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Adsorption of BSA and Hemoglobin on Hydroxyapatite Support: Equilibria and Multicomponent Dynamic Adsorption

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Abstract. Interactions of Bovin Serum Albumin and Hemoglobin with an hydroxyapatite gel (HA-Ultrogel, Sepracor), have been studied separately in batch experiments. The adsorption isotherms are of the Langmuir type and can be used directly to scale column operations.

For adsorption of hemoglobin alone, in column at pH 6.8 (equal to its isoelectric point) we notice that a classical intraparticle transfer model, based on a constant effective diffusion coefficient represents perfectly the symmetrical breakthrough curve. For acid pH values (pH 5.8), Langmuir isotherms of BSA and hemoglobin adsorptions showed a strong curvature, sign of a quite irreversible adsorption and breakthrough curves obtained under these conditions, exhibit a high dissymmetrical shape for both proteins. In that case, a model of diffusion based on the adsorption on two types of independent sites, with two intraparticle transfer coefficients, gives a good representation of the breakthrough for adsorption of both proteins separately.

Binary mixtures of these components were prepared and injected in columns packed with the same support. Competitive Langmuir equation, based on the results obtained in monocomponent batch experiments, give a very good fit to our system. The intraparticle transfer in that case seems to be facilitated, and one effective coefficient alone is enough to predict the breakthrough curves obtained. This behaviour may be the result of an increase of the solution ionic strength, and of the smaller irreversibility feature of the adsorption when proteins are in competition.

Keywords: chromatography, multicomponent, intraparticle diffusion, experimental data, proteins

Introduction

There is considerable industrial interest in isotherm scale-up and optimisation of chromatographic operations used in the recovery and purification of biomolecules. During one of the chromatographic steps of the purification of an enzyme from a bacterial broth, we faced the problem of the competition for adsorption on an hydroxyapatite gel between this enzyme and several other proteins including albumin. Till now, hydroxyapatite supports (HA) have been used in a rather empirical fashion for biomolecule separations. Since their mechanical resistance has been improved (crystals supported on macroporous silica (Bruno et al., 1990) or agarentrapped (Sindhuphak et al., 1984)), they represent a powerful potential for protein and nucleic acid separations in aqueous systems even in High Pressure Liquid Chromatography (Bruno et al., 1990; Hjerten et al., 1988; Lindeberg et al., 1990). A number of studies on chromatographic adsorption have been made to try to understand the interaction mechanisms involved in protein binding and elution (Bernardi et al., 1972; Gorbunoff, 1984a, 1984b; Gorbunoff and Timasheff, 1984; Kawasaki, 1991). But they still remain rather unpredictable since they depend mainly on tridimensional protein structure and on high surface densities or clusters of ionized groups. Furthermore, they have never been quantified. It appeared then very interesting to try to characterize and modelize mono and further multicomponent protein adsorption on such

a support. We chose two proteins, bovin serum albumin (BSA) and bovin hemoglobin (Hb) to realize this study.

The major factors affecting the adsorption processes in a chromatographic operation are the equilibrium and the rate to reach this equilibrium. Here, we measure adsorption isotherms of these two proteins separately on a gel hydroxyapatite support (HA-Ultrogel, IBF-Sepracor) at different pH. Assuming that the overall resistance to diffusion may be represented by film and internal diffusion, we first apply the equilibrium parameters obtained to the simulation of chromatographic adsorption operations of BSA and Hb alone on HA-Ultrogel. Then we study the multicomponent adsorption of both proteins. In order to put them in favourable conditions for competition, we use chemical environment such as we obtain a great adsorption of both of them, in an acid phosphate potassium buffer (pH = 5.8), where their adsorption capacities are similar and high.

Materials and Methods

Materials

HA-Ultrogel was purchased from IBF-Sepracor (Villeneuve-la-Garenne, France). This gel belongs to the class of agar-entrapped supports and HA crystals are made according to Tiselius method (1956). This support is stable for pH range between 4 and 13 and a temperature range of 4 to 121°C. Bead diameters range from 60 to 180 μm.

Proteins used in this study are bovin serum albumin (A-7906) and bovin hemoglobin (H-2625) from Sigma. BSA has a relative molecular mass of 66000 daltons and an isoelectric point (pI) of 4.9, whilst Hb has a relative molecular mass of 68000 daltons and a pI of 6.8. Because of the possible denaturation of proteins, protein solution and support were usually conditioned in a potassium phosphate buffer (KP buffer).

Batch Experiments

A concentrated protein solution is first dialysed against dissolving solution in order to eliminate salts or other pollution that might be in the freeze-dried protein powder. This solution is filtered in order to eliminate non-dissolved materials.

The support is washed in a column by two interstitial volumes of concentrated potassium phosphate buffer (0.5 M) before use and then conditioned with the appropriate solvent. In order not to damage the gel structure we avoid drying it before use and choose to use settled support quantities packed into small graduated microtubes. The volume used is of 1.5 ml. The gel dry weight for each batch experiment is determined afterwards and corrected for weight of the adsorbed proteins. We transfer the gel in a volume V_0 (20 ml) of protein solution at a given initial concentration C_0. The Nalgen tubes in which adsorption takes place are gently shaken for 24 hours at room temperature (20°C). The concentration of the protein solution and the equilibrium pH are then measured. The C^* protein concentration obtained corresponds to a final volume V which includes V_0 plus the external solvent volume in gel volume.

Analysis. BSA initial and final concentrations are measured by UV absorbance at 280 nm. For hemoglobin we use the wavelength of 406 nm. The optical densities are converted to concentrations by reference to calibration data.

Calculation. Adsorption isotherm points are calculated by mass balance in the batch:

$$q^* = \frac{C_0 V_0 - C^* V}{m} \quad \text{with } m = \text{dry weight of HA-Ultrogel.} \qquad (1)$$

Packed-Bed Experiments

We use a glass column of 0.9 cm or 1 cm diameter depending on the experiment. The volumetric flow rate is around 1 ml/min in a downward direction. The support is conditioned with the buffer solvent. At time zero the column is fed with the proteic solution and follow in line the outlet stream by its optical density with an Hitachi spectrophotometer (U-2000) having a flow-through compact cell of 1 mm optical length. The wave lengths used are 280 nm for BSA and 406 nm for Hb. We also collect fractions at the column outlet which are further analysed to check protein concentrations. In the case of binary protein mixtures, the optical densities are measured both at 280 nm and 406 nm in the fractions collected. The latter value gives the hemoglobin concentration alone. According to calibration data of hemoglobin absorbance at 280 nm, we calculate its contribution to the total optical density of the mixture at 280 nm and deduce the absorbance of BSA alone in the sample. Consequently, its concentration by reference to calibration data is determined.

