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Mechanistic model of retention in protein ion-exchange chromatography

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Abstract

A mechanistic model is developed to describe the retention of proteins in ion-exchange chromatography, as a simplified version of a more elaborate colloidal model within which retention is related to protein and stationary-phase structural and functional parameters and eluent composition. The protein parameters are the size and net charge, while incorporation of stationary-phase properties, namely the surface charge density and a short-range interaction energy, allows a more mechanistic interpretation of stoichiometric displacement model (SDM) parameters as well as prediction of retention on different stationary-phase materials. Experimental exploration of the model capabilities was performed on two different PEI-based carboxylic acid cation exchangers. Isocratic experiments using lysozyme were used to estimate the stationary-phase parameters for each material. Predictions of isocratic experiments on chymotrypsinogen A correctly captured the Z slope of the data, along with reasonable absolute retention times. In addition, the correct trends and reasonable quantitative results were predicted for gradient elution of a set of small globular proteins. The mechanistic basis for the model, particularly the explicit inclusion of stationary-phase properties, makes it a powerful tool to use in the selection of materials and optimization of operating conditions.

Keywords: Retention models; Stationary phases, LC; Retention prediction; Proteins

1. Introduction

While chromatography has been used successfully for the separation and purification of proteins in many analytical, preparative and process applications, a mechanistic understanding of the factors governing protein adsorption is lacking. This presents an obstacle to the development of chromatographic separation processes on any other than an empirical basis [1], whereas a quantitative understanding of protein adsorption would provide the basis for the rational design of chromatographic separations. This would allow streamlining of the development process for large-scale applications, currently estimated to average more than $200 million per pharmaceutical product [2].

For the case of ion-exchange chromatography, the dominant mechanism, i.e. charge interactions, is well-established, as are the general ideas that a

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protein should be retained on a sorbent of opposite charge sign and that more strongly charged proteins (where the sign is opposite to that of the sorbent) should be retained more strongly. However, this physical picture fails to explain the retention of proteins on sorbents of like charge [3] or to predict quantitatively the differential retention of two solute proteins, since net charge appears not to be the sole determinant of retention [4,5]. Our aim is to provide quantitative characterization and prediction of protein ion-exchange retention as a function of the physicochemical properties of protein and sorbent. Such a framework would allow not just determination of optimal separation conditions for a given mixture of proteins, but also establishment of quantitative criteria for distinguishing the properties of different sorbents.

The earliest model for protein adsorption onto ion-exchange materials is the stoichiometric displacement model (SDM), first proposed about forty years ago [6], in which adsorption occurs when a protein molecule displaces one or more ions on the sorbent in a strict ion exchange. The basic idea has been revised to include the concentration of ions in solution and the concept of distinct binding sites on the protein [4,7] but has remained quite popular for the correlation of ion-exchange data. The essential result of this model is a linear dependence of the logarithm of capacity factor on the logarithm of ionic strength, with the (negative) slope given by the net charge or number of binding sites on the protein, depending on interpretation. In the literal interpretation of the SDM, the slope parameter does not depend upon sorbent properties and so is not useful for scale-up or even the prediction of elution order.

Several recent models have applied colloidal ideas of particle–particle interactions to protein adsorption. Such an early application was the proposal of a type of chromatography based on adsorption kinetics resulting from the interplay among colloidal van der Waals, electrostatic, and short-range repulsion forces [8]. This "potential barrier chromatography" has not grown into an established mode of chromatography, but the idea of an interplay between electrostatic and van der Waals interactions in protein chromatography persists. For example, a recently developed model has utilized colloidal interactions as a basis for retention in ion-exchange chromatography [9,10]. This model is based on calculating the combined electrostatic and van der Waals contributions to the protein–surface interaction energy, albeit using the simplified geometry of two flat plates, for which analytic solutions exist to the governing continuum equations. Despite the geometric simplicity, this model successfully correlates ion-exchange data both at low ionic strengths, where the retention is dominated by electrostatic attraction, and at higher ionic strengths. The logarithm of the capacity factor is predicted by this model to be proportional at low ionic strengths to the inverse square root of ionic strength [9], as opposed to the inverse first power of ionic strength predicted by the stoichiometric displacement model. An interesting point is that the same experimental data that were correlated on a log–log plot of capacity factor versus ionic strength [7] can also be correlated on a log–square root plot [9,10], which indicates the ambiguities inherent in multiparameter correlations using logarithmic plots.

Predicting changes in retention with environmental conditions or with changes in sorbents requires a model with parameters that can be estimated independently from the experimental data set that it describes. Detailed incorporation of both protein and surface characteristics into models of protein–surface interactions has been accomplished in limited applications with recent advances in computational capabilities. For the interaction of proteins and charged surfaces, the means to describing adsorption has been through calculation of the electrostatic interaction energy between a protein molecule and a planar charged surface [11]. It has been found that a particular protein can have both favorable and unfavorable electrostatic interactions with a charged surface, depending on orientation [11–14]. Such results can explain retention of proteins on surfaces of like net charge [3], but for less eclectic situations, simplified models of the protein, represented as a sphere with its lower-order moments, can be useful in describing the electrostatic interaction approximately [15].

