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Chromatography of Proteins on Charge-Variant Ion Exchangers and Implications for Optimizing Protein Uptake Rates

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Abstract

Intraparticle transport of proteins usually represents the principal resistance controlling their uptake in preparative separations. In ion-exchange chromatography two limiting models are commonly used to describe such uptake: pore diffusion, in which only free protein in the pore lumen contributes to transport, and homogeneous diffusion, in which the transport flux is determined by the gradient in the total protein concentration, free or adsorbed. Several studies have noted a transition from pore to homogeneous diffusion with increasing ionic strength in some systems, and here we investigate the mechanistic basis for this transition. The studies were performed on a set of custom-synthesized methacrylate-based strong cation exchangers differing in ligand density into which uptake of two proteins was examined using confocal microscopy and frontal loading experiments. We find that the transition in uptake mechanism occurs in all cases studied, and generally coincides with an optimum in the dynamic binding capacity at moderately high flow rates. The transition appears to occur when protein-surface attraction is weakened sufficiently, and this is correlated with the isocratic retention factor k' for the system of interest: the transition occurs in the vicinity of $k' \sim 3000$. This result, which may indicate that adsorption is sufficiently weak to allow the protein to diffuse along or near the surface, provides a predictive basis for optimizing preparative separations using only isocratic retention data.

Keywords

protein diffusion; protein uptake; dynamic capacity; breakthrough; lysozyme; ribonuclease A

1. Introduction

Prediction of chromatographic behavior is desirable in the scale-up and design of protein purification strategies. The models used would optimally cover column loading followed by selective elution, and for this purpose should account for both thermodynamic (retention and static capacity) as well as transport information. In the case of preparative ion-exchange chromatography of proteins, the limiting transport step is usually intraparticle diffusion. Because of this, and the frequent absence of complicated adsorption phenomena, extraparticle transport is modeled adequately with representations that typically account for convection, axial dispersion and external mass transfer resistances. With the exception of convection, the

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effects of these phenomena are typically estimated using empirical or theoretical correlations or neglected due to their relatively small influence compared to that of intraparticle transport.

Intraparticle transport is dictated not only by the adsorbent pore structure but also by the coupling of transport with adsorption, particularly when operation is in the non-linear region of the adsorption isotherm. Each of the models commonly used assumes an underlying mechanism that governs overall transport. The pore diffusion model assumes non-adsorbed protein in the pore lumen to be free to diffuse while in equilibrium with immobile adsorbed protein [1,2]. For near-rectangular isotherms typical of protein adsorption in ion-exchange at low salt concentrations, pore diffusion is characterized by a self-sharpening protein front within the adsorbent during uptake, which reduces to the shrinking core model in the limit of a fully rectangular isotherm [3–7]. The homogeneous diffusion model, in contrast, assumes that all protein in the particle, adsorbed and in the pore lumen, is mathematically indistinguishable and able to diffuse, with the protein flux a function of the gradient in the overall protein concentration [1,8,9]. The intraparticle concentration gradients predicted by the homogeneous diffusion model are typical of those seen for simple Fick's law behavior and therefore much more diffuse than for the pore diffusion model in the presence of a sharp isotherm. The transport model can also account directly for the coupling between free and adsorbed protein via the chemical potential in what is sometimes referred to (inaccurately; [10]) as a Maxwell-Stefan formulation [11]. Alternative models include dual transport mechanisms that allow for separate diffusion of bound and unbound protein [12–14]. The flexibility afforded by the incorporation of additional parameters can make better fitting of experimental data possible but calls the effectiveness of model discrimination into question.

For any of these transport models, the diffusivities are typically obtained by fitting the model to experimentally determined rate data obtained by measuring external protein concentrations as a function of time in a packed column or stirred batch system. Carta et al. [15] reviewed various methods for determining diffusivities experimentally, showing that different procedures generally yield reasonable results if the transport model is known. In some cases, however, fitting uptake data to different diffusion models does not provide enough information to distinguish among the models [16,17]. Choosing the incorrect model can cause inaccurate model predictions, in turn affecting scale-up calculations.

Instead of using indirect methods to determine transport rates, confocal microscopy can be used to determine transport rates and the underlying transport mechanism in chromatographic adsorbents directly. This is done by capturing the intraparticle uptake profiles and fitting the observed concentration gradients to a representative model, with most attention being paid to the association of a sharp or a diffuse front to the pore diffusion or the homogeneous diffusion model respectively [18–25]. This method has been used to explore the roles of solution conditions, adsorbent structure and protein structure in determining the transport mechanism. Specifically, in some situations the uptake mechanism has been observed to change with solution conditions, primarily an increase in ionic strength [18–22,26]. The change in uptake mechanism was accompanied by an enhancement in the overall transport rate, as reflected in higher effective diffusivities in batch uptake experiments [19].

The change in uptake mechanism appears to have important implications for process chromatography as breakthrough profiles at higher flow rates are significantly sharper at ionic strengths at which the homogeneous diffusion model applies [18,20]. Harinarayan et al. [26] found similar behavior in identifying maxima in the dynamic binding capacities of monoclonal antibodies (mAbs) as a function of conductivity (hence ionic strength). They suggested that the enhanced transport with increasing conductivity resulted from the screening of electrostatic repulsion between the diffusing proteins and those already adsorbed. However, they also presented evidence from confocal microscopy suggesting a change in uptake mechanism for

solution conditions corresponding to the enhanced transport. In an earlier study, Hubbuch et al. [21] also demonstrated that the enhancement in uptake can be tuned to increase the transport of one protein relative to that of another, adding another dimension to separation design strategies.

The evidence therefore suggests a connection between the change in uptake mechanism and improved column performance, making identification of the basis for the change in mechanism desirable; this is the principal goal of this work. Several possibilities have been suggested. Hubbuch et al. [27] associated the change in uptake mechanism with a qualitative assessment of binding strength based on confocal microscopy observations. A second possibility is an electrokinetic contribution to transport [19], based on the observation that in that study the change in mechanism was seen only in adsorbents of high surface charge density. Another possibility is the electrostatic exclusion mechanism [26], but this seems more plausible for large proteins such as mAbs in narrow-pore adsorbents than for small proteins in wide-pore adsorbents, for which the effect has also been seen [19].

