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Solution properties of polyelectrolytes

VII[a]. Non-ideal mechanisms in size-exclusion chromatography of synthetic polyions, peptides and proteins

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ABSTRACT

Chromatographic data for sodium polystyrene sulphonate were obtained on both silica- and polymer-based size-exclusion supports using mobile phases of various pH and ionic strength. Deviations of the elution volume were observed towards both lower and higher values relative to the reference calibration graph obtained with uncharged standards. An empirical correlation is proposed in order to account for all the secondary effects observed. The general applicability of this correlation was further tested for chromatographic data obtained for a series of peptides and proteins on a silica-based support under very different eluent conditions. Deviations from ideal elution behaviour such as ion-exclusion and hydrophobic effects were analysed in the light of this approach.

INTRODUCTION

High-performance aqueous size-exclusion chromatography (HPASEC) has attracted increasing attention in recent years because of the possibilities it offers both in basic biochemistry and for biotechnological applications. It has proved to be a powerful tool in the separation of biopolymers (peptides, proteins, polynucleotides, etc.) [1] and macromolecular assemblies (viral particles, liposomes, etc.) [2,3] using typically mild, non-agressive mobile phase conditions which preserve the native structure and functionality of the solute. However, considerable experimental evidence has shown that the elution mechanism of most biopolymers on HPASEC supports deviates from a pure size-exclusion mechanism, mainly owing to a number of secondary effects, including ion exclusion and ion exchange, hydrophobic interaction and hydrogen bonding, originating from specific solute–matrix interactions [4].

[a] For Part VI, see ref. 14.

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Although these effects have turned out to be advantageous in some instances and have been exploited to improve the separation of macromolecules of similar hydrodynamic volume, it is in general desirable to minimize (if not cancel) them, particularly for characterization purposes. Considerable efforts have been made in this derection in the last decade by both manufacturers, to design supports as inert as possible without a decrease in gel performance, and by chromatographers, to manipulate the mobile phase conditions rationally to any given separation. At the same time, different theoretical approaches have been elaborated attempting to quantitate the aforementioned secondary effects. At present, however, it seems that the total suppression of these effects has not yet been achieved and that there is no theory capable of predicting them in a completely satisfactory manner, especially for biological macromolecules, characterized by widely differing geometry and spatial configuration, surface topology and charge distribution. For this reason, most attempts to account for secondary effects are based on experimental evidence obtained on packing materials of very different nature with model charged macromolecules, *e.g.*, linear polyelectrolytes such as sodium polystyrene sulphonate (NaPSS) or poly(sodium acrylate).

Dubin and co-workers [5–7] proposed a model to predict ion-exclusion effect based on the reduction in the pore volume accessible for polyions, calculating a repulsion volume as a function of an electrostatic potential of the stationary phase. The same group also recently proposed [8] the use of a hydrophobicity index related to the hydrophobic effect. Mori [9] established an empirical correlation between the repulsion volume and eluent ionic strength. Other attempts have been made to obtain a parameter representative of the size and shape of a biopolymer (or macromolecular assembly). Thus, the product $M[\eta]$, where M and $[\eta]$ are the macromolecule molecular weight and intrinsic viscosity, respectively, and a number of modifications of this product [5,6,9–14] and other macromolecular dimensions representative of a variety of geometries have been suggested [15–18]. Finally, some theoretical models for ion exclusion have been presented, most of them based on the Poisson–Boltzmann equation to calculate the electrostatic interaction for a charged polymer near a charged wall [19,20].

In this paper, we propose an empirical correlation for analysing in a general manner secondary effects in the HPASEC of both model polyelectrolytes and biopolymers. This approach interprets solute–matrix attractive–repulsive interactions in terms of (bio)polymer–support compatibility, making use of a thermodynamic formalism previously developed for uncharged polymers [21,22]. In order to evaluate the general applicability of this correlation, we first analysed the chromatographic behaviour of NaPSS on silica- and polymer-based supports using different mobile phase compositions, and then the same treatment was extended to the chromatographic data reported by Irvine [23] on the elution of a series of peptides and proteins.

