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Determinants of protein retention characteristics on cation-exchange adsorbents

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Abstract

There are currently a large number of commercially available strong and weak cation-exchange adsorbents for preparative protein purification, typically prepared by coupling charged ligands to a mechanically rigid porous bead. Because of the diverse chemical nature of the base matrix (carbohydrate, synthetic polymer, inorganic) and the coupling and ligand chemistry, cation-exchange adsorbents from different suppliers can differ substantially in chemical surface properties and physical structure. The differences in chemical properties can be in ionic capacity, hydrophobicity, the presence of hydrogen bond donors/acceptors, and the nature of the charged functional groups. In order to probe the effects of these factors on protein affinity, the isocratic retention of a set of model proteins was examined on a set of cation-exchange adsorbents to obtain a quantitative assessment of retention differences between adsorbents. Two adsorbent factors were found to be the dominant determinants of overall protein retention: the anion type and the adsorbent pore size distribution. Protein retention on strong cation-exchangers was found to be greater than that on corresponding weak cation-exchangers. Protein retention was increased on adsorbents with pore size distributions that include significant amounts of pore space with dimensions similar to those of the protein solute.

Keywords: Stationary phase, LC; Ion exchangers; Pore size distribution; Proteins

1. Introduction

Chromatography, and ion-exchange in particular, offers the potential for high selectivity, capacity and throughput in large-scale protein purification processes. The evaluation and selection of the adsorbent for a separation problem are critical to obtaining these benefits, and these are determined to a large extent by the nature of the protein–adsorbent interaction. In the case of ion-exchange, this interaction is broadly understood to involve ionic interaction between opposite charges, but at a molecular level the adsorption process is complex. By virtue of their size, proteins have the potential for multiple atomic interactions with the adsorbent surface. Protein–adsorbent interactions can also be envisioned to be affected by the same interactions that stabilize folded proteins and protein association with other proteins or ligands: the pairing of complementary charges, the repulsion of like charges, hydrogen bond formation, and burying of hydrophobic groups. While the electrostatic interaction between opposite charges is of primary importance in ion-exchange chromatog-

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raphy, the other interactions have the potential to influence protein retention and therefore selectivity.

The unpredictable existence and extent of such interactions, combined with the multitude of commercial ion-exchange adsorbent options, means that stationary-phase selection generally proceeds through an empirical screening process, in which a specific separation problem is examined over a set of adsorbents from different suppliers. The benefits of such a screening program can be significant; peaks that are only partially resolved on one adsorbent can be resolved to baseline on a second adsorbent, under identical conditions [1].

A comprehensive screening program to optimize the selection of an ion-exchange adsorbent is time consuming. There are a large variety of competing products from numerous suppliers, and operating conditions such as pH, salt and buffer type, and product capacity must also be considered over this adsorbent set. Furthermore, this empirical approach will result in a set of operating conditions optimized for a given separation problem, but it will not yield a mechanistic understanding of why one adsorbent outperformed another, and so the screening effort will have to be repeated for different protein products. Clearly, increased insight into the protein and adsorbent properties that dictate the magnitude of chromatographic retention would assist this selection process by directing such screening programs toward a more limited and manageable subset of adsorbents. Toward that goal, several groups have proposed models for chromatographic retention on ion-exchange adsorbents [2–13]. The differences among both the protein solutes and the porous stationary phases typically used for protein separation present formidable challenges to using such models predictively unless they are truly mechanistic. This is not yet a reality, though, as a considerable understanding of the molecular and surface structures and of characteristics of both the protein and the adsorbent is needed to represent the many potential non-covalent interactions that constitute the adsorption process.

For protein solutes, the heterogeneous and asymmetric charge distribution over the exterior surface makes the net charge concepts that describe small molecule ion-exchange chromatography less relevant. For example, several studies have noted the adsorption or chromatographic retention of proteins on surfaces with a like net charge [3,14–21]. Consequently, the concept of chromatographic adsorption on ion-exchange stationary phases includes some degree of spatial orientation as a characteristic feature, in which contact occurs through a preferred subset of the amino acid sequence. The amino acids that comprise these regions, variously referred to as ‘‘contact regions’’ [22], ‘‘ionotypes’’ [15] or ‘‘patches’’ [10], have occasionally been well defined [23–27]. However, the size and heterogeneity of protein sequences means that this feature contains considerable ambiguity; a given protein may contain several such regions, giving rise to multiple potential orientations for adsorption. Observed chromatographic retention is then a global average of all such contributions, with the relative importance of each difficult to assess.

The other component of chromatographic adsorption is the stationary phase surface. The chemical and physical heterogeneity of the porous phases typically used for protein chromatography also present a characterization problem of some magnitude; it has received less systematic attention than protein characterization, largely because of the focused priorities of individual manufacturers. A number of different chemistries and synthetic strategies have been developed to prepare the base particles, and subsequently to conjoin spacer arms and charge groups. The physical structures that result are also quite complex, the pore network contained within typically being a poorly defined collection of spaces of which the shape, cross-section and connectivity may vary substantially.

These adsorbent structures are too complex to model realistically in both their physical and chemical structure on length scales that determine protein retention. As an alternative, however, we describe here how differences in protein retention can be correlated with stationary phase physical and chemical structure through comparative retention studies that utilize well-characterized model proteins and a large set of stationary phases that differ in their physicochemical properties.

The physical characteristics of these stationary phases have been described in a previous study [28]. There, the pore size distribution (PSD), the mean pore radius, and the phase ratio (ϕ) were characterized as a function of solute size for a set of SEC and cation-exchange stationary phases using inverse size-

exclusion chromatography (ISEC). In the work reported here, the relative retention of a set of three model proteins (lysozyme, chymotrypsinogen, cytochrome c) was determined for the same set of adsorbents. By defining and characterizing both the key physical properties of the adsorbents and the chromatographic retention, several potential correlates to protein retention were examined, including adsorbent charge density, ligand type, and adsorbent PSD. Because the adsorbents selected vary simultaneously in a number of physicochemical properties, some uncertainty is inherent in any interpretation of retention differences among the set. However, log scale differences in adsorbent retentivity were found; such large differences are not generally indicative of subtle properties, so that strong trends and correlations were found in the data here.

2. Materials and methods

2.1. Chromatographic stationary phases

Ten strong (SCX) and weak (WCX) cation-exchange adsorbents, listed in Table 1, were used for this study. All TosoHaas resins were purchased from TosoHaas (Montgomeryville, PA, USA). Strong and weak ''tentacle'' type cation-exchange adsorbents were purchased from EM Industries (US associate of Merck, Darmstadt, Germany). Spherodex cation-exchange adsorbents were purchased from BioSepra (Marlborough, MA, USA). Sepharose Fast Flow (Sepharose FF) resins were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Cellufine Sulfate was purchased from Amicon (Beverly, MA, USA).

