



Gradient elution behavior of proteins in hydrophobic interaction chromatography with U-shaped retention factor curves

Arch Creasy^a, Joseph Lomino^b, Gregory Barker^b, Anurag Khetan^b, Giorgio Carta^{a,*}

^a Department of Chemical Engineering, University of Virginia, Charlottesville, VA, USA

^b Biologics Process Development, Bristol-Myers Squibb, Hopewell, NJ, USA

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ABSTRACT

Protein retention in hydrophobic interaction chromatography is described by the solvophobic theory as a function of the kosmotropic salt concentration. In general, an increase in salt concentration drives protein partitioning to the hydrophobic surface while a decrease reduces it. In some cases, however, protein retention also increases at low salt concentrations resulting in a U-shaped retention factor curve. During gradient elution the salt concentration is gradually decreased from a high value thereby reducing the retention factor and increasing the protein chromatographic velocity. For these conditions, a steep gradient can overtake the protein in the column, causing it to rebind. Two dynamic models, one based on the local equilibrium theory and the other based on the linear driving force approximation, are presented. We show that the normalized gradient slope determines whether the protein elutes in the gradient, partially elutes, or is trapped in the column. Experimental results are presented for two different monoclonal antibodies and for lysozyme on Capto Phenyl (High Sub) resin. One of the mAbs and lysozyme exhibit U-shaped retention factor curves and for each, we determine the critical gradient slope beyond which 100% recovery is no longer possible. Elution with a reverse gradient is also demonstrated at low salt concentrations for these proteins. Understanding this behavior has implications in the design of gradient elution since the gradient slope impacts protein recovery.

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1. Introduction

Hydrophobic interaction chromatography (HIC) plays an important role in protein purification for the removal of product and process related impurities both in the laboratory and at process scale [1–3]. The process is based on the reversible partitioning of proteins and other biopolymers between a mobile phase containing a kosmotropic salt, such as ammonium sulfate, and a mildly hydrophobic stationary phase. Normally, increasing the concentration of the kosmotropic salt increases partitioning toward the stationary phase, while reducing it increases partitioning toward the mobile phase. The effect of the kosmotropic salt concentration on protein retention in HIC is thus inverse to the well-known effect of kosmotropes on protein solubility in aqueous solutions [4].

Because of the strong dependence of protein retention on salt concentration, HIC is often operated in gradient mode starting at a high salt concentration and ending with a low salt concentration or, often, with no salt [5]. Stripping with water is even recom-

mended [6]. Gradient operation generally improves robustness and helps prevent irreversible binding and/or denaturation as a result of strong interactions with the mobile or stationary phase or with both [5]. Of course, for relatively easy separations, HIC can also be operated in a step mode with an abrupt change from high salt conditions, used for protein binding, to no salt to facilitate elution of strongly retained species. There have also been successful attempts to use HIC resins for protein separations with low concentrations of a kosmotropic salt [7] or even without a kosmotropic salt in the mobile phase [8]. In this case, protein binding results from “hydrophobic affinity” [9], rather than salting in/out effects, which in turn, can be modulated by varying pH or supplementing the mobile phase with certain additives in order to modulate retention [8]. One difficulty is that without addition of a kosmotropic salt, protein binding is often very weak so that, in practice, as noted by Kato et al. [7], a high initial kosmotrope concentration is used.

Although protein retention in HIC is typically regarded as a monotonic function of kosmotropic salt concentration [10], in some cases, protein retention can dramatically increase at very low salt concentrations resulting in a U-shaped retention factor curve. This behavior can become a concern in process operations since using a gradient from high to low salt concentrations can result in con-

* Corresponding author.

E-mail address: gc@virginia.edu (G. Carta).

ditions where the protein instead of eluting becomes trapped in the column, strongly retained at low salt. In this paper, we show that with U-shaped retention factor curves, the normalized gradient slope determines whether the protein will elute in the gradient, will partially elute, or will essentially never elute becoming trapped in the column for conditions where the retention factor increases as the kosmotrope concentration decreases. A local equilibrium analysis of this chromatographic behavior is presented along with predictions based on the numerical solution of the general rate model of chromatography. Experimental results are presented for two different monoclonal antibodies and for lysozyme on Capto Phenyl (High Sub) resin. One of the mAbs and lysozyme exhibit U-shaped retention factor curves and for each of them, we determine the critical gradient slope beyond which 100% recovery is no longer possible. The remaining mAb does not exhibit this behavior and elutes at virtually any gradient slope. This behavior has important implications for designing gradient elutions since the slope of the gradient directly impacts the recovery of the mAb and the fraction of protein left behind which can eventually foul the column.

2. Theory

2.1. Protein retention in hydrophobic chromatography

Melander and Horvath [4] and later Melander et al. [5] showed that the physico-chemical basis of both hydrophobic affinity chromatography at low salt concentrations and HIC at high salt concentrations is explained by the so-called solvophobic theory. Accordingly, the logarithm of the isocratic retention factor is expressed as a summation of Gibbs free energy differences between mobile and stationary phases that are associated with cavity formation, electrostatic effects, and van der Waals interactions. Following the treatment of Melander and co-workers, protein retention can be expressed as a function of salt concentration, C_M , by the following equation:

$$\ln(k' - k'_M) = \ln k'_0 - \frac{b\sqrt{C_M}}{1 + c\sqrt{C_M}} + \lambda C_M \quad (1)$$

where

$$k' = \varphi(\varepsilon_p + K) \quad (2)$$

is the retention factor of the protein, $\varphi = (1 - \varepsilon)/\varepsilon$ is the phase ratio, ε is the extraparticle or external porosity, ε_p is the intraparticle or internal porosity, K is the protein Henry's constant describing protein binding, k'_M is the retention factor of the salt, and k'_0 is the protein retention factor $k' - k'_M$ at $C_M = 0$. If the salt is not bound, then $k'_M = \varphi\varepsilon_p$. Although, as shown by Melander et al. [5], the parameters k'_0 , b , c , and λ are theoretically related to thermodynamic functions, in practice, they can be treated as empirical constants whose values are determined by data fitting. It should be noted, that the solvophobic theory expresses the salt concentration as a molality. In practice, however, if the salt concentrations are relatively low, replacing molalities with molarities results in a relatively small error.