In seeking to establish the validity of such modeling, we have recently concluded an experimental adsorption study of the proteins lysozyme and chymotrypsinogen A at charged quartz interfaces [16]. It was found that colloidal (electrostatic and van der Waals) modeling using mechanistic, structur-

al parameters (along with one fitted model parameter that is independent of the protein type) was able to describe and predict adsorption equilibrium constants over a wide range of ionic strengths with a reasonable degree of success. Surprisingly, the incorporation of complete structure and charge distribution into the model did not improve significantly the agreement between theory and experiment, relative to representations involving only a sphere with its net charge.

The success of the sphere–plate model suggests that a fairly simple construct can be developed for the rapid characterization of ion-exchange chromatographic data while retaining physically meaningful parameters. Here, we present a simplified model for ion-exchange retention that is based primarily on electrostatic attraction but that also includes non-electrostatic interactions, which should be important principally at short range. The key parameters are the size and charge of the protein, as well as the effective charge of the sorbent and an energy parameter characterizing the short-range interactions of proteins with the sorbent. The sorbent charge density and short-range energy are determined experimentally using a well-characterized protein and isocratic measurements over a range of ionic strengths. Parameters determined in this manner are then applied to the prediction of both isocratic and gradient retention times of other proteins based on their structural properties. We also present an example of how such a methodology might be used in the selection of sorbents and operating conditions to optimize a desired separation.

2. Theory

The model to be developed is intended to describe and predict the capacity factor k', defined by

$$k' = \frac{t_R - t_o}{t_o} = K_{eq}\Phi \tag{1}$$

where t_R is the retention time of the solute of interest, t_o is the time for an unretained solute to pass through the column, K_{eq} is the adsorption equilibrium constant, given by the surface concentration divided by the bulk concentration, and Φ is the phase ratio of the column, defined as the surface area available for adsorption per unit mobile-phase volume. The second part of Eq. 1 is valid only for linear chromatography.

The model development here focuses on the equilibrium constant K_{eq}, which has been the subject of our previous studies [12,16]. The simplified model described here engenders several assumptions and relaxes a certain degree of rigor; its utility can be assessed in its application to the description of experimental data. The primary assumption made is that the adsorption equilibrium constant can be expressed as the product of equilibrium constants for electrostatic and short-range contributions, i.e.,

$$K_{eq} = K_{es}K_{sr} \tag{2}$$

This form is suggested by parametric plots of dependence on surface charge density and Hamaker constant (for short-range van der Waals interactions) in more detailed colloidal models [12]. Since the capacity factor can be expressed as the product of the adsorption equilibrium constant and the phase ratio, the logarithm of the capacity factor can be expressed, by combining Eqs. 1 and 2, as:

$$\log k' = \log K_{es} + \log K_{sr} + \log \Phi \tag{3}$$

In order to describe the electrostatic equilibrium constant in a tractable manner that nevertheless retains mechanistic properties, we employ a functional form suggested by the linear superposition approximation (LSA), which provides a solution to the linearized Poisson–Boltzmann equation for two bodies that is exactly correct when they are far apart and is often a reasonable estimate when they are closer together [17,18]. The electrostatic free energy within the approximation used here is given by:

$$\frac{\Delta F}{kT} = \frac{E_o}{kT} e^{-\kappa z} \tag{4}$$

where z is the separation distance and the characteristic electrostatic energy E_o is:

$$E_o = \frac{\sigma Q}{\epsilon\kappa(1 + \kappa R)} \tag{5}$$

Here σ is the surface charge density of the adsorbent, Q is the net charge of the protein, R is the characteristic radius of the protein, ϵ is the solvent dielectric permittivity, and κ is the Debye parameter defined for a z:z electrolyte by:

$$\kappa^2 = \frac{2e^2z^2n_0}{\epsilon kT}$$

where e is the electronic charge, z the ion valency, n_0 the bulk concentration of electrolyte before dissociation, k the Boltzmann constant, and T the absolute temperature. Dependence on the key experimental variable, namely the ionic strength I, enters via n_0, since $I = z^2n_0$ for a symmetrical electrolyte. Using the LSA, the electrostatic equilibrium constant can be computed using the Gibbs excess notion of adsorption [12] to obtain:

$$K_{es} = \kappa^{-1}\left\{ -\frac{E_o}{kT} + \frac{(-E_o/kT)^2}{2\cdot 2!} + \frac{(-E_o/kT)^3}{3\cdot 3!} + \cdots \right\} \quad (6)$$

This sum converges to seven significant figures typically within twenty to thirty terms for E_o between -5 and $-10kT$.

