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Influence of surface modification on protein retention in ion-exchange chromatography Evaluation using different retention models

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ABSTRACT

A large number of different stationary phases for ion-exchange chromatography (IEC) from different manufacturers are available, which vary significantly in a number of chemical and physical properties. As a consequence, binding mechanisms may be different as well. In the work reported here, the retention data of model proteins (α-lactalbumin, β-lactoglobulin A, bovine serum albumin and alcohol dehydrogenase) were determined for three anion-exchange adsorbents based on synthetic copolymer beads with differences in the functional group chemistry. Fractogel EMD DEAE and Fractoprep DEAE consist of functional groups bound to the surface via "tentacles", ToyopearlDEAE by a short linker. Three models which describe chromatographic retention were used to analyse the characteristic parameters of the protein/stationary-phase interactions. The number of electrostatic interaction between the stationary phase and the model proteins, the protein specific surface charge densities and the interacting surface of the proteins with the adsorptive layer of the chromatographic media depend on the surface modification as well as on the molecular mass of the model proteins. In general, protein retention of the model proteins on the weak anion exchangers was found to be greater if the stationary phase carries tentacles and protein mass is above 60 kDa.

1. Introduction

Ion-exchange chromatography (IEC) is widely used for the purification of pharmaceutical proteins. A large number of different stationary phases are available from different manufacturers, which vary significantly in a number of chemical and physical properties. The materials are manufactured by different proprietary processes, which utilize inorganic, synthetic organic, or carbohydrate matrices, as well as a variety of coupling chemistries.

The comparison and selection of chromatographic resins is usually performed during process development and is mainly empirical. 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 the separation process. A better understanding of the interaction between proteins and individual adsorbents would facilitate this selection process.

Several models for chromatographic retention of ion-exchange adsorbents have been proposed [1], which can be divided into stoichiometric and non-stoichiometric models. Stoichiometric models describe the multifaceted binding of the protein molecules to the stationary phase as a stoichiometric exchange of mobile phase protein and bound counterions. This stoichiometric displacement model (SDM) predicts that the retention of a protein under isocratic, linear conditions is related to counterion concentration as follows:

$$\log k = -\left(\frac{z_P}{z_S}\right) \log C_m + \log(\varphi Q)$$

where k is the retention factor and C_m is the concentration of the counterion in the mobile phase. z_P/z_S (=z) is the ratio of the characteristic charge of the protein to the valence of the counterion and represents a statistical average of the electrostatic interactions of the protein with the stationary phase as it migrates through the column.

The relationship has been experimentally demonstrated under conditions commonly employed in the IEC of proteins. The model has been extended to describe protein retention under linear gradient elution conditions (LGE model) [2], as well as under non-linear protein adsorption conditions [steric mass action (SMA) model] [3] for isocratic and gradient chromatography. An extension of the stoichiometric model for the ion-exchange adsorption which accounts for charge regulation was developed recently [4,5].

Although stoichiometric models are capable of accurately describing the behaviour of ion-exchange chromatographic systems, they assume that the individual charges on the protein molecules interact with discrete charges on the ion-exchange

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surface. In reality, retention in ion exchange is more complex and is primarily due to the interaction of the electrical fields of the protein molecules and the chromatographic surface.

During the last decade, several non-stoichiometric models for describing protein retention as a function of the salt concentration in the eluent have been proposed. Two of the models are based on Manning's ion condensation theory [6–9], originally formulated to estimate the properties of cylindrical polyelectrolytes, such as DNA, in a salt solution. Another approach has been taken by Noinville et al. [10], who used protein crystal structures to simulate the adsorption process by calculating screened coulomb and Lennard–Jones interactions over all protein rotations and distances. Quantitative structure–property relationship (QSPR) models have been derived for protein retention in IEC by means of different numerical approaches that attempt to correlate retention to functions of descriptors derived from the three-dimensional structure of the protein [11–13].

More recently theories used in colloid and surface chemistry to describe electrostatic and other interactions have been applied to describe retention properties of proteins in IEC. In these theories, the electrostatic interaction is often calculated from solutions of the Poisson–Boltzmann equation for a system of given geometry [1,14–17].

Ståhlberg and co-worker [14] have developed the electrostatic interaction model which treats the retention process as a Coulomb interaction between a charged sphere and an oppositely charged planar surface in an electrolyte solution. A simplified model (slab model) [15,16], in which the solution of the linearized P–B equation for two oppositely charged flat plates is used, has been shown to describe the change in retention of proteins in IEC as a function of salt concentration in the mobile phase. The slab model predicts that the logarithm of the retention factor varies linearly with the reciprocal square root of the ionic strength of the eluent. The slope of the straight line depends on the protein charge density, as well as on the interacting area between the protein and the stationary phase. A recent modification of the slab model includes the charges that are induced at the protein surface by the electrostatic field of the chromatographic surface, and is therefore called the charge regulated slab model [17].

Roth et al. [18] solved the linearized Poisson–Boltzmann equation numerically for a sphere interacting with an oppositely charged surface by using a functional form suggested by the linear superposition approximation. The results were used to develop a mechanistic model within which the retention is related to protein and stationary-phase structural and functional parameters, as well as eluent composition. The protein parameters are size and net charge, while incorporation of stationary-phase properties, namely the surface charge density and the short-range interaction energy, allows for a more mechanistic interpretation of SDM parameters.