Experiments are stopped when the support is saturated by the protein solution.

Results and Discussion

1. Properties of the Hydroxyapatite

One of the first users of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) for protein chromatography was Tiselius in 1956. Bernardi et al. (1972) studied adsorption/desorption of several amino acids and polypeptides, and these studies were carried on by Gorbunoff (1984a, 1984b), Gorbunoff and Timasheff (1984) and Kawasaki (1991) on more than 20 proteins. Crystallographic and experimental observations lead to the existence of two types of adsorbing sites:

— The first, called calcium site, is composed by a lack of hydroxyl ion on the crystal surface inserted by two calcium ions. These ions carrying positive charges can be compared to an anion exchanger site on which a phosphate anion from a phosphate buffer and a phosphate or carboxylic group on a protein can adsorb. This takes place on a crystallographic surface called "a" by Kawasaki (1991).
— The second type of site is a phosphate one. One can consider two different phosphate sites consisting of a lack of calcium ions, one surrounded by six negatively charged oxygen atoms belonging to three crystal phosphates, the other formed by three oxygen atoms belonging to three other crystal phosphates. These "cation exchanger" sites are not localised on the same crystal surface as calcium sites. This surface is named "c" (Kawasaki, 1991).
— Conclusions on the *behaviour of acid and neutral proteins* on hydroxyapatite were made by Gorbunoff (1984a, 1984b); Gorbunoff and Timasheff (1984) and Kawasaki (1991) deduced from elution experiments in column.
— At a neutral pH, and acid protein like BSA (isoelectric point pI < 5) would bind chiefly on calcium sites by its carboxylic groups. It is only eluted by anions such as phosphate ions, but not by chloride ions Cl^- which have no ability to form complexes with calcium. It is not eluted by monovalent cations such as Na^+ or K^+ and furthermore divalent cations (Ca^{2+} or Mg^{2+}) reinforce its adsorption and create new adsorption links.
— In the same conditions, a neutral protein like hemoglobin (pI ≈ 7) would adsorb on the two types of sites. It is eluted by phosphate anions and monovalent cations but not by divalent cations (Ca^{2+} or Mg^{2+}) which would reinforce its binding to calcium sites.

2. Equilibrium Isotherms of BSA and Hb

Conditions of isothermal adsorption experiments are given in Table 1. Figures 1 and 2 show the effect of pH on BSA and Hb adsorptions respectively, for a constant KP buffer concentration of 10 mM. For a pH of 7.5, BSA was not retained anymore on this support. Experiments of BSA elution on HA-Ultrogel, identical to those achieved by Gorbunoff and Timasheff (1984) on hydroxyapatite crystals were performed to appreciate the mechanism of protein adsorption and are given elsewhere (Fargues, 1993).

Figure 1 shows that BSA adsorption increases when the pH of the solution decreases. For pH 7.5, 6.75 and 5.7, the BSA carries a net charge less and less negative

Table 1. Langmuir parameters calculated from experimental results for BSA and Hb.

Experimental conditions		Langmuir parameters	
	pH	q_s (mg/g dry)	K (ml/mg)
BSA	5.7	67 (±2)	19 (±2)
	6.75	17.4 (±0.6)	0.7 (±0.1)
Hb	5.75	76 (±2)	165 (±40)
	6.7	110 (±5)	4.5 (±0.8)
	7.4	65 (±4)	1.3 (±0.3)

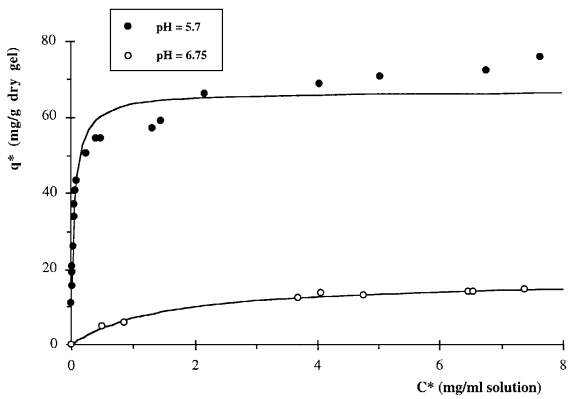

Figure 1. Effect of pH on equilibrium adsorption isotherms for BSA on HA-Ultrogel in KP buffer 10 mM. Continuous lines correspond to Langmuir equation.

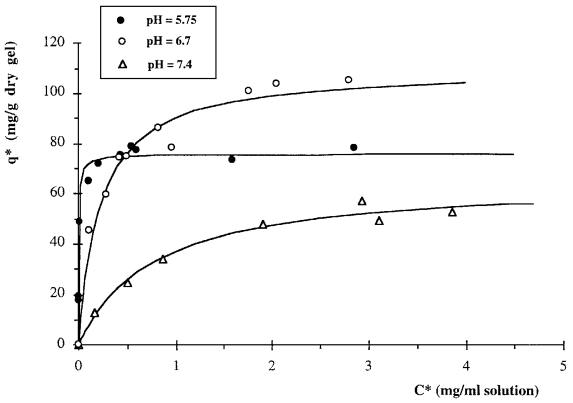

Figure 2. Effect of pH on equilibrium adsorption isotherms for hemoglobin on HA-Ultrogel in KP buffer 10 mM. Continuous lines correspond to Langmuir equation.

(respectively -15, -10 and -2) and also the ionic strength of the buffer decreases (respectively 0.026 M, 0.02 M and 0.01 M) due to a decreasing concentration in divalent phosphate species of the buffer, HPO_4^{2-} ions. Generally, the global charge of a protein is only an indication to the electrostatic interactions that may occur, but it has been proved that it is rarely the main parameter of the retention: local high concentrations of same charges are the leader of adsorption and sterical hindrance prevent all the ionised groups on the protein surface to interact with a support (Norde, 1986; Regnier, 1987). It has even been noticed a maximal adsorption for some proteins near their isoelectric points, explained by a minimal denaturation of the protein at this pH value when it exhibits its most compact shape (Norde, 1986). At its pI, the molecule presents also a minimum net charge, which minimises electrostatic repulsions with an ionic surface (Koutsoukos et al., 1982; Kopaciewicz et al., 1983). We notice the same behaviour here, where adsorption is the highest for the smallest net charge. But the fact that no adsorption occurs under pH 7.5, where it exhibits a net charge of -15, would show that few interactions are developed by anion exchange on the calcium sites. Some experiments on BSA elution by potassium salts (KCl and KP buffer) as made by Gorbunoff and Tinasheff (1984) were achieved in columns and confirmed the differences between HA-Ultrogel and hydroxyapatite crystals: the BSA is eluted by the same molarity of KCl solution than KP solution (Fargues, 1993). From what was said in Part 1, if chloride ions elute the BSA it means that this protein is not mainly adsorbed on calcium sites. It probably binds partly on the two types of sites, but with a predominant adsorption on phosphate sites. Great adsorption of the BSA at pH 5.7, is then explained by the contribution of more numerous positive local charges present on this macromolecule. The weaker ionic strength of the buffer also goes in the direction of a better adsorption.

