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Lateral interaction between electrostatically adsorbed and covalently immobilized proteins on the surface of cation-exchange sorbents

Chitra K. Ratnayake[a,*], Fred E. Regnier[b]

[a]*PerSeptive Biosystems Inc., 500 Old Connecticut Path, Framingham, MA 01701, USA*

[b]*Department of Chemistry, Purdue University, West Lafayette, IN, USA*

Abstract

This paper examines the nature of chromatographic separations on a weak cation-exchange material in which immobilized proteins coats 50% or less of the sorbent surface. It was found that although these sorbents still function as cation exchangers, covalently immobilized proteins frequently contribute to the ion-exchange behavior of some protein analytes. Chromatographic retention of analytes was equal to or greater on immobilized protein derivatized columns than underivatized sorbents. Anionic proteins, in contrast, were not adsorbed, indicating that immobilized proteins were acting synergistically with ionic stationary phase groups to enhance retention. It was concluded that electrostatic adsorption is a prerequisite for analyte protein/immobilized protein interactions of sufficient magnitude to impact ion-exchange separations. Large differences in protein resolution were observed on columns that were identical in all respects except for the immobilized protein, further confirming that analyte/immobilized protein interactions were unique to the interacting pair. The extent of interaction was also influenced by concentration of the immobilized protein in the case of lysozyme. Interactions between the analyte and immobilized protein were found to occur between both the same two proteins and dissimilar species. It was concluded that these phenomena are due to lateral interactions between immobilized proteins and analyte proteins subsequent to electrostatic adsorption of the analyte on the underivatized surface of ion-exchange sorbents.

Keywords: Stationary phases, LC; Sorbents; Proteins

1. Introduction

Half a century of research on protein structure has established that proteins are semi-rigid and unique in size, shape, and surface heterogeneity. This has led to the conclusion [1] that in surface mediated separations (i) only a portion of the surface of the protein can simultaneously interact with the sorbent surface, (ii) the portion of the exterior of a protein which is most complementary to the surface will dominate adsorption, (iii) structural variations outside this contact region have a lower probability of impacting the separation process, (iv) the chromatographic contact region on the surface of protein will vary between separation modes, (v) molecular modelling might be able to predict the chromatographic behavior of proteins [2]. This relatively simple "footprint model" of surface mediated separations explains why proteins of very similar amino acid composition, size, and isoelectric point can separate very differently on ion-exchange, hydrophobic interaction, reversed-phase, affinity, and size-exclusion columns [1]. One of the most troubling features of chromatographically based protein separations is the non-linear retention characteristics encountered in preparative systems at high loading. As in the case of non-linear adsorption isotherms observed with gas–solid systems, this behavior can

*Corresponding author.

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be explained in terms of alterations in the phase ratio [3], steric (crowding) induced changes in the orientation of molecules to a surface [4], adsorption of multiple layers of protein on the sorbent surface, and intra-molecular interactions between molecules of the target species [5]. In fact, formation of multiple layers of protein at a surface is generally thought to be the result of inter-molecular interactions. A recent report that lysozyme and α-chymotrypsinogen A do not bind in linear chromatographic behavior, requires a different explanation for inter-molecular interactions. The prospect that inter-molecular interactions at surfaces are the result of lateral interactions between proteins seems not to have been considered.

It has recently been established [6] that when lysozyme is covalently immobilized on a weak cation-exchange sorbent through either carboxymethyl or acrylic acid stationary phase groups that the sorbent surface is only approximately 50% saturated. This leads to several questions; (i) can the residual uncoated cation-exchanging surface of these sorbents be used for cation-exchange chromatography and (ii) if so, do the covalently immobilized proteins participate in the chromatographic separation. The objective of this paper is to examine these two questions using a variety of cation-exchange sorbents that vary ten-fold in ion-exchange capacity.