Both the slab model and the mechanistic model make use of characteristic parameters of the protein, as well as of the stationary phase, to describe the retention process. Key determinants of protein retention were found to be the adsorbent pore size distribution, the ionic group and the type of surface chemistry.

Although some data are available for different ion exchangers, little data have been published on the retention of tentacle modified phases. No investigations have been done on weak anion-exchange resins carrying tentacles. In the work reported here, the retention of the four model proteins α-lactalbumin (α-Lac), β-lactoglobulin A (β-LacA), bovine serum albumin (BSA) and alcohol dehydrogenase (ADH) was determined for three anion-exchange adsorbents (Toyopearl DEAE; Fractogel EMD DEAE, Fractoprep DEAE). These chromatographic stationary phases differ in the composition of the base matrix and/or in the structure of the attached ligands.

The base matrix of Fractogel EMD and Toyopearl resins consists of crosslinked methacrylate (particle size: 40–90 μm). Fractoprep beads are crosslinked vinyl ether copolymers (particle size: 30–150 μm). In contrast to Toyopearl DEAE, the surface modification of Fractogel EMD DEAE and Fractoprep DEAE exploits a cerium(IV)-catalyzed graft polymerization as described by Müller [19]. These grafted polyelectrolyte chains (tentacles) exhibit motional freedom and allow additional interactions with proteins, which are not observed with standard-type anion exchangers [20]. The pore dimensions of the anion exchangers as determined by inverse size-exclusion chromatography (ISEC) were found to be in the same order of magnitude as described for the corresponding cation exchangers [21].

Using different retention models several characteristic parameters of the protein/stationary-phase interactions were determined and correlated with structural differences of the retentive layers.

2. Theory

2.1. Stoichiometric displacement model

The SDM was first formulated by Boardman and Partridge [22] and has since then been used in several alternative formulations, all based on the stoichiometric concept [3,23,24]. The main assumption inherent in the SDM is that only electrostatic interactions are responsible for the adsorption/desorption process of molecules. The binding process, in absence of specific salt binding effects, results in equilibrium of the eluents and the polyions of the sample between the mobile and the stationary phase. The equation, which is used to analyse the retention data, is derived from the biochemical description of this equilibrium:

$$\log k = -\left(\frac{z_P}{z_S}\right)\log C_m + \log(\varphi Q) \quad (1)$$

$$Q = \left(\frac{A}{z_S}\right)^{N_I} K_b \quad (2)$$

In a $\log k - \log C_m$-plot the slope of the straight line gives the number of the interacting groups between the resin and the protein. By using a monovalent salt as the eluent ion, the slope directly represents the number of exchange sites. The y-intercept represents the logarithm of the phase ratio (φ) multiplied with a constant Q, which is related to the equilibrium constant (K_b) for the exchange reaction according to Eq. (2).

2.2. Mechanistic model of retention

The model has been developed by Roth et al. [18] to describe the retention of proteins in IEC and predict the retention factor k.

In this work, a comparison of different chromatography resins in terms of their protein specific surface charge density (σ_M) and characteristic short-range interactions (w_0) is of interest.

In the model, the equilibrium constant (K) is the product of a constant for the electrostatic interactions (K_{es}) and a constant for the short-range interactions (K_{sr}). The calculation of K_{es} is based on the solution of the linearized Poisson–Boltzmann equation for defined geometries. The short-range interactions include each non-electrostatic mechanism of adsorption, such as van der Waals interactions, attractive and repulsive solvation effects, and steric effects. The central equations of this model are

$$\log k = \log K_{es} + \log K_{sr} + \log \Phi = \log K - z \times \log C_m \quad (3)$$

$$K_{es} = \kappa^{-1}\left\{-\frac{E_0}{kT} + \frac{(-E_0/kT)^2}{2\times 2!} + \frac{(-E_0/kT)^3}{3\times 3!} + \dots\right\} \quad (4)$$

$$E_0 = \frac{\sigma_M Q_P}{s\kappa(1+\kappa R_{h,P})} \quad (5)$$

$$K_{sr} = e^{(-w_D R_{h,P}/kT)} \quad (6)$$

The evaluation of the experimental data is based on $\log k - \log C_m$-plots and allows the calculation of K_{eq} ($K_{eq} = k/\Phi$), K_{sr} ($K_{sr} = K/\Phi$) and K_{es} ($K_{es} = K_{eq}/K_{sr}$). Using Eqs. (4)–(6) the protein specific surface charge density of the resin (σ_M) and the characteristic short-range interaction energy (w_0) are calculated. These values offer clues to help pinpoint the differences in binding mechanism between the different stationary phases, when the molecular mass of the model proteins increases.