The short-range equilibrium constant encompasses all non-electrostatic mechanisms for adsorption, including the van der Waals interactions and attractive and repulsive solvation and steric effects, all of which are expected to operate primarily at short range for molecular protein–surface interactions. Since the dimensions (of length) of the equilibrium constant have been incorporated into K_{es}, the short-range contribution must be dimensionless and is characterized by the free energy difference w, denoting the depth of an energy well at short range.

The dependence of the short-range interaction on physical parameters can be approximated by expressing the free energy change in a form suggested by well-defined contributions to the interaction. This purpose is served by the van der Waals interaction of a sphere and a plate at close range, for which the interaction energy is approximately:

$$\Delta F_{vdw} \approx -\frac{AR}{6z} \quad (7)$$

where A is the Hamaker constant, typically 1.5 to $2kT$ for the interaction of proteins with other materials in water [19]. Therefore, we allow the well depth to reflect the scaling with respect to size, i.e.,

$$w = w_oR \quad (8)$$

and if the characteristic energy can be determined experimentally for one protein of known size, then that for other proteins can be estimated via Eq. 8. Through this characteristic energy, the model can reflect the effect of different sorbent properties in terms of their Hamaker constants. Different scalings could be used alternatively to account for solvation or other interactions, if the size dependence of the interaction energy were known, but the linear dependence given in Eq. 8 is used in the absence of such information; the qualitative trend is certainly appropriate for contributions such as hydrophobic interactions. The short-range equilibrium constant is obtained simply as:

$$K_{sr} = e^{-w/kT} = e^{-w_oR/kT} \quad (9)$$

This framework has two important attributes. First, it provides a good approximation to the more accurate numerical solution for the sphere–plate system [15], yet retains the main physical features and parameters. Second, it allows for a physical interpretation of the stoichiometric displacement model (SDM), commonly used for correlation of ion-exchange data by an equation of the form:

$$\log k' = \log K - Z \log I \quad (10)$$

where Z is the quantity normally associated with the protein net charge or number of binding sites, K is a constant, and I is the ionic strength [4,6]. At the ionic strengths in which we are primarily interested, the short-range interactions should not be a function of salt concentration, so comparing Eq. 10 to Eq. 3 gives:

$$\frac{d[\log k']}{d[\log I]} = \frac{d[\log K_{es}]}{d[\log I]} = -Z \quad (11)$$

The quantity K in Eq. 10 can then be identified as the product of the phase ratio Φ and the short-range equilibrium constant K_{sr}.

Use of this model requires experiments on a new sorbent to be performed with a well-characterized protein for the purpose of estimating parameters. From isocratic experiments spanning a small range of ionic strengths, the slope Z is obtained from Eq. 11. Using the size and net charge of the well-characterized protein, the effective sorbent charge density is obtained implicitly from Eqs. 5 and 6. An initial guess, coupled with a Newton–Raphson iteration, provided rapid estimation of the surface charge density for all cases studied here. Using an estimate of the phase ratio, the values of the electrostatic equilibrium constant (obtained directly from Eq. 6 once σ has been estimated), and the retention data, the short-range contribution can then be computed from Eq. 3.

Predictions then can be made regarding the retention of the same protein under slightly different salt conditions or a different protein under similar conditions. The short-range energy w is calculated from the short-range equilibrium constant via Eq. 9, which provides the means to calculate the short-range contribution for another solute, i.e., by scaling with respect to size. The electrostatic equilibrium constant is calculated with Eqs. 5 and 6, using the charge density estimated with the test solute.

Given the quantities K (which, in this model, amounts to the product $K_{sr}\Phi$) and Z in Eq. 10, the retention time t_R of a protein in a salt gradient can be estimated as [20]:

$$t_R - t_o = \left\{ \left[I_i^{Z+1} + \frac{t_o K(Z+1)(I_f - I_i)}{t_G} \right]^{\left(\frac{1}{Z+1}\right)} - I_i \right\} \frac{t_G}{(I_f - I_i)} \quad (12)$$

where t_G is the total gradient time, I_i is the initial ionic strength (i.e., at the beginning of the gradient), I_f is the final ionic strength, and t_o is the dead time for an unretained solute to elute. While formally valid only where the linear relationship (Eq. 10) holds over the entire salt concentration range of the gradient, this expression can be applied in general by using the values of parameters K and Z near the point of elution. At salt strengths much less than that at which the protein elutes, the adsorption equilibrium constant and consequently k' will be large and no significant degree of elution will occur. Since the purpose is to make a priori predictions, the point of elution is initially unknown; consequently, an initial guess is made and iteration is performed until the calculated elution time is consistent with the ionic strength.