The adsorbents selected differ in particle morphology, the chemical nature of the base matrix, the spacer-arm chemistry, the anionic ligand, and the ligand density. The physicochemical properties of these adsorbents, as given by the manufacturers, are summarized in Table 1, with a more detailed description provided elsewhere [28]. The surface charge densities shown for the adsorbents were estimated using the phase ratios calculated for the smallest probe molecule, glucose, in the inverse SEC characterization of the pore size distribution [28]. In view of the different morphologies of the materials studied, there is some uncertainty in these values, but not enough to affect the conclusions drawn in this

Table 1
Physicochemical properties of the cation-exchange stationary phases as provided by the suppliers; surface charge densities were calculated based on phase ratios for the smallest probe molecule (glucose) used in inverse SEC measurements of pore size distributions [28]

Stationary phase	Anionic ligand	Base matrix	Ion exchange capacity (μmol/ml)	Surface charge density (μmol/m^2)
EM Industries				
EMD SO_3^- M	Sulfoisobutyl polyacrylamide	Methacrylate	Not given	–
EMD COO^- M	Polyacrylic acid Polyacrylamide	Methacrylate	Not given	–
TosoHaas Toyopearl®				
SP-650 M	Sulfopropyl	Methacrylate	120–170	4.4–6.3
SP-550 C	Sulfopropyl	Methacrylate	120–180	0.66–0.99
CM-650 M	Carboxymethyl	Methacrylate	80–120	2.8–4.2
BioSepra				
SP Spherodex	Dextran sulfate	Silica	131	1.7
CM Spherodex	Carboxymethyl	Silica	131	0.86
Amersham Pharmacia Biotech				
SP Sepharose FF	3-(2-Hydroxypropoxy)-1-propanesulfonic acid	Agarose	180–250	3.6–5.0
CM Sepharose FF	Carboxymethyl	Agarose	90–130	2.1–3.0
Amicon				
Cellufine sulfate	Sulfate ester	Cellulose	8	–

work. For the SCX adsorbent set, there is significant variation among the chemical structures of the anionic ligands, with the SP designation commonly used in naming SCX adsorbents being applied to structures other than sulfopropyl. Overall, the adsorbents comprise a diverse set that represent several of the synthetic strategies currently used in commercial chromatographic adsorbent manufacture. The set contains four pairs of adsorbents for which the manufacturer supplies both strong and weak cation-exchangers: BioSepra SP and CM Spherodex M, Pharmacia SP and CM Sepharose Fast Flow, EM Industries EMD SO_3^- M and EMD COO^- M, and TosoHaas Toyopearl SP and CM-650 M. Two strong cation-exchangers, Amicon Cellufine Sulfate and TosoHaas Toyopearl SP-550 C, have no corresponding weak counterpart. Cellufine Sulfate is unique in having a very low charge density (8 μmol/ml of adsorbent), at least an order of magnitude lower than typical cation-exchange stationary phases.

2.2. Protein samples and preparation

Lysozyme (chicken egg white), α-chymotrypsinogen A (aCT, bovine pancreas) and cytochrome c (bovine heart) were purchased from Sigma (St. Louis, MO, USA), and were used as received. The protein size and charge properties are given in Table 2. Protein solutions were prepared by dissolving 10 mg protein per ml of 10 m*M* sodium phosphate at a pH of 7, and filtered through Millipore Millex-GV 0.22-μm filters (Bedford, MA, USA).

2.3. Instrumentation

Glass columns of 20 cm×1.0 cm I.D. (AP-1) were purchased from Waters (Milford, MA, USA). The isocratic cation-exchange chromatography of the standard proteins was performed on a BioCad 20 system from PerSeptive Biosystems (Framingham, MA, USA), equipped with a 100-μl sample loop. The BioCad, mobile phases and protein sample were maintained at a temperature of 4–6°C within a Fisher Scientific Isotemp cold box (Pittsburgh, PA, USA).

2.4. Adsorbent preparation and column packing

To prepare each adsorbent, an aliquot was settled and decanted 3 times in 1 *M* NaCl in 10 m*M* sodium phosphate, pH 7, prior to packing. After the third decantation, the slurry volume was adjusted to produce an adsorbent suspension of approximately 60–70%. The suspension was added to the column, and then flow packed at 4 ml/min (300 cm/h). Small amounts of adsorbent were added or removed to produce packed beds of 6±0.6 cm length.

2.5. Isocratic chromatography to determine k′ values

Isocratic retention times were obtained for each protein and adsorbent pair at varying NaCl concentrations in 10 m*M* sodium phosphate buffer, pH 7. The highest NaCl concentration was 1 *M* for lysozyme, 0.5 *M* for α-chymotrypsinogen A and cytochrome c. These concentrations were sufficient to prevent protein retention on the adsorbents tested.

Table 2
Summary of the size and charge properties of the proteins used

	Lysozyme	aCT	Cytochrome c
Molecular mass	14 300	25 700	11 600
Equivalent radius (Å)[a]	15.9	19.4	15.2
Calculated p*I*[b]	9.32	8.5	9.5
Positive charges[c]	17	19	23
Negative charges[c]	9	15	15
Net charge[c]	+8	+4	+8
Calculated dipole moment (Debye)[c]	72	516	325
Ave. charge density (μC/cm^2)[c]	+4.2	+1.4	+4.4

[a] Radius of sphere of equivalent volume.
[b] Calculated p*I* values from the Expert Protein Analysis System (ExPASy) server of the Swiss Institute for Bioinformatics (SIB).
[c] At pH 7.

The lowest concentration for each protein–adsorbent pair was determined empirically by adjusting the NaCl concentration until the resulting k' value approached or exceeded 100. Therefore the range of k' values obtained for each protein on each column spanned about three orders of magnitude.

Isocratic elution at different sodium chloride concentrations was achieved by blending mobile phases A and B using the BioCad system to achieve the desired composition, in randomized order. Ten column volumes of mobile phase was used between successive runs to equilibrate the column prior to injection. Mobile phase A was 10 m*M* sodium phosphate, pH 7. For lysozyme and cytochrome c chromatography, mobile phase B was 1 *M* NaCl in 10 m*M* sodium phosphate, pH 7, and for α-chymotrypsinogen A, it was 0.5 *M* NaCl in 10 m*M* sodium phosphate, pH 7. The volumetric flow-rate was 2 ml/min. Injection volumes were 100 μl. Peak detection was by UV, at a wavelength of 280 nm for lysozyme and cytochrome c chromatography; for α-chymotrypsinogen A 220 nm was used to obtain the necessary sensitivity. Isocratic elution of proteins with high k' values results in broad peaks, and the values of t_R reported here are based on the peak maxima, calculated as the average of duplicate injections. The column void time was determined by using the retention time of the protein at high sodium chloride concentrations (1 or 2 *M*).