The study reported here was designed to test the hypothesis that the change in mechanism is a function of adsorbent charge density [19], and it consequently made use of a set of strong cation-exchange adsorbents that differed only in their charge density. This allowed the direct determination of how charge density affects overall transport, retention and capacity while eliminating adsorbent structural differences, and together with previously reported results, it allows definition and prediction of the onset of the change in mass transport. Past charge density studies have predominantly been outside the field of ion-exchange adsorbents [28,29], but work on ion-exchange adsorbents found that the overall adsorbent capacity did not increase for charge densities above a specific threshold, while retention did increase with increasing ligand density [30]. However, transport effects were not evaluated in that work. The present study, in contrast, includes structural and functional characterization of the adsorbents with varying charge density and provides an extensive picture of the transport and adsorption phenomena in these and other materials.

2. Materials and Methods

2.1 Protein Samples and Preparation

Monobasic anhydrous sodium phosphate (Sigma-Aldrich, MO, USA) and formic acid (Acros Organics, NJ, USA) were used to prepare 10 mM buffers at pH values of 7.0 and 3.8 respectively. Sodium chloride was added to increase the ionic strength. In this work total ionic strength is used, accounting for the contributions of both the buffer species and sodium chloride. Before use buffers were pH adjusted and filtered using a VacuCap 60 filter unit with a 0.2 µm Supor membrane (Pall Corporation, NY, USA).

Lysozyme (chicken egg white), ribonuclease A type II-A (bovine pancreas) and cytochrome c (horse heart) were purchased from Sigma (St. Louis, MO, USA) and were used as received. Salient properties of these proteins can be found in Table 1. All are basic proteins with a positive charge at physiological pH and are similar in size. Protein solutions were prepared by dissolving the protein in the buffer of choice, adjusting the pH of the solution using sodium hydroxide or hydrochloric acid, and filtering with Millipore Millex-GV 0.22 µm filters (Bedford, MA, USA). Solution concentrations were determined using UV absorbance at 280 nm (Lambda 4B spectrophotometer, Perkin-Elmer, Wellesley, MA, USA, or DU 640 spectrophotometer, Beckman Coulter, Fullerton, CA, USA) or the micro-bicinchoninic acid (BCA) assay from Pierce Biotechnology (Rockford, IL, USA). Standard curves for the BCA assay were created using solutions of the protein being studied rather than the bovine serum albumin standard provided by the manufacturer. The BCA test was used only to determine lysozyme concentrations in isotherm measurements and it was found to be inaccurate at higher protein

concentrations. Because of this, BCA test results were used only for protein concentrations below 1.2 mg/mL.

Fluorescently labeled protein solutions were prepared by conjugating the mono-reactive fluorescent dye Cy5 (PA35001, GE Healthcare, United Kingdom) to the protein of interest. A 20 mg/mL solution of protein was prepared containing the protein in a pH 9.3, 0.1 M sodium carbonate solution. This solution was added to an Eppendorf tube containing the unconjugated dye, mixed well, wrapped in aluminum foil to block light, and placed on a rotating rack for 30 minutes. Unconjugated dye was separated from the dye-protein complex using a 1.0 cm ID, 30 cm FLEX-COLUMN (Kontes Glass Co., Vineland, NJ, USA) gravity-packed with Sephadex G-25 size-exclusion gel (GE Healthcare) that had been equilibrated with a minimum of 10 column volumes of eluent. This labeled protein solution was then diluted 1:50 into an unlabeled protein solution to produce a solution containing <0.5% label on a molar basis as determined by UV/vis spectrometry.

2.2 Stationary Phases

Five strong cation-exchange adsorbents, custom-synthesized by Tosoh Bioscience (Yamaguchi, Japan), were used. They were prepared on a methacrylate base matrix with chemical properties similar to those of commercially available SP-650 C and SP-550 C materials (Tosoh Bioscience, Montgomeryville, PA, USA). The particles were functionalized with a sulfopropyl group, and differed only in their ion-exchange capacities, which were determined by acid-base titration by the manufacturer to be 0.04, 0.07, 0.11, 0.17 and 0.22 meq/mL. These charge densities significantly extend the range of the corresponding commercial ion-exchange adsorbents, viz. 0.14–0.18 meq/mL.

Adsorbents were washed three times with a 1.0 M sodium hydroxide solution or a 1.0 M sodium chloride solution. Adsorbents used in isotherm determination were subsequently washed three times with a buffer of the desired ionic strength for equilibration. Glass AP microcolumns 5 mm × 50 mm (Waters, Milford, MA, USA) were gravity-packed with a 70% v/v particle slurry and then flow packed up to 120% of the maximum flow rate for the experiment with 1.0 M sodium chloride or 1.0 M sodium hydroxide. Once the particles were completely compacted the adjustable frit was moved to rest firmly against the particle bed.

2.3 Particle Size Determination

The particle size distributions of the charge variant adsorbents were measured by confocal microscopy using a LSM 510 microscope (Carl Zeiss, Germany) equipped with a 40x C-Apochromat (NA 1.2) water-immersion lens (Carl Zeiss) and a HeNe laser (5 mW) (LASOS Lasertechnik GmbH, Germany). Lysozyme labeled with Cy5 was allowed to bind to the particles overnight. The particles were then placed in a glass-bottom dish (MatTek Corporation, Ashland, MA) and covered with a cover slide to prevent movement. The confocal microscope was used to capture successive 2D images of a large number of particles in the x - y plane in z increments of 7 μ m. These images were then digitally collapsed into one x - y image, providing the largest radial distance of each particle in one image regardless of its location in the focal plane of the microscope. The 7 μ m z increment corresponds to errors in measuring the radius on the order of 0.2 μ m (0.006% relative error). The collapsed images were analyzed using Adobe Photoshop 6.0 (Adobe Systems, CA, USA) with the Fovea Pro 2.0 Plug-In (Reindeer Graphics, Inc., NC, USA). This analysis allowed particle radii to be found from calculated particle areas after eliminating potential sources of error due to particles in contact, shards of particles or non-spherical particles.