3. Materials and methods

All of the stationary-phase syntheses and the gradient chromatographic experiments described below were performed at the Johannes Gutenberg-Universität (Mainz, Germany), whereas the remainder of the chromatographic experiments were performed at the University of Delaware. Except where otherwise noted, the materials and equipment described herein were those utilized at the former location.

The chromatographic supports were prepared from a sample of MinUSil 30 μm crystalline quartz that was kindly donated by Pennsylvania Glass Sand Company. This non-porous material was used to eliminate any pore-size effects on retention. Toluene and methanol were obtained from E. Merck (Darmstadt, Germany). The other chemicals used in the stationary-phase synthesis, including γ-glycidoxypropyltrimethoxysilane (GLYMO), diisopropylethyleneamine (DIPEA), dicumyl peroxide, and butanedioldiglycidylether (BUDGE), were obtained from Aldrich Chemie (Steinheim, Germany). The polyethyleneimine polymers PEI2 and PEI6 (M_r ca. 230 and 600, respectively) were a gift of Dr. Fred Regnier, Purdue University. Water used was either HPLC-grade from J.T. Baker (Phillipsburg, NJ, USA) or deionized in-house (Millipore, Eschborn, Germany). Salts, including sodium phosphate monobasic, sodium phosphate dibasic, and sodium chloride, were also obtained from J.T. Baker. Proteins were obtained from Sigma (St. Louis, MO, USA) and used without further purification.

For the chromatographic experiments at the University of Delaware, some materials were obtained from different sources. Sodium chloride was obtained from Aldrich (Milwaukee, WI, USA). Sodium phosphate monobasic and dibasic were obtained from Fisher Scientific (Fair Lawn, NJ, USA). House-

distilled water was deionized with a Milli-Q Plus cartridge system (Millipore, Medford, MA, USA).

Stationary phases embodying weak cation-exchange functionalities were produced in this work and used for chromatographic experiments. The product was a carboxylic acid exchanger resulting from the reaction of a cyclic anhydride with a crosslinked polyethyleneimine (PEI) layer [21]. Typically, a sample of 15 g of MinUSil was added to 100 ml of a 50:50 (v/v) mixture of distilled and deionized (DI) water–concentrated (37%) hydrochloric acid. The resulting dispersion was stirred at room temperature for 2 h in order to maximize the conversion of siloxane to silanol groups. Subsequently, the particles were washed in a Teflon membrane cartridge with DI water until the pH of the effluent was equal to the pH of the water, after which they were washed with methanol and dried under vacuum at approximately 60°C to remove physisorbed water and prevent condensation of the subsequent organosilane modifier.

The silanization was performed in accordance with previous protocols [22]. About 10 g of silica were placed in a reaction flask and alternately dried in vacuum and purged with nitrogen gas. Meanwhile, 2 ml of GLYMO and 100 ml of DIPEA were added to 50 ml of dry toluene and placed under nitrogen. The amount of GLYMO was such as to be in approximately twenty-fold excess with respect to the silanol groups. The reactants were then added to the particles through a septum using a syringe. The mixture was stirred and maintained under nitrogen atmosphere (using a balloon) for 18 h at room temperature, after which they were removed and rinsed in the membrane cartridge with dimethylformamide (DMF) and methanol.

This pretreatment produced an epoxide that was next reacted with polyethyleneimine (PEI) to generate an amine coating. Batches were separately produced using the 230 and 600 molecular masses. For this reaction, 2 g of PEI were suspended in 48 ml of DMF and sonicated for 15 min, after which the solution was added to 4 g of the glycidoxypropylsilyl support and allowed to react, with stirring, for 48 h. After this time, the products were successively washed with the original solution, DMF, methanol, and DI water; following this, they were dried under vacuum for 30 min. A crosslinking reaction followed in which the particles were put into a solution containing 2 g of BUDGE in 48 ml DMF, allowed to sit at room temperature overnight, then heated in a water bath at 80°C for 1 h [23]. BUDGE is an example of a family of epoxides that crosslink by bonding to primary and secondary amines on the PEI [23,24]. The crosslinked products were washed with methanol and dried under vacuum.

The PEI-bonded or -coated and crosslinked products have been used as stationary phases in both anion exchange and hydrophobic interaction modes [23–26]. In order to obtain a cation-exchange surface, the amines were reacted with a cyclic anhydride [21]. For about 4 g of the cationic PEI product, a solution was prepared containing 1 g of succinic anhydride in 20 ml of DMF with 250 ml of DIPEA as a proton scavenger. The particles were then added to this solution, which was heated at 60°C with rotation of the flask for 6 h. The products were washed successively with methanol, DI water, a small amount of triethylamine, DI water, and methanol. A last step of vacuum drying for 30 min completed the process. The structure of the resulting material is depicted in Fig. 1. It was packed using a Haskel (Burbank, CA, USA) air-driven fluid pump, a 40-ml slurry reservoir, and nitrogen gas for pressure into columns of dimensions 33×4.6 mm I.D. (Bischoff, Leonberg, Germany).