2.3. Charge regulated slab model

The model developed by Ståhlberg is based on the mathematical, physical and chemical description of two oppositely charged slabs immersed in an electrolyte solution. By combination of the mathematical expression for electrostatic interactions between two oppositely charged slabs and in consideration of the variable distance between these two bodies (thermodynamic definition of the retention factor involves the integral of the exponential interaction energy over the separation distance) the following equation results:

$$\ln k = \frac{\sigma_P^2 A_P}{F\sqrt{2RT\varepsilon_0\varepsilon_r(1-K_P)}} \times \frac{1}{\sqrt{I}} + \ln\left(\frac{A_S d}{V_D}\right) \quad (7)$$

In a $\ln k$ vs. $1/\sqrt{I}$-plot the slope is given by

$$\text{slope} = \frac{\sigma_P^2 A_P}{F\sqrt{2RT\varepsilon_0\varepsilon_r(1-K_P)}} \quad (8)$$

Ståhlberg developed this model in order to describe the retention of proteins in IEC. To prove the accuracy of this model, he compared the calculated net charge of proteins from chromatographic experiments with titrimetrically determined protein net charges. His results indicated that the chromatographically measured protein charges are comparable to those obtained from titrimetric experiments.

The aim of this work is the determination of the protein surface (A_P) interacting with the resin (Ståhlberg approached A_P with half of the total surface of the protein = $0.5\,A_{P0}$) and the characteristic width of the adsorptive layer (d) multiplied with the surface of the stationary phase (A_S) to characterize and compare the different resins.

3. Experimental

3.1. Materials

Sodium chloride, Tris and ethanol were obtained from Merck (Darmstadt, Germany). Sodium acetate and hydrochloric acid were obtained from Carl Roth (Karlsruhe, Germany.) All chemicals were used without further purification. All buffers were prepared at room temperature ($23 \pm 2\,^{\circ}C$) using deionised water from a Millipore Milli-Q system and were filtered with 0.22 μm bottle-top filters. Buffers were prepared using 20 mM Tris/HCl and were adjusted to pH 8 using 1 M sodium hydroxide. A high-salt version containing 1 M NaCl was prepared in addition, and intermediate ionic strengths were obtained by mixing the high- and low-salt buffers.

3.2. Proteins

BSA and α-lactalbumin from *Bos taurus* were purchased from Fluka (Buchs, Switzerland). β-LacA and ADH from *baker's yeast* were purchased from Sigma–Aldrich (Germany). α-Lac, β-LacA and ADH were used without further purification. BSA was further purified before use. Purification of BSA was carried out near the isoelectric point of BSA, using a Resource Q 6 mL column, in sodium acetate with different sodium chloride concentration as buffer system (A: 10 mM pH 5.3; B: 10 mM + 0.5 M NaCl pH 5.3) and linear gradient elution [0–100% B in 20 column volumes (CVs)] [25,26].

The properties of the proteins are summarized in Table 1. The molecular masses and isoelectric points are taken from the data sheet of the manufacturers. The net charge of the proteins at pH 8 is calculated according to Mosher et al. [27] and compared with experimentally determined titration curves [28]. The hydrodynamic radii of the proteins are taken from literature [29–31]. The surface charge density (σ_P) of the proteins was calculated as

$$\sigma_P = \frac{Q_P(\text{pH}\,8)}{S_{P,R_{h,p}}}$$

3.3. Adsorbents

The three weak anion-exchange chromatographic media were a gift from Merck. The phase ratios of the stationary phases were experimentally determined. The ionic capacities were determined by Merck (Toyopearl DEAE = 124 mequiv./L; Fractogel EMD DEAE = 78 mequiv./L; Fractoprep DEAE = 80 mequiv./L). The K_P values for BSA (0.24) and β-LacA (0.37) were taken from the literature [1], for ADH a value of 0.24 and for α-Lac a value of 0.37 were estimated.

3.4. Isocratic retention runs

The retention factor $k = (t_R - t_0)/t_0$ is a dimensionless measure of protein retention under linear adsorption conditions, where t_R is the retention time at a given salt concentration and t_0 is the retention time of the protein under non-adsorptive conditions. Retention times were measured on a Superformance 50–10 column (10 cm length × 1 cm I.D.) (Götec Labortechnik, Mühltal, Germany) packed with the adsorbent of interest using isocratic elutions at NaCl concentrations up to 1 M and a flow rate of 0.5 mL/min (38.2 cm/h) or 1 mL/min (76.4 cm/h). A pulse injection of 20 μL of protein solution at 1 mg/mL was applied using an ÄKTA purifier chromatography workstation with the response detected by a GE UV-900 detector at 280 nm. The ionic strength during the elution runs was controlled by in-line mixing of the low- and high-salt buffers. The ionic strength of the protein sample was adjusted using PD-10 desalting columns (GE Healthcare, Germany) and the appropriate elution buffer. The sequence of the runs was randomized. Unicorn 5.01 software was used for data collection and evaluation (GE Healthcare).

4. Results and discussion

Three models for IEC are used to correlate retention data of a set of well-characterized model proteins with stationary phase's physical and chemical structure properties.

4.1. Evaluation of the stoichiometric displacement model

Plots of $\log k$ vs. $\log C_m$ for the model proteins on the different stationary phases result in almost linear dependencies indicating that the SDM is a good approximation for this relationship in the range of mobile phase compositions investigated (Fig. 1). Due to experimental uncertainties retention factors smaller than 0.2–0.3 were not used to determine the z value from the slope of the regression line as well as the y-intercept. Table 2 summarizes the z-values and y-intercepts used for the evaluation of the SDM.