At high values of C_M , the last term on the right hand side of Eq. (1) becomes dominant and this equation reduces to:

$$\ln(k' - k'_M) = \ln k'_0 - \frac{b}{c} + \lambda C_M = A + \lambda C_M \quad (3)$$

which gives the exponential dependence of protein retention on salt concentration normally observed in HIC. At low C_M values, the third term on the right hand side of Eq. (1) becomes less important and k' values that decrease as C_M increases are predicted resulting in a U-shaped curve with a minimum k' that depends on the specific values of $\ln k'_0$, b , c , and λ . This possibility was experimentally

observed for several proteins by Melander et al. [5] using NaClO_4 as the mobile phase modulator in HIC and by Machold et al. [11] for lysozyme on a number of HIC resins using ammonium sulfate as the modulator. In addition to the solvophobic theory, other adsorptive interactions can also lead to U-shaped retention factor curves in HIC. For example, hydrophobic binding and protein unfolding on the resin surface can lead to strong retention at low salt concentrations, weaker interactions at intermediate kosmotrope concentrations and strong binding again at high kosmotrope concentration. Additionally, U-shaped retention factor curves can be a result of the interplay of hydrophobic and electrostatic interactions which has been observed for protein binding to multimodal chromatography media by Melander et al. [12], Nfor et al. [13], Kallberg et al. [14], and Lee et al. [15]. Finally, U-shaped retention factor curves as a function of modifier concentration have also been observed in reverse phase chromatography (RPC) and attributed to secondary interactions with the stationary phase backbone [16].

2.2. Protein retention in gradient elution

2.2.1. Local equilibrium model

Protein retention in gradient elution can be predicted from the isocratic retention factor using the local equilibrium theory for conditions where the binding isotherm is linear and the mass transfer kinetics is fast. The theory is well known [2,17–19] and only a brief outline is presented here.

With a linear gradient, the salt concentration at a particular time, t , and distance x from the column entrance is given by:

$$C_M = C_{M,0} + \beta \left[t - \frac{x}{v} (1 + k'_M) \right] \quad (4)$$

where $C_{M,0}$ is the initial salt concentration, β is the gradient slope, and v is the mobile phase interstitial velocity. The gradient slope is given by $\beta = (C_{M,f} - C_{M,0})/t_G$ where $C_{M,f}$ is the salt concentration at the end of the gradient and t_G is the duration of the gradient in time units. Taking the differential of Eq. (4), we obtain:

$$\frac{dC_M}{dx} = \frac{\partial C_M}{\partial t} \frac{dt}{dx} + \frac{\partial C_M}{\partial x} = \beta \frac{dt}{dx} - \frac{\beta}{v} (1 + k'_M) \quad (5)$$

Movement of the protein through the column is described by the chromatographic velocity

$$v_c = \frac{dx}{dt} = \frac{v}{1 + k'(C_M)} \quad (6)$$

where $k'(C_M)$ is the protein retention factor at C_M . Combining Eqs. (5) and (6) yields:

$$\frac{dC_M}{dx} = \frac{\beta}{v} (1 + k') - \frac{\beta}{v} (1 + k'_M) = \frac{\beta}{v} (k' - k'_M) \quad (7)$$

In turn, this equation can be written as:

$$\frac{dC_M}{d\gamma} = k' - k'_M \quad (8)$$

where $\gamma = \beta x/v$ is a normalized gradient slope. The latter can also be expressed in the terms of the duration of the gradient in column volumes units, CV_G , as $\gamma = \varepsilon (C_{M,f} - C_{M,0})/CV_G$. Eq. (8) can be integrated with initial condition $C_M|_{\gamma=0} = C_{M,0}$ to determine the salt concentration, $C_{M,R}$ at which the protein elutes. The corresponding elution time is found from Eq. (4) as:

$$t_R = \frac{C_{M,R} - C_{M,0}}{\beta} + \frac{x}{v} (1 + k'_M) \quad (9)$$

This equations can be rewritten as:

$$CV_R = \varepsilon \left[\frac{C_{M,R} - C_{M,0}}{\gamma} + (1 + k'_M) \right] \quad (10)$$

where $CV_R = t_R \varepsilon v / x$ is the number of column volumes at elution. An analytical solution of Eq. (8) is easily obtained if k' is described by Eq. (3) and is given by [17,20]:

$$C_M = -\frac{1}{\lambda} \ln [e^{-\lambda C_{M,0}} - A \lambda \gamma] \quad (11)$$

where $A = k'_0 e^{-b/c}$. Numerical integration of Eq. (8) is however required in the general case of Eq. (1).

2.2.2. Rate model

The theory described in Section 2.2.1 can only describe situations where band broadening is small, which are encountered when small resin particles are used, when the intraparticle diffusivity is high, or when the residence time is high. In practice, however, when dealing with larger proteins, band broadening is substantial and the peak elutes over a range of salt concentrations rather than at the single value predicted by the local equilibrium model. Nevertheless, even for those conditions the local equilibrium model is expected to describe the general trends with respect to the peak mean retention time. A model taking into account band broadening is needed however for a more precise description of the elution behavior, especially when conditions are such that only a portion of the injected protein elutes in the gradient.

Two well-known rate models were used in this work to describe these scenarios. The first, referred to as the pore diffusion model, describes the adsorption/desorption rate with a differential mass balance for the stationary phase. The second, referred to as the LDF model, describes the adsorption/desorption rate based on the linear driving force approximation, using a liquid phase concentration driving force. The model equations are as follows:

Pore diffusion model

$$\frac{\partial q_i}{\partial t} = \frac{D_{e,i}}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial c_i}{\partial r} \right) \quad (12)$$

$$r = 0 \rightarrow \partial c_i / \partial r = 0 \quad (12a)$$

$$r = r_p \rightarrow c_i = C_i \quad (12b)$$

$$\frac{\partial \bar{q}_i}{\partial t} = -\frac{3D_{e,i}}{r_p} \frac{\partial c_i}{\partial r} \Big|_{r=r_p} \quad (12c)$$

LDF model

$$\frac{\partial \bar{q}_i}{\partial t} = \frac{15D_{e,i}}{r_p^2} \left(C_i - \frac{\bar{q}_i}{\varepsilon_p + K} \right) \quad (13)$$

In these equations, $q_i = \varepsilon_p c_i + K c_i$ is the local concentration of species i at each point in the particle, $\bar{q}_i$ is the value of q_i averaged over the particle volume, $D_{e,i}$ is the effective pore diffusivity, and r_p is the particle radius. In both cases, these equations are coupled with the following differential balance for the column:

$$\varepsilon \frac{\partial C_i}{\partial t} + (1 - \varepsilon) \frac{\partial \bar{q}_i}{\partial t} + \varepsilon v \frac{\partial C_i}{\partial x} = 0 \quad (14)$$

$$x = 0 \rightarrow C_i = C_{i,F}(t) \quad (14a)$$

where $C_{i,F}$ is the feed concentration. Both models neglect axial dispersion and film mass transfer resistance. This is not appropriate for all situations but is expected to be valid at values of the reduced velocity $v' = v d_p / D_0$ greater than about 100, where d_p is the particle diameter and D_0 is the protein diffusivity, since, for these conditions, band-broadening is controlled by intraparticle transport [2]. For our experiments v' had values between 800 and 8000, so that our assumption seems justified.