3. Results and discussion

3.1. Relative and normalized retention on strong and weak cation-exchange adsorbents

The k' values obtained for each protein on the ten adsorbents were compared at different NaCl concentrations using log–log plots of k' against the NaCl concentration. This form of presentation is most often reported when the isocratic retention of proteins on ion-exchange adsorbents is evaluated and compared within the stoichiometric displacement model (SDM) [23,26,29,33], but its use here is not intended to imply assumption of any specific retention model. The data are shown for lysozyme in Fig. 1, for α-chymotrypsinogen A (aCT) in Fig. 2, and for cytochrome c in Fig. 3. These graphs show

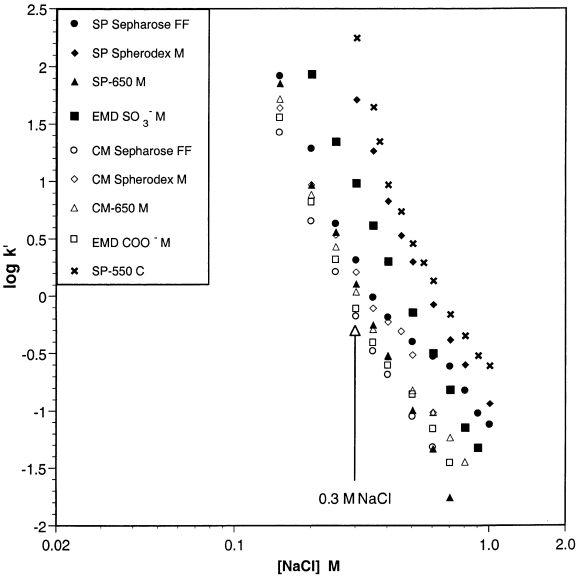

Fig. 1. Log k' vs. log [NaCl] for lysozyme on the strong and weak cation-exchange adsorbents listed. The arrow indicates the NaCl concentration (0.3 *M*) at which k' values are compared for the different adsorbents. Each column was 60 mm×10 mm I.D.; lysozyme was eluted isocratically at a flow-rate of 2 ml/min in 10 m*M* sodium phosphate, pH 7, at varying concentrations of sodium chloride. Detection was by UV at 280 nm.

the decrease in isocratic k' values as salt concentration is increased; for these log–log treatments the result is a series of lines with negative slopes. A common feature of the graphs is that the lines for the different adsorbents are largely parallel, with little crossover, and they therefore have very similar slope values. Some crossover occurs at the higher salt concentrations, where the slopes of the lines may also flatten. At these salt concentrations, electrostatic interactions may be augmented or supplanted by secondary interactions, such as van der Waals or hydrophobic interactions, which are expected to result in curvature of these plots at high salt concentration [30].

The parallel nature of the lines permits a quantitative measure of the retention differences between adsorbents to be expressed through a simple ordering of the k' values obtained for each protein at a given NaCl concentration. The NaCl concentration selected for each protein was the lowest value for which a data point was available for each adsorbent. Therefore, for lysozyme, the comparison was made at 0.3

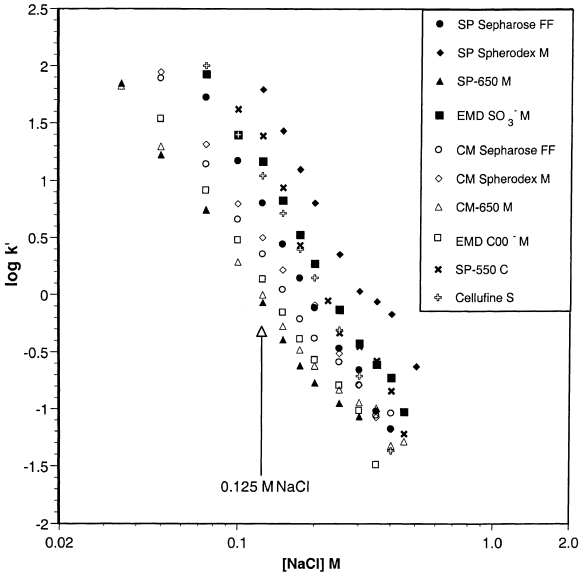

Fig. 2. Log k' vs. log [NaCl] for aCT on the strong and weak cation-exchange adsorbents listed. The arrow indicates the NaCl concentration (0.125 *M*) at which k' values are compared for the different adsorbents. Each column was 60 mm×10 mm I.D.; aCT was eluted isocratically at a flow-rate of 2 ml/min in 10 m*M* sodium phosphate, pH 7, at varying concentrations of sodium chloride. Detection was by UV at 220 nm.

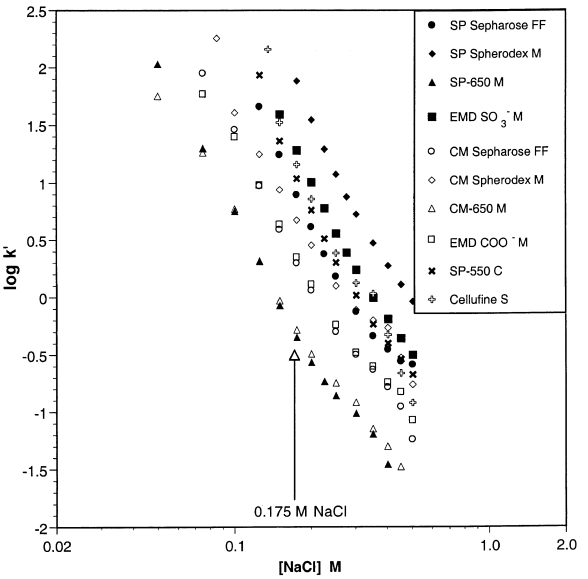

Fig. 3. Log k' vs. log [NaCl] for cytochrome c on the strong and weak cation-exchange adsorbents listed. The arrow indicates the NaCl concentration (0.175 *M*) at which k' values are compared for the different adsorbents. Each column was 60 mm×10 mm I.D.; cytochrome c was eluted isocratically at a flow-rate of 2 ml/min in 10 m*M* sodium phosphate, pH 7, at varying concentrations of sodium chloride. Detection was by UV at 280 nm.

M NaCl, for aCT at 0.125 *M* NaCl, and for cytochrome c at 0.175 *M* NaCl. These comparison concentrations are indicated by arrows in Figs. 1–3, and the k' values for each protein at the common concentrations are given in Table 3. For all three proteins examined, very large differences in retention were found among the different adsorbents. At the comparison concentrations, retention ranged from nearly unretained ($k'<1$) to very strongly retained ($k'>50$). This initial set of retention measurements provides a database from which to begin correlation of differential retention to differences in adsorbent surface and physical properties.

A collective examination of the k' values at the common comparison points given in Table 3 shows that the adsorbent set spans a wide range of retentivity. The strongest retention was exhibited by four adsorbents (SP Spherodex, TosoHaas SP-550 C, Cellufine S and EMD SO_3^- M), the k' values for which are >10 at the comparison concentrations for all three test proteins. The weakest retention was found for five adsorbents (CM Spherodex, CM Sepharose Fast Flow, TosoHaas SP-650 M, TosoHaas CM-650 M and EMD COO^- M) with k' values <5 at the comparison concentrations for all three test proteins. In all cases, these k' values were at least 2-fold and typically 10-fold lower than those on the strongly retentive adsorbents. The retention on the SP Sepharose Fast Flow fell intermediate to these two groupings, with k' values between 1 and 10 for the three proteins.