2.4 Transmission Electron Microscopy (TEM)

The charge variant adsorbents were equilibrated overnight in 5 mg/mL lysozyme solution to allow binding of protein molecules to the adsorbent. This served to increase the electron density of the pore region and consequently enhance the image contrast under EM. The adsorbent particles were transferred into a 1.2 mm x 200 μ m high-pressure freezing (HPF) flat specimen carrier (Leica Microsystems, Cat. No. 16706898), and 3 μ L of a 20% solution of dextran (MW 40,000; Sigma, St. Louis, MO) was added as a space filler. HPF of the adsorbents was carried out at a cooling rate of at least 14 000 $^{\circ}$ C/s in a Leica EMPACT (Leica Microsystems, Vienna, Austria). The samples were then freeze-substituted with 1% osmium tetroxide in acetone in a Leica EM Automatic Freeze Substitution system (Leica Microsystems), which was programmed to maintain -90° C for 72 h, warmed at 5 $^{\circ}$ C/h to -30° C and held for 2 h, and then warmed at 5 $^{\circ}$ C/h to room temperature. Upon reaching room temperature, samples were rinsed three times in 100% anhydrous acetone and then slowly infiltrated with EMBED 812 using 1:3, 1:1, and 3:1 (v/v) solutions of resin/acetone and two changes of 100% resin at 3 h intervals. Polymerization in EMBED 812 was carried out at 60 $^{\circ}$ C for 3 days.

Serial sectioning of the samples was performed on a Reichert-Jung Ultracut E. Sections of ca. 50 nm were stained in uranyl acetate methanol solution for 5 minutes and Reynolds lead citrate for 5 minutes. Images were collected using a Zeiss CEM 902 TEM equipped with a Megaview II digital camera (Soft Imaging System, Boulder, CO) operating at 80 kV. Different regions of the particles in the sample block were sampled for exploration of potential structure variations. Natural features were used as landmarks to locate specific positions on each section.

2.5 Adsorption Isotherms

Adsorption isotherms were measured using batch uptake. Wiretrol II capillary mini-pipettes (Drummond Scientific Company, Broomall, PA) were used to measure the amount of adsorbent for each sample. The particles were allowed to settle in the mini-pipettes and the settled particle height was measured and converted to volume of hydrated particles. This conversion, 0.002 mL adsorbent/mm height, was assumed to be the same as that determined for commercially available SP-650 C and SP-550 C, which were measured to be the same despite the intraparticle differences [18]. The measured adsorbent was placed into Eppendorf tubes along with protein solutions of known volume and concentration and rotated overnight for a minimum of 12 hours. The Eppendorf tubes were then centrifuged and the protein concentration in the supernatant protein solution was determined. The amount of protein adsorbed at equilibrium was determined by mass balance.

2.6 Isocratic Retention

Isocratic retention measurements for lysozyme and cytochrome c were performed at pH 7.0 and for ribonuclease A at pH 7.0 and 3.8. Isocratic elution was performed on a Waters 2695 chromatography workstation with the response detected by a Waters 2996 photodiode array detector. Isocratic retention times for proteins on the given adsorbents were determined for a range of ionic strengths. Protein solutions were between 1 and 20 mg/mL with ionic strengths ranging from 0.02–1.15 M. Ionic strength was controlled during the runs by in-line mixing of the low and high ionic strength buffers. Prior to sample injection, the column was equilibrated with a minimum of 10 column volumes of running buffer. A 20–30 μ L sample of protein solution was injected at an eluent flow rate of 0.2–0.3 mL/min. After the peak had emerged the column was washed with either 1.0 M sodium hydroxide or sodium chloride. The order of the runs was randomized. The first moment, determined by numerical integration of the solute peak, was used to determine the retention time, t_r , of the protein. The retention factor, k' , was determined using t_0 based on the retention time of the protein at a non-retained condition (high ionic strength).

2.7 Frontal Loading

Frontal loading was performed on an ÄKTA Explorer 100 (GE Healthcare) using either the sample pump or a 150 mL Superloop at superficial velocities of 120 and 600 cm/hr respectively. Protein solutions at a concentration of ca. 2 mg/mL were used for the experiments. The UV response of the feed protein concentration was determined by bypassing the column and monitoring the detector response. The column was then equilibrated with 10 column volumes of the desired ionic strength buffer and the protein solution was loaded directly onto the column while the output was monitored. When the response reached 80% of the feed solution concentration, the run was terminated and the column was washed with 1.0 M sodium hydroxide for 10 column volumes.

2.8 Protein Uptake by Confocal Microscopy

Labeled protein uptake into chromatographic adsorbents was monitored using laser scanning confocal microscopy using a flow cell and an LKB Bromma positive displacement HPLC pump with solvent flow controlled via a multi-way valve as described previously [19]. A flow rate of 1 mL/min was used for all experiments, so the concentration of the protein in the flow cell can be considered constant, considering that the flow cell volume is ca. 5 μ L. The initial protein concentration for all experiments was ca. 2 mg/mL. The particles were washed in the flow cell with 1.0 M NaOH and equilibrated for a minimum of one minute at a flow rate of 1.0 mL/min. The high flow rate aids in keeping the particles stationary during the experiment. The real-time images of the labeled protein entering the adsorbents were recorded for later analysis.

The most extensive quantitative analysis of the confocal data was for results in the limit of a sharp uptake front, where behavior is consistent with the shrinking core model [6]. This occurs when the isotherm is near-rectangular, and the analysis therefore assumes that the adsorbed protein concentration in the region behind the front remains constant as the front progresses. Behavior similar to this was observed in a subset of the experiments, and the radial position of the protein front as a function of time was obtained from confocal images.

