Determinants of Protein Elution Rates from Preparative Ion-Exchange Adsorbents

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Abstract

The rate processes involved in elution in preparative chromatography can affect both peak resolution and hence selectivity as well as practical factors such as facility fit. These processes depend on the physical structure of the adsorbent particles, the amount of bound solute, the solution conditions for operation or some combination of these factors. Ion-exchange adsorbents modified with covalently attached or grafted polymer layers have become widely used in preparative chromatography. Their often easily accessible microstructures offer substantial binding capacities for biomolecules, but elution has sometimes been observed to be undesirably slow. In order to determine which physicochemical phenomena control elution behavior, commercially available cellulosic, dextran-grafted and unmodified agarose materials were characterized here by their uptake and elution profiles at various conditions, including different degrees of loading. Elution data were analyzed under the assumption of purely diffusion-limited control, including the role of pore structure properties such as porosity and tortuosity. In general, effective elution rates decreased with the reduction of accessible pore volume, but differences among different proteins indicated the roles of additional factors. Additional measurements and analysis, including the use of confocal laser scanning microscopy to observe elution within single chromatographic particles, indicated the importance of protein association within the particle during elution. The use of protein stabilizing agents was explored in systems presenting atypical elution behavior, and L-arginine and disaccharide excipients were shown to alleviate the effects for one protein, lysozyme, in the presence of sodium chloride. Incorporation of these excipients into eluent buffer gave rise to faster elution and significantly lower pool volumes in elution from polymer-modified adsorbents.

Keywords: Ion-exchange chromatography, polymer-modified adsorbents, cellulosic media, protein elution, excipients, confocal laser scanning microscopy

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1. Introduction

The design of chromatographic processes incorporates multiple facets that can be optimized. Extensive attention is typically paid to protein uptake or dynamic binding capacities, which are readily quantifiable, but less attention is paid and fewer metrics are reported for protein elution when purification methods are developed for biologics. Elution is a critical step in process performance in that it determines separation selectivity and the pool volume containing the product. Attention to elution is therefore necessary to maintain high recoverability of products and enable reuse of packed beds.

Few previous reports have focused directly on analyzing effective rates of elution. Concentration profiles in affinity-based separations have been modeled for desorption-controlled elution [1,2], but the application of such methods to analyzing experimental data remains sparse, and diffusion-controlled elution appears not to have been investigated explicitly for bind-and-elute processes.

The elution process can depend on the composition of the eluent, the physical particle structure of the adsorbent, the nature and amounts of the adsorbates, or some combination of these factors. With the advent of polymer-modified materials, i.e., adsorbents that possess covalently attached or grafted polymer extenders within the pore space, the protein capacities achievable can be considerably higher than for conventional adsorbents [3–5]. An example is the group of dextran-modified agarose adsorbents: SP Sepharose XL (SP XL), a polymer-derivatized analog of SP Sepharose Fast Flow (SP FF), and Capto S, which differs from SP XL in the cross-linking density of the base matrix and spacer-arm length [6,7]. Cellulosic media, such as the HyperCel family of adsorbents, also allow increases in desirable adsorptive characteristics while not being strictly of a polymer-modified variety [8]. In both polymer-modified and cellulosic media, the matrix acts as a three-dimensional volume for sorption in a gel-like structure. A common feature these resins share is the reduction in size of the pore lumen, in which protein is able to diffuse freely. It has been shown that charged polymer extenders grafted on a surface tend to collapse toward that surface in environments of increased total ionic strength (TIS) due to screening of repulsion between like charges [9,10]. This would increase the size of the pore lumen in polymer-modified phases during elution carried out at increased TIS, making the dimensions of the lumen uncertain.

The addition of polymer extenders to macroporous adsorbents has been shown to increase protein transport rates during loading and hence dynamic capacities of proteins. This has been shown for comparisons between UNOsphere S and Nuvia S, a polymer-derivatized version of UNOsphere [5,11], and between SP Sepharose FF and SP Sepharose XL, the latter being a polymer-modified counterpart of the former [6,12]. In these studies it was observed that both the adsorptive capacity and the rate of uptake were significantly higher in the polymer-modified material than in the non-polymer-modified counterpart. This rapid transport has been explained by diffusion of protein sorbed in the polymer phase, described as solid or homogeneous diffusion, even under relatively strong binding conditions where uptake would be dominated by pore diffusion in conventional non-polymer-modified adsorbents [4,13]. However, in the absence of an electrostatic driving force retaining the protein in the polymer layer, such transport is unlikely during elution from these stationary phases, with the protein likely to be

released into in the pore lumen. Diffusion of macromolecular solutes has been measured through pores lined with polyelectrolytes even in the absence of a direct electrostatic attraction [14,15], but such behavior in chromatographic materials has received less attention.

In this work we studied the elution behavior of three model proteins, lysozyme, lactoferrin and a monoclonal antibody, from cation-exchange media with and without polymer modification. Our underlying hypothesis was that sorbed protein is desorbed rapidly in the presence of the eluent, with the rate-limiting step determining the overall elution rate being simple pore diffusion out of the particle. While the data appear to support the hypothesis in general, the biophysical properties of the protein are found to have an appreciable influence in some cases and warrant careful consideration in optimizing elution procedures.