The analytical solution of both rate models in Ref. [21] was used to describe the isocratic elution behavior yielding nearly indistinguishable results. The LDF model was thus used to describe the LGE runs. In this case, Eqs. (13) and (14) were solved numerically as

described by Creasy et al. [22]. The analytical solution of the LDF model for LGE in Ref. [20] was used to corroborate the validity of the numerical solution for cases where complete elution of the peak was predicted.

3. Experimental

3.1. Materials

The resin used in this work is Capto Phenyl (High Sub), which was obtained from GE Healthcare (Uppsala, Sweden). The particle diameter of the sample used (volume average $d_p = 78 \mu\text{m}$) was obtained from microphotographs. The intraparticle porosity ($\varepsilon_p = 0.91$) and the mean pore radius ($r_{\text{pore}} = 30 \text{ nm}$) were obtained by inverse size chromatography (iSEC) using dextran standards according to the method in Ref. [23] as described in Ref. [24].

The proteins used in this work are lysozyme ($pI \sim 11$, MW $\sim 15 \text{ kDa}$), obtained from MilliporeSigma (St. Louis, MO, USA), and two monoclonal antibodies, mAbA ($pI \sim 8.2$, MW $\sim 150 \text{ kDa}$), and mAbB ($pI \sim 8$, MW $\sim 150 \text{ kDa}$). Both mAbs were highly pure with undetectable aggregate levels. However, based on their retention on a Butyl Sepharose HIC column (GE Healthcare), mAbB is much more hydrophobic than mAbA. Chemicals used in buffer preparation (trisodium phosphate, phosphoric acid, and ammonium sulfate) were obtained from MilliporeSigma (St. Louis, MO, USA) and Fisher Scientific (Fair Lawn, NJ, USA).

3.2. Methods

All buffers were prepared by mixing trisodium phosphate and ammonium sulfate with distilled-deionized water to yield 30 mM Na^+ and the desired ammonium sulfate molarity. The pH was then adjusted to 7.2 by stirring in drop-wise concentrated phosphoric acid. Lysozyme solutions were prepared by adding weighed amounts of the protein to these buffers. mAb solutions were prepared by size exclusion chromatography with a HiPrep 26/10 desalting column from GE Healthcare (Piscataway, NJ, USA).

Isocratic elution experiments were conducted by slurry packing the resin into a $0.5 \times 5 \text{ cm}$ Tricorn column from GE Healthcare according to the resin manufacturer packing instructions. Packing quality was verified using pulse injections of 0.8 M NaCl in water with 0.4 M NaCl in water as eluent. The extraparticle or external porosity of this column was determined to be $\varepsilon = 0.43$ based on the elution of 2000 kDa dextran. Protein samples (20 μL , containing 11 g protein/L for the mAbs and 5 g protein/L for lysozyme) were injected and eluted isocratically at a flow rate of 0.5 mL/min (153 cm/h) using an Acuity H-Class UPLC system from Waters (Milford, MA, USA) with UV monitoring at 280 nm. This system has low dead volumes providing more accurate results for the smaller column used in these experiments. The ensuing chromatograms were used to determine the protein retention factor from the first moment of the eluted peak after adjustment for the extracolumn volume.

LGE experiments were carried out with a $0.5 \times 20 \text{ cm}$ Tricorn column packed with the resin as described above to a bed height of 19.3 cm using an AKTA Pure 25 system at a flow rate of 0.5 mL/min (153 cm/h) with UV monitoring at 280 nm along with conductivity monitoring of the salt gradient. Because of the larger column used for these experiments, the larger dead volumes in this system compared to the UPLC system did not compromise the accuracy of the results. The extraparticle porosity of this column was $\varepsilon = 0.41$ based on the elution of 2000 kDa dextran. As for the isocratic runs, protein samples (100 μL , containing 11 g protein/L for the mAbs and 5 g protein/L for lysozyme) were injected at either 1 M ammonium sulfate for lysozyme or 0.2 M ammonium sulfate for the mAbs and