For the hemoglobin, adsorption reaches a maximum at pH 6.7, near the isoelectric point of the protein ($pI_{(Hb)} = 6.8$) (Fig. 2). According to De Bruin et al. (1969), the net charge of Hb would be around $+13$ at pH 5.75, $+5$ at $pH = 6.7$ and -1 at $pH = 7.4$. In the present case, the high adsorption found when Hb carries a positive net charge would lead to the conclusion that adsorption on HA-Ultrogel would occur mainly by cation exchange on phosphate sites. At the lowest pH (5.75) the high positive net charge of the protein leads to a strong electrostatic interaction with the support which is shown by the strong curvature of the isotherm. We have no definitive explanation for the variation of the maximum adsorption capacity between pH 6.7 and 5.75. This decrease is perhaps due to a partial denaturation of the tetrameric hemoglobin molecule.

The solid lines in Figs. 1 and 2 correspond to Langmuir fit, which is the equation most commonly used to describe adsorption phenomena in various systems:

$$q^* = \frac{q_s K C^*}{1 + K C^*} \tag{2}$$

with

q^* = equilibrium concentration of adsorbed solute (mg or mol/unit adsorbent)
q_s = maximum concentration of adsorbed solute (mg or mol/unit adsorbent)
C^* = equilibrium solution concentration of solute (mg or mol/unit solution)
K = Langmuir equilibrium constant or affinity parameter (unit solution/mg or mol).

Despite the fact that the assumptions involved in Langmuir isotherms are seldom satisfied in the case of protein adsorption (Norde, 1986) it provides a good fit to many systems. Its main assumptions are:

— one molecule adsorbs on one site and the number of sites is limited;
— the adsorption is reversible;
— there are no lateral interactions between adsorbed solute molecules.

It has been used successfully by several authors to describe BSA adsorption (Leaver, 1984; Skidmore and Chase, 1988; Skidmore et al., 1990; Yoshida et al., 1993, 1994). Huang and Horvath (1987) used it for peptides adsorption and Arve and Liapis (1987) or Whitley et al. (1989) for proteins on several ion exchangers.

The Langmuir coefficients obtained are shown in Table 1. They give a very good fit of Hb adsorption at all pH values. The adsorption capacity of the protein decreases at pH 5.75 compared to pH 6.7 but the K affinity parameter increases: adsorption isotherm becomes nearly rectangular as discussed above and perhaps partly irreversible, which could be due to some denaturation of the protein. Concerning the adsorption of BSA, the Langmuir equation represents the isotherm at pH 6.75. At pH 5.7, a good fitting cannot be obtained mainly due to the fact that experimental points are not on a plateau. This is probably due to the fact that it can bind on the two types of sites a and c. As adsorption on phosphate sites would be the most important, it is probably the one that occurs mainly for small concentrations of BSA. When the concentration increases, then the contribution of the second type of sites (calcium sites) becomes more visible. This is shown in Fig. 1 by the points for $C > 4$ g/l. As for Hb the K value is higher at low pH.

This study provides parameters which are directly usable for scale-up of chromatographic columns. We conclude here on the relative similarity of behaviour of the two proteins on HA-Ultrogel despite their very different isoelectric points ($pI_{Hb} = 6.8$, $pI_{BSA} = 4.9$). Albumin and hemoglobin in mixtures probably compete for adsorption on HA-Ultrogel by their common adsorption on phosphate sites, by cation exchange. For adsorption in potassium phosphate buffer, 10 mM/pH = 5.7, they exhibit identical maximum capacities which would support the use of a competitive Langmuir equation for binary adsorption:

$$q_{s\,(\text{hemoglobin})} = 76\ \text{mg/g} = 0.00112\ \text{mmol/g} \quad (\text{MW}_{\text{Hb}} = 68000\ \text{daltons})$$

$$q_{s\,(\text{albumin})} = 67\ \text{mg/g} = 0.00102\ \text{mmol/g} \quad (\text{MW}_{\text{BSA}} = 66000\ \text{daltons}).$$

3. Packed-Bed Studies: Dynamic Adsorption

Frontal experiments were performed to test whether isotherm results of BSA and Hb uptake on HA-Ultrogel, as determined in stirred tank, could be used directly to describe and further predict adsorption in packed bed of this support.

(a) Single-Component Adsorption Fronts. Experimental conditions are given in Table 2. Given the low adsorption of BSA at neutral pH, we perform the experiments with BSA at pH 5.8 only.

Breakthrough curve of the hemoglobin at pH 6.8 exhibits a symmetrical shape, which is perfectly fitted by a classical model based on a single and constant effective particle diffusion coefficient D_e (Fig. 3). The model developped to describe this curve is based on the use of equilibrium isotherms of Langmuir type as described before (Eq. (2)) and on two physical limitations of kinetics, both diffusion of the protein from the bulk solution to the particle surface and diffusion within the gel particles. It also includes axial dispersion in the column. The equations used are the following:

• *Differential mass balance of component i*:

$$u_i \frac{\partial C_i}{\partial z} + \frac{\partial C_i}{\partial t} + \rho_a \frac{1-\varepsilon}{\varepsilon} \frac{\partial \bar{q}_i}{\partial t} = D_{\text{ax}} \frac{\partial^2 C_i}{\partial z^2} \quad (3)$$

Table 2. Packed-bed experimental conditions for mono-component adsorption fronts.