Comparison of the z-values for the different proteins shows, that there is nearly no difference between the values of the three resins determined for the two small proteins α-Lac (z-values from 2.71 to 3.18) and β-LacA (z-values from 4.82 to 5.51). Similar results for these two proteins on strong anion exchanger were obtained

Table 1
Summary of the size and charge properties of the proteins used.

Protein	m [kDa]	pI	Charge at pH 8	$R_{h,P}$ [nm]	Surface [nm^2]	σ_P [C/m^2]	A_{P0} [m^2/mol]
α-Lac	14	4.5	−6.15	1.92	11.52	0.0214	2.78×10^7
β-LacA	37	5.1	−23.67	2.16	14.66	0.0647	3.53×10^7
BSA	67	5.0–5.2	−17.13	2.72	23.24	0.0300	5.60×10^7
ADH	141	5.4	−17.86	3.5	38.48	0.0186	9.27×10^7

pI = isoelectric point.

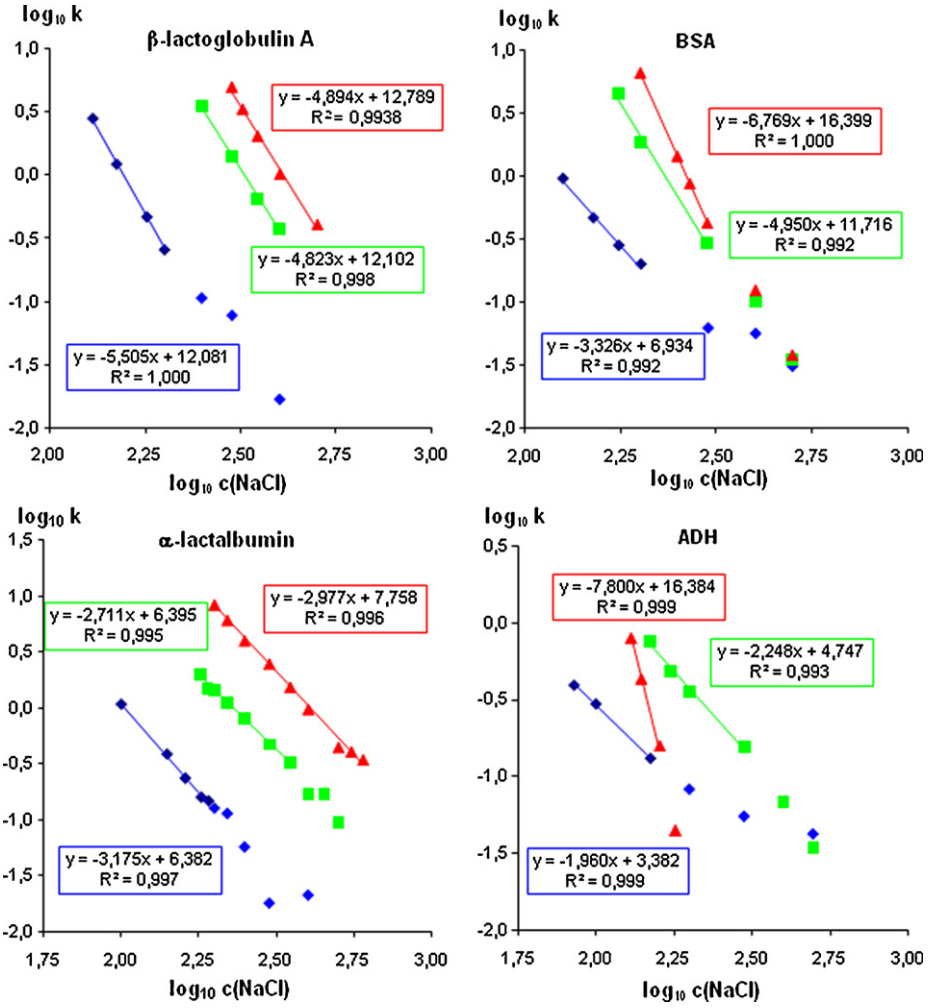

Fig. 1. Log k – log C(NaCl) plots for the four model proteins. Comparison of experimental isocratic retention data of the protein on (♦) Toyopearl DEAE, (■) Fractogel DEAE and (▲) Fractoprep DEAE at pH 8.0. The lines are the results of a linear regression analysis.

recently [32]. A large number of investigations using small model proteins on different cation exchangers also resulted in largely parallel lines in a log–log plot and therefore have very similar z-value [21,33]. These results indicate that the z-value is determined predominantly by the model protein properties.

The two larger proteins BSA and ADH show increasing z values starting from Toyopearl DEAE to Fractogel EMD DEAE and Fractoprep DEAE. Under the basic assumption that the z-values represent the number of interacting groups on the resin, the results of the isocratic elution experiments show clearly, that the usage of a resin with tentacle-technology maximize the electrostatic interactions between the resin and the model proteins. The larger the tested protein, the higher the number of groups, that participate in the ion-exchange process. These additional electrostatic interactions were

Table 2
Model parameters (SDM) for the four model proteins on the media Toyopearl® DEAE, Fractogel® EMD DEAE, and Fractoprep® DEAE estimated from isocratic retention data.