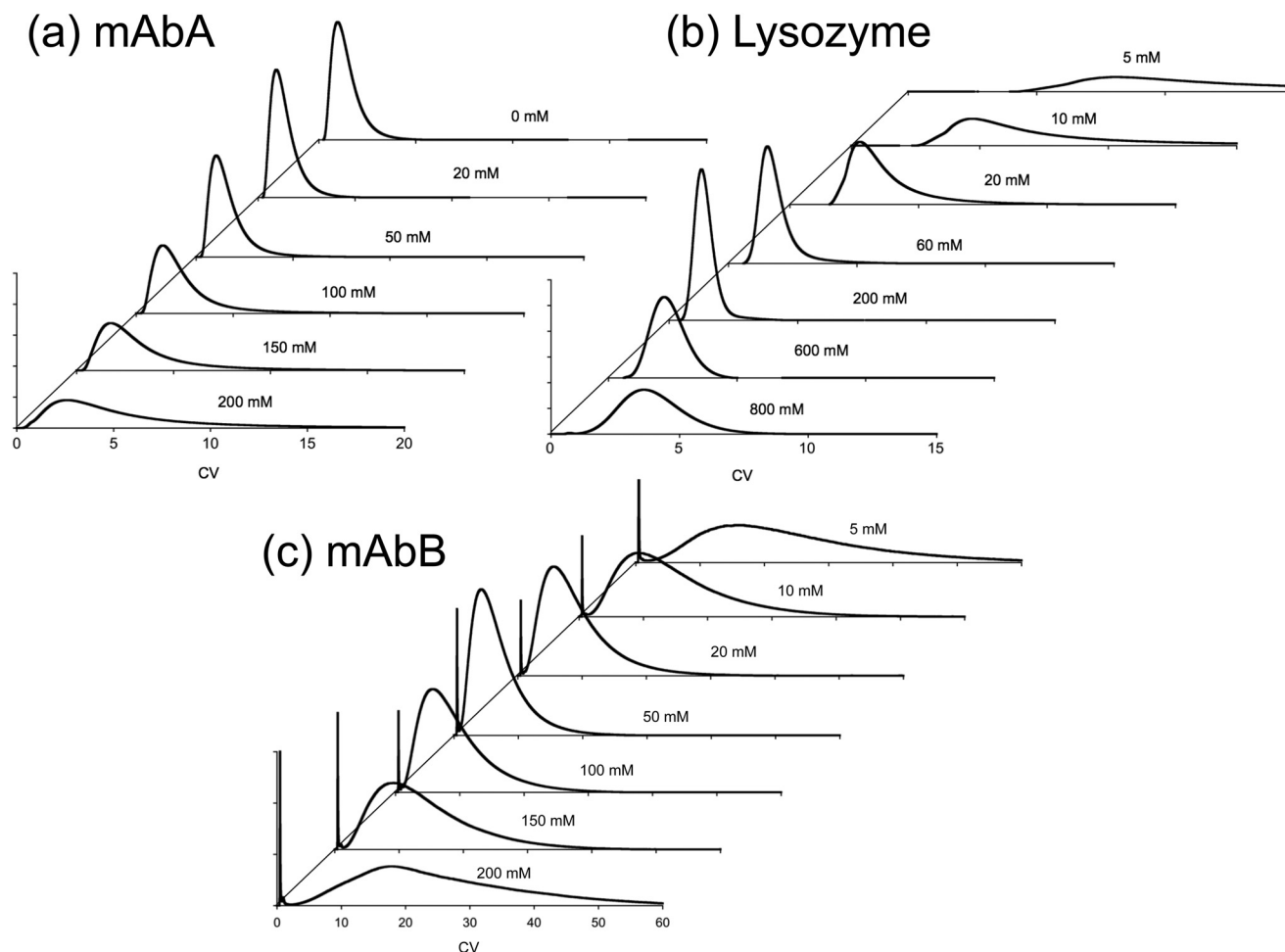


Fig. 1. Isocratic elution peaks obtained for (a) mAbA, (b) lysozyme, and (c) mAbB at the ammonium sulfate concentrations indicated for each curve.

eluted with a linear gradient to 0 M ammonium sulfate followed by a 5 CV hold step for the mAbs and a 3 CV hold step for lysozyme at 0 M ammonium sulfate. UV280 and conductivity were recorded and converted to the corresponding protein and ammonium sulfate concentrations using independently obtained calibration curves. Appropriate shifts of UV and conductivity signals were made to take into account the dead volumes between columns and detectors. The ammonium sulfate concentration at elution was determined from the first moment of the eluted peaks. The protein recovery was obtained by material balance. A clean-in-place step with 0.1 M sodium hydroxide was used to regenerate the column after each LGE run. A large regeneration peak was observed for cases where low recovery was observed during LGE virtually proportional to the amount of unrecovered protein. All experiments were conducted at room temperature, $21 \pm 1^\circ\text{C}$.

4. Results

4.1. Isocratic elution behavior

Fig. 1 shows the isocratic elution behavior of the three proteins used in this work. As seen in Fig. 1 a, mAbA could be eluted isocratically at different ammonium sulfate concentrations from 0.2 M to 0, with essentially 100% recovery in each case. This protein exhibited the normally expected behavior of decreasing retention with decreasing ammonium sulfate concentrations. On the other hand, both lysozyme and mAbB exhibited a minimum retention

Table 1

Parameters of the solvophobic model (Eq. (1)) obtained from the isocratic retention factor for the proteins used in this work with C_M in mol/L and $\varepsilon = 0.43$.

Parameter	mAbA	Lysozyme	mAbB
$\ln(k'_0)$	0.566 ± 0.118	5.53 ± 0.26	6.17 ± 0.24
b	1.95 ± 1.98	42.0 ± 7.14	45.7 ± 7.4
c	1.35 ± 5.30	4.74 ± 1.12	5.74 ± 1.20
λ	12.1 ± 1.6	4.64 ± 0.59	17.9 ± 1.0

at an intermediate ammonium sulfate concentration and neither protein could be eluted at 0 M ammonium sulfate. The lysozyme peaks are considerably sharper than those obtained from mAbB as a result of the smaller size and larger diffusivity. mAbB is more strongly retained than lysozyme at the higher ammonium sulfate concentration suggesting a greater hydrophobic character.

Fig. 2 shows the retention factors calculated from the isocratic elution runs of Fig. 1 as a function of the ammonium sulfate concentration. While k' for mAbA increases monotonically with C_M , both lysozyme and mAbB exhibit U-shaped retention factor curves. This behavior is especially pronounced for mAbB, which exhibits a strong minimum at about 0.06 M ammonium sulfate. The lines in this figure are based on the solvophobic model, Eq. (1), with the parameters $\ln k'_0$, b , c , and λ obtained by least squares fit using Kaleidagraph v. 4.5 (Synergy Software) and summarized in Table 1. k'_M was set equal to 1.21, based on the iSEC data.

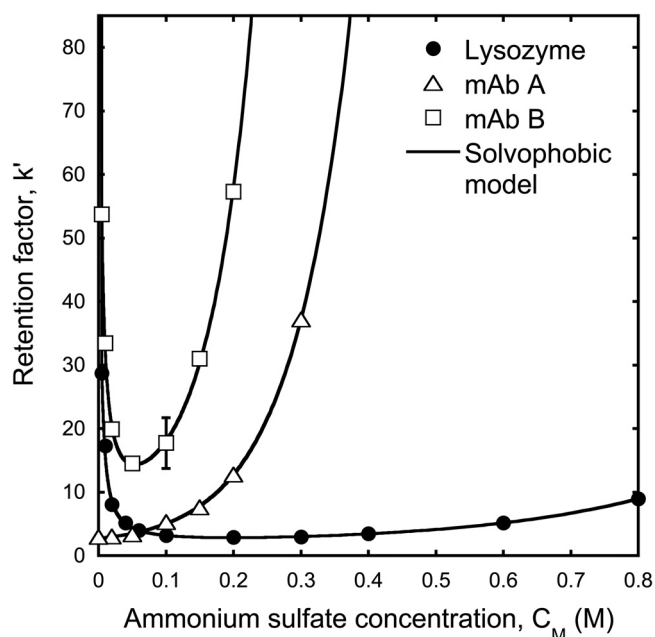


Fig. 2. Retention factors obtained from the data in Fig. 1 for mAbA, Lysozyme, and mAbB as a function of the ammonium sulfate concentration. All solutions contained 10 mM trisodium phosphate adjusted to pH 7.2 with phosphoric acid. The lines are based on the solvophobic model, Eq. (1), with parameters in Table 1.