The analysis [3–6] was based on the normalized position of the front, $\eta = R_f/R$, with R_f being the radial position of the front. If external mass transfer is accounted for, the speed of the front, $d\eta/dt$, is described by

$$\frac{\varepsilon_p D_p c_b}{R^2 q_s} = \left[\left(1 - \frac{1}{Bi} \right) \eta^2 - \eta \right] \frac{d\eta}{dt} \quad (1)$$

where ε_p is the accessible particle porosity, D_p is the intraparticle diffusivity, c_b is the concentration of the protein in the bulk, R is the adsorbent radius, q_s is the adsorbed protein concentration in equilibrium with the bulk concentration, Bi is the Biot number ($k_f R / \varepsilon_p D_p$), and k_f is the external mass transfer coefficient. k_f was determined from [31]

$$k_f = 1.15 \frac{u}{\varepsilon_b} Re^{-1/2} Sc^{-2/3}, \quad (2)$$

where u is the superficial velocity, ε_b is the bed porosity, Re is the Reynolds number ($u d_p \rho / \varepsilon_b \mu$), Sc is the Schmidt number ($\mu / \rho D_m$), D_m the molecular diffusivity and μ / ρ the kinematic viscosity of the mobile phase. Equation 1 was solved using the Matlab routine ODE15S. The effective diffusivity, $D_e = \varepsilon_p D_p$, was fitted to the experimental data using the Matlab non-linear least squares fitting routine NLINFIT. The diffusivity of lysozyme was taken as 1.101×10^{-6} cm²/s [32], and q_s was determined from the isotherm results. The accessible particle porosity ε_p could not be determined directly because the quantities of adsorbent were insufficient, but it can be estimated from the maximum static capacities in the adsorption

isotherms on the charge variants, SP-550C and SP-650C by assuming that these capacities scale linearly with the accessible surface area per particle volume, which depends on the pore size distribution [16,18,33]; this in turn provides a relation to the accessible porosity. The accessible porosities of SP-550 C and SP-650 M are 0.29 and 0.49 respectively for a molecule of 1.6 nm in radius [34], which provides an estimate approximately in the range 0.35–0.4 for the accessible porosity of the adsorbents studied here.

3. Results and Discussion

The main thrust of this work is elucidation of the mechanisms of coupled diffusion and adsorption using the set of charge variant materials in order to test the hypothesis [19] that the ligand charge density is the principal determinant of the uptake transport mechanism. Several forms of structural and functional characterization of the adsorbents are presented before the transport studies. The structural studies are intended to verify the comparability of the materials in that the principal difference is in the ligand density, while the functional studies, specifically of adsorption characteristics, are both of fundamental interest as well as being useful in interpreting the transport experiments, which include both confocal microscopy and column breakthrough studies.

3.1 Structural Characterization

Figure 1 shows the particle size distributions on a volume-weighted basis for the five charge variant adsorbents. The distributions are symmetric and overlap reasonably closely, as would be expected from their being prepared on the same base matrix. Table 2 shows the average diameter and standard deviation of each adsorbent along with the number of particles sampled, again showing the similarity of the particle size distributions around an average diameter of 109.6 μm . Staby et al. [35] determined the particle size distributions of commercially available Toyopearl SP-650 C and SP-550 C and found them to be much wider, with a mean particle size of 80 μm for both adsorbents using Coulter counting and of 93 and 78 μm for SP-650 C and SP-550 C respectively using SEM. Transport modeling can account for the particle size distribution of the adsorbent [36–38], but the adsorbents studied here lie within sufficiently narrow distributions to allow monodispersity to be assumed.

Intraparticle structural information can be obtained under realistic chromatographic conditions using inverse size exclusion chromatography [34,39,40], but insufficient quantities of the charge variant materials were available for reliable measurements. Instead, local rather than averaged information was obtained by TEM. A number of forms of structural heterogeneity were observed at different levels. First, although all variants were prepared on the same base matrix, the pore structures revealed by the imaging turned out to be unexpectedly diverse, with the pore volume fraction and dimensions being noticeably different among the different adsorbents. Structural variability within the particles was observed for all the resins except at the 0.07 meq/mL charge density, with the four heterogeneous adsorbents displaying two representative pore morphologies, viz. sparsely scattered pores vs. larger, more interconnected pores (Figure 2). A layered structure was seen in the 0.17 and 0.22 meq/mL adsorbents, each containing sparser pore coverage in the outer layer near the particle surface. It is not clear whether these observations are fully representative of the respective adsorbents, and if so, whether the differences are sufficiently pronounced to have functional consequences. As seen below, there appear to be clear systematic trends in functional properties among the different adsorbents, so differences of the kind revealed by the TEM studies may not manifest themselves in the more highly averaged measures used for bulk property measurements.

3.2 Isocratic Retention

Retention factors k' for lysozyme, cytochrome c and ribonuclease A on the charge variant adsorbents are shown in Figure 3 as a function of the ionic strength I . For all the adsorbents, retention followed the order ribonuclease A (pH 3.8) > lysozyme > cytochrome c > ribonuclease A (pH 7.0). This follows the trend of protein charges as shown in Table 1, but the significantly stronger retention of lysozyme than of cytochrome c at pH 7, which has frequently been seen previously [30,41,42], can not be explained by the net charge or indeed by detailed electrostatic calculations [43]. The stronger lysozyme retention could stem from the intrinsically stronger electrostatic binding of arginine than of lysine; lysozyme is rich in arginine residues and cytochrome c in lysines [42]. Wu and Walters saw the trend reverse itself as a function of ligand density on silica supports [30]. They attributed the difference to the binding of phosphate to cytochrome c [44], and verified this by showing that cytochrome c retention was higher using non-phosphate buffering species.

For each protein, retention on the adsorbent of lowest charge density, 0.04 meq/mL, was lower than that on the other adsorbents, but the retention data for the remaining adsorbents show little dependence on charge density. Table 3 shows the parameters from fitting each k' data set in Figure 3 to the ionic strength I in terms of the stoichiometric displacement model [45]

$$k' = K_{sd} I^{-Z}. \quad (3)$$

The use of this model does not imply any mechanistic interpretation other than to note that changes in slope are related to the properties of the protein and adsorbent and should change as the physical properties change. The results in Table 3 show that the Z values for the 0.04 meq/mL retention data are consistently lower than those of the remaining adsorbents for all data sets except for cytochrome c. There are no discernible trends in the other slopes.