2. Materials and methods

2.1. Chemicals

LiChrospher Si 1000 (10μm, 1000 Å) was purchased from E. Merck (Darmstadt, Germany). γ-Glycidoxypropyltrimethoxysilane, acrylic acid, acrylamido-2-methyl-1-propanesulfonic acid (AMPS), Ce(IV)sulfate, and dicyclohexyl carbodiimide (DCC) were purchased from Aldrich (Milwaukee, WI). 3-sulfo-N-hydroxysuccinimide (S-NHS) was purchased from Pierce (Rockford, IL). Lysozyme (chicken egg white), cytochrome *c* (horse heart), α-chymotrypsinogen A (bovine pancreas), soybean trypsin inhibitor (STI), conalbumin (CON) (chicken egg), carbonic anhydrase (CA) (bovine erythrocyte), β-lactoglobulin (β-LAC) (bovine milk), and myoglobin (MYO) (horse heart) were purchased from Sigma (St. Louis, MO).

2.2. Instrumentation

The particles were slurry packed into stainless steel columns 50×4.6 mm I.D. using high pressure packing pump (Shandon Southern Instrument, Sewickley, PA). Chromatography was performed on BioCad liquid chromatograph (Perseptive Biosystems, Framingham, MA). Absorbance was monitored at 280 nm.

2.2.1. Preparation of diol bonded phase

This step was done in 10 g batches in aqueous medium where the pH and the temperature was sufficient to produce a heavy silylation and also to open epoxides on the silane [7]. Ten grams of LiChrospher Si 1000 (10μm, 1000 A) were transferred into a 250 ml three-necked round bottom flask fitted with a mechanical stirrer and briefly degassed in a 100 ml solution of 6 *M* HCl by purging nitrogen through the solution. Then the slurry was stirred for 24 h at room temperature, vacuum filtered and washed with water to neutrality. The activated silica was then transferred into a one-necked round bottom flask and 100 ml of 10% aqueous solution of γ-glycidoxypropyltrimethoxysilane adjusted to pH 3.5 was added. the silica mixture was heated to 90°C for 2 h while stirring. After cooling, the solution was filtered, washed with water and then with THF and dried in vacuo.

2.2.2. Polyacrylate stationary phase synthesis

A modification of the method developed by Mino and Kaizerman [8] was used. One gram of diol silica was transferred into a 100 ml three-necked round bottom flask fitted with a mechanical stirrer and 50 ml of deionised water was added. Addition of 3 ml of acrylic acid was followed by purging with nitrogen through while stirring. The mixture was briefly sonicated to enhance the removal of air from the pores of silica. Then 0.5 g of Ce(IV) sulfate was added to the reaction flask and heated to 50°C for 7 h under nitrogen. The mixture was then cooled to room temperature and filtered in a sintered-glass funnel, washed thoroughly with water and 50 ml quantities of 0.5 *M* H_2SO_4. When the silica beads turned pure

white, it was washed again with water to neutrality and finally rinsed with THF and dried under vacuum.

2.3. Protein immobilization

2.3.1. Activation of carboxylic acid groups of the stationary phase

Acrylate grafted silica 0.1 g was transferred into a 20 ml polypropylene tube and 2 ml of 9.28 mg/ml solution (5 equiv.) of sulfo-N-hydroxy succinimide added. The mixture was degassed by purging with nitrogen for 10 min. Then 2 ml of dicyclohexyl carbodiimide (DCC) (8.85 mg/ml in dioxane, 5 equiv.) was added. Mixing was continued for 3 h, silica was centrifuged out, and the supernatant was discarded. The silica was then washed thoroughly with water and with dioxane to remove N,N′-dicyclohexyl urea and excess DCC. After a further dioxane wash, the silica was rinsed with water and dried.