EXPERIMENTAL

Chemical and reagents

Dextran samples obtained from Pharmacia (Uppsala, Sweden) of molecular weight 10 000, 40 000, 83 000, 177 000 and 500 000 g mol^{-1} were used as standards for uncharged polymers. The chromatographic low-molecular-weight range was covered

using poly(ethylene oxide) (PEO) standards of molecular weight 2000 and 4000 g mol^{-1}, purchased from Fluka (Darmstadt, Germany). NaPSS samples were dialysed fractions of commercial standards from Pressure Chemical (Pittsburgh, PA, USA) of molecular weight 1600, 4000, 16 000, 31 000, 88 000, 177 000 and 354 000 g mol^{-1} with polydispersity lower than 1.1. All reagents used in the preparation of buffers were of analytical-reagent grade from Merck (Darmstadt, Germany). High-performance liquid chromatographic grade water (Merck) was tested conductimetrically daily as reported elsewhere [13].

Viscosity

Viscosity values for uncharged polymers in pure water at 25.0°C were obtained using the following viscosimetric equations: $[\eta] = 97.8 \cdot 10^{-3} M^{0.50}$ ml g^{-1} for dextrans [24] and $[\eta] = 2.0 + 0.016\ M^{0.76}$ ml g^{-1} for PEO [25], where M = molecular weight. The effect of ionic strength and pH on the viscosity of non-ionic polymers was neglected [6,13,14]. Concerning NaPSS, the intrinsic viscosities of the polyion p at finite salt (c_s) and polyion (c_p) concentrations, denoted by $[\eta]_{p,c_p,c_s}$ were calculated using a recently proposed general equation whose validity has been demonstrated for a wide range of solvent compositions, [13,14,26,27].

Chromatographic measurements

The Waters Assoc. liquid chromatographic equipment used has been described elsewhere [13]. Ultrahydrogel-250 (U-250) column packed with hydroxylated polymethacrylate-based gel of 250 Å nominal pore size and a silica-based Protein I-250 column were used. The interstitial packing volume and total pore volume were 5.5 and 5.1 ml for the U-250 and 5.9 and 6.1 ml for the I-250 column, as measured with high-molecular-weight dextran and ^2H_2O, respectively.

Buffers of pH 7.0 and 5.9 (phosphate) and 4.0 (acetate) were used as eluents, in all instances following degassing and filtration through regenerated cellulose 0.45-μm pore diameter filters from Micro Filtration Systems (Dublin, CA, USA).

The column was equilibrated overnight prior to starting any experiment. Polymer solutions were always prepared using the corresponding mobile phase as solvent. The volume injected was 100 μl in all instances, covering an NaPSS concentration range from 0.1 to 10 g l^{-1}. The calibration graphs for uncharged standards were obtained by extrapolation to zero concentration of peak elution volumes obtained for at least three injected concentrations.

RESULTS AND DISCUSSION

The most common approach used to analyse secondary effects in HPASEC of polyelectrolytes is based on the comparison of calibration graphs obtained under the same experimental conditions for both the polyion under study and uncharged polymers as a reference. The choice of an appropriate quantity for the hydrodynamic volume of the charged macromolecule is, however, the subject of some controversy, as has been mentioned previously [16–18]. In this work, we used for this purpose the product $M[\eta]_{p,c_p,c_s}$ as a useful representative polyelectrolyte size parameter under any experimental conditions at finite concentration of polyion and salt, c_p and c_s. We have recently reported on the chromatographic behaviour of NaPSS independently on

polymer-based [13] and silica-based [14] supports as a function of a number of chromatographic variables.

We next present a direct comparison of the elution of NaPSS on both types of supports for the same mobile phase compositions. Fig. 1 depicts the calibration graphs obtained for this polyelectrolyte on either a I-250 or a U-250 column using (A) 0.02 *M* and (B) 0.05 *M* acetate buffer (pH 4.0) as eluent; Fig. 1. also includes the calibration graphs for uncharged polymers (dextrans and PEO) as a reference for ideal SEC. This comparison may be of particular interest taking into account that I-250 has been a commonly used silica-based column for biopolymer separation [28,29] whereas U-250 is a relatively recent soft, polymer-based support [2,13,30,31] with the advantages of a wide range of pH stability and a higher inertness as far as residual charge density is concerned [32].