Colloidal models of retention suggest that Z is related to the surface charge density of the adsorbent, the charge of the protein, and the size of the protein [46–49], and such models typically predict increasing Z with increasing ligand density. This trend was verified in the work of Wu and Walters but is not seen for the adsorbents here. This indicates that simple colloidal models can not capture retention behavior completely. The most likely source of error is the continuum representation of the adsorbent surface and its characterization solely in terms of the surface charge density. Discrete charge representations have also been used [43,50–52] but do not allow simple analytic approximations to be developed easily.

3.3 Adsorption Isotherms

Adsorption isotherms of lysozyme on the charge variant materials at ionic strengths of 50 and 100 mM are shown in Figure 4. At both ionic strengths, the plateau values of the isotherms increase with the charge density up to a threshold beyond which higher charge densities do not provide added static capacity. Because of this, the 0.11, 0.17 and 0.22 meq/mL adsorbents provide approximately the same capacity at a given ionic strength. As expected, an ionic strength increase from 50 to 100 mM results in a lower capacity on each adsorbent due to screening of the electrostatic attraction that drives adsorption in ion-exchange, as reflected also in the k' trends. Wu and Walters [30] also saw a plateau in lysozyme capacity with increasing charge density, as seen here. The lower capacity at low charge densities is most likely due to weaker adsorption stemming from the lower charge density, although the k' data suggest that this can not explain the difference in the plateau levels between the 0.07 meq/mL material and the three materials of higher ligand density.

3.4 Intraparticle Transport Visualized by Confocal Microscopy

3.4.1 Lysozyme Uptake—Confocal microscopy images of labeled lysozyme uptake into the five charge variant materials are shown in Figure 5. Each micrograph was taken approximately 150 seconds after the start of the uptake and displays a slice along the $x-z$ axis of the adsorbent, i.e., the incident and emitted light are transmitted vertically in each image. Uptake patterns at the lower ionic strengths are indicative of a pore diffusion mechanism as reflected by the sharp protein front diffusing into the adsorbent and the absence of protein in the center of the particle. At higher ionic strengths the sharp protein front is replaced by a diffuse front that becomes more diffuse with increasing time (data not shown); the diffuse fronts are consistent with a homogeneous diffusion mechanism. The departure from the sharp front is first seen in the 0.04 meq/mL adsorbent at 100 mM ionic strength but in the other four charge variants only at 200 mM. This indicates that the solution conditions as well as the charge density play roles in determining the transport mechanism.

The speed of the protein front entering the adsorbent was determined within the framework of the shrinking core model as described earlier at ionic strengths of 50 and 100 mM. The confocal images confirm that the shrinking core model applies to all the conditions used except for the 0.04 meq/mL adsorbent at 100 mM, so the diffusivity obtained for the latter should be used with caution. The effective diffusivities, $D_e = \varepsilon_p D_p$, of lysozyme into the five adsorbents were determined from the data fits and are provided in Figure 6. Because the TEM images of these adsorbents discussed earlier indicate that differences in the intraparticle pore structure may be present both among and within particles, experiments at 100 mM were duplicated on different individual particles under the same conditions in order to capture diffusivity differences among particles. The error bars shown on the 100 mM ionic strength data represent the resulting 95% confidence intervals, and are much larger than the errors associated with fitting the data. Given this uncertainty, there is no definitive trend in the diffusivity values as a function of either ionic strength or charge density under conditions where the shrinking core model applies.

As discussed in Section 2.8, the porosities of the adsorbents are not known, so the pore diffusivities can not be calculated reliably. However, the estimated accessible porosity of lysozyme of 0.35–0.4 would place the D_p values in Figure 6 close to the upper end of the range of $5\text{--}10 \times 10^{-7} \text{ cm}^2/\text{s}$ obtained on SP-650 C using confocal microscopy and batch uptake for ionic strengths similar to those studied here [53]. Pore diffusivities of lysozyme in SP-550 C were in the range $2\text{--}5 \times 10^{-7} \text{ cm}^2/\text{s}$ for ionic strengths of 6–100 mM [19]. That the results shown here appear to exceed the free solution diffusivity of lysozyme indicates that transport is not exclusively by pure pore diffusion.

3.4.2 Ribonuclease A Uptake—Images of ribonuclease A uptake into the charge variant materials for selected conditions at pH 7 (Figure 7) show a different pattern to that seen previously with lysozyme. The uptake behavior at low ionic strength is similar to that seen for a pore diffusion mechanism, but the sharp front entering the adsorbent is complemented by a bright ring that represents an ‘overshoot’ in the radial intensity profile. The overshoot exists at ionic strengths of 20 mM or less and disappears between ionic strengths of 20 and 50 mM for all of the charge variants, giving rise to a diffuse front similar to that for the shrinking core mechanism.

The overshoot phenomenon seen here, which has been reported to appear in the transition region between the shrinking core and the diffuse front patterns [18,19], has been a source of some controversy, with the evolution of interpretations summarized well by Carta et al. [15]. Initial arguments invoked electrokinetic phenomena in various forms [19,54], but more recent theoretical and experimental studies in various groups have implicated displacement mechanisms involving labeled and unlabeled proteins [55–57]. Specifically, conjugation of a label such as Cy5 to a protein via a lysine residue replaces a positive charge at neutral pH by

a negative charge, making the charge on the labeled protein different to that on the unlabeled one by -2. As a result, for a positively charged protein in cation exchange, the labeled form adsorbs more weakly than the unlabeled one, and indeed the two forms can be separated under suitably chosen conditions for ion-exchange [56,57]. During uptake, the labeled protein can be displaced by the unlabeled one, which is present in large excess, giving rise to the overshoot behavior in the presence of an otherwise sharp front [55,56]. However, an overshoot has also been seen in experiments using unlabeled protein and multi-photon [19] or intrinsic fluorescence microscopy [58], and long-time observations show the labeled protein concentration to increase rather than to decrease as would be expected for displacement [19]. Therefore, while the presence of an overshoot provides strong support for the displacement theory driven by the presence of labeled protein, it is possible that the phenomenon is more complex.