2.3.2. Protein coupling to the activated surface

A 40 mg/ml solution of protein in 0.1 *M* $NaHCO_3$, pH 7.5 was used. To the activated acrylate sorbent, 2 ml of the protein solution was added and continuously agitated for 24 h at room temperature. After the silica was separated by centrifugation, it was washed according to the following sequence:

1. 0.1 *M* phosphate buffer pH 7.0 (1×5 ml).
2. 1.0 *M* NaCl in 0.1 *M* phosphate buffer pH 7.0 (1×5 ml).
3. 0.1 *M* phosphate buffer pH 7.0 (1×5 ml).
4. 10% ethylene glycol (1×3 ml).
5. 0.1 *M* phosphate buffer pH 7.0 (1×5 ml).

Elemental analyses (C, H, N, S) on silica-based stationary phases were performed by H.D. Lee, Purdue University Department of Chemistry Microanalysis Laboratory.

2.4. Chromatographic evaluation

Portions of the protein immobilized silica were packed into 50×4.6 mm I.D. stainless steel columns. Conditions used for chromatography were: 15 min linear gradient elutes from 10 m*M* phosphate buffer, pH 7.0 to 1.0 *M* NaCl in 10 m*M* phosphate buffer, pH 7.0 at a flow-rate of 1.0 ml/min.

Protein samples were prepared in 10 m*M* phosphate buffer, pH 7.0 at a concentration of 4.0 mg/ml. Cation-exchange behavior and "*Z* numbers" were examined using a mixture of α-CHYM A, CYT *c*, and LYS. Resolution of α-CHYM A, CYT *c*, and LYS were examined at 0.5 ml/min, 1.0 ml/min, and 2.0 ml/min. "*Z* numbers" of protein immobilized sorbents were determined using retention times of proteins at different concentrations of NaCl in phosphate buffer, pH 7.0.

3. Results and discussion

3.1. Sorbent synthesis

Two types of sorbents were used in these studies; both of which were on based on a 1000 Å pore diameter "diol silica" support. Diol silica was obtained by silylation of 1000 Å pore diameter LiChrospher silica support with γ-glycidoxypropyltrimethoxysilane followed by acidic hydrolysis of the resulting oxirane containing bonded phase. In the sorbent designated AC-1, the ionic stationary phase was prepared in a two-step process involving oxidative cleavage of the diol and oxidation of the resulting aldehyde to a carboxylic acid group [9]. Carboxylic acid ligand density in this sorbent was approximately 2.72 μmol/m^2 as determined by elemental analysis. The second sorbent, designated as AC-6 (see preceding communication), was prepared by a Ce(IV) initiated graft polymerization of a acrylic acid onto the diol bonded phase. Carboxyl stationary phase density in AC-6 was 11.67 μmol/m^2 as determined by elemental analysis. See Table 1 for column descriptions and abbreviations.

3.2. Protein immobilization

Protein immobilization was achieved by activation of stationary phase carboxyl groups in the AC-1 and AC-6 sorbents with sulfonylated N-hydroxysuccinimide followed by protein coupling at pH 7.5. Protein immobilized on the AC-6 sorbent and the designation used to identify each protein bonded materials are as follows; myoglobin (AC–MYO),

Table 1
Full descriptions of the columns used in this study

Column abbreviation	Description
AC-6	Ion-exchange sorbent made reacting LiChrospher 1000 diol (10 μm, 1000 Å) with 6% acrylic acid
AC–LYS	Lysozyme immobilized on AC-6
AC-1–LYS	Lysozyme immobilized on conventional weak cation-exchange sorbent prepared by oxidizing the diol coating of LiChorpher 1000 Diol
AC–CYT *c*	Cytochrome *c* immobilized on AC-6
AC–α-CHYM A	α-chymotrypsinogen immobilized on AC-6
AC–MYO	Myoglobin immobilized on AC-6
AC–BSA	Bovine serum albumin immobilized on AC-6

cytochrome *c* (AC–CYT *c*), α-chymotrypsinogen A (AC–α-CHYM), bovine serum albumin (AC–BSA), and lysozyme (AC–LYS). Lysozyme was also immobilized on sorbent AC-1 and designated as AC-1–LYS) (Table 1).