Fig. 1A shows that at c_s = 0.02 *M* the polyion–matrix electrostatic repulsion is indeed stronger for I-250 than for U-250, as deduced from the divergence between the calibration graphs for NaPSS and the uncharged standards. At moderately higher eluent ionic strength, c_s = 0.05 *M* (Fig. 1B), ion exclusion is cancelled in the case of U-250 whereas a substantial divergence still remains for I-250. Note that for the polymer-based support polyion and reference calibrations are not completely congruent, probably owing the appearance of a salt-induced matrix–solute hydrophobic interaction, shifting the elution volume to higher values than those expected for ideal behaviour. In this regard, Mori [9] has recently reported some hydrophobic retention of NaPSS on derivatized silica supports using mobile phases with relatively high ionic strength. On the other hand, the resolution observed for I-250 is higher than that for

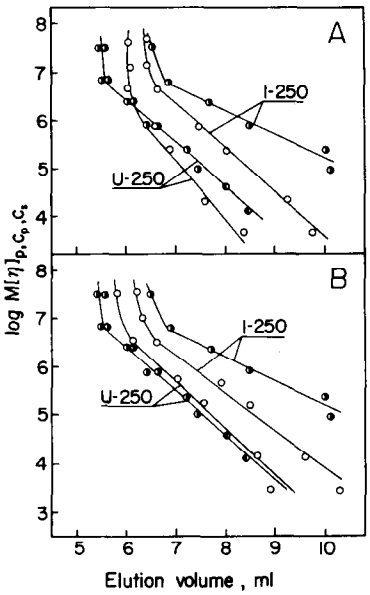

Fig 1. Comparison of calibration graphs obtained for (○) NaPSS and standard uncharged polymers (◑, dextrans; ●, PEO) on a silica-based column (I-250) and a polymer-based column (U-250) at c_p = 10 g l^{-1} using (A) 0.02 *M* and (B) 0.05 *M* acetate buffer (pH 4.0) as eluents.

U-250, so that the former could be more suitable for compromised separations of either polyelectrolytes of close molecular weight or ionomers. It is also worth commenting that in the limit of total exclusion different elution volumes can be obtained for the same value of $M[\eta]_{p,c_p,c_s}$ depending on the mobile phase composition. In this regard, it has been pointed out [33] that for the elution of uncharged polymers in organic media, different polymer–solvent interactions (represented by either the exponent a in the viscometric equation or the second virial coefficient) determine different polymer–gel interactions and, consequently, different elution volumes may correspond to similar hydrodynamic volumes.

As can be observed in the different calibration graphs in Fig. 1, the elution volume of NaPSS can be shifted, depending on c_p and on mobile phase ionic strength and pH, towards either higher or lower values relative to the reference calibration. The same behaviour has been widely observed experimentally in the past for uncharged polymers in organic binary mixed eluents [34]. In both instances, *i.e.*, aqueous SEC of polyions and SEC of synthetic polymers using organic mobile phases, the deviations (to higher or lower elution volumes) from the reference calibration graph (assumed to correspond to a pure size-exclusion mechanism) can be attributed to polymer–matrix interactions, although it is evident that the molecular causes for these secondary effects must be different.

Based on this phenomenological similarity between both types of systems, we next derive an expression which affords a new approach to the analysis of secondary effects in the SEC of charged (bio)polymers.