The HPLC instrumentation was centered on a Waters 600 multisolvent delivery system (Milford, MA, USA). Injections were made using a 20-μl injection loop (Rheodyne, Cotati, CA, USA). Detection was performed with an Isco (Lincoln, NE, USA) V^4 absorbance detector at 280 nm. Output was

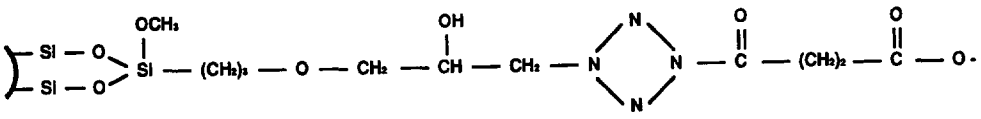

Fig. 1. Structure of PEI-based carboxylic acid stationary phase.

recorded and analyzed with Isco software on a personal computer.

All solutions were passed through a 0.2-μm filter (Gelman, Ann Arbor, MI, USA) after preparation. Before and intermittently (automatically controlled by the HPLC instrument) during use, solutions were sparged with helium. After the system had been flushed with water, the columns were conditioned for at least half an hour with the ionic strength to be used for isocratic experiments or the starting solution for gradient experiments. All solutions were based on 0.01 *M* phosphate buffers at pH 7, with NaCl added to achieve the desired ionic strength. In gradient mode, a linear gradient was employed from no added salt to 1 *M* NaCl over 5 min. For the experiments at Mainz, a large number of proteins and other solutes were injected sequentially. The minimum elution time was taken to be the dead time of the column. For the experiments at the University of Delaware, the dead time of the column was determined using standards that have been found [16] not to adsorb to negatively charged surfaces: pepsin and N-acetyl tryptophan.

4. Results and discussion

4.1. Isocratic experiments

Ionic strength-mediated retention was observed on the PEICX cation exchangers, as expected. For the stationary phase based on the M_r 230 PEI (PEICX2), the results of a number of isocratic experiments are summarized in Fig. 2. These experiments were performed at the University of Delaware, and complemented several data points that had been obtained on the same column at the University of Mainz almost two years earlier. Reasonable agreement was obtained between the two sets of data, providing assurance that the adsorbent was stable.

Similar results were obtained with the stationary phase synthesized using the higher molecular mass polymer support (PEICX6); these are plotted in Fig. 3. Some minor differences in retention exist between the two phases, i.e., the *Z* slopes are a little larger on the PEICX6 column and the intercepts are higher on the PEICX2 column, although these differences are not statistically significant. Elemental analysis of the

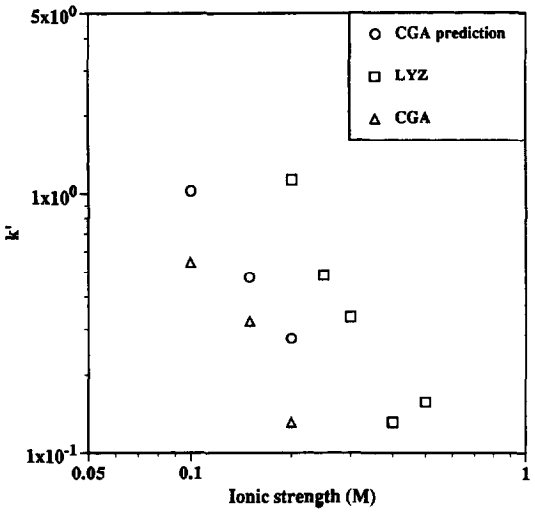

Fig. 2. Isocratic cation-exchange HPLC retention on PEICX2 stationary phase, showing comparison of observed chymotrypsinogen A retention with that predicted by mechanistic extrapolation of lysozyme results.

two materials failed to provide any useful data, as the low surface area of the non-porous support resulted in measured elemental compositions too low to be meaningful.

Hen egg-white lysozyme would seem to be a good example of a well-characterized protein. It was

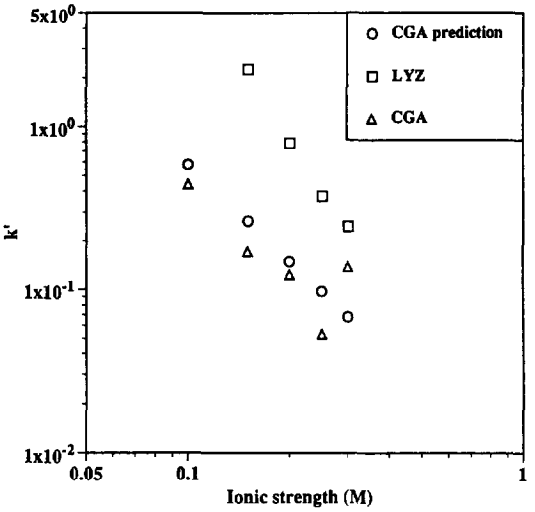

Fig. 3. Isocratic cation-exchange HPLC retention on PEICX6 stationary phase, showing comparison of observed chymotrypsinogen A retention with that predicted by mechanistic extrapolation of lysozyme results.