The overall retention as characterized by k' is generally considered to be determined by two factors, the intrinsic adsorption equilibrium in the linear region of the isotherm, given by the equilibrium constant *K*, and the accessible surface area, given by the phase ratio ϕ. Thus, $k' = K\phi$, and in order to ascertain the contribution of the intrinsic protein–stationary phase equilibrium, it is necessary to normalize the k' values by the respective phase ratios. Phase ratio values [28] for each adsorbent are given in Table 4. These phase ratios were calculated for solutes of viscosity radius $R_\eta = 1.77$ nm and $R_\eta = 2.65$ nm, values that correspond closely to

Table 3
k' values for three proteins demonstrating the ranking of ten strong and weak cation-exchangers; k' values for lysozyme were determined at 0.3 *M* NaCl, aCT at 0.125 *M* NaCl, and cytochrome c at 0.175 *M* NaCl

Cation-exchange adsorbent	k' value		
	Lysozyme	aCT	Cytochrome c
Strong retention			
BioSepra SP Spherodex	52.0	62.7	75.9
TosoHaas SP-550 C	158.5	24.7	11.0
Cellufine Sulfate	–	11.1	14.6
EMD SO_3^- M	9.7	14.8	19.3
Intermediate retention			
Pharmacia SP Sepharose FF	2.1	6.4	7.9
BioSepra CM Spherodex	1.6	3.2	4.8
Weak retention			
Pharmacia CM Sepharose FF	0.7	2.3	2.0
TosoHaas SP-650 M	1.3	0.9	0.5
TosoHaas CM-650 M	1.1	1.0	0.5
EMD COO^- M	0.8	1.4	2.2

those of lysozyme, cytochrome c, and aCT (R_η of 1.85 nm, 1.63 nm, and 2.50 nm, respectively [31]). The resulting normalized retention plots of log *K* vs. log [NaCl] are shown in Fig. 4 for lysozyme, Fig. 5 for aCT, and Fig. 6 for cytochrome c. As with the k' values, comparison of the normalized retention across the adsorbent set at a given NaCl concentration is shown in Table 5.

Retention values normalized for phase ratio differences can be taken as comparative estimates of binding affinity. Some changes in the ordering are observed as a result of the normalization, but this normalization does not greatly reduce the large differences among adsorbents. Although the determination of phases ratios by ISEC requires various assumptions [28], the remaining differences in k' values are too large to be explained by this potential

Table 4
Phase ratios as a function of solute size for ten strong and weak cation-exchangers

Cation-exchange adsorbent	ϕ (m^2/ml)	
	$R_\eta = 1.77$ nm	$R_\eta = 2.65$ nm
BioSepra SP Spherodex	62.5	55.2
BioSepra CM Spherodex	68.4	41.7
EMD SO_3^- M	55.4	37.1
EMD COO^- M	36.7	28.8
Pharmacia SP Sepharose FF	43.6	39.6
Pharmacia CM Sepharose FF	39.2	37.3
TosoHaas SP-650 M	22.5	20.7
TosoHaas CM-650 M	23.9	21.9
TosoHaas SP-550 C	64.3	34.9
Cellufine Sulfate	Not applicable	Not applicable

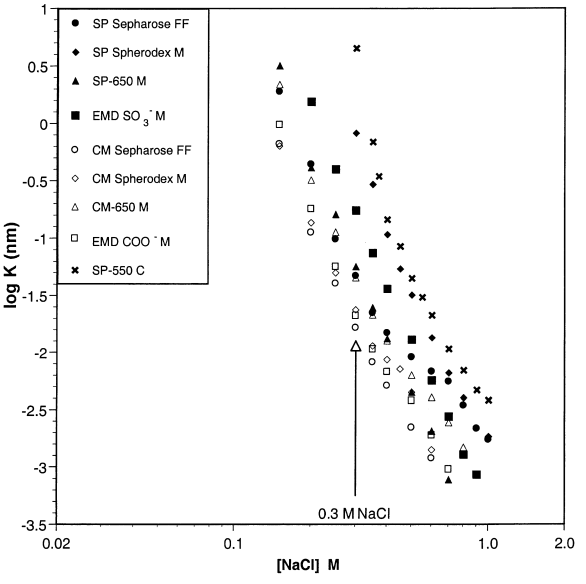

Fig. 4. Log *K* vs. log [NaCl] plots for lysozyme on the strong and weak cation-exchange adsorbents listed. The arrow indicates the NaCl concentration (0.3 *M*) at which *K* values are compared for the different adsorbents. *K* values were calculated by dividing the k' values given in Fig. 1 for lysozyme by the phase ratio for each adsorbent shown in Table 4 for a solute with R_η of 1.77 nm.

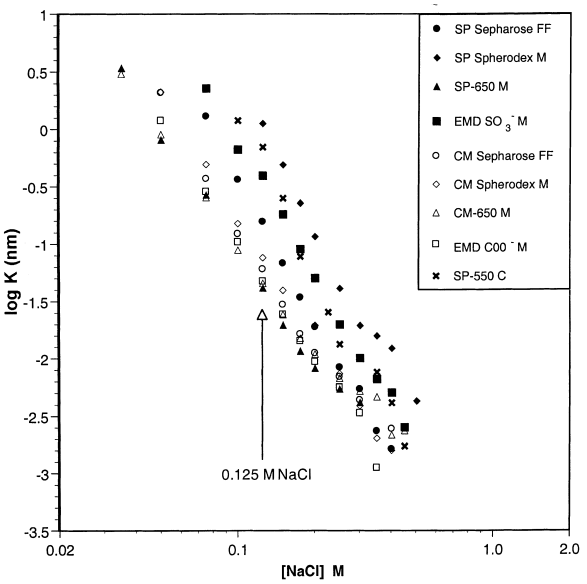

Fig. 5. Log K vs. log [NaCl] plots for aCT on the strong and weak cation-exchange adsorbents listed. The arrow indicates the NaCl concentration (0.125 M) at which K values are compared for the different adsorbents. K values were calculated by dividing the k' values given in Fig. 2 for aCT by the phase ratio for each adsorbent shown in Table 4 for a solute with R_η of 2.65 nm.