2. Materials and Methods

2.1. Buffers and Protein Solutions

Monobasic sodium phosphate (NaH_2PO_4) and sodium bicarbonate (NaHCO_3) were purchased from Fisher Scientific (Fair Lawn, NJ) and used to prepare 10 mM sodium phosphate and 50 mM sodium bicarbonate buffer solutions at pH 7 and pH 9, respectively. The total ionic strengths (TIS) of different solutions were adjusted using NaCl. L-arginine and trehalose were purchased from Fisher Scientific (Fair Lawn, NJ) and used to make buffer solutions containing 500 mM of either excipient in addition to 1 M NaCl and 50 mM sodium bicarbonate at pH 9.

Hen egg white lysozyme was purchased from Sigma-Aldrich and bovine lactoferrin was obtained from DMV-International. A lactoferrin stock solution was first purified using SP Sepharose FF to remove trace impurities before the subsequent use for controlled elution experiments. The monoclonal antibody (mAb) used was obtained from Genentech, Inc. in formulation buffer concentrated to 30-40 mg/mL. Solutions of lysozyme and lactoferrin were prepared by dissolving lyophilized protein in the desired TIS-controlled buffer and concentrating using 10K Amicon centrifugal filters. Concentrated samples were rediluted with buffer and reconcentrated three times, and the final protein solutions were filtered using 0.22 μm syringe filters. Protein concentrations were determined using UV spectrophotometry on a Thermo Scientific NanoDrop 2000. Target protein concentrations for stock solutions were 2.5 ± 0.2 mg/mL for binding capacity and protein elution studies and 200-300 mg/mL for phase behavior experiments. Protein properties, including the isoelectric point, molecular weight, effective radius, and free-solution diffusion coefficient, are shown in Table 1.

2.2. Stationary Phases

Prepacked 25 mm x 7 mm i.d. HiTrap columns containing SP Sepharose FF, SP Sepharose XL and Capto S, all strong cation exchangers, were purchased from GE Healthcare Life Sciences. A prepacked 50 mm x 5 mm i.d. S HyperCel column was obtained from Pall Life Sciences. The physical properties of the resins are summarized

in Table 2. SP FF comprises an agarose base matrix functionalized with sulfonate groups attached to a short spacer arm. SP XL uses the same agarose base matrix as SP FF, but also contains covalently attached 40 kDa dextran extenders. Both the dextran and the agarose base matrix carry sulfonate functionalization, substantially expanding the volume available for adsorption within this material as compared to its non-polymer-modified counterpart but concomitantly reducing the pore space available for transport. Capto S contains similar 40 kDa extenders to those present in SP XL but the cross-linking density of the agarose base matrix is higher than for the Sepharose materials [7], resulting in a slightly smaller pore lumen [16]. S HyperCel is comprised of a fully cellulosic base matrix without any defined polymer modification [8], but it exhibits properties comparable to those seen in polymer-modified stationary phases [17], including increased capacity and enhanced uptake rates.

2.3. Dynamic Binding Capacity (DBC) Measurements

Breakthrough experiments were performed on an ÄKTA Explorer 100 system (GE Healthcare) for each resin-protein system for which elution behavior was to be analyzed. All DBC measurements were performed at a linear velocity of 120 cm/hr, pH 7 and 20 mM TIS. To ensure consistency, DBC measurements were compared to values obtained previously for lysozyme and lactoferrin at these conditions on SP FF, SP XL and Capto S [12].

Protein loading was continued until at least 25% of the feed concentration was measured in the eluate, monitored by absorbance at 280 nm. Protein was eluted using buffered solutions at 1 M TIS for lysozyme and the mAb and at 1.5 M TIS for lactoferrin, due to an increased affinity of the latter protein on cation exchangers. Collection and spectroscopic measurement of eluted fractions showed close to 100% recovery of loaded protein in all cases.

DBC values were determined by integrating the area above the breakthrough curve up to 10% breakthrough and subtracting the system dead volume obtained by performing a breakthrough run without a column in line. Loadings for elution experiments followed the same procedures but were based on a fraction of the DBC values determined, depending on the system.

2.4. Column Elution and Determination of Apparent Elution Diffusivities

Columns were loaded with protein using the same operating conditions as in the determination of the DBCs. Multiple loading factors were employed, corresponding to 10, 60 and 100% of the DBC value at 10% breakthrough for each system. A 5-column volume wash step preceded a 100% step elution to an elevated TIS condition (1 M TIS, 1.5 M TIS, or 1 M NaCl + 500 mM excipient). Due to the high protein concentrations in the eluate, the UV detector was saturated at times, causing a cutoff of the elution curve around 3000 mAU. Therefore, mass balances could not be performed by integration under the UV curve and instead were carried out by collecting and analyzing eluate fractions.