Protein	KP buffer 10 mM	$(OD)_o$ (1 mm)	C_o (g/l)	m (g dry gel)	V_r (ml)	V_p (ml)	Flow-rate (ml/min)
BSA	pH = 5.7	0.325 (280 nm)	4.9	2.56	14.8	5.9	0.73
Hb	pH = 6.8	0.332 (406 nm)	0.501	0.72	4.63	1.85	1.034
	pH = 5.8	0.314 (406 nm)	0.47	0.84	5.5	2.2	1.03

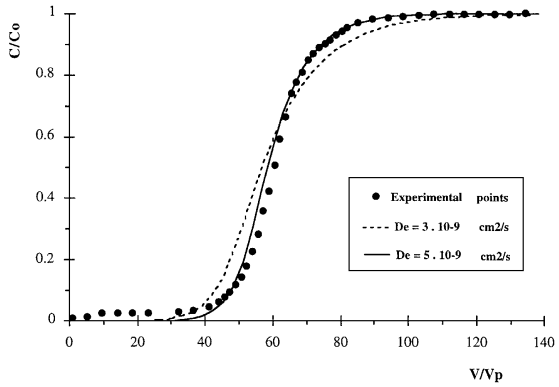

Figure 3. Experimental and calculated breakthrough curves of hemoglobin on HA-Ultrogel in KP buffer 10 mM, pH = 6.8. Solid and dashed lines are model results corresponding to $k_f = 10^{-3}$ cm/s and respectively $D_e = 5 \cdot 10^{-9}$ cm^2/s and $D_e = 3 \cdot 10^{-9}$ cm^2/s.

where

ρ_a = density of solid phase
$\bar{q}_i$ = average adsorbed solute i concentration in the adsorbent particle $= \frac{3}{r_o^3}\int_0^{r_o} r^2 q_i(r)\,dr$
ε = extraparticle bed porosity = 0.4.
u_i = intersticial velocity

The axial dispersion coefficient is calculated from the Peclet number based on particle diameter, $\mathrm{Pe_{ag}}$, which is near 1:

$$D_{\mathrm{ax}} = \frac{u_i d}{\mathrm{Pe_{ag}}} \ (d = \text{particle mean diameter}).$$

• *Film and particle diffusion*

Film Diffusion. Mass transfer to the surface of the adsorbent is governed by a film model characterized by a mass transfer coefficient k_f. Accumulation of the protein i in the gel at each point in the bed is expressed by:

$$\frac{d(\rho_a \bar{q}_i)}{dt} = a_s k_f (C_i - C_i^*), \qquad (4)$$

where C_i^* is the liquid phase concentration in equilibrium with q_i at the solution/particle interface ($= q_i(r_o)$). Molecular diffusion coefficients D of BSA and hemoglobin being very similar ($D \approx 6 \cdot 10^{-7}$ cm^2/s according to Cussler (1984) and Tyn and Gusek (1990)), we use only one value for the external coefficient k_f. It is calculated according to the correlation given by Kataoka et al. (1972) which corresponds to our hydrodynamic conditions.

Diffusivity within the Particle. A bead of HA-Ultrogel is made of numerous hydroxyapatite crystals entrapped in an agarose gel (IBF, Product information 200909IBF). It is considered as an homogeneous material in which the solute diffuses in a manner described by a constant effective particle diffusion coefficient, D_e. This coefficient takes into account internal porosity and tortuosity for protein i. Mass balance on a slice dr of the particle gives the relation:

$$\frac{\partial q_i}{\partial t}(r,t) = D_{ei}\left(\frac{\partial^2 q_i}{\partial r^2} + \frac{2}{r}\frac{\partial q_i}{\partial r}\right) \qquad (5)$$

where q_i = local concentration in the particle, function of time and position r. D_{ei} will be adjusted on the experimental monocomponent curves.

• *Boundary conditions*

In fluid phase:

for $t = 0$, $\quad C_i(z) = 0$

for $z = 0$, $\ \forall t$, $\quad u_i C_i = u_i C_{io} + D_{\mathrm{ax}} \dfrac{\partial C_i}{\partial z}$

for $z = L$, $\ \forall t$, $\quad \dfrac{\partial C_i}{\partial z} = 0$

In solid phase:

for $t = 0$, $\quad q_i(r) = 0$

for $r = 0$, $\ \forall t$, $\quad \dfrac{\partial q_i}{\partial r} = 0$

for $r = \dfrac{d}{2} = r_o$, $\ \forall t$, $\quad \dfrac{d(\rho_a q_i)}{dr} = \dfrac{k_f}{D_e}(C_i - C_i^*)$

This model has been implemented in our laboratory (Chue et al., 1993) and successfully used to represent the adsorption on QMA-Sphérosil and DEAE-Sphérodex supports of the BSA and hemoglobin separately, respectively under pH 7 and 9 (Schanen et al. (1992)).

In our case, Fig. 3 shows that the best fit is with $D_e = 5 \cdot 10^{-9}$ cm^2/s, value very close to the intraparticle transfer coefficient found for hemoglobin by Schanen et al. (1992) which was $D_e = 3 \cdot 10^{-9}$ cm^2/s.

At pH 5.8, we notice that adsorption fronts for hemoglobin (Fig. 4) as for BSA (Fig. 5) exhibit a great tail towards the plateau value, around $C/C_o = 0.95$, whereas the initial part is much steeper. Simulations by the previous classical model give a poor agreement, and the internal coefficient should then be smaller than

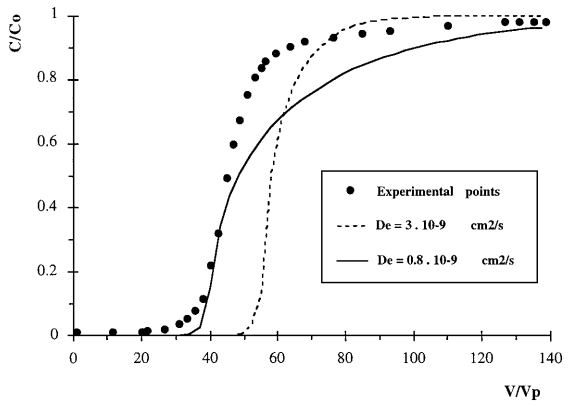

Figure 4. Experimental and calculated breakthrough curves of hemoglobin on HA-Ultrogel in a KP buffer 10 mM, pH = 5.8. Solid and dashed lines are model results corresponding to $k_f = 10^{-3}$ cm/s and respectively $D_e = 0.8 \cdot 10^{-9}$ cm^2/s and $D_e = 3 \cdot 10^{-9}$ cm^2/s.