among the first proteins whose structures were determined by means of X-ray crystallography. Furthermore, its small dipole moment at pH 7 [27] results in its electrostatic interactions being described accurately by the approximations made here [18]. Consequently, we use lysozyme to characterize the stationary phase in terms of the two properties σ and w_o, indicative of its electrostatic and short-range interactions, respectively. A best-fit line through the PEICX2 data in the ionic strength range of 0.2 to 0.4 M leads to the parameter values shown in Table 1. The slope Z, along with a characteristic radius for lysozyme of 16.0 Å and a net charge of +8, gives an estimate of the surface charge density of -4.3 $\mu C/cm^2$. This value is within the range estimated for a variety of typical adsorbent materials under similar conditions (e.g. Refs. [16,28–31]).

The value of K for lysozyme represents the product of the phase ratio and its short-range equilibrium constant. The phase ratio Φ can be estimated from the specific area of the adsorbent (1.8 m^2/g for MinUSil 30, P. Brode, personal communication), the density of the particles, and a void fraction (assumed equal to 0.35) to be $6.7 \cdot 10^{-4}$ $Å^{-1}$. This should be considered to be an upper bound, as the accessible area for protein adsorption is probably less than the area determined by nitrogen gas adsorption measurements, and also because the polymer coating is likely to have smoothed the surface and reduced its area. Nonetheless, since only scalings in K_{sr} and K are considered, the exact value of Φ is of secondary importance. For the same reason, the absolute values of capacity factor, while subject to some uncertainty (through any uncertainty in the value of t_o) for the low values measured, are not as important for interpretation as their trends with respect to protein, ionic strength, and adsorbent.

The short-range equilibrium constant is equal to 12.2 for lysozyme in this analysis of the PEICX2 results. This value corresponds to a short-range energy $w = 2.5kT$, or $w_o = 0.156kT/Å$ (assuming a characteristic radius for lysozyme, based on its molecular volume, of 16.0 Å).

Table 1
Experimentally determined parameters on cation-exchange columns

		Z	$K \times 1000$ ($Å^{-1}$)
PEICX2	Lysozyme	3.03	8.2
	Chymotrypsinogen A	2.02	5.8
PEICX6	Lysozyme	3.26	4.4
	Chymotrypsinogen A	2.17	3.0

The characterizations of the sorbent based on lysozyme can be used to make predictions concerning the chymotrypsinogen A (CGA) isocratic data. By calculating the equilibrium constant at two ionic strengths very close in value, using the sorbent surface charge density and the size and charge of chymotrypsinogen A, a predicted slope of $Z = 1.89$ is obtained. Likewise, from the value of w_o and the size of CGA, a short-range equilibrium constant of 20.6 is obtained, leading to a predicted value of $12.0 \cdot 10^{-3}$ $Å^{-1}$ for K. Comparison with a fit of the CGA data in Fig. 2, for which the experimentally determined slope is $Z = 2.02$ and the intercept $K = 5.8 \cdot 10^{-3}$ $Å^{-1}$, indicates that the predicted slope is in good agreement with the experimental data, but that the trend in the short-range equilibrium constant is contrary to expectation. However, as shown in Fig. 2, while the predictions overestimate the actual values somewhat, they show correctly the trend in the difference between the lysozyme and chymotrypsinogen A data.

Since the intercept K is merely a product of the phase ratio and the short-range equilibrium constant, the retention data show a short-range interaction for CGA that is actually weaker than that for lysozyme. In light of experimental results using the same two proteins [16] for which the non-electrostatic contributions to adsorption increased with protein size, this trend is somewhat surprising. However, at short range a number of subtle interactions, including minor or major conformational change, salt binding, and changes in the hydrogen bonding state are all possible. Furthermore, the data are in a region where the electrostatic contribution should be dominant. Perhaps a clearer indication of the expected effect emerges from isocratic anion-exchange chromatographic data for a set of proteins with molecular masses ranging from 12.4 to 69 kDa; these showed apparent intercepts (apparent since they were not computed explicitly in the published work) that displayed a strong positive correlation with molecular mass, but the values seemed to be a strong function of salt type (among all univalent salts) [32].