Independent of this uncertainty, though, is the question of whether the presence of the overshoot still allows the appearance of a diffuse front to be interpreted as a transition in uptake mechanism for results such as those in Figure 7. Confocal microscopy results for a basic protein (lysozyme) labeled with acidic fluorophores clearly show diffuse fronts at higher ionic strengths despite the presence of labeled and unlabeled proteins as well as relatively sharp isotherms [19]. Therefore, the presence of a diffuse front in this system appears to be a reliable indicator of a homogeneous diffusion mechanism, and the results of Teske et al. [57] suggest that this will be the case whenever the labeled protein is less strongly retained than the unlabeled one. Additional support for inferring a change in uptake mechanism that reflects an increase in the uptake rate is provided by uptake measurements on pure unlabeled protein in batch and breakthrough experiments; some additional data of this kind are presented below.

All the transport studies shown thus far were performed at pH 7, but pH is an important experimental variable that can be used to manipulate protein retention, as is apparent from Figure 3. Uptake of labeled ribonuclease A at pH 3.8 was therefore studied into two of the charge variants, representative of low and high surface charge density. Images of this uptake into the 0.04 meq/mL and 0.17 meq/mL charge variant materials at pH 3.8 are shown in Figure 8, with the results for each material presented at a pair of ionic strengths bracketing a transition in the uptake front profile from sharp to diffuse. These transitions occur between 100 and 150 mM for the 0.04 meq/mL adsorbent and between 300 and 350 mM for the 0.17 meq/mL adsorbent; these ionic strength ranges are much higher than those necessary for the corresponding transitions at pH 7.0. Evidence of an overshoot in the uptake profile is seen on the 0.17 meq/mL adsorbent at 300 mM.

3.5 Frontal Loading

Frontal loading (breakthrough) experiments provide both an assessment of the importance of the transport mechanism for process chromatography and a measure of transport rates independent of the need for labeling. Frontal loading results are shown in Figure 9 for lysozyme on each adsorbent at two superficial velocities, 120 and 600 cm/hr, and at three ionic strengths, 50, 100 and 150 mM. The normalized outlet concentration is presented as a function of the mass of lysozyme loaded per column volume. Overall, the breakthrough curves for each adsorbent shift to the left as the ionic strength is increased, following the trend of the static capacity. Additionally, breakthrough at 120 cm/hr is sharper than at 600 cm/hr, indicating the increasing significance of mass transfer limitations at increasing flow rates. The results for the 0.11, 0.17 and 0.22 meq/mL adsorbents show some variability despite their similar static capacities and pore structures; these discrepancies presumably reflect the small differences in these properties as well as column porosity differences.

The dynamic binding capacity (DBC) at 10% breakthrough, $Q_{10\%}$, for each adsorbent is shown as a function of ionic strength and flow rate in Figure 10. At 120 cm/hr, $Q_{10\%}$ increases with

increasing charge density in much the same manner as the static capacity. The increase is most pronounced from 0.04 to 0.07 meq/mL and smaller with further increases above 0.07 meq/mL, with only small differences among the adsorbents of highest charge density. At 600 cm/hr, the DBC is uniformly lower than at 120 cm/hr because of the reduced contact time for mass transfer. The overall DBC trend is somewhat similar to that described above, but the 0.07 meq/mL adsorbent differs less from those of higher charge density adsorbents than at the lower flow rate.

A noteworthy effect of flow rate that is apparent in Figure 10 is in the dependence of $Q_{10\%}$ on ionic strength. At a flow rate of 120 cm/hr $Q_{10\%}$ decreases monotonically with ionic strength, following the same pattern as static capacity as expected. However, at 600 cm/hr, the curves are much flatter, and indeed $Q_{10\%}$ attains a maximum at intermediate ionic strength for the 0.07, 0.11 and 0.17 meq/mL adsorbents. This maximum occurs at ionic strengths near the lower end of the range over which the transition in uptake mechanism is observed. The maxima seen are shallow, and a maximum was not observed for the 0.04 meq/mL adsorbent under the conditions examined, although this may be because the uptake transition in this adsorbent was observed below 100 mM ionic strength. Much higher maxima have been reported for mAbs [26], perhaps indicating a role for protein size. Nevertheless, that the dynamic binding capacity does not mirror the decline seen in the static capacity as the ionic strength is increased from 50 to 100 mM indicates an enhancement in intraparticle transport despite the absence of a clear trend in the diffusivities in Figure 6.

The maximum in the DBC seen at higher flow rates is of great practical importance for preparative separations in that it suggests that conditions corresponding to lower static capacity may in fact allow higher dynamic capacities to be attained at high flow rates, where throughputs are higher. In this way column productivity, which can nominally be expressed as the product of DBC and volumetric flow rate, can be increased substantially over the values attainable under conditions where DBC alone is optimal, viz. low flow rate and low ionic strength. Given the potential practical impact of the maximum in the DBC, it is important to try to identify its origins and hence to predict the conditions under which it can be found in general for a given adsorbent-protein pair.

3.6 Origins of Change in Uptake Mechanism and Maximum in Dynamic Binding Capacity

Maxima in the DBC as a function of ionic strength such as those described above have been observed previously, as discussed in the Introduction, and have been associated with the effects of ionic strength on intraparticle transport [19,20,26,27]. Although the maximum in the DBC appears to be correlated with the transition in the transport mechanism, the ultimate origin of this behavior in both the structure of the stationary phase and the operating conditions remains incompletely understood. For the charge variants studied here, the transition for lysozyme uptake at pH 7.0 occurs between an ionic strength of 50 to 100 mM for the 0.04 meq/mL adsorbent and between 100 and 200 mM for the remaining adsorbents. The maximum in dynamic binding capacity is seen at the lower end of this range, indicating that the physical processes driving the two are related. This is also the case for lysozyme uptake on SP Sepharose FF [20] and SP 650 C [53], where the transition occurred between 50 and 100 mM ionic strength. The DBC maxima for the mAb set on Sepharose media occurred over a range of ionic strengths that was a function of pH and was correlated with the calculated net charge on the respective molecules [26].