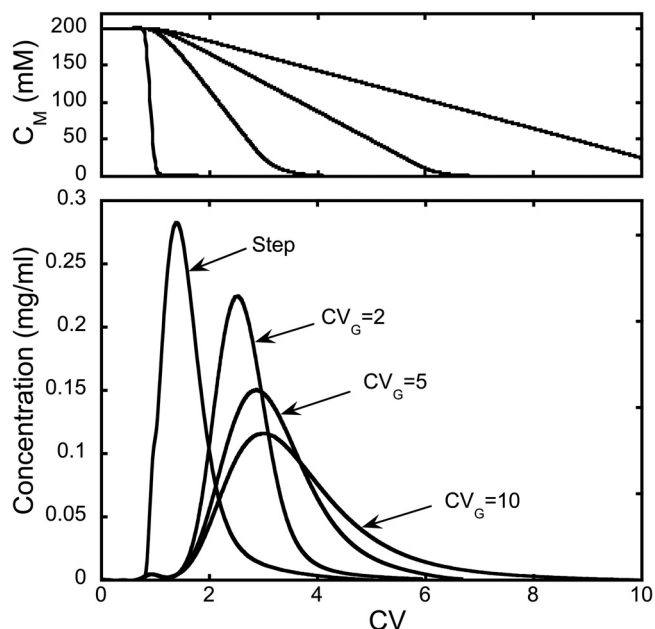


Fig. 3. LGE results for mAbA with linear gradients from 0.2 to 0 M ammonium sulfate. CV_G is the duration of the gradient in column volumes units. The gradient profiles shown in the top panel are obtained from the conductivity signal using a calibration curve.

4.2. LGE behavior

Figs. 3–5 show the LGE results for the three proteins used in the work. As seen in these figures different behaviors are exhibited by the three different proteins as a function of the gradient slope. For mAbA (Fig. 3) elution is obtained with essentially 100% recovery even with very sharp gradients. The elution peak becomes sharper and elutes at lower ammonium sulfate concentrations as the gradient slope increases (or the gradient volume, CV_G , decreases). Even with a step change to 0 M ammonium sulfate, essentially complete

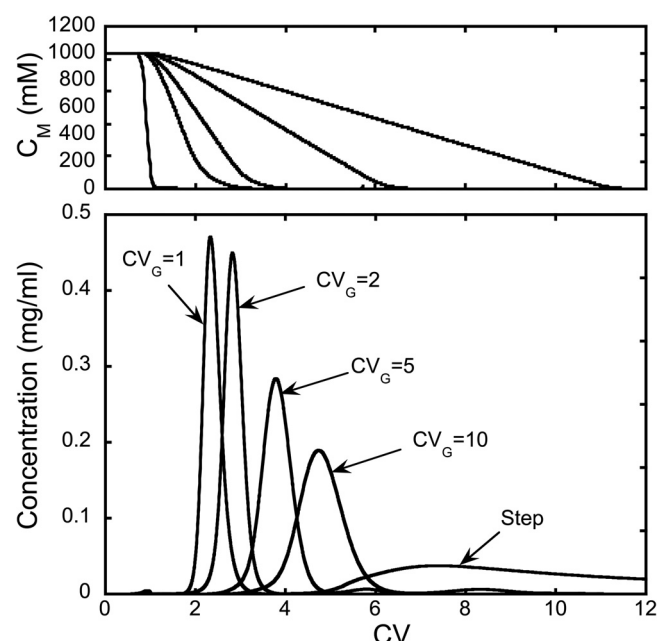


Fig. 4. LGE results for lysozyme with linear gradients from 1 to 0 M ammonium sulfate. CV_G is the duration of the gradient in column volumes units. The gradient profiles shown in the top panel are obtained from the conductivity signal using a calibration curve.

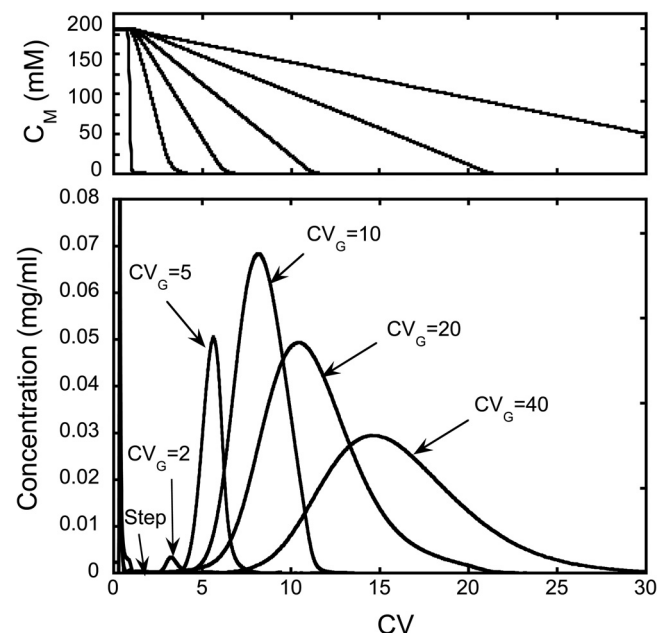


Fig. 5. LGE results for mAbB with linear gradients from 0.2 to 0 M ammonium sulfate. CV_G is the duration of the gradient in column volumes units. The gradient profiles shown in the top panel are obtained from the conductivity signal using a calibration curve.

elution is obtained with the elution peak emerging from the column immediately after the ammonium sulfate step.