3.3. Chromatographic evaluation

The question being examined in these studies was whether weak cation-exchange chromatography sorbents which had been partially coated with covalently immobilized protein could still function in ion-exchange chromatography and if so, would the immobilized protein play any role in the retention process. These questions were examined in three ways; by (i) ionic strength gradient elution of protein mixtures under conditions which give ion-exchange separations on the AC-1 and AC-6 sorbents, (ii) comparing the resolution of protein mixtures on underivatized and the protein derivatized sorbents, and (iii) contrasting analyte *Z* numbers [6] on the derivatized and underivatized sorbents.

3.3.1. Retention time

Chromatographic retention, i.e. the capacity factor (k'), is related to the available surface area (A_s) of a sorbent by the equation

$$k' = K_d(A_s/V_m) \quad (1)$$

where K_d is the chromatographic distribution coefficient and V_m is the volume of the mobile phase. Assuming that immobilized proteins do not function in the ion exchange adsorption of proteins, the available surface area (A_s) of protein derivatized sorbents would be

$$A_s = A_t - A_p \quad (2)$$

where A_t is the total surface area and A_p is the surface area occupied by the protein. Based on previous work [6] it is thought that $A_s = 0.5\ A_t$. Although elution of protein analytes were achieved by gradient elution in these studies, it is to be expected from the equations above that if analytes are adsorbed to residual cation-exchange surface that elution times will be shorter than those of the underivatized sorbent.

It is shown in Fig. 1 and Table 2 that this is not the case. All the AC-6 derivatized sorbents retained basic proteins in the cation-exchange mode at least as well as the underivatized ion-exchange sorbent. Furthermore, the separation achieved with the BSA derivatized sorbent appears to be superior to that with the underivatized ion-exchange sorbent. The AC–LYS column retained the three test proteins, in a 12 min separation, lysozyme is retained approximately 1 min longer on the AC–LYS, AC–BSA, and AC–MYO columns than on the AC-6 cation exchanger. Cytochrome *c* is also retained slightly longer by these columns. This allows us to conclude first that the residual surface of a sorbent not derivatized with protein is capable of cation-exchange chromatography.

Fig. 1 and Table 2 show that protein derivatized, weak cation-exchange sorbents adsorb cationic proteins at low ionic strength and release them during the course of ionic strength gradient elution. It is apparent from these results that derivatization of the weak cation-exchange sorbents AC-1 and AC-6 with

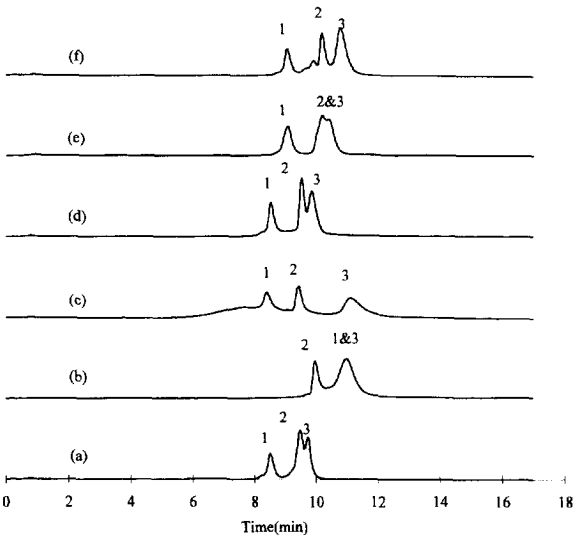

Fig. 1. Resolution of α-chymotrypsinogen A (1), cytochrome *c* (2), and lysozyme (3) by AC-6 (a), AC–LYS (b), AC–α-CHYM A (c), AC–CYT (d), AC–MYO (e), and AC– BSA (f). Columns 50×4.6 mm I.D: mobile phase: A, 10 m*M* phosphate buffer pH 7.0; B, 1.0 *M* NaCl in 'A'; flow-rate: 1.0 ml/min; gradient: 0–100% B in 15 min; detection: UV 280 nm.