Within the hypothesis that pore diffusion is the rate-limiting step for elution, the tails of the measured elution profiles were used to estimate the apparent pore diffusivities,

D_e . This was based on the model for unsteady-state diffusion from a sphere initially of uniform concentration to the surroundings, accounting for a finite rate of mass transfer at the surface [18]. As shown in the Supplementary Material, incorporation of this process into column models of different degrees of rigor leads to prediction at long times of an exponentially decreasing tail of the elution curve of the form

$$C_{\text{eluate}} = C_1 e^{-\beta_1 D_e t / R^2} \quad (1)$$

where R is the radius of the adsorbent particle and β_1 is the first eigenvalue obtained from the characteristic equation for the unsteady-state diffusion problem,

$$\beta_1 \cot \beta_1 + \text{Bi} - 1 = 0 \quad (2)$$

The Biot number, $\text{Bi} = kR/D_e$, reflects the relative importance of intraparticle and extraparticle transport resistances; high values ($\text{Bi} > 10$) indicate that the intraparticle resistance is dominant [19], as is usually the case in preparative chromatography of proteins. The mass transfer coefficient at the particle surface, k , was obtained from the correlation of Carberry [20] for flow in a packed bed,

$$k = \frac{1.15 D_0^{2/3}}{(2R/u)^{1/2} \nu^{1/6}} \quad (3)$$

where D_0 is the free-solution diffusivity of the protein, u is the linear velocity of the interstitial fluid and ν is the kinematic viscosity, taken to be $10^{-6} \text{ m}^2/\text{s}$. Values of k of $2\text{--}6 \times 10^{-4} \text{ cm/s}$ were predicted for the systems studied. The effective elution rate, or apparent pore diffusivity, D_e , was extracted by using the nonlinear least squares fitting routine, NLINFIT, in Matlab to solve Equations 1 and 2 simultaneously. The data were placed on a logarithmic scale for ease in assessing the goodness-of-fit, which was also determined by the mean squared error.

2.5. Inverse Size Exclusion Chromatography and Size Exclusion Chromatography

Inverse size exclusion chromatography (ISEC) experiments using dextran probes were carried out to determine apparent intraparticle porosities during elution for specific resin-protein systems. Measurements were made on SP XL under low- (20 mM TIS) and high-salt (1.5 M TIS) conditions. The procedures followed those used in previous work using the same experimental setup and similar methods were used to assess pore dimensions, by analysis of the calibration curves relative to the distribution coefficients for each dextran solute [8,10]. ISEC data for SP FF [10], Capto S [16] and S HyperCel [8] have previously been investigated using this technique under different solution conditions. Mean pore radii were determined from the first moment of the fitted pore size distributions and are reported in Table 3, along with accessible particle volumes for each resin-protein pairing (see Section 3.1.2). Retention volumes of uncharged dextran standards were used to determine the accessible fraction of particle volume, $\epsilon_{p,\text{acc}}$, from

$$\epsilon_{p,\text{acc}} = \frac{V_r - V_0}{V_c - V_0} \quad (4)$$

where V_r is the retention volume of a given solute, V_c is the total column volume and V_0 is the column void volume determined using a 3000 kDa dextran standard, which is fully excluded from all the stationary phases used here.

Standard size exclusion chromatography was also performed on lactoferrin fractions on a 24 mL Superdex 75 column (GE Healthcare) to determine if protein oligomerization occurs at high salt, as lactoferrin has previously been shown to form tetramers above sodium chloride concentrations of about 200 mM TIS [21]. Dextran standards of known radii were used as molecular size markers to determine size differences for lactoferrin under low- (20 mM TIS) and high-salt (1 M TIS) running conditions.

2.6. Instantaneous Phase Behavior of Lysozyme in Sodium Chloride Solutions

Experiments were performed to determine the phase boundary for lysozyme in the presence of sodium chloride in a manner similar to that reported previously [22,23]. Samples were prepared by mixing appropriate amounts of low- and high-salt buffered solutions (between 0 and 3 M NaCl in 10 mM sodium phosphate at pH 7 or in 50 mM sodium bicarbonate at pH 9) in 0.5 mL tubes to a volume of 170-190 μ L. Ten to thirty μ L of concentrated lysozyme solution (200-300 mg/mL) was pipetted into the tubes for a final volume of 200 μ L and aspirated for at least 30 seconds afterward; in most cases, samples were then vortexed for an additional 30 seconds. At sufficiently high protein concentrations instantaneous precipitation was observed, but at intermediate concentrations the precipitate redissolved after vortex mixing. The boundary for instantaneous phase separation was taken as the highest protein concentration in which the precipitate that formed began to resolubilize into solution following vortex mixing.

2.7. Confocal Laser Scanning Microscopy Imaging of Single-Particle Elution

SP FF or XL particles were packed into a flow cell designed for use in a confocal microscope [24] and the form of elution fronts of fluorescently-labeled protein was observed as described previously [17]. Lysozyme was fluorescently labeled with DyLight-650 NHS Ester (Thermo Scientific, 62266: amine-reactive dye packs) in a 1-2% labeling ratio following protocols supplied by the manufacturer. Reservoirs of equilibration buffer (10 mM sodium phosphate, pH 7), of a solution of fluorescently-labeled protein (2 ± 0.2 mg/mL), high-salt buffer (1 M TIS), and high-salt buffer containing excipient (1 M NaCl, pH 9, 500 mM L-arginine) were used to load and elute protein successively, in order to allow imaging of elution behavior using a Zeiss 5 LIVE DUO high-speed confocal microscope equipped with a 40x C-Apochromat (NA 1.2) water-immersion lens (Carl Zeiss). The protein solution was recirculated through the flow cell for 40-45 minutes at 1 mL/min to ensure full saturation and the particles were then washed for one minute with equilibration buffer before the high-salt eluent was fed. Images were captured every 5 seconds from the time of switching to the eluent feed until no fluorescently-labeled protein remained visible inside the particle or no appreciable decrease in fluorescence was observed.