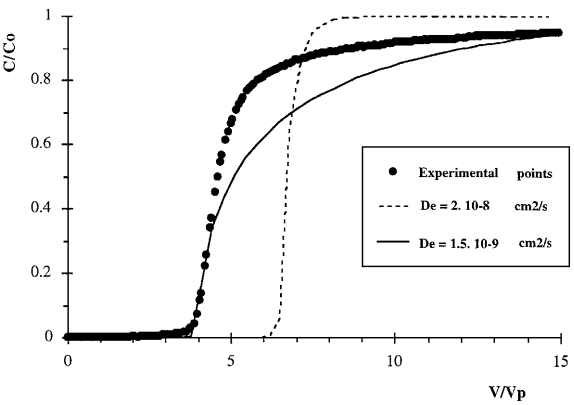

Figure 5. Experimental and calculated breakthrough curves of BSA on HA-Ultrogel in a KP buffer 10 mM, pH = 5.7. Solid and dashed lines are model results corresponding to $k_f = 10^{-3}$ cm/s and respectively $D_e = 1.5 \cdot 10^{-9}$ cm^2/s and $D_e = 2 \cdot 10^{-8}$ cm^2/s.

the values found by other authors: Concerning the hemoglobin, we found $D_e = 0.8 \cdot 10^{-9}$ cm^2/s, value six times smaller than the one used for pH = 6.8. Concerning the BSA, $D_e = 1.5 \cdot 10^{-9}$ cm^2/s, to be compared to $D_e \approx 2 \cdot 10^{-8}$ cm^2/s obtained by Graham et al. (1987) and Van der Wiel (1989) respectively on Sephadex A-50 and QMA-Spherosil. A lower value would produce a premature breakthrough and exaggerate the tail observed towards the plateau value; a higher one such as $2 \cdot 10^{-8}$ cm^2/s does not represent anymore the experimental tail, and leads to a very delayed breakthrough (Fig. 5).

Such disymmetrical shapes were noticed already in the case of macromolecule adsorption, where sterical hindrance as well as a tendency for an irreversible adsorption play a huge role in intraparticle transfer limitations. It was observed by Tsou and Graham (1985) and Graham et al. (1987) for BSA adsorption on a gel-like structure support, by Skidmore et al. (1990) for BSA on S Sepharose FF, or Saunders et al. (1989) for Phenylalanine on a strong cation exchange resin. It was also demonstrated that accessibility to all paths in the bead differs from one protein to another regardless to its size (Kopaciewicz et al., 1987; Skidmore et al., 1990). Models considering a restricted internal diffusion have been developed in the case of the immobilization of enzymes (Carleysmith et al., 1980; Clark et al., 1985; Hossain et al., 1986, 1992). They take into account a reduction of the effective cross section of the pores with local concentration of immobilized enzyme.

To explain the results obtained here, we should remember that the shape of the adsorption isotherms in that conditions for both proteins was very favourable, nearly rectangular (Figs. 1 and 2 for pH around 5.8) and a consecutive irreversibility of the adsorption occurs in the external layers of the particle. This leads to great sterical hindrance for adsorption into the deeper pores of the bead. This irreversibility feature may be the result of a denaturation of the proteins at this pH value. It was effectively shown by Kondo et al. (1992) that great changes in secondary structure of Hb during adsorption (reduction of α-helix content) increased when the pH decreased. On the contrary, Hb is not denatured at neutral pH, corresponding to its isoelectric point (pI = 6.8): the protein exhibits a denser shape, native one, leading probably to reduced sterical effects (Andrade, 1986), and adsorption isotherm is less favourable (Fig. 2, pH 6.8).

To take this reduced transfer kinetics into account, we propose instead of Eq. (5), a kinetic model based on the existence of two types of sites in the particle, sites 1 and 2, leading to two simultaneous intraparticular and independent kinetics for the adsorption:

$$\frac{dq}{dt} = \alpha k_1 (q^* - q_1) + (1 - \alpha) k_2 (q^* - q_2) \qquad (6)$$

with the linear approximation for the two particle diffusion coefficients (Gluekauf, 1955):

$$k_1 = \frac{15 \bar{D}_1}{r_o^2} \quad \text{and} \quad k_2 = \frac{15 \bar{D}_2}{r_o^2}$$

and

$$\alpha = \text{fraction of site 1.}$$

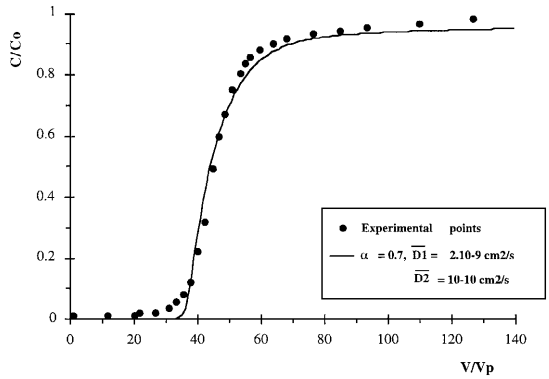

Figure 6. Experimental and calculated breakthrough curves of hemoglobin on HA-Ultrogel in a KP buffer 10 mM, pH = 5.8. Solid lines are model results corresponding $k_f = 10^{-3}$ cm/s and to Eq. (6) with $\alpha = 0.7$, $\bar{D}_1 = 2 \cdot 10^{-9}$ cm^2/s and $\bar{D}_2 = 1 \cdot 10^{-10}$ cm^2/s.

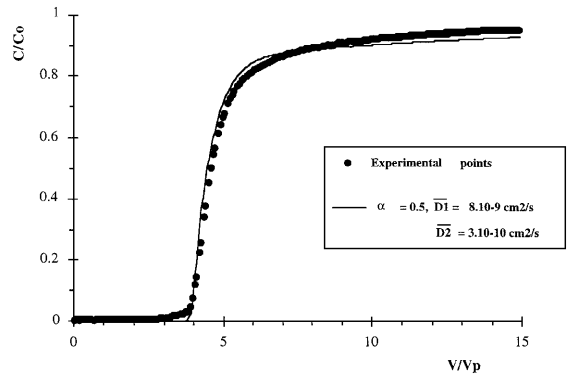

Figure 7. Experimental and calculated breakthrough curves of BSA on HA-Ultrogel in a KP buffer 10 mM, pH = 5.7. Solid lines are model results corresponding to $k_f = 10^{-3}$ cm/s and to Eq. (6) with α = 0.5, $\bar{D}_1 = 8 \cdot 10^{-9}$ cm^2/s and $\bar{D}_2 = 3 \cdot 10^{-10}$ cm^2/s.

This qualitative model, based on three parameters, gives a very good fit to our experiments at pH 5.8 (Fig. 6 for Hb and Fig. 7 for BSA). The simulations show that the rapid adsorption on site 1 can be represented by a diffusion coefficient 20 times higher than the lower one. We also notice that adsorption for BSA is faster than for hemoglobin, which reflects a smaller denaturation state. The results show the opportunity of this modeling.

(b) Binary Adsorption Fronts. In order to analyse with the same solution dilution both hemoglobin and albumin concentrations (by spectrophotometry), we use a ratio $\frac{C_{\text{albumin}}}{C_{\text{hemoglobin}}} = \frac{C_{2o}}{C_{1o}}$ constant and equal to 10 in the binary mixtures.