protein did not diminish their macromolecular ion-exchange characteristics. In fact, the separation achieved with the BSA derivatized sorbent appears to be superior to that achieved with the underivatized AC-6 ion exchange sorbent. It will also be noted that on all the protein derivatized columns except AC–CYT at least one of the three test proteins, lysozyme, cytochrome *c*, or α-chymotrypsinogen, was retained longer than on underivatized AC-6. For example, in a 12 min separation, lysozyme is retained more than 1 min longer on the AC–LYS while α-chymotrypsinogen was retained almost 2.5 min longer. The AC–BSA column also retains lysozyme 1 min longer, but shows only 0.5 min increase with cytochrome *c* and α-chymotrypsinogen. AC–MYO increased the elution time of all three test analytes by approximately 0.5 min. These results allow us to conclude that covalently immobilized proteins which only partially cover the surface of a weak cation-exchange sorbent (i) may contribute to the ion-exchange chromatographic behavior of certain protein analytes, (ii) but not to the same extent with all analytes, and (iii) some immobilized proteins, such as cytochrome *c*, do not interact with protein analytes at all.

Further studies with anionic proteins such as soybean trypsin inhibitor, conalbumin, β-lactoglobulin, and myoglobin, showed that these proteins were not retained on either the underivatized AC-6 weak cation exchanger, the AC–BSA column, or the AC–LYS column [data not shown]. This means that the immobilized proteins are not acting alone as stationary phases. Electrostatic adsorption is a prerequisite for analyte protein/immobilized protein interactions of sufficient magnitude to impact ion-exchange separations.

3.3.2. *Influence of immobilized protein concentration*

A series of sorbents based on AC-6 with increasing concentration of immobilized lysozyme were prepared by varying the coupling time. The concentration of immobilized protein in mg/m^2 and the designator, in parentheses, are as follows; 1.22 (AC-6-1), 1.34 (AC-6-2), 2.0 (AC-6-3), and 2.16 (AC-6-4). Protein concentration was determined by elemental analysis. With the most lightly loaded AC-6-1 sorbent, i.e. 1.22 mg/m^2, retention of all three proteins increased without appearing to change selectivity (Fig. 2). There was also a substantial increase

Table 2
The effect of immobilized proteins on some selected parameters

Column	Retention time (t_R)			Selectivity (α)	
	α-CHYM A	CYT *c*	LYS	α-CHYM A–CYT c	CYT *c*–LYS
AC–LYS	10.98	9.97	10.98	0.91	1.10
AC–CYT *c*	8.52	9.52	9.85	1.13	1.03
AC–α-CHYM	8.39	9.42	11.10	1.13	1.19
AC–MYO	9.06	10.19	10.39	1.13	1.06
AC–BSA	9.04	10.18	10.79	1.13	1.06
AC-1–LYS	10.54	9.15	11.62	0.86	1.28

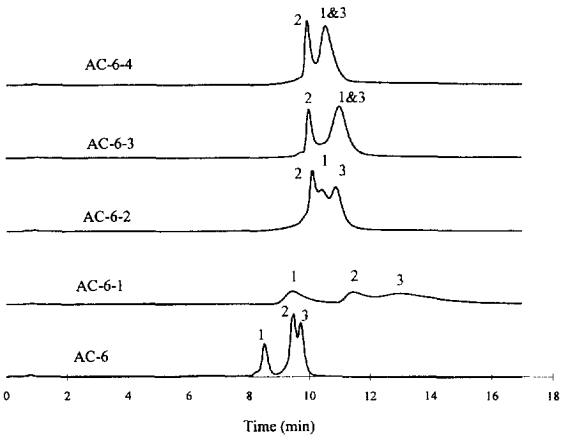

Fig. 2. The selectivity differences of lysozyme immobilized columns; AC-6 (polymeric weak cation-exchange sorbent), AC-6-1, AC-6-2, AC-6-3, and AC-6-4 are sorbents with immobilized lysozyme ligand density of 1.22, 1.34, 2.0, and 2.16 mg/m^2, respectively. Conditions are as given in Fig. 1.