3. Results and Discussion

3.1. Apparent Elution Diffusivities

3.1.1. Estimation from Elution Data

Protein concentration profiles in the eluate following step elution were used to determine both the number of column volumes necessary for near-complete recovery and the apparent pore diffusivities during elution by application of Equations 1 and 2. Figure 1 depicts an example of both raw elution chromatograms and adjusted data used for model analysis for the case of lysozyme elution from Capto S. The number of column volumes necessary for near-complete elution (when the UV signal returned to 100 mAU above the baseline) is obtained directly from the raw data for each curve, and the values obtained are shown in Table 4. The raw elution data for cases other than that in Figure 1 are provided in Figures S1–S3 in Supplementary Material. For most cases, three loading factors, namely 10, 60 and 100% of the DBC for each system (Table 4), were used to determine if the extent of column loading affected elution rates and volumes. Altering this factor had a significant impact on the elution pool volume as expected, due to the larger amounts of protein to be eluted. Only lysozyme was used at 100% loading factors for all systems due to constraints in protein availability. In general, for equivalent loadings, near-complete elution was achieved much sooner in the non-polymer-modified SP FF, while more column volumes were required for recovery in polymer-modified and cellulosic media. Solute size also appears to affect this metric, with mAb eluting from the Capto S adsorbent displaying the longest elution time at 60% loading factor, for instance.

The adjusted data in Figure 1 were truncated to just after the end of detector saturation (the plateau region in the raw data at approximately 3000 mAU) and plotted on semilog axes, with linearity, at least at longer times, indicating a satisfactory fit to the model. The elution model was fit only to absorbance values below 85% of the maximum (saturated) UV signal to ensure linearity between detection measurements and true protein concentrations in the eluate. Despite the appreciable differences in elution volume with different column loadings, the fits to the post-saturation portion of the elution profile show only slight differences in the effective diffusivities. The diffusivities determined by analysis of the elution profiles are tabulated in Table 5. Similarly to the column volumes required for near-complete elution, the general trend remains in which the non-polymer-modified material shows the fastest elution, followed by, for the most part, an order-of-magnitude reduction in the rate for polymer-modified and cellulosic media.

The salt concentrations used for elution above were chosen to be high enough to eliminate protein retention and thereby allow elution to occur under the most favorable conditions. Less extreme salt concentrations may be used in practice, and Figure 2 shows elution curves for lactoferrin from SP FF at a 60 % loading factor for 1, 1.5 and 2 M TIS. Even at 1 M TIS the elution curve is much wider, with a longer tail, than at 1.5 M. Lactoferrin has a k' value of about 1 on SP FF at 1 M TIS [6], which under linear adsorption conditions should lead to a diffusivity smaller than that under unretained conditions by a factor of about $k'+1 \sim 2$. Analysis of the elution profiles yields effective

elution diffusivities of 2.8×10^{-8} , 5.9×10^{-8} and 7.0×10^{-8} cm²/s at 1, 1.5 and 2 M TIS elution, respectively, so although adsorption is unlikely to be linear at the high protein concentrations encountered early during elution, the predicted reduction in the elution diffusivity during emergence of the tail is reasonable. Even though there is a slight increase in the elution diffusivity and decrease in the number of column volumes needed for elution from 1.5 to 2 M TIS elution, 1.5 M TIS was selected for all lactoferrin step elution experiments as this effect was not observed in any other exchanger studied. In addition, 2 M TIS is a condition more representative of the full regeneration of ion-exchange stationary phases and not typical of bind-and-elute mode operation, so the use of a lower TIS condition is more appropriate in these cases. Regardless of the elution TIS condition, close to 100% recovery was observed in all lactoferrin elution experiments.

Values of the Biot number for the different resin-protein systems were of order 10 to 200, the high values indicating dominance of the intraparticle transport resistance. While the Biot numbers for all resins were of a similar order of magnitude, those for SP FF were generally the lowest, reflecting the generally higher pore diffusivities in the very open agarose structure.

The fitting of the effective diffusivities to Equation 1 is premised on their being pore diffusivities, as the high-salt conditions of elution would make adsorption on the pore walls or the polymer extenders negligible. What is less clear for the polymer-modified media is whether desorbed protein is totally excluded from the polymer, but this seems a reasonable assumption given ISEC results that show all but the smallest dextran probes being excluded from the polymer layer in the absence of an electrostatic driving force for adsorption [14]. Indeed, the results for lysozyme and lactoferrin are similar in magnitude to shrinking-core (pore) diffusivities previously determined for SP FF during protein uptake [25]. However, the elution diffusivities are slightly higher than the uptake values, which were determined at low ionic strengths, where diffusion is coupled to adsorption that is strong enough to be considered irreversible.