For lysozyme (Fig. 4), the elution behavior is analogous to that of mAbA for the shallower gradient slopes. However, a different result is obtained with a step change to 0 M ammonium sulfate, with the peak eluting isocratically only many CVs after the ammonium sulfate step. The elution peak is very shallow in this case as a result of the long retention in the column. Finally, for mAbB (Fig. 5), complete elution occurs only with relatively shallow gradients. Gradients with duration of 10 CV or less resulted in incomplete

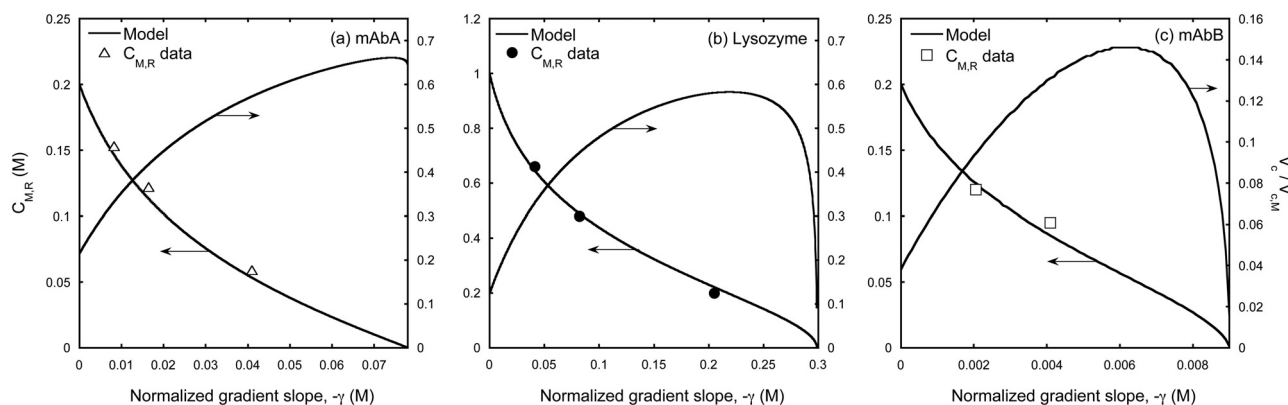


Fig. 6. Plots of the ammonium sulfate concentration at elution, $C_{M,R}$, vs. the normalized gradient slope, $-\gamma$, for (a) mAbA, (b) lysozyme, and (c) mAbB based on the local equilibrium model. The vertical right-hand side axis gives the protein chromatographic velocity v_c divided by the chromatographic velocity of the modulator, $v_{c,M}$. Conditions are the same as those in Figs. 3–5. Experimental $C_{M,R}$ values based on the ammonium sulfate concentration at the first moment of the protein peak are shown only for conditions where protein recovery was essentially 100%.

elution, with much of the protein remaining trapped in the column at the end of the gradient and even during the ensuing hold step. Almost no elution is obtained with a 2 CV gradient and no elution at all was seen even after 16 CVs using a step change to 0 M ammonium sulfate.

The behaviors exhibited by the three proteins appear to be qualitatively consistent with the corresponding k' trends observed isocratically. The normal elution behavior of mAbA is a consequence of the monotonically increasing k' values. In this case, lowering the ammonium sulfate concentration always results in reduced retention and, thus, increased chromatographic velocity. As a result, the peak always elutes within the gradient. For the other two proteins, but especially for mAbB, because of the U-shaped retention factor plot, the chromatographic velocity increases during the initial portion of the gradient allowing the protein to move down the column but decreases as the protein is exposed to the final portion of the salt gradient. Whether the protein reaches the column outlet or not depends on the gradient slope. If the gradient slope is too high (10 CVs or less in the case of mAbB), lower ammonium sulfate concentrations overtake the protein in the column increasing retention and causing the chromatographic velocity to decrease. At this point the protein becomes trapped in the column in the down-sloping region of the retention factor plot resulting in low or no recovery even for long CVs. The behavior is less striking for lysozyme, since the retention factor plot is much broader with a less pronounced minimum. Even so, the extremely sharp gradient generated by a step change to 0 M ammonium sulfate at the column entrance results in a strong retention of the protein in the column.

4.3. Prediction of LGE behavior from isocratic data

4.3.1. Local equilibrium model results

Fig. 6a–c show the ammonium sulfate concentrations at peak elution, $C_{M,R}$, vs. the normalized gradient slope, $-\gamma$, for the three different proteins calculated according to Eqs. (1) and (8)–(10) using the parameters in Table 1. The corresponding protein chromatographic velocity normalized by the modulator chromatographic velocity, $v_c/v_{c,M} = (1 + k'_M)/(1 + k')$, is also shown in these figures. v_c is the rate at which the protein band moves through the column at each $C_{M,R}$ -value. Qualitatively similar behaviors are observed for all three proteins at low values of $-\gamma$ (i.e. for shallow gradients) and experimental values of $C_{M,R}$ (shown only for the LGE runs that gave essentially 100% recovery) are in good agreement with the model predictions. On the other hand, distinctly different behaviors are predicted for the three proteins at high values of $-\gamma$ (i.e. for steep gradients). For mAbA, as $-\gamma$ increases the protein chromatographic

velocity increases monotonically, reaching a high value close to the chromatographic velocity of the ammonium sulfate modulator as $C_{M,R}$ approaches zero. Thus, the model predicts that mAbA will elute within the gradient or shortly after the gradient regardless of gradient slope. On the other hand, for both lysozyme (Fig. 6b) and mAbB (Fig. 6c), the chromatographic velocity decreases sharply at high values of $-\gamma$ as a result of the U-shaped retention factor curve. In the case of mAbB, in particular, the chromatographic velocity is close to zero at $C_{M,R} = 0$ suggesting that, for a given initial modulator concentration, a critical gradient slope exists beyond which this protein becomes trapped in the column and does not elute over reasonable time scales. From Fig. 6c, the critical value of $-\gamma$ is about 0.009 M, which corresponds to a gradient duration $CV_G \sim 9$. Indeed, as seen in Fig. 5, gradient durations smaller than 10 CVs resulted in decreasing recovery with little or no elution for very steep gradients.

4.3.2. Rate model results

Detailed predictions based on the rate model were made only for mAbB, which exhibited the greatest deviations from the normally expected behavior. This model requires the protein effective diffusivity, D_e , which was obtained by matching the experimental isocratic elution curves to the analytical solution. A value of $D_e = (1.0 \pm 0.2) \times 10^{-7} \text{ cm}^2/\text{s}$ provided a good fit for all isocratic elution runs. Fig. 7 shows representative examples at 0.05 and 0.2 M ammonium sulfate. As seen from this figure there is little difference between the pore diffusion model and the LDF model.