The present study was motivated by the suggestion [19] that the transition was seen to occur (up to ionic strengths of 100 mM) only for adsorbents of relatively high surface charge density. That the transition occurs at a lower ionic strength for the 0.04 meq/mL adsorbent than for those of higher ionic capacity clearly disproves this hypothesis. An alternative hypothesis can be developed based on the observation that the adsorbents in which transitions were observed

up to 100 mM ionic strength [19] were those for which linear isocratic k' values [42] were appreciably lower at a given ionic strength than those for which no transition was seen. This hypothesis is qualitatively consistent with the earlier transition and lower k' values seen in the present work on the 0.04 meq/mL adsorbent than on those of higher charge density. The association of the change in uptake mechanism with binding strength was also suggested previously [27], but the binding strength in that work was assessed based qualitatively on confocal microscopy observations. We seek here to examine the hypothesis more quantitatively in order to show that retention measurements can be used as a guide to predict not only where the transition in transport mechanism occurs but also where the maximum in dynamic binding capacity may be realized.

Figure 11 shows the location of the transition in uptake mechanism in the context of isocratic retention results for all the systems examined in this work as well as those studied elsewhere for which appropriate data are available. These include lysozyme at pH 7 on the charge variant adsorbents, SP Sepharose FF and Toyopearl SP-650 [20,42,53] and ribonuclease A at pH 7 and 3.8 on the charge variant adsorbents. The uptake transition measurements were performed on SP-650 C [53], but are compared to the available retention data on SP-650 M [42], which differs only in particle size. Because the accessible range of k' measurements is at higher ionic strengths than the range for useful breakthrough experiments, the k' data have been extrapolated linearly (on the log-log plot) to lower ionic strengths, consistent with the functional form suggested by the stoichiometric displacement model [45]. Points represented by stars are located at conditions for which confocal experiments were performed to test for the transition in transport mechanism; open stars represent conditions for which a sharp front was observed while filled stars represent points for which a diffuse front was observed.

The results indicate that, for the systems studied, the transition in each system occurs in a log k' range roughly between 2 and 4. This rather wide range can be narrowed considerably by observing that sharp uptake profiles are seen for essentially all conditions for which $\log k' > 3.5$ and diffuse profiles for $\log k' < 4$. This relatively narrow range is especially noteworthy given that there are several sources of uncertainty in the estimated k' values, especially those associated with the extrapolation; the steep k' curves for ribonuclease A at pH 3.8 are particularly susceptible to this. Nevertheless, the correlation holds over relatively narrow ionic strength ranges varying from < 50 mM to > 300 mM. This correlation suggests that a simple set of isocratic retention measurements can be used as the basis for predicting conditions for the change in uptake mechanism and for narrowing down conditions for optimizing DBC as a function of flow rate. Indeed, the k' vs. ionic strength curve can be estimated with reasonable accuracy from a small set of gradient elution experiments coupled with a parametric equation relating k' to ionic strength [59,60].

The relation between the ionic strength range for the transition in uptake mechanism and that at which the DBC maximum occurs appears to reflect a trade-off between the loss of static capacity and the acceleration in uptake rate as the ionic strength is increased. The initial slope of the isotherm is directly related to k' , and the plateau value of the isotherm typically decreases as k' decreases with increasing ionic strength (see, e.g., Figure 4). The practical utility of the faster uptake associated with homogeneous diffusion depends on the survival of a sufficiently high plateau in the adsorption isotherm, i.e., a sufficiently high static capacity, on the high ionic strength side of the transition. The transition appears to occur for k' values of order 10^3 , which are high enough to give near-rectangular isotherms that can result in practically useful static and dynamic capacities. Such capacities may, however, not be obtained if the range of the transition in uptake mechanism and the slope of the k' vs. ionic strength plot are sufficiently high; if this is so, the plot may be manipulated by changing the pH (e.g., ribonuclease A in Figure 3) if protein stability permits.

The mechanistic basis for the correlation between protein retention and the transition in uptake mechanism remains uncertain. One possibility is that it is related to the intermolecular repulsion mechanism discussed earlier [26], but this mechanism seems more likely for large proteins such as mAbs on a narrow-pore material such as SP Sepharose XL, and less likely to be significant for a small protein such as lysozyme on a wide-pore material such as SP-650 C. A more likely explanation is that, since k' is a measure of protein–surface interactions, the transition is made possible by the weaker adsorption, which may allow the protein to diffuse in the vicinity of the surface, whether by direct surface diffusion or by successive desorption, diffusion and readsorption. The rapidity of the uptake may be due either to the freedom of even adsorbed protein to diffuse, or to some other mechanism such as an electrokinetic contribution to transport.

If the k' relationship is indeed an indication that protein-surface interactions determine the uptake transition, k' may in fact be a surrogate for a more rigorous measure. Specifically, $k' = K_{eq}\phi$, where K_{eq} is the adsorption equilibrium constant and ϕ the phase ratio. K_{eq} is a more fundamental measure of the protein-surface interaction, so it may be more appropriate for use in correlating the uptake mechanism transition. The results can be transformed with knowledge of the phase ratio [34], but this could not be measured for the charge variant materials because insufficient quantities were available. However, the range of phase ratios observed for proteins on most materials is likely to span much less than an order of magnitude [34], so the k' predictor should retain its utility for most adsorbents. A likely exception is the class of polymer-modified adsorbents that have emerged over the past decade or two, which can show very high static and dynamic capacities and for which the retention and transport mechanisms may differ from those in more traditional materials.

4. Conclusions

There are three main contributions of this work. The principal ones are associated with protein transport mechanisms in ion-exchange adsorbents and have important practical implications for large-scale bioprocessing. The key observation is the quantitative relation of the transition in transport mechanism from a sharp to a diffuse front to solution conditions that correspond to $\log k'$ values approximately in the range 2–4, although it may be possible to narrow this range to 3.5–4. This relationship suggests that retention experiments can provide a relatively simple approach for finding the transition in the uptake mechanism. We test the predictive value of this result elsewhere [53] for lysozyme uptake on Toyopearl SP-550 C.