Protein	Toyopearl DEAE		Fractogel EMD DEAE		Fractoprep DEAE	
	z	y-Intercept	z	y-Intercept	z	y-Intercept
α-Lac	3.18	6.38	2.71	6.40	3.00	7.76
β-LacA	5.51	12.08	4.82	12.10	4.89	12.79
BSA	3.33	6.93	4.95	11.72	6.77	16.40
ADH	1.96	3.38	2.25	4.75	7.80	16.38

Table 3
Stationary phases parameters used for calculating the phase ratio according to MMR model.

	$S_{s.p.}$ [m²/mL]	V_C [mL]	S_{total} [cm²]	Φ [1/Å]
Toyopearl DEAE	26.00	3.77	9.80×10^5	2.64×10^{-3}
Fractogel DEAE	76.50	4.163	3.18×10^6	8.25×10^{-3}
Fractoprep DEAE	159.50	4.477	7.14×10^6	1.89×10^{-2}

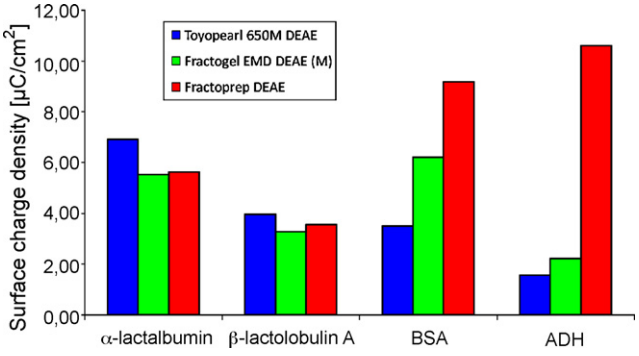

Fig. 2. Calculated protein specific surface charge density (σ_M) for the three different stationary phases.

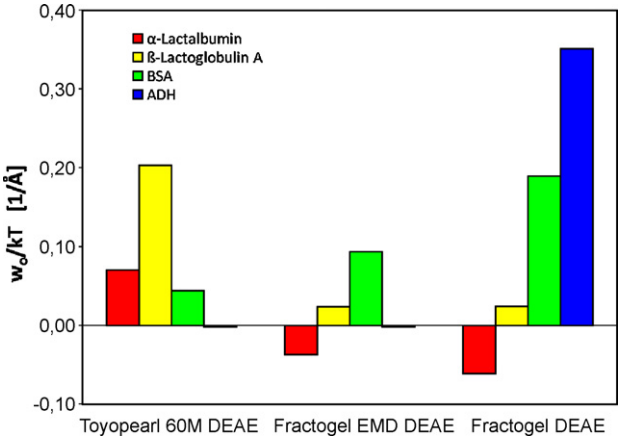

Fig. 3. Calculated characteristic energy of short-range interactions (w_0) for the four model proteins on the three different stationary phases.

also postulated by DePillips and Lenhoff [21], but not observed for the small proteins used in their studies [21,34].

4.2. Evaluation of the mechanistic model of retention

The phase ratios for this model are calculated with the characteristic parameters of the resins shown in Table 3. The surface area of the resin (S_r) and the density were determined by Merck, the other values were determined experimentally. The compression of the resin during packing procedure was 20% for all stationary phases used in this study.

The use of the SDM does not imply any mechanistic interpretation other than to note that changes in the slope are related to the properties of the protein and the adsorbent and should change as the physical properties change. The MMR model allows the calculation of the protein specific surface charge densities of the resin (σ_M) (i.e. the charges of the stationary phase, which are involved in the binding of the different proteins) as well as the constant for additional short-range interactions (K_{sr}) and allows for a more mechanistic interpretation of SDM parameters.

The evaluation of the MMR is based on the values for the slopes and y-intercepts shown in Table 4. Compared to the SDM the x-axis is scaled to $\log_{10} C$(NaCl) [mol/L] resulting in different y-intercepts but identical slopes. The calculated σ_M values are shown in Fig. 2.

The difference in the σ_M-values for one protein between Toyopearl DEAE and Fractoprep DEAE grows with increasing molecular mass of the protein used (0.81 for α-Lac, 0.90 for β-LacA, 2.62 for BSA and 6.75 for ADH). The protein specific surface charge densities increase for BSA and ADH, when a resin with tentacles is used. A protein, which interacts with a surface, such as that of Toyopearl DEAE resin (the functional groups are crosslinked to the stationary phase via short spacers), cannot access every DEAE group, because of its own size and the charge distribution on the surface of the protein. The protein will be hindered sterically by itself and other proteins. Contrary to this tentacles will maximize the accessible surface of the resin. Due to the flexibility of the polyelectrolyte chains, the steric hindrance effect is reduced. This effect should be more pronounced for larger proteins. A proof for this assumption is that the protein specific surface charge densities of the resins decrease or remain constant for the small proteins α-Lac and β-LacA. When a model protein with higher molecular mass, such as BSA and ADH is used, the σ_M-values always increases for the tentacle resins.