3.1.2. Interpretation of Apparent Pore Diffusivity Values

The values obtained for the pore diffusivities can be interpreted within the theoretical framework in which the values are related to the corresponding free-solution diffusivities, D_0 , by [26]

$$\frac{D_e}{D_0} = \frac{\varepsilon_p \phi_p}{\tau_p} \quad (5)$$

where ε_p is the intraparticle porosity, ϕ_p is the diffusional hindrance coefficient and τ_p is the tortuosity factor. The dependence on porosity, which more strictly reflects the accessible porosity $\varepsilon_{p,acc}$ and therefore depends on solute size, simply captures the space available for diffusion. The hindrance coefficient accounts for hydrodynamic drag as a solute diffuses in a narrow pore and is related to the relative sizes of the solute and the pore [27–29], with a tighter fit giving rise to lower values. Most of the theoretical relationships that have been derived are for spherical particles in cylindrical pores, but especially for polymer-derivatized materials, in which the "pore wall" is in fact a polymer containing solvent, these values are highly questionable. Although transport through

such pores has been modeled [14], considerable uncertainty surrounds suitable values of ψ_p other than that they are of order unity. The tortuosity factor, while being a function of the pore geometry, is not necessarily explicitly dependent on solute or pore size, although the change in pore accessibility with solute size introduces such a dependence, again with considerable uncertainty.

Values of $\epsilon_{p,acc}$ were measured directly by inverse SEC for both polymer-modified and non-polymer-modified adsorbents. Figure 3 shows results for SP FF at low ionic strength and SP XL at high and low ionic strengths. These data clearly show a higher pore accessibility of SP FF than of SP XL for any individual dextran probe, due directly to the effect of the polymer extenders in SP XL. The results also show the greater accessibility of SP XL at high than at low TIS due to screening of charge repulsion between ligands and consequent collapse of the extenders [10,13]. Accessible particle volumes for the proteins studied are given in Table 3, determined by the calibration curves obtained from ISEC and the Stokes radii for each protein seen in Table 1. Accessible particle volumes were also found for Capto S [16] and S HyperCel [8] based on previously generated ISEC data.

Figure 4 shows the dependence of the normalized apparent pore diffusivities during elution on the accessible particle volume at high salt. Different plot markers represent the different stationary phases and connected lines represent data for the same protein. The general trend is one of increasing normalized diffusivities with increasing accessible particle volumes, but the dependence is not uniformly linear as suggested by Equation 5. This may reflect experimental uncertainties but also more fine-grained architectural differences among the different media, including differences in pore size distribution as distinct from just the porosity values, which may affect the hindrance and tortuosity factors.

Despite these uncertainties, one trend in Figure 4 that seems counterintuitive is that the normalized diffusivities increase with increasing protein size. Lysozyme has a molecular weight approximately 10 times smaller than that of a monoclonal antibody, and one would assume that a smaller molecule such as this would encounter less hindrance in pore diffusion during elution [28], but the opposite effect is suggested by the data.

The additional dependence on ψ_p and τ_p is more problematic and is investigated by a more absolute comparison of the values of the ratio τ_p/ψ_p (Figure 5), determined from Equation 5 using the normalized elution diffusivities and therefore reflecting the high salt concentrations used for elution. The hindrance coefficients are difficult to estimate, especially when the protein size and mean pore radius are very close together, e.g., mAb in dextran-modified media. However, especially in cases involving polymer-derivatized media the hindrance coefficients would be expected to be much closer to unity than expected from the theoretical results for solid pore walls [27–29], so the ratio plotted in Figure 5 can be expected to reflect primarily the trends in the tortuosity factor, where values of 2 to 6 are reported to be reasonable [30]. The values for the mAb are at the low end of this range, but those for lactoferrin and lysozyme appear somewhat high for consistency with the diffusional model, especially in light of the much lower values for the mAb in the same adsorbents.

The result for lactoferrin can be explained by the formation of tetramers at and above sodium chloride concentrations of 200 mM [21], which increases the size of the

eluting species and hence the free-solution diffusivity, D_0 (Table 1). Additional sets of normalized data for lactoferrin, accounting for the revised D_0 value and the adjusted accessible porosity $\epsilon_{p,acc}$ at high salt, are shown in Figures 4 and 5. The trend lines based on the tetramer are much more consistent than the monomer case with those for the mAb. SEC measurements performed on Superdex 75 on unpurified and purified lactoferrin at high and low concentrations of sodium chloride (see Figure S4, Supplementary Material) suggest that the formation of tetramer is reversible and hence that the true trendlines for lactoferrin in Figures 4 and 5 may lie somewhere between the tetramer and monomer lines. However, at the high lactoferrin concentrations in the pore after desorption, mass-action considerations would be expected to favor tetramer formation.

For both the mAb and lactoferrin, Figure 5 shows a slight increasing trend of τ_p/ψ_p with $\epsilon_{p,acc}$. This may reflect differences in the pore structures of the different adsorbents, but if anything one might expect a decreasing tortuosity with the more open structures encountered at high porosities, particularly for SP FF. A possible explanation emerges from the combination of the overall protein loading and porosity for each material, which together determine the estimated local pore concentration immediately following desorption, i.e., when the high-salt front penetrates a protein-loaded particle. Using lysozyme as an example, the resulting values for this concentration, plotted against the accessible intraparticle porosity for each material (Figure 6), suggest that in the cellulosic and polymer-modified media, where average pore sizes are the smallest and the binding capacities are high, there is a transient period when the local pore concentration can be on the order of several hundred mg/mL. This concentration would decay as elution proceeds and protein leaves the stationary phase to enter the interstitial mobile phase, but the dependence of protein diffusivities on concentration [31] may explain the higher diffusivities at lower porosities implied by the counterintuitive trends for the mAb and lactoferrin in Figure 5.