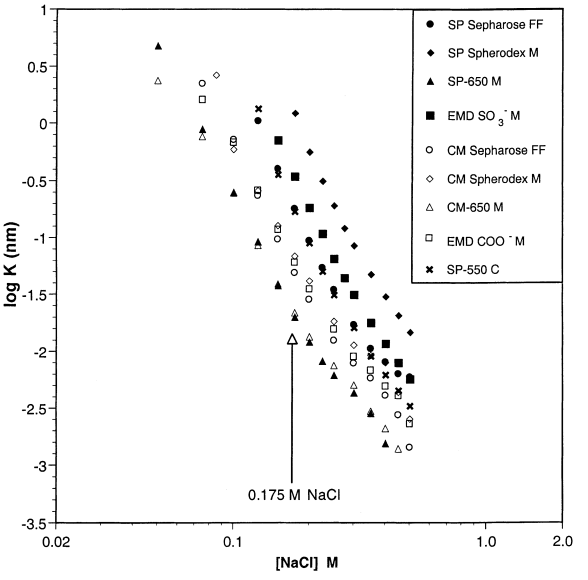

Fig. 6. Log K vs. log [NaCl] plots for cytochrome c on the strong and weak cation-exchange adsorbents listed. The arrow indicates the NaCl concentration (0.175 M) at which K values are compared for the different adsorbents. K values were calculated by dividing the k' values given in Fig. 3 for cytochrome c by the phase ratio for each adsorbent shown in Table 4 for a solute with R_η of 1.77 nm.

source of error. Therefore, although of direct relevance, the 2–3-fold variation in phase ratios can account for only a small portion of the >50-fold range observed in k' values. The remaining retention differences among adsorbents must then be related to differences in stationary phase surface chemistry and morphology.

3.2. Comparison of adsorbent retention

A number of protein or adsorbent properties can be considered as potential contributors to retention differences; these include sequence and structural features of the test proteins, secondary (non-electrostatic) interactions, the adsorbent charge density, the charged ligand type, and the adsorbent pore size distribution. A discussion of these factors follows.

3.2.1. Protein sequence and structure

Key protein properties, such as the number of charged groups, their type, distribution and accessibility, are clearly important for retention in an absolute sense [3,5,15,23], and specifically for the proteins used here, where the retention on all adsorbents follows the trend lysozyme>cytochrome c>aCT. The results for each protein are also consistent in that the curves on the different adsorbents are largely parallel, indicating that the slope is determined predominantly by protein properties; this is captured in the so-called Z parameter of the SDM [23,26,29,32], although we do not invoke that model here.

Explaining these trends quantitatively is not yet possible, but several factors should be considered to move beyond the notion of net charge, the simplistic nature of which is reflected in the consistently stronger retention of lysozyme than that of cytochrome c despite their similar net charge at pH 7. One factor is the charge distribution, which, as discussed earlier, has often been invoked, especially in the suggested role of local charged regions on adsorption [10,15,22–27]. A second one, which has received less attention, is the role of amino acid composition, insight into which can be obtained from the binding of monomers and homopolymers of

Table 5
Normalized retention (K) values for three proteins demonstrating the ranking of ten strong and weak cation-exchangers. The K values for lysozyme were determined at 0.3 M NaCl, aCT at 0.125 M NaCl, and cytochrome c at 0.175 M NaCl

Cation-exchange adsorbent	K value ($\times 10^2$)		
	Lysozyme	aCT	Cytochrome c
Strong retention			
BioSepra SP Spherodex	83.3	113.5	123.6
TosoHaas SP-550 C	454.2	70.8	17.1
Cellufine sulfate	–	–	–
EMD SO_3^- M	17.5	39.9	34.9
Intermediate retention			
Pharmacia SP Sepharose FF	4.7	15.8	18.1
BioSepra CM Spherodex	2.4	7.7	6.9
Weak retention			
Pharmacia CM Sepharose FF	1.7	6.1	5.0
TosoHaas SP-650 M	5.7	4.2	2.0
TosoHaas CM-650 M	4.6	4.6	2.2
EMD COO^- M	2.1	4.8	6.1

arginine and lysine to heparin, a sulfated glycosaminoglycan [37]. Titration calorimetry and equilibrium dialysis demonstrated that arginine and arginine-containing peptides bound more strongly to heparin than the analogous lysine species. Heparin affinity chromatography with poly-Arg and poly-Lys peptides showed that these observed binding differences also resulted in increased retention for the Arg-containing peptides in a NaCl gradient. Possible mechanisms that were proposed to explain these interaction differences were stronger hydrogen bonds and more exothermic electrostatic interactions between sulfate and the guanidino group of arginine than the ammonium group of lysine. Based on a qualitative interpretation of the interactions in terms of hard–soft acid base principles, the soft acid–soft base interactions of the guanidino cation and sulfate anion are expected to be inherently more stable than the hard acid–soft base interaction of the ammonium cation and sulfate anion. These findings highlight the fact that structural and electrochemical differences between arginine and lysine result in different sulfate binding affinities; for the interaction of blocked arginine and lysine with heparin, $K_{d,Lys}/K_{d,Arg}$ values ranged from 2.05 to 2.76. Such differences would contribute to the systematic differences in retention seen among our three test proteins, unlike the traditional view of protein cation-exchange chromatography in which the coulombic interactions of the charged lysine or arginine with the opposite charges of sulfate or carboxylate groups on the adsorbent are equivalent. For example, the stronger arginine–sulfate interaction may mean that the number of arginine residues and the ratio of arginine to lysine may be important determinants of overall retention on SCX adsorbents. Certainly in the study here, the retention of lysozyme, which contains 11 arginines, is much stronger than those of aCT or cytochrome c, which contain 4 and 2, respectively. Further work is needed to address this in more detail.

Despite the evident importance of protein properties in determining absolute extents of retention, they do not appear in this study to be critical determinants of the relative retention across the adsorbent set. For the adsorbents used here, a similar classification of protein retention as strong, intermediate, and weak was observed for all three test proteins, indicating that the observed trends in relative retention are related primarily to the adsorbent properties, and are largely independent of protein structural features. For the proteins examined, retention appears to be independent of the physiological role of a protein as enzyme or proenzyme (lysozyme and aCT, but not cytochrome c) or of substrate type (polysaccharide or protein).

3.2.2. Non-electrostatic interactions

Hydrophobic or other non-electrostatic interactions

with the spacer arm or base matrix have been cited to explain retention differences between adsorbents [32,33], and protein retention on anionic stationary phases was found to increase as the hydrophobicity of the supports was increased [34]. Since ion-exchange adsorbents have been used for hydrophobic interaction chromatography at elevated salt concentrations, non-electrostatic interactions can certainly contribute to overall retention. Of interest here is whether these contributions are particular to the adsorbents displaying the strongest protein retention, and would therefore account for the enhanced retention they exhibit. The chemical diversity of the synthetic and natural polymers used to prepare these four adsorbents (Table 1) would argue against a common additional mode of interaction, particularly of the magnitude necessary to differentiate the retention of this group of four from the other adsorbents. Because three of the four adsorbents have chemically similar analogs (WCX) with much weaker protein retention, any postulated additional hydrophobic interactions would have to result from hydrophobic interactions with the anionic ligands on the SCX versions, and not the base matrix itself.