As the charge density (in mequiv./L) of the adsorbents are comparable and well above the critical values estimated previously [33,35] the spatial and geometric positions of the charged ligands relative to the protein surface take on added significance. A detailed analysis of the binding sites of lysozyme to different cation exchanger [36,37] showed a re-orientation of the protein on non-flexible surface modifications with a change in the pH. This adaption of the protein orientation for optimal binding was not observed for the flexible ligands in Fractogel resins, because the preferred interaction sites are already bound by the flexible tentacles.

Fig. 3 shows the calculated characteristic energy of short-range interactions for the model proteins with the three stationary phases. The short-range interaction energy of Toyopearl DEAE increases from α-Lac to β-LacA and decreases when using a protein larger than β-Lac A. Contrary to this Fractogel EMD DEAE shows this decrease only when a protein as large as ADH is used and finally the short-range interaction energy of Fractoprep DEAE always increases.

Table 4
MMR model parameters for the four model proteins on the media Toyopearl DEAE, Fractogel EMD DEAE, and Fractoprep DEAE estimated from isocratic retention data.

Protein	Toyopearl DEAE		Fractogel EMD DEAE		Fractoprep DEAE	
	z	y-Intercept	z	y-Intercept	z	y-Intercept
α-Lac	3.18	−3.14	2.71	−1.74	3.00	−1.17
β-LacA	5.51	−4.49	4.82	−2.26	4.89	−1.90
BSA	3.33	−3.05	4.95	−3.14	6.77	−3.91
ADH	1.96	−2.50	2.25	−2.00	7.80	−7.02

The calculation of the characteristic energy is based on van der Waals interactions. The different sorbent properties in terms of their Hamaker constants are reflected by this characteristic energy, which should be independent of the protein size. The analysis clearly shows that this is not the case and additional short-range interactions and entropic effects have to be taken into account. Hydrophobic interactions with the spacer arms or base matrix have been cited to explain retention differences [38,39] although no correlation between ligand hydrophobicity and retention was observed by DePhillips and Lenhoff [21]. Protein/base matrix interactions as well as desolvation effects may also modulate electrostatic interactions [34].

Although the short-range interactions are small and under binding conditions are low shadowed by the much stronger electrostatic interactions, they can introduce significant amplification or attenuation that has a measurable impact on retention.

Under non-binding conditions (high salt) the short-range interactions should dominate the retention behaviour of the proteins. As the tentacle layer covers a large part of the pores an exclusion of proteins due to entropic effects should be observed and an increase in the repulsive short-range interactions is expected with an increase in protein size. For BSA and ADH this increase of the characteristic energy on the tentacle resins is observed compared to the non-tentacle material.

4.3. Evaluation of the charge regulated slab model

A fit of the experimental data results in a linear dependence of the $\ln k - 1/\sqrt{I}$-plot with correlation coefficients between 0.983 and 0.999. This fact leads to the assumption, that the fit of the experimental data is a good approximation to describe the interactions during the ion-exchange process. Fig. 4 shows the evaluated straight lines for β-LacA and Table 5 summarizes the model parameters for all four proteins.

The calculated interacting surface area of the protein increases for every model protein, when a resin with tentacles is used. The higher the molecular mass of the investigated protein, the larger is the difference between the Toyopearl DEAE resin and the Fractoprep DEAE resin. For better comparison, the A_P-values are divided by the total surface area of the protein (A_{P0}). The A_P/A_{P0} value is a rough estimate of the percentage of surface area used during the binding event only, because a spherical conformation and an even charge distribution of the protein is assumed. The value could be a measure for the charge distribution and the binding orientation of proteins with similar molecular masses.

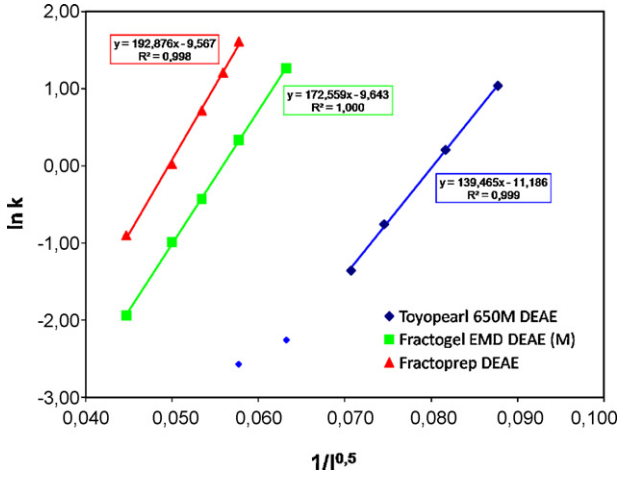

Fig. 4. Ln k – $1/\sqrt{I}$-plots for β-Lac A. Comparison of experimental isocratic retention data of the protein on (♦) Toyopearl DEAE, (■) Fractogel DEAE and (▲) Fractoprep DEAE at pH 8.0. The lines are the results of a linear regression analysis.