A similar procedure can be applied to the data on the PEICX6 column. Because of the slightly higher Z slope displayed in the lysozyme data, a larger stationary-phase surface charge density of -4.7 $\mu C/cm^2$ describes the data in the context of the LSA model. The subsequent prediction of slope for CGA on the PEICX6 material is very close to that observed experimentally (Fig. 3). The key difference between the two supports is the reduction in the short-range equilibrium constants, as can be seen by comparison of K values in Table 1. The short-range interactions are very weak on this support, and, as a result, the fact that the CGA short-range equilibrium constant is again found to be less than that for lysozyme is insignificant with regard to predicting k' data.

While the numerical agreement between the predicted and measured retention times is different for the two similar cation exchangers, it can be considered good for both phases. The agreement in slopes is very good; the ratio of slopes for lysozyme and CGA (roughly 3:2) is different from the ratio of net charges (8:4), a result that would not be predicted by models where Z is considered to be equal or proportional to net charge. A previous study [5] has shown that the correlation between the net charge of proteins and their retention times, albeit for gradient chromatography, is rather weak.

The dependence of the capacity factor on short-range interactions is found to be rather weak, particularly for PEICX6. The hydrophilic polymeric coating is expected to display relatively weak short-range attraction for proteins. The tendency of polyethylene oxide to resist protein adsorption has been argued to be dependent on a high chain density and chain length [33]. Both of these features would be enhanced in the material made from a higher molecular mass polymer, so it is possible that the steric repulsion between proteins and chains is increased for the 600 molecular mass PEI, thus leading to a very small short-range energy and the weaker retention observed on this material. It is also possible that the materials are merely crosslinked differently. These types of effects should have little bearing on electrostatics, which are relatively long-range interactions, as is indicated by the experimental results.

Although a better understanding is required to assess the dependence of the short-range equilibrium constant on protein properties, the results of this study are encouraging, as they demonstrate not only the ability to predict retention times a priori but also the ability to assign greater mechanistic significance to the functional form of the frequently used SDM. The ability to predict elution order based on molecular properties is in itself a useful capability, and these results represent a step in that direction.

4.2. Gradient experiments

While isocratic experiments are most useful for extracting information regarding adsorption in chromatography, some form of gradient is used in achieving most protein separations. With optimization of gradient conditions and slope, separation of multicomponent samples into individual components can be achieved rapidly and efficiently. The required optimization is, however, a trial-and-error process that is often categorized as the "art" of chromatography. A mechanistic understanding of the dependence of retention on solvent strength, as well as other column parameters affecting transport and kinetics, would aid greatly in the optimization process.

Gradient chromatograms were obtained for several proteins on the same PEICX2 column used for the isocratic experiments described above. As was seen in the comparison of lysozyme and chymotrypsinogen A, electrostatic effects are dominant, with the retention times increasing in the same rank order as the protein net charge (Table 2). Furthermore, the retention times are distinct enough that their separation factors, defined for two solutes 1 and 2 as:

$$\alpha = \frac{k_2'}{k_1'} \tag{13}$$

are significant and should lead to good separation. Similar results were observed for PEICX6, based on the higher molecular mass polymer. It should be noted that all of the proteins used in these experiments are relatively close in molecular mass; even if there were significant short-range interactions occurring, there would be little distinction among them from size-dependent effects.

The characterization of the sorbent with the lysozyme isocratic experiments can be used with the integration to gradient elution given by Eq. 13 to

Table 2
Measured retention times for proteins on PEICX2 and PEICX6 columns

Protein	Net charge	PEICX2 (s)	PEICX6 (s)
Lysozyme	+8	101	83
Cytochrome C	+5	65	59
Ribonuclease A	+4	50	37
Myoglobin	0	18	14

For each case, a linear gradient of 0–1.0 *M* NaCl (each in 0.01 *M* buffer) was applied over 5 min, using a flow-rate of 1.0 ml/min.

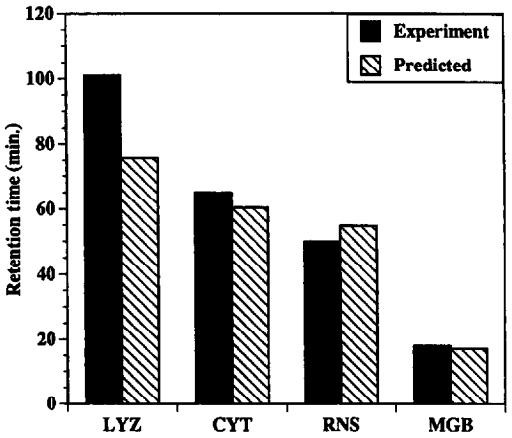

Fig. 4. Measured vs. predicted gradient retention times for several proteins on the PEICX2 column. Predictions based on parameters extracted from lysozyme isocratic data, along with physical properties of the proteins involved.