Comparison of the anionic ligand structures (Table 1) does not show a correlation between ligand hydrophobicity and retention. For example, the six carbon spacer-arm on the SP Sepharose FF would be expected to be more hydrophobic than the sulfopropyl group of the SP-550 C, or the sulfated carbohydrates used for SP Spherodex and Cellufine Sulfate. Yet retention is in inverse order as hydrophobicity, with the SP Spherodex, Cellufine Sulfate and SP-550 C much more retentive than SP Sepharose FF. In addition, the SP-550 C and SP-650 M contain identical matrix chemistry and anionic ligands, yet the SP-550 C is very retentive while the SP-650 M is not. For the adsorbents examined here, hydrophobic interactions may make some contribution to retention, but are not the primary determinant of the large retention differences between them.

3.2.3. Adsorbent charge density

Systematic empirical studies to measure the effect of adsorbent charge density on protein retention are difficult to conduct because the necessary adsorbent sets, varying only in ligand density, are not commercially available. In one such study, Wu and Walters [35] examined the chromatographic retention of lysozyme and cytochrome c on cation-exchange materials with ligand densities over the range of 10–500 μmol/g. Protein capacity and retention (isocratic k' values) increased significantly as a function of ligand density only up to a threshold value at which the average distance between charged ligands on the adsorbent surface was comparable to the protein diameter. Further increases in ligand density had little effect on protein capacity or retention. This is consistent with protein retention concepts discussed above, in which protein interactions with the charged stationary phase surface occur through relatively small localized areas on the protein surface. Introduction of additional adsorbent surface charges will enhance protein retention only if they are able to participate in additional protein–adsorbent interactions.

Kopaciewicz et al. [34] also examined the effect of charge density, by comparing the gradient elution of four proteins on a series of PEI anion-exchange materials. They found that retention times increased over a threefold range of charge density (250–750 μmol/g). Although these charge densities are quite high and therefore the findings appear contrary to the work of Wu and Walters, the authors noted that up to 50% of the nitrogen groups are inaccessible and only a small fraction of the accessible amines may be ionized. The usable charge densities of these adsorbents are thus difficult to ascertain, making it difficult to reach definitive conclusions.

The data shown here, while not systematically addressing charge density as a retention variable, are in agreement with the conclusions of Wu and Walters: above a threshold amount, increased charge density and ionic capacity do not necessarily result in increased protein retention. The ionic capacities of the adsorbents tested here span at least two orders of magnitude in charge capacity and about an order of magnitude in surface charge density (Table 1), with the lowest surface charge density shown corresponding to a ligand spacing of about 16 Å, about half the effective diameter of the proteins used. No strong correlation between ionic capacity and retention is obvious from the data. Examples of weak retention with average to high ionic capacity are evident, as well as the opposite case, strong retention

with low to average ionic capacities. For instance, SP Spherodex showed the strongest SCX retention, although its ionic capacity (131 μmol/ml) is at the low end of the range for Toyopearl SP-650 M (120–170 μmol/ml) and less than that of SP Sepharose Fast Flow (180–250 μmol/ml). Similarly, the ionic capacity of Cellufine Sulfate is given as 8 μmol/ml, placing it at the low end of the cation-exchange adsorbents examined, by at least an order of magnitude. Despite this low charge density, retention of both aCT and cytochrome c was very strong on Cellufine Sulfate, exceeded only by those on EMD SO_3^- M and SP Spherodex. In terms of surface charge density, a good example of the poor correlation with retention is that of the two Toyopearl SCX materials, where the much more retentive SP 550C has a significantly lower surface charge density than SP 650 M.

The relative unimportance of high charge density seen here suggests that the number of Coulombic interactions can be maximized with just a subset of the charged ligands available on typical chromatographic adsorbents. These adsorbents all appear to provide ligand densities above the threshold value described by Wu and Walters at which ligand spacing is comparable to protein size. The spatial and geometric positions of the charged ligands relative to the protein surface thus take on added significance. Maximizing the number of Coulombic interactions, and hence retention, would involve mutually favorable positioning and geometries so that as many as possible of the charged patches on the protein surface can interact simultaneously with adsorbent groups of opposite charge. This may be achieved with lower charge density if the adsorbent ligands are oriented and positioned optimally, whereas poorer positioning would require increasing the charge density. Determining the relative roles of charge density and ligand positioning in a given stationary phase is extremely difficult, but it is clearly related to the topography of the base matrix and to the pore size distribution (Section 3.2.5). An additional corollary is that our interpretation of the role of ligand density is inimical to most colloidal electrostatic models, which predict increased retention by a non-specific increase in the adsorbent field strength, which results from increased charged ligand density.

3.2.4. Anion type

The CM adsorbents were among the least retentive of the adsorbents tested here: as shown in Table 3 and Figs. 1–3, comparison of WCX and SCX pairs shows that protein k' values for three of the four CM adsorbents were lower than for the corresponding SCX versions. A similar comparative study of the retention of cytochrome c on Baker WCX and SCX adsorbents also showed stronger retention on the SCX version, particularly when step gradients were used [36]. For the exception observed here, TosoHaas SP and CM-650 M, where protein retention on the WCX and SCX pair were similar, the retention on the SCX version was much weaker than those on the other SCX adsorbents. From these experimental data, the reduced retention on the weak cation-exchangers would appear to make these adsorbents advantageous for analytical or preparative separations where either protein stability or solubility is reduced in high salt, making operation in low salt mobile phases preferable.

The differences in normalized retention seen between three of the four SCX and WCX pairs point toward intrinsically stronger interactions between the proteins and the sulfate anions than between the proteins and the carboxylate anions. Differences between SCX and WCX are usually considered to lie in the pH range of operation, with strong and weak referring to acid or base strength rather than to binding strength. At the pH of 7 used in this study, both the carboxylate and sulfate anions on the adsorbent should be fully deprotonated and carry a full $-1e$ charge, so protonation of the stationary phase is not a satisfactory explanation for the retention differences.

The differences in retention on sulfate and carboxylate ligands are analogous to the specific differences discussed earlier ([37]; Section 3.2.1) in ion-pairing preferences and interaction strength between arginine and lysine residues on sulfated ligands. Again the contrast is with the conventional view of ion exchange as being determined simply by the net charges of the participating groups. Fromm et al. [37] did not investigate binding to carboxylate ligands, but the retention differences on SCX and WCX can be understood qualitatively in terms of a second and much more general examination of ion pairing preferences [38–41] in which differences in

the hydration of ions in aqueous solution were used to correlate ion charge density with the solubility of ion-pairs and with ion-pairing preferences. Small monovalent ions of high charge density (kosmotropes) bind water molecules strongly relative to the bulk water, thereby structuring and immobilizing the surrounding water. In contrast, large monovalent ions of low charge density (chaotropes) bind water molecules weakly, leaving this loosely bound water more mobile than the bulk water. Both ion-pairing preferences and interaction strength arise from these influences on water structure. The pairing of two oppositely charged kosmotropes or two oppositely charged chaotropes in aqueous solution is favorable, with a stronger interaction for the kosmotrope pair. The removal of strongly held water from the small kosmotrope ions is energetically unfavorable, but the association of oppositely charged kosmotropes is driven by the more effective charge neutralization of the resulting kosmotrope–kosmotrope neutral salt. The large chaotrope–chaotrope pair formation is driven by the release of the weakly held water molecules, which are then available to form stronger water–water interactions. Mixed chaotrope–kosmotrope ion pair formation is unfavorable since the charge interaction and subsequent release of weakly bound water from the chaotrope does not compensate for the work required to strip water from the small kosmotrope ion.