The results for lysozyme remain anomalous, based on the inconsistent trends for the elution diffusivity (Figure 4) and the high apparent tortuosity (Figure 5). One possible explanation is that lysozyme, the smallest of the model proteins used, can access micropores in these resins that are inaccessible to the mAb or lactoferrin and that these pores have higher tortuosities. However, the very high absolute values seen for lysozyme in Figure 5 and the relatively high value in as open a structure as SP FF makes this seem unlikely. An alternative explanation is explored in the next section.

3.2. Phase Behavior of Lysozyme Under Elution Conditions

Maintaining protein solubility is crucial in preventing on-column aggregation and fouling, as well as ensuring product stability. Salting-out characteristics of proteins, such as lysozyme and monoclonal antibodies, have been studied outside of the column environment [22,32,33], but not typically in conditions that would mimic those of a typical elution from an ion-exchange material. The transient conditions that exist in the early stages of elution are somewhat similar in nature to these studies, even though phase separation cannot be readily observed directly.

Figure 7 shows the confocal microscopy profiles gathered during elution of DyLight-650-labeled lysozyme from SP FF and XL. The higher intensity at the center of

the particle at $t = 0$ s can be explained as an overshoot attributed to differences in retention of labeled and unlabeled lysozyme [34]; if loading were carried out for a longer period, the intensity would be more uniform throughout the particle cross-sections [35]. The sequences show gradual depletion of the initial reservoir of protein in each particle from the outer edge, but there are clear differences between the results for the two materials. For SP FF, the outside edge of the intensity profile during exit of protein from the particle is diffuse, as would be expected for diffusive transport of protein in solution. For SP XL, the profile is very different, with a sharp retreating front that can be described as a shrinking-core profile, with the very center of the particle retaining a relatively high fluorescence intensity until the last captured frames of elution. The time required for apparent full elution of protein from SP XL is also more than 5 times longer than what was required for SP FF. Similar elution behavior of lysozyme to that in SP XL was observed in S HyperCel, which is not polymer-derivatized but shares similar characteristics with SP XL, such as high binding capacity and low accessible porosity. As was seen in Figure 6, such conditions result in extremely high protein concentrations in the pore space at the start of elution, and this is further compounded by the presence of a high salt concentration. This may lead to very rapid phase separation in which the protein in the pore, instead of being in free solution, is present as a dense phase, presumably a precipitate or gel. Elution from the particle would then first require redissolution of the dense phase, which would occur at its outer edge, giving rise to the kind of elution profiles seen for SP XL in Figure 7. The data in Figure 6 indicate that this kind of behavior is least likely in SP FF among the materials studied, consistent with the diffuse edges seen for this material in Figure 7.

High salt and high protein concentrations are of course prime conditions for phase separation, and lysozyme has previously been shown to phase-separate instantaneously under comparable conditions to those used here for elution (1-2 M sodium chloride, > 70 mg/mL protein) [22]. Additional such measurements were made here, with instantaneous phase boundaries for lysozyme in sodium chloride solutions (at pH 9) shown in Figure 8. The leftmost curve is the aggregation boundary representative of normal elution conditions, which lies at around 60 mg/mL lysozyme for a sodium chloride concentration of 1 M, the condition used for elution experiments. Protein concentrations of higher than 60 mg/mL were difficult to achieve in the phase separation measurements due to the nature of the experiments and the limit to which the protein could be concentrated.

That behavior similar to that of lysozyme was not seen for the other two proteins presumably reflects the different physicochemical properties of different proteins. Even for lysozyme in SP XL, elution is complete after 2-3 minutes (Figure 7), so the effect would require instantaneous or near-instantaneous phase separation. Different proteins have different protein- and salt-concentration thresholds for such phase separation, and even where phase separation occurs it may do so only via a relatively slow nucleation step [22,32]. Another possible factor is that the restricted dimensions of the pore space may make formation of a consolidated dense phase difficult, especially for larger proteins.

3.3. Elution Using Excipients

The kind of behavior seen for lysozyme and the mechanism proposed above raise the question of whether any remedy is available to improve elution rates in such systems. One route to addressing the challenges of combined high protein and salt concentrations is to perform elution at lower salt concentrations while ensuring that k' is low enough to eliminate essentially all retention, and indeed this is typical in industrial practice. For lysozyme elution from S HyperCel, for example, there is a modest but clear improvement when the salt concentration is reduced, as assessed by both the time for complete elution and the emergence of a more diffuse boundary as seen by confocal microscopy (Figure S5, Supplementary Material). However, the high capacities and small pore volumes that are characteristic of polymer-functionalized media will still lead to very high pore concentrations after desorption, and although solubilities may be higher at lower salt concentrations, phase change may still be possible.

Excipients, such as disaccharides and amino acids, are commonly used for formulation applications to stabilize proteins and prevent aggregation, e.g., trehalose and L-arginine have been shown to have stabilizing effects in protein systems [36–39]. L-arginine has also been used to enhance elution behavior of antibodies in Protein A [40,41], hydrophobic interaction and cation-exchange systems [42], showing a reduction in eluted aggregates.