in band spreading. One of the unexpected and currently unexplainable observations associated with this study was that increases in retention were smaller at higher protein loading than at 1.22 mg/m^2. As noted, elution order on the underivatized AC-6 sorbent was from α-chymotrypsinogen, and cytochrome c, to lysozyme in increasing order. With increasing lysozyme loading the retention time of α-chymotrypsinogen increased until it coeluted with lysozyme on the 2.16 mg/m^2. In this regard, α-chymotrypsinogen was much more sensitive to immobilized protein concentration than lysozyme and cytochrome c which increased in retention only slightly at higher protein concentration.

3.3.3. Resolution

It was concluded that resolution measurements would be a better indicator of chromatographic differences between underivatized AC-6 sorbent and the protein derivatized sorbents than to report column efficiency, i.e. theoretical plates, or selectivity. Resolution of the analyte pairs α-chymotrypsinogen A/cytochrome c, α-chymotrypsinogen A/lysozyme, and cytochrome c/lysozyme with the various immobilized protein columns is seen in Table 3. In virtually all cases, resolution was different on the immobilized protein columns than on the underivatized weak cation exchanger. The most dramatic case was the decline in resolution of the α-chymotrypsinogen A/lysozyme pair from 2.85 on the AC-6 column to zero on the AC–LYS column. In contrast, resolution of the cytochrome c/lysozyme pair increased from 0.62 on the AC-6 column to 2.50 on the AC–α-CHYM A column. These large differences in resolution on columns that are identical in all respects except the immobilized protein are further confirmation of the fact that immobilized protein plays a role in the chromatographic behavior of protein analytes.

3.3.4. Z numbers

This section of the paper explores the nature of the interactions described above by application of the stoichiometric displacement model (SDM) of retention for ion-exchange chromatography. The SDM is used to predict the number of charged groups on a protein that interact with the surface of an ion-exchange sorbent. This model is based on the concept that reversible electrostatic association of a protein with a sorbent may be described by the equilibrium constant (K_b)

$$K_b = [P_b][S_o]^z/[P_o][S_b]^z \quad (3)$$

where $[P_o]$ is the protein concentration in solution, $[S_b]$ is the concentration of bound displacing ion,

Table 3
R_s values of three basic proteins obtained with immobilized protein columns

Column	$R_{s\,\alpha\text{-CHYM A–CYT }c}$	$R_{s\,\alpha\text{-CHYM A–LYS}}$	$R_{s\,\text{CYT }c\text{–LYS}}$
AC-6	2.13	2.85	0.62
AC–LYS	1.45 (−0.68)[a]	–	1.45 (0.82)
AC–BSA	3.06 (0.93)	3.4 (0.55)	1.20 (0.58)
AC–MYO	2.08 (−0.05)	2.57 (−0.28)	0.40 (−0.22)
AC–α-CHYM A	1.50 (−0.63)	2.79 (−0.06)	2.50 (1.88)
AC–CYT	3.03 (0.9)	3.21 (0.36)	0.43 (−0.19)

[a] $\Delta R_s = R_s$ (AC–Protein) − R_s(AC-6).