The elution volume of an uncharged polymer on a SEC support can be expressed through the basic equation

$$V_e = V_0 + K_{SEC} V_p \tag{1}$$

where V_e is the experimental elution volume of the sample, V_0 and V_p are the dead volume and the total pore volume of the column, respectively and K_{SEC} is the distribution coefficient for pure SEC. The elution behaviour of a polyelectrolyte on the same support when secondary effects take place can be described by

$$V'_e = V_0 + K'_{SEC} V_p \tag{2}$$

where V'_e is the experimental elution volume of the polyion and K'_{SEC} is a new distribution coefficient accounting for the different contributions to the separation mechanism. If we assume that V_e and V'_e correspond to the elution volume of an uncharged polymer and a polyelectrolyte having the same $M[\eta]_{p,c_p,c_s}$, then K'_{SEC} can be divided into two contributions and expressed as

$$K'_{SEC} = K_{SEC} K_p \tag{3}$$

where K_p is a partition coefficient for secondary effects. It must be noted that in this context K_p can be higher, equal to or lower than unity, therefore accounting for any type of deviation (to higher or lower elution volumes) from the reference calibration (see Fig. 1). In other words, $K_p > 1$ means that the polyion is eluted at a higher elution volume than a reference uncharged polymer of the same $M[\eta]_{p,c_p,c_s}$, this sec-

ondary effect being based on polyelectrolyte–support net favourable interactions (*e.g.*, hydrogen bonding, hydrophobic interactions). On the other hand, $K_p < 1$ implies a situation where unfavourable interactions between the polyion and the matrix (essentially electrostatic repulsion) take place and, consequently, the polyelectrolyte is eluted at a lower elution volume than the reference polymer. Obviously, $K_p = 1$ is considered as pure size exclusion, *i.e.*, when no secondary effect is observed.

Substitution of K'_{SEC} from eqn. 3 into eqn. 2 yields

$$V'_e = V_0 + K_{SEC}K_pV_p \quad (4)$$

Alternatively, the experimental elution volume of the polyelectrolyte can be expressed as

$$V'_e = V_0 + K_{SEC}V^*_p \quad (5)$$

where

$$V^*_p = K_pV_p \quad (6)$$

is a variable denoting a virtual pore volume, *i.e.*, a corrected V_p "effectively seen" by the macromolecule because of the secondary effects taking place. Some comments deserve to be made on this parameter, which can be regarded as a descriptor of the elution behaviour of (bio)polymers. Although V_p and V^*_p have the same units, the former corresponds to the geometrical volume of the pores whereas the latter, rather than having a strictly geometrical meaning, should be correlated with the time of residence of the sample inside the pores. In other words, V^*_p will be higher than V_p when the polyion residence time in the stationary phase is longer than that for an uncharged reference polymer of the same hydrodynamic volume, owing to polyelectrolyte–support attractive interactions. In contrast, V^*_p will be lower than V_p when the polyion resides inside the stationary phase for a shorter time than a reference polymer of the same hydrodynamic volume, owing to repulsive interactions with the matrix. In this regard, and only for the deviations towards lower elution volumes relative to the reference calibration, V^*_p could be considered equivalent to the effective pore volume reported by Dubin and Tecklenburg [5], V'_p which is a measure of the portion of the pore available to the polyion.

Concerning the procedure for the calculation of V^*_p values, assuming that V'_e and V_e values connected through a horizontal line in the calibration plots are compared, rearrangement of eqns. 1 and 5 yields

$$V^*_p = (V'_e - V_0)(V_e - V_0)^{-1}V_p \quad (7)$$

where V'_e corresponds to the experimental polyion elution volume and V_e to the elution volume obtained for a hypothetical uncharged standard of the same $M[\eta]_{p,c_p,c_s}$. As an example, Table I summarizes the V^*_p values together with the related parameters V'_e, K'_{SEC} and V_e calculated for NaPSS samples of different molecular weight on U-250 at $c_p = 10$ g l^{-1} with 0.02 *M* phosphate buffer (pH 5.9) as eluent. It can be observed that V^*_p varies with the molecular weight of the polyion. In order to attempt to find an empirical correlation accounting for this variation, we used an

TABLE I

MOLECULAR WEIGHT DEPENDENCE OF V_p^* FOR NaPSS AND RELATED PARAMETERS INVOLVED IN ITS CALCULATION

Chromatographic conditions: phosphate buffer (pH 5.9, c_s = 0.02 *M*) as eluent; c_p = 10 g l^{-1}.