Additional data also included in Figure 8 show that the addition of 500 mM trehalose or L-arginine causes a significant shift in the aggregation boundary to higher sodium chloride concentrations. Due to the pK_a of the primary amino group and the buffering effect of the relatively high concentration of L-arginine, a pH of 9 was used for all the excipient experiments to ensure consistency. Although the data in Figure 8 are limited to protein concentrations below about 60 mg/mL, extrapolation of these lines down to 1 M sodium chloride suggests that phase separation would still occur following desorption since the local protein concentrations would still be about 300-400 mg/mL for polymer-derivatized and cellulosic absorbents. However, the pronounced shift in the boundaries suggests that the addition of excipients can still assist in reducing protein association within the pores following elution.

This conjecture was tested by confocal microscopy observations similar to those shown in Figure 7. Figure 9 shows time-series images for elution of DyLight-650-labeled lysozyme from SP FF with and without the inclusion of 500 mM L-arginine at pH 9. The L-arginine case shows diffuse edges similar to those without excipient at pH 7 (Figure 7), as would be expected for simple diffusive transport. The “shrinking-core” profile seen at pH 9 without excipient is presumably due to phase separation allowed by the lower net charge of lysozyme at the higher pH. The addition of 500 mM L-arginine completely eliminates this effect, even though the pH remains at 9 in both cases.

Figure 10 shows time-series images for elution of DyLight-650-labeled lysozyme from SP XL with and without the inclusion of 500 mM L-arginine at pH 9. Both cases can be qualitatively described as displaying a “shrinking-core” profile during elution. While elution without the use of L-arginine at both pH 7 (Figure 7) and pH 9 shows a very sharp front bounding a high-intensity core, a more diffuse front is observed around the periphery for elution with L-arginine. Figure 11 shows radial intensity profiles during lysozyme elution from SP XL with no excipient and with 500 mM L-arginine at pH 9, all

normalized to the same relative intensity scale. Comparison of the third panel for each case (105 s in the excipient-free case and 50 s in the L-arginine case) shows a broader intensity “peak” and a wider baseline in the L-arginine case, indicative of a more diffuse area around the sharp front. Qualitatively, this is the only indication that the use of the excipient is aiding in the elution of lysozyme by providing additional protein stabilization and resistance to association. However, a very large difference is seen in the time scales of elution for each case. While elution at pH 9 and pH 7 takes approximately 250 seconds and approximately 160 seconds for completion respectively, elution with the use of L-arginine is complete after about 80 seconds, about half of what was observed for the base case of pH 7 and no excipient.

A practical test of the potential benefit of using an excipient during elution for a system such as that of lysozyme is to perform column elution experiments directly. Figure 12 shows lysozyme step elution experiments conducted in the presence of 1 M NaCl and 500 mM trehalose or L-arginine at pH 9 on SP FF and XL. Conditions such as the loading and flow rate were kept the same in each case as those in the prior column elution experiments (Figure 1 and Supplementary Materials). Apparent elution diffusivities as well as the pool volumes needed for near-complete elution are shown in Table 6. While excipients had little to no effect on apparent elution diffusivities or volumes for SP FF, apparent diffusivities were increased and elution volumes were significantly reduced for SP XL, consistent with the presence of a significant phase-separation effect in this system in the absence of excipient.

The confocal data in Figure 10 show that the addition of L-arginine is not sufficient to eliminate phase separation fully during elution in stationary phases with constricted pore volumes, as the sharp intensity front was not eliminated. However, it still provides a significant benefit in the reduction of pool volumes as observed in the column elution results, presumably because of a significant shift in the distribution of protein between dense and solution phases.

4. Conclusions

Protein elution is a critical step in preparative separations that merits similar attention to that of its counterpart, protein uptake. As with the design of protein loading, the ease and rate of elution depend on the structure of the adsorbent material and the operating conditions. In the absence of anomalous effects, elution appears to be controlled simply by pore diffusion out of individual particles. However, our results show that for some systems elution can also depend very strongly on the physicochemical properties of the proteins themselves. Slow elution rates and larger pool volumes are undesirable for bind-and-elute separations, but by tailoring elution schemes for certain systems, such as modifying elution operating conditions or utilizing protein stabilizing agents, the overall processing step can be improved greatly.

Our results show that polymer-derivatized and cellulosic cation-exchange adsorbents may present poorer elution performance than materials with higher accessible intraparticle porosities, such as SP FF. While these materials with higher binding capacities and faster uptake than traditional macroporous resins do have benefits with respect to efficiency and overall throughput, there is a tradeoff between optimization of uptake and elution. The findings reported here may aid in elucidating

poor elution performance and may offer pathways to ameliorating such issues. In addition to the use of excipients explored here, elution using pH steps with lower salt concentrations may help to reduce the possibility of phase separation such as that inferred here for lysozyme; *k'* data can be used to ensure that retention is negligible under the conditions used, minimizing the retardation effect of residual retention. Other approaches may include reducing column loadings and using shallower salt gradients. Such strategies can be assessed during stationary-phase selection to keep necessary downstream steps and processing time to a minimum.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

