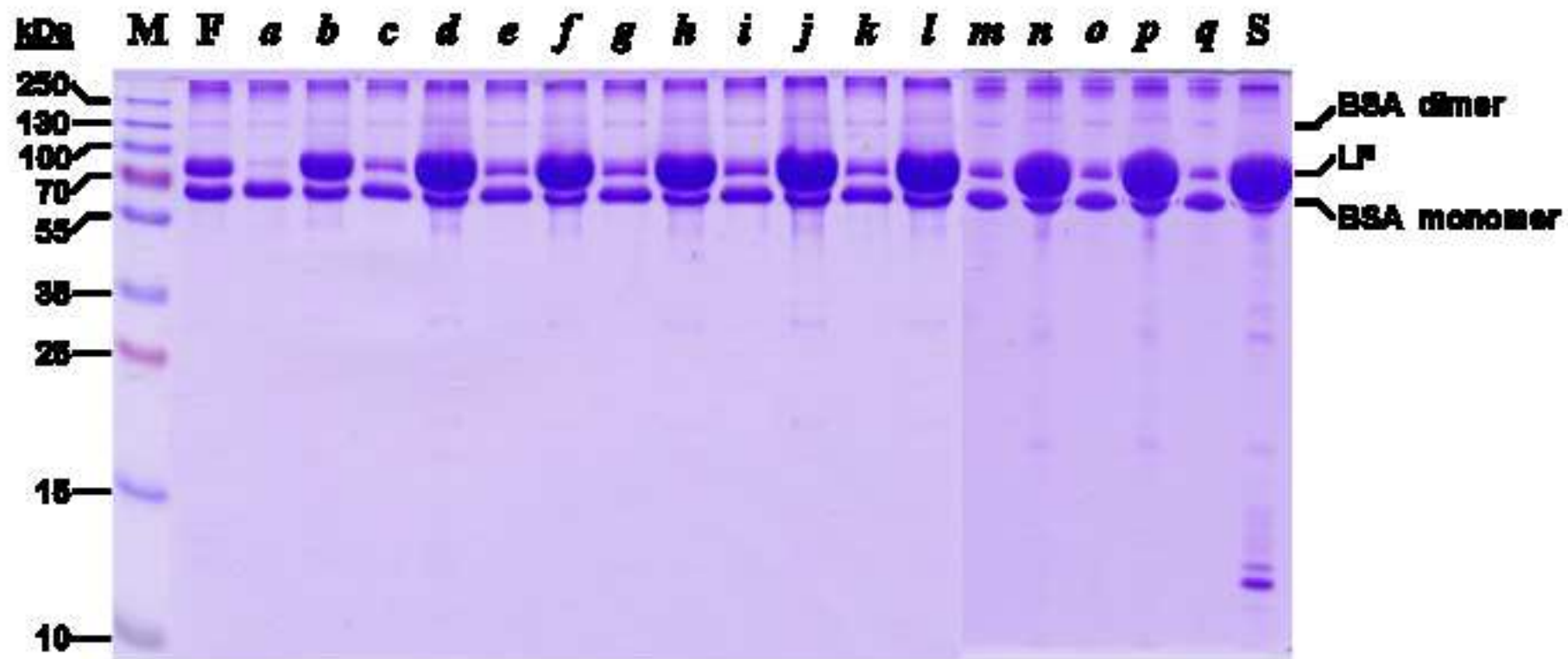


Table 1. Characterization of the Sepharose CL6B based thermoCEX supports prepared and used in this work. See text for details.

Parameter	ThermoCEX-CL-6B			
	A1	A2	A3	A4
<i>Support ID</i>	A1	A2	A3	A4
<i>Base matrix</i>	Sephacryl CL-6B (lot 10061497)			
<i>Particle size distribution</i>	45 – 165 μm (98%)			
<i>Globular protein fractionation range, M_r</i>	$1 \times 10^4 - 4 \times 10^6$ 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>				
<i>‘NIPAAm:tBAAm:AAc’ (mM)</i>	900:50:25	900:50:50	900:50:100	900:50:150
<i>‘NIPAAm:tBAAm:AAc’ ratio</i>	90:5:2.5	90:5:5	90:5:10	90:5:15
<i>Immobilized copolymer composition of thermoCEX support:</i>				
<i>‘NIPAAm + tBAAm’ content ($\mu\text{mol/g}$ dried support)^{b,c}</i>	4227	4177	4131	3974
<i>‘NIPAAm:tBAAm’ ratio^d</i>	90:10	88:12	85:15	81:19
<i>NIPAAm content ($\mu\text{mol/g}$ dried support)</i>	3804	3676	3511	3219
<i>tBAAm content ($\mu\text{mol/g}$ dried support)</i>	423	501	620	755
<i>Ion exchange capacity ($\mu\text{mol H}^+/\text{g}$ dried support)^e</i>	330	469	582	811
<i>‘pNIPAAm + tBAAm + AAc’ content ($\mu\text{mol/g}$ dried support)</i>	4557	4646	4713	4785
<i>‘NIPAAm:tBAAm:AAc’ ratio</i>	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>				
<i>NIPAAm + tBAAm + AAc</i>	15.1	15.0	14.5	14.1
<i>NIPAAm</i>	13.7	13.2	12.6	11.6
<i>tBAAm</i>	27.4	32.5	40.2	49.0
<i>AAc</i>	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
	B1	B2	B3	B4	C1
<i>Support ID</i>					
<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