$[P_b]$ is bound protein, $[S_o]$ is the concentration of the displacing ion in solution, and Z is the number of ions displaced when a protein binds to the sorbent [10]. It has been shown that this equilibrium may be related to chromatographic capacity factor (k') by the equation

$$\log k' = Z \log(1/[S_o]) + \log I \quad (4)$$

where $I = K_b \phi [S_b]^z$. The constants K_b and $[S_b]^z$ are defined above and the constant ϕ is the chromatographic phase ratio. Plots of Eq. 4 allow Z values, which are thought to be the number of charged groups on a protein analyte that interact with the surface of an ion-exchange sorbent, to be obtained graphically from the slope.

It is seen in Table 4 that the Z number for immobilized protein sorbents is larger than with AC-6 in several cases. For example, the Z number for α-chymotrypsinogen A is from 0.6 to 2.2 units higher in all cases. It is with the AC–LYS column that the Z number is elevated 2.2 units. The interpretation of these results is that at least a part of the increased retention of α-chymotrypsinogen A is due to a positive electrostatic interaction between the analyte and immobilized lysozyme involving a charged group, or groups, not involved in electrostatic adsorption to the sorbent surface. Fractional Z numbers are explained as being due to either partial ionization of the group(s) involved or molecular motion causes charged groups to be in transitory contact with each other. In contrast, the fact that the retention time of lysozyme is over 1 min longer on the AC–LYS column than the AC-6 column with relatively little change in the Z number indicates that some other force is at work to increase retention in this case. This most likely is due to some other type of interaction such as hydrophobic or hydrogen bonding. Unfortunately, the SDM has not been developed to the point that it may be used for the analysis of interactions other than electrostatic.

Table 4
Retention characteristics of immobilized protein columns

Column	Z numbers		
	α-CHYM A	CYT *c*	LYS
AC-6	3.05	4.20	3.55
AC–LYS	5.23	4.19	3.71
AC–CYT *c*	3.63	3.90	3.77
AC–BSA	3.72	4.20	3.75
AC–MYO	3.73	4.19	3.9

3.4. Implications

It has been demonstrated above that proteins covalently immobilized on the surface of a chromatography sorbent which only partially coat the surface of the sorbent may alter the chromatographic behavior of protein analytes. Although these immobilized proteins are not free to move across the surface of a sorbent, i.e. linear and some rotational diffusion is restricted, it is assumed that there is some similarity to electrostatically adsorbed proteins. The phenomena reported above could have important implications in both analytical and preparative chromatography.

First, there is the prospect that irreversibly adsorbed or denatured macromolecular analytes which have accumulated on a chromatography column will alter column selectivity. It is apparent that reproducibility of separation methods could be compromised by "dirty" columns.

Second, it is possible that at higher protein loading, lateral interactions between the same or dissimilar analyte species could impact the chromatographic behavior of some analytes. As opposed to covalently immobilized proteins which are diffusionally restricted, electrostatically adsorbed proteins would be free to diffuse and form complexes in which the energy of interaction is maximized. This would mean that in preparative separations of complex mixtures at high sample loading, the chromatographic behavior of early eluting species could be strongly impacted by large amounts of later eluting constituents. If true, this means that the chromatographic behavior of some species will be determined by other components in the mixture and not by their own adsorption properties alone. This makes it particularly difficult to model preparative chromatographic separations of complex mixtures.

4. Conclusions

It may be concluded from the data presented above that (i) weak cation-exchange sorbents partially coated with covalently immobilized protein can

still function in the cation-exchange mode, (ii) proteins immobilized on these sorbents can influence the chromatographic behavior of cationic proteins being separated in the cation-exchange mode, (iii) when immobilized proteins impact chromatographic behavior they do so by interacting with proteins in a manner that increases chromatographic retention, (iv) the extent of the interaction can depend on the concentration of immobilized protein, (v) interactions between the analyte and immobilized protein may occur between both the same two proteins and dissimilar species, (vi) electrostatic adsorption is a prerequisite to further interaction(s) with immobilized proteins and (vii) lateral interactions between the analyte protein/immobilized protein is responsible for the contribution of immobilized proteins to the chromatographic behavior of protein analytes.

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