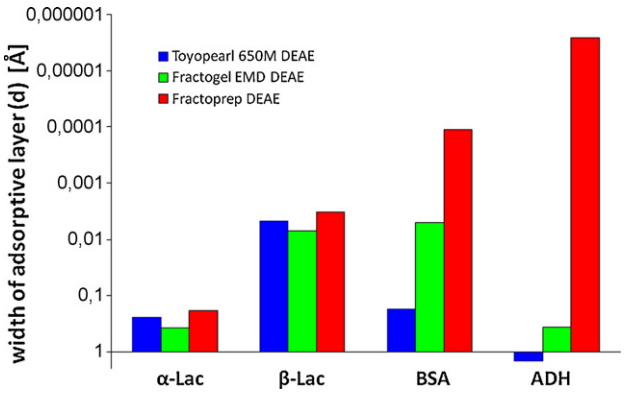

Fig. 5. Characteristic width (d) of the adsorptive layer for the four model proteins on Toyopearl DEAE, Fractogel DEAE and Fractoprep DEAE.

The results (Table 5) for the A_P values clearly show, that the tentacle modification leads to higher differences in the surface area of the protein, which participate in the ion-exchange process, when the molecular mass of the protein increases. A comparison of the A_P/A_{P0} values of the Toyopearl DEAE resin and the Fractoprep DEAE resin for small proteins, like α-Lac and β-LacA, shows an increase of the interacting surfaces of the proteins with the resin by a factor of 1.47 for α-Lac and 1.15 for β-LacA. As the molecular mass of the protein increases to 67 kDa (BSA) and finally to 141 kDa (ADH) the difference in the interacting surface between these two resins increases from 2.5 to 4.5. This is a consequence of the tentacle modification. When the size of the protein increases, the tentacles are able to reach more and less accessible charged groups on the protein surface in comparison to small rigid functionalized spacers.

Calculations by Steels et al. [40] showed that a protein can penetrate into a polymer brush like the tentacle layer when sufficient attractive interactions are formed. The engulfment of the protein should lead to an A_P/A_{P0} value close to 1, which is only observed for α-Lac and ADH on Fractoprep. An uneven charge distribution on the protein surface can explain the lower A_P/A_{P0} value for the tentacle resins. Repulsions between local positive surface charges and the positive tentacle charges will disturb the orientation of the tentacles near the protein surface and repel the polymer chain locally. The low A_P/A_{P0} value for β-Lac on all three resins might be a result of the very asymmetrical charge distribution and the high surface charge density (see Table 1).

The y-intercept can be identified as $\ln(A_s d/V_0)$, where V_0 is the dead volume of the column and A_s is the surface area of the stationary phase that is accessible to the protein. The alterations in the values of the y-intercept directly represent the change in ($A_s d$), because the dead volume of the column is a constant in this equation (Fig. 5).

For α-Lac and β-LacA the difference of the d values between the resins is low (2×). This difference increases with increasing molecular mass of the protein (1.6×10^3 for BSA; 5.6×10^6 for ADH).

The value of d can be estimated as the width of the adsorption layer given by the distance between the loci where the protein concentration is half of the maximum value [15–17] and is a measure of the compactness of the protein layer held near the surface. The d values for Toyopearl 650 M DEAE correlate with the surface charge density of the proteins, the larger the σ_P values the smaller the d value. When many charges of opposite sign are involved in the exchange process, the width of the adsorptive layer gets smaller and vice versa. The strong decrease in the d-values for the tentacle materials indicates a very compact protein layer near the surface of the stationary phase for larger proteins.

Table 5
Model parameters (slab model) for the four model proteins on the media Toyopearl DEAE, Fractogel EMD DEAE, and Fractoprep DEAE estimated from isocratic retention data.

Resin	Protein	Slope	A_P [m²/mol]	A_P/A_{P0} [%]	y-Intercept	d [Å]
Toyopearl DEAE	α-Lac	74	1.85×10^7	67	−7.29	2.47×10^{-1}
	β-LacA	139	3.82×10^6	11	−11.19	4.60×10^{-3}
	BSA	84	1.32×10^7	24	−7.55	1.77×10^{-1}
	ADH	42	1.67×10^7	18	−5.43	1.47×10^{0}
Fractogel DEAE	α-Lac	85	2.13×10^7	77	−5.68	3.79×10^{-1}
	β-LacA	173	4.72×10^6	13	−9.64	7.00×10^{-3}
	BSA	151	2.39×10^7	43	−9.96	5.10×10^{-3}
	ADH	66	2.64×10^7	28	−5.68	3.67×10^{-1}
Fractoprep DEAE	α-Lac	110	2.75×10^7	99	−5.55	1.89×10^{-1}
	β-LacA	193	5.28×10^6	15	−9.57	3.30×10^{-3}
	BSA	210	3.33×10^7	60	−12.95	1.10×10^{-4}
	ADH	188	7.50×10^7	81	−17.00	2.63×10^{-6}

The slab model is based on electrochemical layers, which interact with each other. With the tentacle modification the charges are ordered in several layers extending from the surface of the retentive layer to the surface of the base particle and cannot be regarded as a single and discrete layer. In addition, an immersion of the protein in the retentative layer is not considered in the slab model.