The second contribution is the related one of elucidating the conditions leading to a maximum in dynamic binding capacity as a function of the ionic strength. This maximum was found only at an elevated linear flow rate and was not present where the flow rate was so low that the DBC tracked the static capacity. Our results suggest that the maximum is seen near the onset of the transition in uptake mechanism and it can thus be correlated with the same $\log k'$ relationship as the transition. The exact nature of the flow rate dependence has yet to be evaluated but should be explored via modeling and further experiments. Knowledge of this component would allow effective optimization of column operating conditions based on a relatively small number of focused experiments.

A final contribution applies to the specific behavior of the charge variant adsorbents, the results for which indicate that it is possible to manipulate the adsorbent charge density so as to obtain specific capacity and retention characteristics while maintaining comparable transport behavior. However, the sensitivity of retention and capacity to charge density is seen only at fairly low charge density, consistent with the capacity trends reported previously [30], making this a relatively unlikely factor to use in manipulating retention, except perhaps of strongly bound moieties such as viruses.

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Figure 1.
Volume-based particle size distribution for 0.04 (○), 0.07 (□), 0.11 (△), 0.17 (●), and 0.22 (■) meq/mL adsorbents.

Figure 2. Representative pore features from separate particles showing (a) an open pore structure with widely interconnected pores and (b) relatively sparsely scattered pores.

Figure 3. k' results for (a) lysozyme and cytochrome c at pH 7.0 and (b) ribonuclease A at pH 3.8 and 7.0. Symbols as in Figure 1.

Figure 4. Lysozyme isotherms on charge variant materials at (a) 50 mM and (b) 100 mM ionic strength. Symbols as in Figure 1.

Figure 5. Confocal microscopy imaging of Cy5 labeled lysozyme diffusing into the different charge variant adsorbents. Each micrograph was taken approximately 150 seconds after the initial protein entrance into the adsorbent. Adsorbent particles are ca. 50 μm in radius.

Figure 6.

Lysozyme effective diffusivities obtained from front profile fits at 50 (?) and 100 (?) mM. Error bars on 100 mM data represent the 95% confidence interval determined from two separate observations.

Figure 7. Uptake of Cy5 labeled ribonuclease A into charge variant materials at different ionic strengths at pH 7.0 as viewed by confocal microscopy.

Figure 8. Uptake of Cy5 labeled ribonuclease A uptake into charge variants with ligand densities of 0.04 and 0.17 meq/mL at different ionic strengths and pH 3.8 as viewed by confocal microscopy.

Figure 9.

Frontal loading profiles for lysozyme at pH 7.0 at ionic strengths of 50, 100 and 150 mM and flow rates of 120 and 600 cm/hr for adsorbents with charge densities of 0.04 (—), 0.07 (···), 0.11 (---), 0.17 (-·-) and 0.22 (---) meq/mL.

Figure 10. Dynamic binding capacity, $Q_{10\%}$, at superficial flow rates of (a) 120 cm/hr and (b) 600 cm/hr. Symbols and lines as in Figure 1 and Figure 9 respectively.

Figure 11.

Isocratic retention factors for lysozyme at pH 7 on charge variant materials, SP Sepharose FF (8) and SP-650 M ($\blacktriangledown$), and ribonuclease A retention factors on charge variant materials at pH 3.8 and 7.0. Symbols for the charge variant materials are as in Figure 1, with retention factors in the order ribonuclease A (pH 7) < lysozyme (pH 7) < ribonuclease A (pH 3.8). SP Sepharose FF and SP-650 M retention results are from DePhillips and Lenhoff [42]. Stars represent the conditions at which confocal microscopy was performed for verification of the transition in uptake mechanism; open and filled stars represent sharp and diffuse fronts respectively.

Table 1

Properties of proteins used.

Protein	Molecular weight [g/mol]	Partial specific volume [g/mL]	pI	Net charge	
				pH 7.0	pH 3.8
Lysozyme	14300 [61]	1.422 [62]	11.10 [63]	~7.4 [63]	~13.8 [63]
Ribonuclease A	13400 [62]	1.414 [62]	9.45 [64]	~3.9 [64]	~12.2 [64]
Cytochrome c	12640 [62]	1.374 [62]	10.04 [65]	~7.1 [65]	~12.0 [65]

Table 2

Particle size distribution results: mean particle diameter, standard deviation and sample size.

Charge density, [meq/mL]	Mean diameter (μm)	σ (μm)	n
0.04	108.6	7.6	209
0.07	107.8	6.8	681
0.11	109.2	6.8	478
0.17	111.8	9.4	300
0.22	112.0	8.6	377

Table 3

Least-squares fitted parameters for isocratic retention results using the stoichiometric displacement model $k' = K_{sd}I^{-Z}$.

Charge density [meq/mL]	Lysozyme, pH 7.0	Cytochrome c, pH 7.0	Ribonuclease A, pH 7.0	Ribonuclease A, pH 3.8
	Z	Z	Z	Z
0.04	3.9±0.3	4.6±0.3	3.6±0.2	6.6±1.1
0.07	4.9±0.5	5.0±0.4	4.2±0.3	10.4±1.3
0.11	6.2±0.6	5.4±0.3	4.4±0.2	10.4±1.1
0.17	5.0±0.3	5.4±0.3	4.5±0.3	12.3±1.7
0.22	5.1±0.3	4.8±0.3	4.4±0.3	12.5±2.5
	log K_{sd}	log K_{sd}	log K_{sd}	log K_{sd}
0.04	-1.2±0.2	-2.9±0.2	-3.1±0.2	-2.1±0.2
0.07	-1.3±0.2	-2.6±0.2	-2.8±0.2	-2.1±0.3
0.11	-1.9±0.3	-2.6±0.2	-2.8±0.2	-1.7±0.2
0.17	-1.4±0.1	-2.6±0.2	-2.8±0.2	-2.1±0.3
0.22	-1.4±0.1	-2.3±0.2	-2.9±0.2	-2.0±0.5

Errors in Z and log K_{sd} represent the 95% confidence interval as determined from the linear fits of the experimental data