Fig. 8 shows numerical predictions of the elution curves for the experiments of Fig. 5 using the LDF model. Comparing Figs. 5 and 8 shows that model predictions are in reasonable agreement with the experimental data. In particular, the numerical model predicts incomplete elution with gradient lengths of 10 CVs or less and virtually no elution for a 2 CV gradient or for a step change to 0 M ammonium sulfate. Note that for 5 and 10 CV gradients, the numerical model predicts extremely slow elution of the protein trapped in the column at the end of the gradient, which is not observed in the experiment. This small discrepancy is likely due to the difficulty of describing retention at 0 M ammonium sulfate. Since, experimentally, the protein did not elute isocratically at 0 M ammonium sulfate, the retention factor at $C_M = 0$ is extrapolated from the fit of the solvophobic model and is thus affected by considerable uncertainty. A higher value of $\ln k'_0$ around 6.9 would eliminate this discrepancy. Some of the simplifications inherent in the model used here may have also contributed to the differences observed between predicted and experimental results. For example, in addition to neglecting axial dispersion for the protein, our LDF model

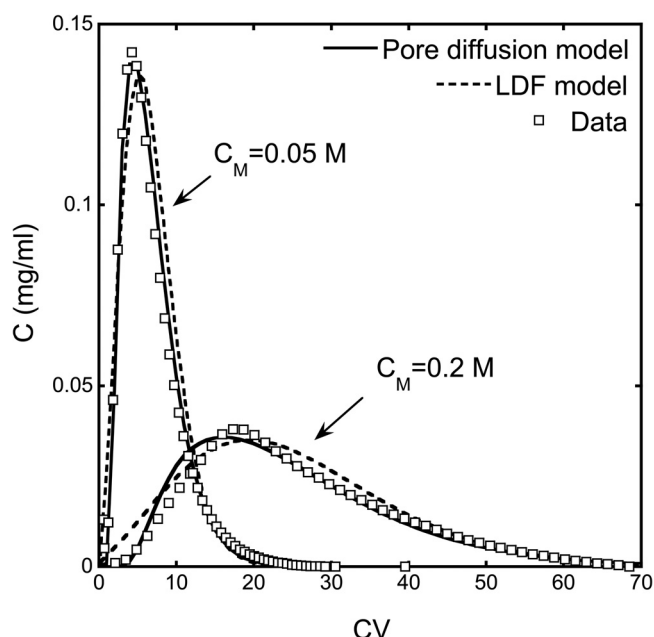


Fig. 7. Comparison of experimental isocratic elution curves of mAbB at 0.05 and 0.2 M ammonium sulfate concentrations with curves calculated according to both the pore diffusion model and the LDF model using a fitted value of $D_e = (1.0 \pm 0.2) \times 10^{-7} \text{ cm}^2/\text{s}$. The curves are calculated with the analytical solution of Eqs. (12)–(14) in Ref. [21]. Experimental conditions are the same as in Fig. 1c.

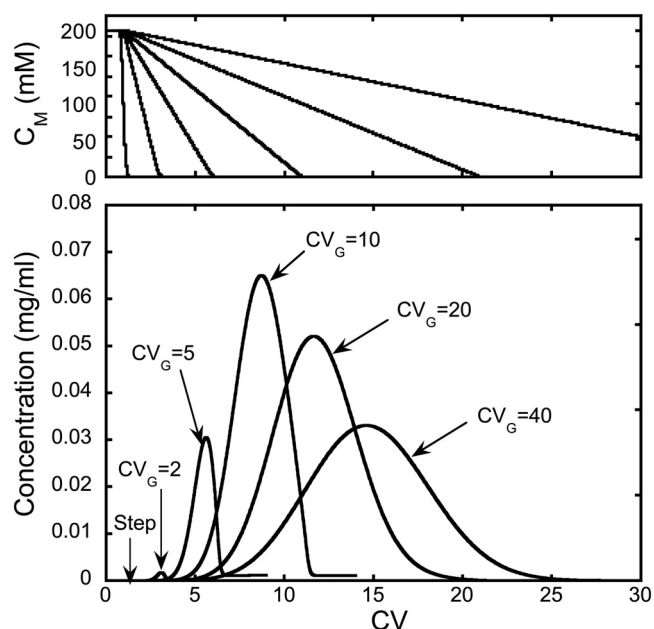


Fig. 8. Predicted elution profiles for mAbB with linear gradients from 0.2 to 0 M ammonium sulfate using the LDF rate model with a D_e value of $1 \times 10^{-7} \text{ cm}^2/\text{s}$ for the protein and a retention factor described by eq. 1 with parameters in Table 1 (bottom panel). CV_G is the duration of the gradient in column volumes units. The top panel shows the gradient profiles at the column outlet predicted using a D_e value of $1 \times 10^{-5} \text{ cm}^2/\text{s}$ for ammonium sulfate. Conditions simulated are the same as those in Fig. 5.

also neglects dispersion of the salt gradient in the column and in the mixer upstream of the column. As a consequence, the model predicts gradients that are more linear than experimentally observed especially for steep gradients, which, in turn may affect the protein elution profile.

Fig. 9 compares predicted and experimental protein recoveries as a function of gradient duration. In both cases, the percentage

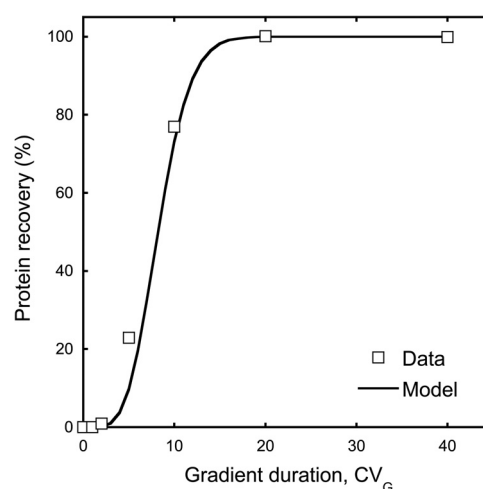


Fig. 9. Comparison of experimental and predicted protein recovery for mAbB with linear gradients from 0.2 to 0 M ammonium sulfate. Model and experimental conditions are the same as those in Figs. 5 and 8.

recovery was calculated from the eluted peak area. As seen in this figure, the rate model is in excellent agreement with the data predicting incomplete elution at $CV_G \leq 10$ and essentially no recovery at $CV_G \leq 2$.