Figure 12

Table 1: Physical characteristics of proteins used

Property	Lysozyme	Lactoferrin (monomer)	Lactoferrin (tetramer)	mAb
pI	11.4 [43]	8.8 [44]	-	~8
M _w (kDa)	14.3 [45]	78 [46]	312	144 [47]
Stokes radius (nm)	2.0	2.3	4.4	5.7
D ₀ (10 ⁻⁷ cm ² /s)	11 [48]	9.6 [21]	4.9 [21]	3.8 [49]

Table 2: Physical properties of stationary phases as reported by manufacturers

Resin	Base matrix	Functional group	Mean particle size (μm)	Ionic capacity ($\mu\text{mol/mL}$)
SP Sepharose FF	Cross-linked agarose	SO_3^- on 6-carbon spacer	90	180-250
SP Sepharose XL	Cross-linked agarose with dextran extenders	SO_3^- on 6-carbon spacer	90	180-250
Capto S	Highly cross-linked agarose with dextran extenders	SO_3^- on 2-carbon spacer	90	110-140
S HyperCel	Cross-linked cellulose	SO_3^-	75	59-84

Table 3: Intraparticle porosities for different proteins, and mean pore radii, based on ISEC analysis. Base conditions: 20 mM TIS, no added NaCl; high salt: [NaCl] = 1-1.5 M.

Resin	Mean pore radius (nm)	$\epsilon_{p,acc}$				Mean pore radius in high salt (nm)	$\epsilon_{p,acc}$ at high salt			
		lysozyme	lactoferrin (monomer)	lactoferrin (tetramer)	mAb		lysozyme	lactoferrin (monomer)	lactoferrin (tetramer)	mAb
SP Sepharose FF	24.7	0.71	0.67	0.64	0.60	24.7	0.69	0.67	0.54	0.47
SP Sepharose XL	5.8	0.30	0.24	0.07	0.03	9.6	0.44	0.40	0.20	0.14
Capto S	4.8	0.19	0.14	0.02	0.01	7.1	0.34	0.30	0.10	0.06
S HyperCel	4.4	0.18	0.14	0.03	0.01	4.4	0.19	0.14	0.02	0.01

Table 4: Dynamic binding capacities and eluent volume, in units of column volumes, required for elution (to 100 mAU) for each protein, resin and loading condition (loading performed at pH 7, 20 mM TIS)

Resin	Dynamic binding capacity (mg/mL)			10% loading factor			60% loading factor			100% loading factor		
	lysozyme	lactoferrin	mAb	lysozyme	lactoferrin	mAb	lysozyme	lactoferrin	mAb	lysozyme	lactoferrin	mAb
SP Sepharose FF	108	25	17	2.2	2.0	1.2	3.1	4.0	2.8	3.9	n/a	n/a
SP Sepharose XL	211	45	28	2.8	1.9	1.5	4.9	3.1	3.1	7.0	n/a	n/a
Capto S	175	94	91	3.7	2.8	4.7	5.2	4.9	9.4	7.2	n/a	n/a
S HyperCel	133	158	n/a	4.6	3.1	n/a	5.2	4.6	n/a	6.2	n/a	n/a

Table 5: Fitted elution diffusivities for each protein, resin and loading condition (loading performed at pH 7, 20 mM TIS). Best fit results shown along with 95% confidence intervals.

Resin	Effective elution diffusion coefficient, D_e (10^{-8} cm ² /s)								
	10% loading factor			60% loading factor			100% loading factor		
	lysozyme	lactoferrin	mAb	lysozyme	lactoferrin	mAb	lysozyme	lactoferrin	mAb
SP Sepharose FF	13 ± 0.50	5.6 ± 0.047	9.6 ± 0.73	15 ± 0.42	5.9 ± 0.043	8.2 ± 0.41	13 ± 0.94	n/a	n/a
SP Sepharose XL	8.7 ± 0.20	6.4 ± 0.13	9.1 ± 0.96	6.5 ± 0.28	6.0 ± 0.13	6.1 ± 0.36	5.0 ± 0.30	n/a	n/a
Capto S	5.1 ± 0.13	5.3 ± 0.15	2.9 ± 0.25	4.5 ± 0.16	3.8 ± 0.087	2.0 ± 0.053	3.3 ± 0.11	n/a	n/a
S HyperCel	1.0 ± 0.079	1.5 ± 0.051	n/a	1.3 ± 0.029	1.2 ± 0.032	n/a	1.3 ± 0.029	n/a	n/a

Table 6: Elution characteristics for lysozyme on SP Sepharose materials (60% loading factor) with and without the use of excipients

Condition	SP Sepharose FF		SP Sepharose XL	
	Elution diffusivity, D_e (10^{-8} cm ² /s)	Column volumes required for elution (to 100 mAU)	Elution diffusivity, D_e (10^{-8} cm ² /s)	Column volumes required for elution (to 100 mAU)
pH 7, no excipient	15 ± 0.42	3.1	6.5 ± 0.28	4.9
pH 9, no excipient	15 ± 3.5	3.1	3.3 ± 0.045	7.1
pH 9, 500 mM trehalose	11 ± 0.81	3.1	4.0 ± 0.086	6.3
pH 9, 500 mM L-arginine	16 ± 2.9	2.6	6.7 ± 0.39	4.8