The development of the breakthrough curves for BSA and hemoglobin for the three sets of experimental conditions described in Table 3 are shown in Figs. 8, 9, and 10. These breakthrough profiles are typical of a competition for adsorption between two species and breakthrough volume of each component as well as the height of the plateau can be calculated

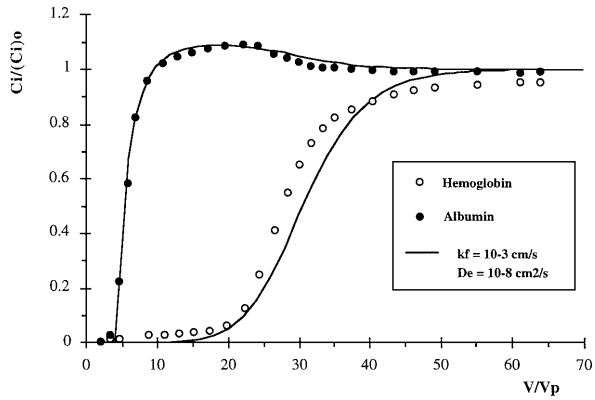

Figure 8. Experimental breakthrough curves of bovin albumin and hemoglobin mixture (set no. 1: $C_{1o} = 0.51$ g/l;$C_{2o} = 4.75$ g/l) on HA-Ultrogel in KP buffer 10 mM, pH = 5.8. Solid lines are model results corresponding to an optimized intraparticle coefficient: $D_e = 10^{-8}$ cm^2/s $- k_f = 10^{-3}$ cm/s.

according to the equilibrium theory (De Vault, 1943). BSA, which affinity for the support is ten times smaller than the hemoglobin's one (illustrated by K values, Table 1) sees its concentration rise above its inlet

Table 3. Packed-bed experimental conditions for binary adsorption fronts.

Set no.	C_{1o} (g/l)	C_{2o} (g/l)	C_{2o}/C_{1o}	m (g dry gel)	V_r (ml)	V_p (ml)	Flow-rate (ml/min)
1	0.51	4.75	9.3	0.98	5.82	2.33	0.99
2	0.76	7.52	9.9	1.83	9.86	3.9	1.12
3	1.04	9.22	8.9	1.98	11.23	4.5	1.09

(Protein 1 = hemoglobin, Protein 2 = BSA).

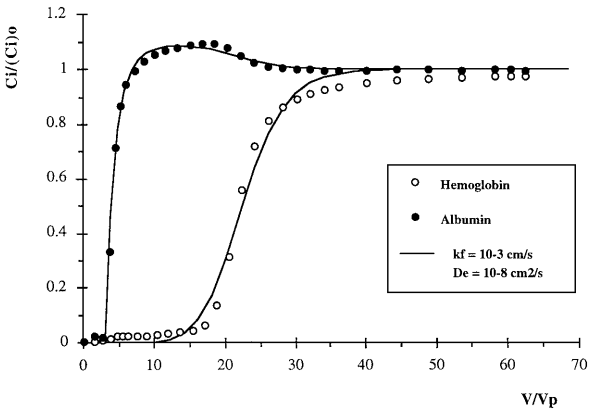

Figure 9. Experimental breakthrough curves of bovin albumin and hemoglobin mixture (set no. 2: $C_{1o} = 0.76$ g/l; $C_{2o} = 7.52$ g/l) on HA-Ultrogel in KP buffer 10 mM, pH = 5.8. Solid lines are model results corresponding to an optimized intraparticle coefficient: $D_e = 10^{-8}$ cm^2/s $- k_f = 10^{-3}$ cm/s.

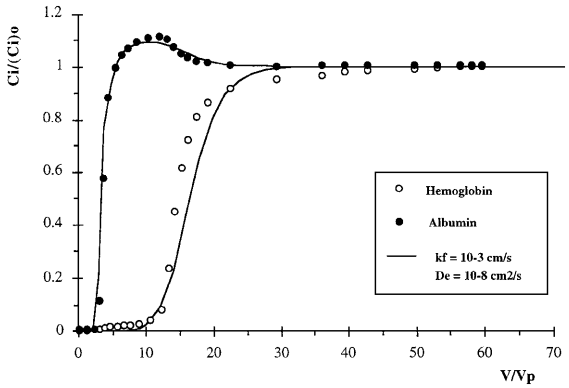

Figure 10. Experimental breakthrough curves of bovin albumin and hemoglobin mixture (set no. 3: $C_{1o} = 1.04$ g/l; $C_{2o} = 9.22$ g/l) on HA-Ultrogel in KP buffer 10 mM, pH = 5.8. Solid lines are model results corresponding to an optimized intraparticle coefficient: $D_e = 10^{-8}$ cm^2/s $- k_f = 10^{-3}$ cm/s.

value, due to displacement phenomena by hemoglobin molecules. Since the fraction of the amount adsorbed of hemoglobin becomes higher than that in the fluid phase, the weaker component (BSA) becomes in excess in the fluid phase and is then pushed to the front of the column, proceeding faster than Hb. To describe this competitive adsorption, we apply the competitive Langmuir form. For component i:

$$q_i^* = \frac{q_{si} K_i C_i^*}{1 + \sum_{i=1}^{n} K_i C_i^*} \tag{7}$$

Langmuir parameters q_{si} and K_i are those determined in the single-protein batch experiments. Additional assumptions adapted to the competition should be fulfilled:

— proteins in competition should exhibit the same maximum adsorption capacities;
— they should interact in the same way with the support;
— they should have equal sizes and distribution of charges on their surfaces.

In our case, the thermodynamic consistency which implies same maximum adsorption capacities q_{si} is fulfilled (q_{si} values in mol/g), as well as common adsorption on phosphate sites.

Table 4 gives the amounts of each protein bound to the support by mass balance from the breakthrough curves compared to the equilibrium adsorption calculated from the competitive Langmuir model. The competitive model gives a good fit with the competition occurring between hemoglobin and albumin on phosphate sites at pH = 5.8. We notice also that the breakthrough profiles are much sharper than the ones observed in the single component fronts even if we still observe a little tail on Hb breakthrough. Using the classical intraparticle model (Eq. (5)) leads to very good simulations, but with a ten times higher diffusion coefficient than found in single adsorption experiments: $D_e = 10^{-8}$ cm^2/s.

Table 4. Column adsorption results compared to batch experiment prediction (by competitive Langmuir equation).

Set no.	C_{1o} (g/l)	q_{1o} batch (mg/g)	q_{1o} column (mg/g)	Adsorption (%)	C_{2o} (g/l)	q_{2o} batch (mg/g)	q_{2o} column (mg/g)	Adsorption (%)
1	0.51	36.7	35.3	96	4.75	34.2	40.4	118
2	0.76	35.5	37.1	105	7.52	35.2	38.2	109
3	1.04	37.6	36.7	98	9.22	33.4	41.9	125

Conclusions

The competitive Langmuir equation, determined simply from monocomponent adsorption in stirred-tank, describes perfectly the competition between BSA and Hb for adsorption on HA-Ultrogel. This model should be tested for the competitive adsorption of other proteins on this support, proteins binding in a different way on hydroxyapatite sites.