4.4. Reverse gradients

A final consideration is whether, given the U-shape retention plots, lysozyme and mAbB can be eluted with a “reverse gradient” (i.e. positive gradient) after loading in 0 M ammonium sulfate. Fig. 10a and b show the results for lysozyme and mAbB, respectively, the former using either a gradient or a step from 0 to 1 M ammonium sulfate and the latter using a gradient or step from 0 to 0.2 M ammonium sulfate. In both cases, the protein elutes essentially completely during the gradient but elutes isocratically only many CVs after the step change in ammonium sulfate concentration has passed through the column. The results are consistent with the U-shaped retention plots. For lysozyme with a positive 2 CV gradient from 0 to 1 M ammonium sulfate, Eqs. (1) and (8) predict $C_{M,R} = 0.45 \text{ M}$ vs. the experimental value of $C_{M,R} = 0.33 \text{ M}$, while for mAbB with a positive 20 CV gradient from 0 to 0.2 M ammonium sulfate, these equations predict $C_{M,R} = 0.075 \text{ M}$ vs. the experimental value of $C_{M,R} = 0.068 \text{ M}$. In both cases, the local equilibrium model predicts late elution for the extremely steep gradient ensuing from the corresponding step changes.

5. Conclusion

U-shaped retention factor curves observed for proteins in HIC with a highly hydrophobic stationary phase are shown to result in quantitatively different elution behaviors dependent on the gradient slope, when the initial and final modulator concentration bracket the retention factor minimum. Starting with a high ammonium sulfate concentration, the protein can completely elute in the gradient, be partially eluted, or the elution peak can disappear altogether as the gradient slope is increased. Normal elution peaks are obtained for shallow gradients while distorted peaks with reduced protein recovery are obtained when the gradient slope is increased above a certain critical value. The interplay of protein and modulator chromatographic velocities and the temporal variation of the modulator concentration at the column entrance is responsible for this behavior. A local equilibrium model can be used to explain the qualitative trends and based on measurements of the isocratic retention factor while a rate model accounting for band broad-

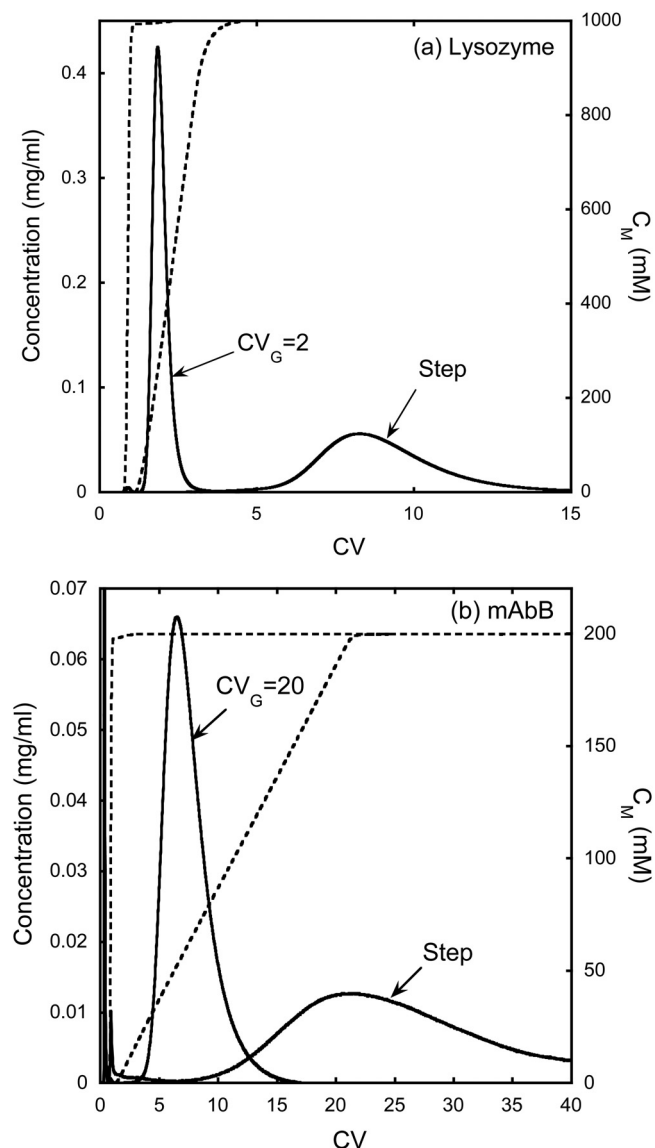


Fig. 10. Elution results for (a) lysozyme eluted with a 2 CV gradient or a step gradient from 0 to 1 M ammonium sulfate and (b) mAbB eluted with a 20 CV gradient or a step gradient from 0 to 0.2 M ammonium sulfate, both following loading in 0 M ammonium sulfate. Solid lines show the protein concentration based on the UV280 signal and dashed lines the ammonium sulfate concentration based on the conductivity signal. Essentially 100% protein recovery is obtained with the 2 CV gradient for lysozyme and with the 20 CV gradient for mAbB.

ening is shown to be capable of quantitative predictions under a broad range of conditions. Lastly, we have shown that proteins that exhibit a U-shaped retention factor plot can be loaded at low ammonium sulfate concentrations and eluted at a higher ammonium sulfate concentration provided that a sufficiently shallow gradient is applied to the column. From a practical perspective, while U-shaped retention factor curves have been observed previously, our theoretical development provides the means to understand their impact of the dynamic of the chromatography column and helps explain the phenomenon of disappearing elution peaks observed in HIC with steep gradients. The related models can be used to select gradients that avoid undesirable behaviors by manipulating either the gradient slope or the final modulator concentration or both.

Since the focus of this work is on the column dynamics that arise from U-shaped retention factor curves, the molecular basis for retention in the absence of ammonium sulfate was not directly addressed. Nevertheless, we can advance some hypotheses. One

possibility is that this phenomenon is correlated with the effects of ammonium sulfate on protein solubility. Although the Hofmeister series is typically used to classify salts with respect to their ability to desolvate proteins and promote their binding in HIC, the actual salting-out properties can vary dependent on the salt concentration range. For example, as shown by Green [25], while increasing ammonium sulfate concentrations above 0.5 M reduce the solubility of carboxyhemoglobin, increasing the salt concentration in a range below about 0.5 M actually increase its solubility. It is possible that the reversed salting-out properties of ammonium sulfate in the low concentration range facilitate elution of our proteins with an increasing ammonium sulfate gradient. Another, related possibility is that the protein unfolds on the hydrophobic surface in absence of the stabilizing effects of ammonium sulfate, resulting in strong retention. In this case, adding ammonium sulfate in low concentration could allow the protein to refold causing it to elute. We plan to explore such correlations more fully in a future communication addressing the effects of pH, salt type and concentration on protein solubility and on retention on resins with different hydrophobicity.

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