Applying these chaotrope/kosmotrope concepts to chromatographic retention requires the various protein and adsorbent ionic groups to be classified appropriately. The carboxylate head groups on the cation-exchange chromatographic ligands are kosmotropes, while the arginine guanidinium and lysine ε-amino groups are chaotropes [41]. Stronger interactions between arginine or lysine and the adsorbent alkyl sulfate groups, as compared to the alkyl carboxylate groups, would be expected if the alkyl sulfate is less kosmotropic than the alkyl carboxylate group, or is a chaotrope. Several additional pieces of evidence related to hydration effects support the assertion that this is indeed the case:

1. the difference in Jones Dole viscosity B coefficients between sulfate and acetate predicts weaker ion–water interactions for sulfate [41];
2. vibrational spectroscopy of sodium methyl sulfate finds that water is weakly bound, suggesting methyl sulfate is a chaotrope [42];
3. combined quantum-continuum calculations show that the hydration free energy of acetate is more negative than that of methyl sulfate [43].

Thus the basis for the increased chromatographic retention on SCX than on WCX includes not just the electrostatic effects, but also the role of the surrounding water molecules. Continuum electrostatic methods typically used to calculate the free energy of binding processes do not predict different interaction energies [43] in the absence of a detailed treatment of solvation effects.

3.2.5. Adsorbent pore size distribution and protein retention

Differences in the morphology and microscopic surface properties of the adsorbent set examined here may present very different possibilities for the spatial orientation of the protein solutes relative to the adsorbent surface. The adsorbent surfaces include an extended layer of polyelectrolyte tentacles for the EMD materials [44], a network of cross-linked agarose helices for the Sepharose surface [45], and derivatized dextran chains within and spanning the pore interior for the Spherodexes [46]. The wide-pore TosoHaas 650 M materials may be the closest representation of a ''flat'' surface, because the dimensions of the pores are very large in relation to the size of the proteins used here.

These morphological features could affect chromatographic retention by permitting differences in the number and/or strength of the electrostatic interactions between protein and adsorbent. Because $K \sim e^{-\Delta G/RT}$, a small increase in the interaction energy between the protein and adsorbent could engender a large change in the k' value. The pore size distributions [28] for the strong cation-exchange adsorbents tested here are shown in Fig. 7. A correlation between the adsorbent pore size distribution (PSD) and protein retention can be observed in this data set. A clear example is seen in the comparison of protein retention on the TosoHaas SP-550 C and the SP-650 M adsorbents, where the k' values on SP-550 C are 20–40 times greater than those obtained with the SP-650 M, despite equivalent chemical composition and charge density. Although the mean pore diameter of the SP-650 M is nearly an order of magnitude greater than that of the SP-550 C, surface area differences (Table 4) do not account for the retention difference. However, the full PSD for

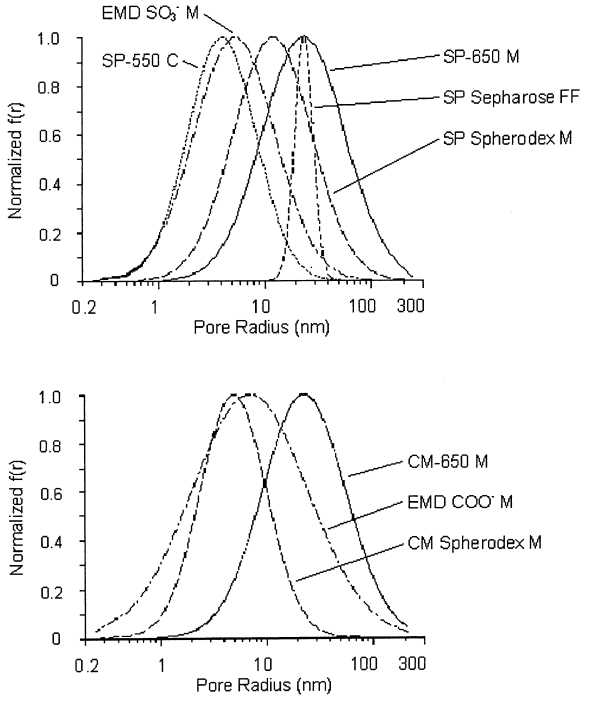

Fig. 7. Pore size distributions for the strong and weak cation-exchange adsorbents listed.

these adsorbents shows that the SP-550 C material contains significant pore volume contained in pores with dimensions similar to those of the protein solutes (3–5 nm), whereas the majority of the pore volume of the SP-650 M is contained in very large pores, with little volume in pores with dimensions approximate to those of the protein solutes. As the PSD represents the only significant difference between these chemically identical adsorbents, the large population of smaller pores in the SP-550 C adsorbent could be envisioned to play a role in the enhancement of protein retention. From this perspective, a protein molecule in such a narrow pore would place more of its exterior surface in close and continuous proximity to the charged ligands on the adsorbent. This ''surrounding'' of the protein by charged adsorbent groups may increase the probability that protein–adsorbent interactions are maximized, in both the number and the interaction strength. This effect would be lacking in the SP-650 M, the very wide pores of which would not permit simultaneous surrounding interactions. This hypothesis is supported by observations of preferential adsorption of lysozyme in scratches on polished silica [47].

Although differing in the underlying chemical structure and physical morphology, the polyelectrolyte networks within the EMD and Spherodex adsorbents provide the functional equivalents of the small pores found in the SP-550 C material. This can be seen in Fig. 7, confirming that the effective PSDs of these adsorbents show significant small-pore volume. The polymerization reactions used to prepare these adsorbents result in significant occlusion of the pore space [28], forming a network of charged polymers within the pore. Protein solutes of the sizes used here in these pores would have a high probability of encountering pore space in which they would be enveloped or surrounded by adsorbent charge groups, the mobility of the tentacles or dextran strands helping to contribute to this ''engulfment''.

The differences in PSD and the corresponding ability to provide stronger or more extensive adsorbent–protein interactions also provide a reasonable explanation of the retention differences seen with SP Sepharose Fast Flow. This adsorbent has a narrow PSD, does not contain the population of small pores seen in the EMD, Spherodex or SP-550 C adsorbents, and does not exhibit the enhanced retention. Another interesting case is the highly retentive Cellufine Sulfate; it is considered by the manufacturer to be non-porous, but is marginally permeable to dextran standards (K_d values ~0.05 for dextrans of molecular mass 20 000–40 000, data not shown) and so may permit some entry of small proteins, or the surface topography may provide invaginations and cavities to serve as effective ''small-pore'' space.