Concerning the kinetics of protein transfer from liquid phase to the adsorption sites, we conclude that a great intraparticle diffusion limitation occurs when proteins adsorb separately at pH 5.8. This is the result of sterical hindrance due to the layer of protein adsorbed quite irreversibly. The gel structure of our support also probably leads to the existence of very small pores inaccessible during the time of a chromatographic experiment. In that case, a model of intraparticle diffusion based on the existence of two types of sites, adsorbing proteins simultaneously and with two independent kinetics, leads to a perfect fit of our results. It is qualitatively a very good model to fit huge limitation of intraparticle transfer, as occurs much of the time when protein adsorption is concerned.

When the two proteins adsorb simultaneously we obtain a good agreement with a constant and single diffusion coefficient. It seems that internal transfer is made easier by the adsorption of two types of proteins in competition. One idea for this acceleration of the internal mass transfer could be the decrease in the hemoglobin denaturation. The internal hydrophobic and Coulomb forces are effectively important for the preservation of the structure of the protein and it was shown that the addition of a salt or an organic solvent to a protein solution, enhancing these internal interactions, stabilise the structure of the protein (Norde, 1986; Regnier, 1987). Here the presence at high concentration of the highly ionised BSA would lead in an increase of ionic strength, and the hemoglobin molecule exhibiting then a more compact shape would progress easier into the pores of the hydroxyapatite particles.

Nomenclature

α	Fraction of site 1 in Eq. (6)
a_s	External specific surface of the adsorbent particle, cm^{-1}
BSA	Bovin Serum Albumin
C^*	Equilibrium solution concentration of protein, mg or mol/unit solution
C_i	Liquid phase concentration of component i in column, mg/l solution
C_i^*	Liquid phase concentration of component i in equilibrium with the adsorbed concentration at the interface solution/particle $q_i(r_o)$, mg/l solution
C_{io}	Inlet solution concentration of component i in column runs, mg/l solution
C_o	Initial solution concentration of protein in batch experiments, mg or mol/unit solution
D	Molecular diffusivity, cm^2/s
d	Particle mean diameter, cm
D_{ax}	Axial dispersion coefficient, cm^2/s
$\bar{D}_1$	Intraparticle diffusion coefficient for site 1 (Eq. (6)), cm^2/s
$\bar{D}_2$	Intraparticle diffusion coefficient for site 2 (Eq. (6)), cm^2/s
D_{ei}	Intraparticle diffusion coefficient for component i, cm^2/s
ε	Extraparticle bed porosity
HA	Hydroxyapatite
Hb	Hemoglobin
K	Langmuir equilibrium constant or affinity parameter, unit solution/mg or mol
k_f	External mass transfer coefficient, cm/s
K_i	Langmuir equilibrium constant for component i, unit solution/mg or mol
KP	Phosphate potassium (buffer)
m	Dry weight of support, g
μ	Dynamic viscosity, kg/s.m
MW	Molecular weight, daltons
OD	Optical density
Pe_{ag}	Peclet number based on particle diameter
pI	Isoelectric point
q^*	Equilibrium concentration of adsorbed solute, mg or mol/unit adsorbent
q_1	Concentration of adsorbed solute on site 1 (Eq. (6))
q_2	Concentration of adsorbed solute on site 2 (Eq. (6))
q_i	Local concentration of adsorbed solute i in the particle, mg/g dry adsorbent
q_i^*	$q_i(r_o)$: Concentration of adsorbed i component at the interface solution/particle in equilibrium with C_i^*, mg/g dry adsorbent
q_{io}	Concentration of adsorbed i component in equilibrium with C_{io}, mg/g dry adsorbent
q_s	Maximum concentration of adsorbed solute: adsorption capacity, mg/g dry adsorbent
q_{si}	Adsorption capacity for component i, mg/g dry adsorbent

$\bar{q}_i$ Average adsorbed concentration of component i in the particle, mg/g dry adsorbent
r Position in the particle, cm
ρ_a Density of solid phase, g/l
r_o Particle mean radius, cm
t Time
u_i Interstitial velocity, cm/s
V Effluent volume during a column run (ml) or final volume of protein solution in batch experiments, ml
V_o Initial volume of protein solution in batch experiments, ml
V_p Breakthrough volume of unretained compound ($= \varepsilon.V_r$), ml
V_r Volume of packed bed, ml
z Position in the column

References

Andrade, J.D. (Ed.), *Principles of Protein Adsorption*, vol. 2, Plenum, N.Y., 1986.

Arve, B.H. and A.I. Liapis, "Modeling and Analysis of Biospecific Adsorption in Finite Bath," *AIChE Journal*, **33**(2), 179–193 (1987).

Bernardi, G., M.G. Giro, and C. Gaillard, "Chromatography of Polypeptides and Proteins on Hydroxyapatite Columns: Some Developments," *Biochim. Biophys. Acta*, **278**, 409–420 (1972).

Bruno, G., F. Gasparrini, D. Misiti, E. Arrigoni-Martelli, and M. Bronzetti, "High Performance Liquid Chromatographic Separation of Biomolecules Using Calcium Phosphate Supported on Macroporous Silica Microparticles," *J. Chromatogr.*, **504**, 319–333 (1990).

Carleysmith, S.W., P. Dunnill, and M.D. Lilly, "Kinetic Behaviour of Immobilized Penicillin Acylase," *Biotechnology and Bioengineering*, **XXII**, 735–756 (1980).

Chue, K.T., G. Grévillot, and D. Tondeur, "Electrosorption on Activated Carbon Bed," "*Fundamentals of Adsorption*," *Proceedings of the Fourth International Conference on Fundamentals of Adsorption*, Kyoto, 1993.

Clark, D.S., J.E. Bailey, and D.D. Do, "A Mathematical Model for Restricted Diffusion Effects on Macromolecule Impregnation in Porous Supports," *Biotechnology and Bioengineering*, **XXVII**, 208–213 (1985).

Cussler, E.L., *Diffusion Mass Transfer in Fluid Systems*, p. 116, Cambridge University Press, 1984.

De Bruin, S.H., L.H.M. Janssen, and G.A.J. Van Os, "Study of the Bohr Groups of Bovine Hemoglobin," *Biochim. Biophys. Acta*, **188**, 207–215 (1969).

De Vault, D., "The Theory of Chromatography," *Journal of Am. Chem. Soc.*, **65**, 532 (1943).