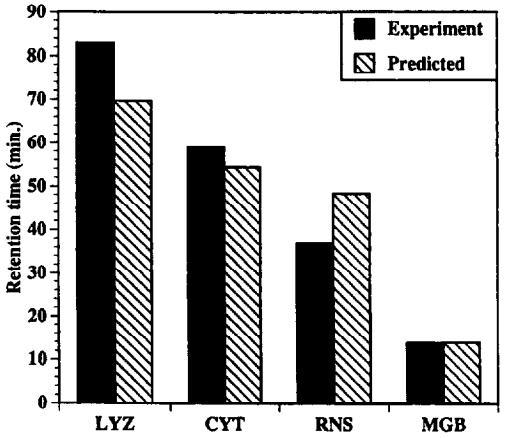

Fig. 5. Measured vs. predicted gradient retention times for several proteins on the PEICX6 column. Predictions based on parameters extracted from lysozyme isocratic data, along with physical properties of the proteins involved.

predict retention times in gradient mode; these are shown in Fig. 4 and Fig. 5. In general, the prediction is good, although there exists an underprediction of retention time for lysozyme and an overprediction for ribonuclease A on each material. The discrepancy for lysozyme could be due to the assumption of a linear dependence of $\log k'$ on $\log I$ with constant slope $-Z$ throughout the entire gradient. Because the shape of the $\log k'$ vs. $\log I$ is typically concave upwards, the linear assumption would underestimate the electrostatic equilibrium constant at ionic strengths less than that at which it is eluted and consequently underpredict the retention time by an amount that would increase with increasing retention time, which is essentially the trend in Table 2. A more rigorous approach would integrate the predicted retention curve as a function of ionic strength from the initial salt concentration to that at the point of elution, but this would diminish the ease with which our approach can be implemented in practice.

The results of the study on the two similar but slightly different stationary phases can be instructive in putting this work into perspective. Of the proteins utilized in the gradient experiments, cytochrome C and ribonuclease A are the most similar in net charge. They are also, along with lysozyme and myoglobin, approximately the same size and molecular mass. For this reason, short-range interactions are not likely to distinguish between them, unless such interactions are specific to some functionality of one of the species. For proteins of similar size, the short-range equilibrium constant is, in effect, wasted. That is, if the short-range interaction is non-selective, then inhibiting it is clearly desired; since all the selectivity is in the electrostatic interactions, it is best to maximize the electrostatic effects through choice of columns and conditions. For the examples studied here, the PEICX6 column would be better thermodynamically for the separation, because the short-

range interaction is weak and the electrostatic distinction is not marred. This idea is reflected in the separation factors for cytochrome C and ribonuclease A that can be calculated from the data provided in Table 2, i.e., roughly $\alpha = 1.5$ for PEICX2 vs. $\alpha = 2.0$ for PEICX6. To improve the separation further, the next step would be to alter the pH or ionic strength to maximize the selectivity, i.e., differences in electrostatic interaction with the charged sorbent.

5. Conclusions

As part of our long-term goal of developing understanding of chromatographic retention of proteins and tools for the rational design of chromatographic separations, a simplified model of charge-dominated adsorption has been introduced. It is based on use of the linear superposition approximation to describe approximately the electrostatic contributions to adsorption, while other contributions are treated by means of a simple scaling with size. A crucial feature of the model is that, despite its simplicity, sorbent properties that can be quantitatively characterized are incorporated: the surface charge density and a characteristic short-range attractive energy.

A cation-exchange sorbent was synthesized as a carboxylic acid derivative of PEI-coated quartz particles. Two versions of this material, differing only in the molecular mass of the PEI precursor, were produced. Isocratic experiments over a range of ionic strengths for lysozyme were used to estimate appropriate values for the two sorbent properties, and used successfully to predict the Z slope of the rate of change of $\log k'$ with $\log I$ in isocratic retention of chymotrypsinogen A for both materials. The PEICX2 material exhibited some short-range interactions, but such interactions were very weak on the PEICX6 column. Contrary to our earlier batch adsorption experiments, in which a size-dependent energy contribution seemed to drive CGA to exhibit greater adsorption relative to lysozyme at increasing ionic strengths, the short-range contribution to CGA adsorption was actually found to be less than that for lysozyme. The prediction of the correct Z slope for CGA is nonetheless an encouraging result, as it cannot be predicted a priori from any other retention model.

Gradient elution allowed for good separation of several positively charged proteins on the cation-exchange sorbents. The elution order for proteins was predicted by the model, and the values were predicted to some extent. The key feature is again the classification of the two materials in terms of electrostatic and short-range, generally size-dependent, interactions; although the surface charge density of the PEICX6 support was found to be slightly higher than that for the PEICX2, more important was the significant reduction in short-range interactions exhibited in the retention data on this material. As a result, greater selectivity of similarly sized proteins was obtained in a shorter elution (i.e., analysis) time. This example shows that the construct for interpreting and predicting chromatographic elution presented here is potentially a very powerful tool for the design and optimization of chromatographic separations.

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