3.2.6. ''Tentacle'' cation-exchange adsorbents

The retention data for the TosoHaas SP-650 M, CM-650 M and the EMD SO_3^- M and COO^- M can be compared to examine the effect of the polymeric ''tentacle'' chemistry on protein retention. All four adsorbents are derivatized from a common base particle, the TosoHaas HW 65 SEC material, the major difference being the presence of the linear polyelectrolyte ''tentacles'', and the resulting high ionic capacity of the EMD adsorbents. Log k' plots for lysozyme, aCT, and cytochrome c on these four adsorbents are shown in Figs. 1–3. The isocratic k'

values obtained at the different reference NaCl concentrations are listed in Table 3.

Retention of the test proteins was similar on TosoHaas SP-650 M, CM-650 M and EMD COO^- M, all having k' values 10 to 40 times smaller than on EMD SO_3^- M. The two EMD tentacle adsorbents, although ostensibly similar in physical structure, with multiple ionic groups on polymer chains extending outward from the adsorbent surface to produce high charge density, exhibit distinctly different retention properties. Some component of this retention difference is related to the anion type, as discussed earlier; however, the tentacle lengths and physical structure could also be envisioned to contribute to retention differences.

Several previous studies of tentacle adsorbents have examined protein capacity, adsorption equilibrium, and the kinetics of protein adsorption [34,48–52]. Emerging as a consensus from these studies is a description of these tentacles as highly flexible polyelectrolyte chains, which can adopt conformations that facilitate the adsorption of proteins. Protein adsorption is depicted as ''multilayer dissolution'' in which the proteins are engulfed within and between the tentacles. This adsorption between tentacles is postulated to allow additional charged areas of the protein surface to interact with the stationary phase, with the stacking of proteins in multiple layers within the tentacles resulting in increased capacity. In fact, this adsorption between tentacles has been inferred by comparing measured protein capacities to theoretical calculated maximum capacities assuming monolayer protein coverage, with multilayer adsorption invoked when measured static capacities exceed the calculated values [48].

The additional contact area between opposite charges on the protein and stationary phase that this engulfment would engender should, in theory, lead to stronger retention than on conventional adsorbents in addition to the increased capacity. Such behavior should also be observed with tentacle adsorbents of different functionality (e.g. anion exchange). However, comparative studies reported in the literature for tentacle and macroparticulate anion-exchange adsorbents do not show significant retention increases with the tentacle morphology [34,48], and in most cases reported show decreased retention.

Similarly, our results show substantial increases in retention only for EMD SO_3^- M and ambiguous results for EMD COO^- M. Our physical characterization of the EMD cation-exchange adsorbents [28] revealed significant differences in the pore structures of the EMD SO_3^- M and EMD COO^- M adsorbents, which is exemplified by the PSDs shown in Fig. 7. Polymerization of the EMD COO^- M had effects consistent with the supplier's representation of the formation of ~10 nm tentacles within a very wide pore (>100 nm) base material. The tentacle polymerization was found to be much more extensive for the EMD SO_3^- M adsorbent, as reflected by a large decrease in the mean pore radius as well as a shift in the PSD toward smaller pore dimensions. This profound reduction in the dimensions of all the pores in the distribution suggests the polymerization of tentacles much longer than 10 nm.

The difference in tentacle lengths between the EMD COO^- M and the EMD SO_3^- M adsorbents provides an alternative explanation for the large retention differences: for the EMD SO_3^- M adsorbent, the much longer tentacles allow for adsorption within and between tentacles as envisioned by the supplier. This manner of adsorption would be facilitated in smaller ''pores'', representing both regions where the tentacles extending outward from the pore walls would nearly or completely span the entire pore, as well as the space between adjacent tentacles. Increasing the tentacle length would result in a larger effective pore volume of this kind. Proteins in these pores would be surrounded by the charged polyelectrolyte tentacles, and the additional electrostatic interaction imparted would increase protein retention.

The significant difference in tentacle lengths observed between EMD SO_3^- M and EMD COO^- M adsorbents may explain the absence of a clear trend in literature reports describing the effect of tentacles on protein retention. The relative dimensions of the tentacles, the resulting PSD, and the protein solute size would attenuate the magnitude of this effect; the shorter tentacles of the EMD COO^- M within a very wide pore base material provide less of this additional electrostatic interaction, with a consequent minor effect on retention. Thus polymerization in a large pore base material would not lead to increased protein retention unless the polymerization reactions produce a significant increase in pore volume of

dimensions similar to that of the protein solute. These potential physical changes in adsorbent PSDs have not received explicit treatment in previous reports on this class of adsorbent.

4. Conclusions

Significant retention differences were observed among the cation-exchange adsorbents. Retention and/or selectivity differences of this magnitude provide multiple potential routes to fine tune these parameters for any given separation, yet complicate the adsorbent selection process by necessitating empirical screens.

Explaining the causes of this variation across the adsorbents from multiple suppliers is challenging, as there are a large number of effects that can contribute to the overall retention quantified by the k' value. Differences in phase ratios among the adsorbents used here can account for ~2–5 fold differences in isocratic retention, leaving substantial retention differences to be ascribed to stationary phase chemistry or morphology. Two factors, the anion type and the adsorbent PSD, appear to be primary determinants of protein retention.

For the SCX adsorbents, those with PSDs centered around dimensions similar to those of the protein solutes display strong protein retention: Amicon Cellufine Sulfate, EMD SO_3^- M, SP Spherodex and TosoHaas SP-550 C. The PSDs that favor strong retention for the small protein solutes used here can be obtained from wide pore base materials by the polymerization of sulfate-containing tentacles or by grafting sulfated dextrans within the pore space. Surrounding the protein solute with sulfate anions maximizes the formation of charge–charge and hydrogen bonding interactions between protein and stationary phase. Placing sulfate anions on short spacer arms in a wide-pore base material, like TosoHaas SP-650 M, appears not to foster such multiple interactions, which may require contact with a larger amount of the protein surface. The adsorbent PSD may therefore have implications for separation selectivity, particularly if the size of the proteins to be separated differs substantially. This observation may be useful to exploit in adsorbent design, which may even be feasible on a customized basis.

For weak cation-exchangers, the effect of PSD on protein retention is discernible, but much weaker. This is exemplified by the CM Spherodex, the morphology of which should favor enhanced interactions, analogous to the SCX version. While protein retention is stronger on CM Spherodex than on the other WCX materials tested, it is much weaker than what can be obtained on SCX materials. Carboxylate anions appear to have intrinsically weaker interactions with the protein solutes than sulfate anions, so that the incremental increase in retention as multiple interactions are made is much smaller.

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