Fargues, C., "Chromatographie des Protéines appliquée à la Purification de la Pénicilline Acylase. Modèlisation de la Colonne d'adsorption sur un Gel d'hydroxyapatite," Thèse de Doctorat, Institut National Polytechnique de Lorraine, Nancy, 1993.

Glueckauf, E., *Trans. Faraday Soc.*, **51**(4), 1178 (1955).

Gorbunoff, M.J., "The Interaction of Proteins with Hydroxyapatite. I Role of Protein Charge and Structure," *Anal. Biochem.*, **136**, 425–432 (1984a).

Gorbunoff, M.J., "The Interaction of Proteins with Hydroxyapatite. II Role of Acidic and Basic Groups," *Anal. Biochem.*, **136**, 433–439 (1984b).

Gorbunoff, M.J. and S.N. Timasheff, "The Interaction of Proteins with Hydroxyapatite. III Mechanism," *Anal. Biochem.*, **136**, 440–445 (1984).

Graham, E.E., A. Pucciani, and N.G. Pinto, "A Comparative Study of Models to Predict Protein Adsorption," *Biotechnology Progress*, **3**(3), 141–145 (1987).

Hjerten, S., J. Lindeberg, and B. Shopova, "High-Performance Adsorption Chromatography of Proteins on 2-μm Spherical Beads of Hydroxyapatite," *J. Chromatogr.*, **440**, 305–313 (1988).

Hossain, Md.M., D.D. Do, and J.E. Bailey, *AIChE Journal*, **32**(2), 1088–1098 (1986).

Hossain, Md.M. and D.D. Do, *Biotechnology and Bioengineering*, **40**, 742–747 (1992).

Huang, J.X. and C. Horvath, "Adsorption Isotherms on High-Performance Liquid Chromatographic Sorbents—I. Peptides and Nucleic Acid Constituents on Octadecyl-Silica," *J. Chromatogr.*, **406**, 275–284 (1987).

Kataoka, T., H. Yoshida, and K. Ueyama, *Journal of Chemical Engineering of Japan*, **5**(2), 132–136 (1972).

Kawasaki, T., "Review: Hydroxyapatite as a Liquid Chromatographic Packing," *J. Chromatogr.*, **544**, 147–184 (1991).

Kondo, A., F. Murakami, and K. Higashitani, "Circular Dichroism Studies on Conformational Changes in Protein Molecules upon Adsorption on Ultrafine Polystyrene Particles," *Biotechnology and Bioengineering*, **40**, 889–894 (1992).

Kopaciewicz, W., M.A. Rounds, and F.E. Regnier, "Retention Model for High-Performance Ion-Exchange Chromatography," *J. Chromatogr.*, **266**, 3–21 (1983).

Kopaciewicz, W., S. Fulton, and S.Y. Lee, "Influence of Pore and Particle Size on the Frontal Uptake of Proteins—Implications for Preparative Anion-Exchange Chromatography," *Journal of Chromatography*, **409**, 111–124 (1987).

Koutsoukos, P.G., C.A. Mumme-Young, W. Norde, and J. Lyklema, "Effect of the Nature of the Substrate on the Adsorption of Human Plasma Albumin," *Colloids and Surfaces*, **5**, 93–104 (1982).

Leaver, G., Ph.D. Thesis, University of Wales, 1984.

Lindeberg, J., T. Srichaiyo, and S. Hjerten, "High-Performance Adsorption Chromatography of Transfer Ribonucleic Acids and Proteins on 2-μm Spherical Beads of Hydroxyapatite—Influence of Sodium Chloride and Magnesium Ions on the Resolution," *J. Chromatogr.*, **499**, 153–163 (1990).

Nicolas-Simonnot, M.O., "Contribution à l'Étude de la Chromatographie Frontale des Protéines par Échange d'Anions. Application à l'Albumine de Sérum Bovin," Ph.D. Thesis, INPL-ENSIC, Nancy, 1991.

Norde, W., "Adsorption of Proteins from Solution at Liquid-Solid Interface," *Advances in Colloid and Interface Science*, **25**, 267–340 (1986).

Regnier, F.E., "The Role of Protein Structure in Chromatographic Behaviour," *Science*, **238**, 319–323 (1987).

Saunders, M.S., J.B. Vierow, and G. Carta, "Uptake of Phenylalanine and Tyrosine by a Strong-Acid Cation Exchanger," *AIChE Journal*, **35**(1), 53–68 (1989).

Schanen, V., K.T. Chue, and G. Grévillot, "A Study of the Saturation Front of a Protein in an Ion Exchange Column. Experiments and Modelisation for Albumin and Haemoglobin," *Prep. 92, Proceedings of the 9th International Symposium on Preparative and Industrial Chromatography*, INPL-SFC, Nancy, France, 1992, pp. 57–62.

Sindhuphak, T., V. Patel and I. Svensson, "Temperature Gradient Chromatography of Transfer RNA on Hydroxyapatite," *J. Chromatogr.*, **314**, 408–412 (1984).

Skidmore, G.L. and H.A. Chase, "A Study of Ion Exchangers for Protein Purification," *Ion Exchange for Industry*, M. Streat (Ed.), pp. 520–532, 1988.

Skidmore, G.L., B.J. Horstmann, and H.A. Chase, "Modelling Single-Component Protein Adsorption to the Cation Exchanger S-Sepharose® FF," *Journal of Chromatography*, **498**, 113–128 (1990).

Tanford, C. "Hydrogen Ion Titration Curves of Proteins," *Electrochemistry in Biology and Medicine*, J. Wiley, N.Y., 1955.

Tiselius, A., S. Hjerten, and Ö. Levin, "Protein Chromatography on Calcium Phosphate Columns," *Archives of Biochem. and Biophys.*, **65**, 132–155 (1956).

Tsou, H.S. and E.E. Graham, "Prediction of Adsorption and Desorption of Protein on Dextran Based Ion Exchange Resin," *AIChE Journal*, **31**(12), 1959–1966 (1985).

Tyn, M.T. and T.W. Gusek, "Prediction of Diffusion Coefficients of Proteins," *Biotechnology and Bioengineering*, **35**, 327–338 (1990).

Van der Wiel, J.P., *Continuous Recovery of Bioproducts by Adsorption*, Academisch Boeken Centrum, De Lier, 1989.

Yoshida, H., H. Nishihara, and T. Kataoka, "Adsorption of BSA on QAE-Dextran: Equilibria," *Biotechnology and Bioengineering*, **41**, 280–286 (1993).

Yoshida, H., H. Nishihara, and T. Kataoka, "Adsorption of BSA on Strongly Basic Chitosan: Equilibria," *Biotechnology and Bioengineering*, **43**, 1087–1093 (1994).