Interestingly calculations for the interaction between a protein and a polymer brush [40] also showed a more compact protein layer when larger proteins are penetrating the brush. The penetration depth decreases with increasing size of the protein whereas the free energy of the system as well as width of the energy dip decreases. The width further decreases when the grafting density is increased. The smaller d values for the Fractoprep material compared to the Fractogel material might indicate a higher grafting density for the Fractoprep material. First results on the characterization of the pore structure of the two materials by inverse SEC support this assumption.

5. Conclusions

Significant retention differences were observed among anion-exchange adsorbents depending on the surface modification chemistry. In general, the tentacle-type resins display a higher affinity.

According to the SDM, the polyelectrolyte chain which represents the tentacle maximizes electrostatic interactions between the resin and the protein as a function of the protein size. As the molecular masses of the proteins increase, more binding sites are involved in the interaction.

Evaluation by the mechanistic model of retention for the tested proteins also indicates that the part of the protein's surface involved in the interaction differs for the different anion exchangers. For Toyopearl DEAE the surface charge density exploited by the protein functional groups becomes smaller for larger proteins and the orientation of the protein due to its charge distribution has a larger impact.

For Fractogel EMD DEAE the protein specific surface charge density for larger proteins like BSA and ADH is always higher than these for the Toyopearl DEAE resin. The higher density of the flexible polyelectrolyte chains on Fractoprep DEAE resin results in an increased overall contact area. In addition, the area on the protein's surface that is involved in the electrostatic binding is larger. This corresponds to a stronger binding for larger proteins like ADH.

For the complete understanding of IEC separations, non-electrostatic interactions should also be considered. For tentacle resins, the molecular mass of the protein has an impact on non-electrostatic interactions. Explaining the reason of this variation across the anion exchangers is challenging, as there are parameters like tentacle length, tentacle density, and pore accessibility that can contribute to this effect but are not well characterized.

All three models treat the binding of the proteins onto the stationary phase as a fully reversible process, in which no conformational change of the protein occurs. A large number of investigations showed that proteins experience structural alterations during interaction with solid surfaces. The extent of conformational changes upon binding depends strongly on the nature of the surface (apolar, polar or charged) and the protein ("hard" or "soft" proteins) [41]. Recent investigations on hydrophobic interaction chromatography showed the significance of conformational changes on the adsorption process of proteins [42,43]. For IEC conformational changes are less pronounced, but should be taken into account for flexible and less stable proteins.

Nomenclature

A	ionic capacity of the resin [mequiv./L]
A_P	interacting surface of the protein [m²/mol]
A_{P0}	surface of the protein [m²/mol]
A_S	protein specific surface of the stationary phase [m²]
$A_S \times d$	"surface" of the stationary phase, which can be penetrated by the proteins [m³]
C_m	concentration of the counter ion in the mobile phase [mol/L]
d	characteristic width of the adsorptive layer [m]
E_0	characteristic electrostatic energy [J]
e	elementary charge [C] = $1.602176487 \times 10^{-19}$ °C
F	Faraday constant [c/mol] = 96485.3399 C/mol
K	equilibrium constant MMR model
K_b	equilibrium constant in SDM
K_{eq}	adsorption equilibrium constant [m; Å]
K_{es}	equilibrium constant for electrostatic interaction [m; Å]
K_{sr}	equilibrium constant for short-range interactions
K_P	constant with a characteristic value for a certain protein at a given pH value and eluent ionic strength
k	Boltzmann constant [J/K] = $1.3806504 \times 10^{-23}$ J/K
k	retention factor
N_A	Avogadro constant [1/mol] = $6.02214179 \times 10^{23}$ mol^{-1}
N_I	number of counterions, which are released upon binding of one protein molecule binds
Q	SDM constant
Q_P	net charge of the protein [C/m²] = $Q \times e \times N_A$
$R_{h,P}$	hydrodynamic radius of the protein [nm, Å]
$S_{P,Rh,P}$	surface of the protein
$S_{s.p.}$	surface of the stationary phase per millilitre packed resin [m²]
S_{total}	surface of the total resin [cm²]
T	absolute temperature [K]
V_C	volume of the column [ml]
V_{MP}	volume of the mobile phase [ml]

V_{SP} volume of the mobile phase [ml]
V_0 dead volume of the column [m^3]
w_0 characteristic short range energy [$J\,mol^{-1}\,nm^{-1}$]
z_P characteristic charge of the protein
z_S valence of the counterion
z $=z_P/z_S$

Greek letters
α selectivity
ε permittivity ($=\varepsilon_0\varepsilon_r$) [F/m]
ε_0 vacuum permittivity [F/m] = $8.854187817 \times 10^{-12}$ F/m
ε_r dielectric constant
κ inverse Debye length [1/m] = $3.27 \times 10^9 \times I^{0.5}$, $T = 298.15$ K
φ phase ratio SD model = V_{MP}/V_{SP}
Φ phase ratio MMR model = S_{total}/V_{MP} [1/Å]
σ_m surface charge density of the resin [C/m^2]
σ_P surface charge density of the protein [C/m^2]

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