M (kilodalton)	V_e (ml)	K'_{SEC}	V_c (ml)	V_p^* (ml)
1.6	8.02	0.497	9.03	3.62
4.0	7.03	0.302	8.23	2.84
16.0	6.46	0.189	7.25	2.78
31.0	6.07	0.112	6.65	2.51
88.0	5.74	0.047	6.05	2.22

approach which regards the chromatographic secondary effects as the result of a competition between polymer–eluent and matrix–eluent interactions. This competition can be described by the preferential adsorption coefficient, λ, widely used in polymer solution thermodynamics and recently applied to SEC of uncharged polymers [21,22]. As (i) the variation of λ with the molecular weight of the solvated polymer is experimentally given by [35]

$$\lambda M^{1/2} = \lambda_\infty M^{1/2} + C \tag{8}$$

where λ_∞ denotes the preferential solvation coefficient at infinite molecular mass and C is a constant, and (ii) λ is proportional to ln K_p [21], and taking eqn. 6 into account, the following expression is obtained:

$$M^{1/2}\ln(V_p^*/V_p) = AM^{1/2} + B \tag{9}$$

where A and B are proportionality constants. This empirical correlation relating the virtual pore volume, V_p^*, with the polyion molecular weight, will be used to test secondary effects for NaPSS and also peptide and protein chromatographic data.

A plot of the first member of eqn. 9 *vs.* $M^{1/2}$ should yield a straight line, for a given mobile phase composition and injected polyion concentration, whose slope reflects the extent of secondary effects. Thus, negative slopes can be attributed to matrix–polyion electrostatic repulsion and positive slopes would indicate favourable interactions (mainly hydrophobic effects and/or hydrogen bonding); if a slope near zero is obtained, secondary effects are counterbalanced and the elution behaviour approaches ideal SEC.

Fig. 2 shows the plots for NaPSS on U-250 obtained under different experimental conditions by varying c_p and pH at constant c_s = 0.02 *M*. V_p^* values were evaluated as mentioned above. As can be seen, at this ionic strength all slopes are negative. A good correlation was observed in all instances, which supports eqn. 9. Note, however, that some deviations appear corresponding to elution volumes outside the linear portion of the calibration graphs. For a given pH, ion exclusion is attenuated as the polyion concentration increases up to c_p = 10 g l^{-1}, a value that was considered as an upper limit to prevent the appearance of viscous fingering [13].

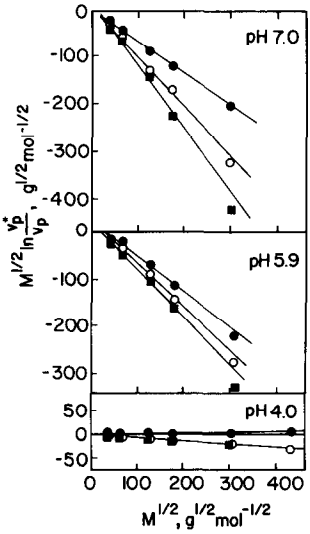

Fig. 2. Plot of eqn. 9 for the chromatographic data obtained for NaPSS on U-250 at c_p values of (■) 0.1, (○) 1.0 and (●) 10 g l^{-1} using 0.02 *M* phosphate (pH 7.0 and 5.9) and acetate (pH 4.0) buffers as eluents.

On the other hand, a decrease in pH causes a diminution in polymer–support electrostatic repulsion, which has been previously explained on the basis of protonation of residual carboxyl groups on the matrix. Thus, at pH 4.0, for c_p = 10 g l^{-1}, a slope near zero is obtained even at this low ionic strength, which allows operation under quasi-ideal SEC conditions. This confirms the low activity of this gel, especially when compared with other commercially available hydrophilic supports.

In order to test eqn. 9 in the zone of the plot corresponding to positive slopes (hydrophobic and other effects), the ionic strength of the mobile phase was increased to 0.2 *M*. Fig. 3 shows a comparison of the chromatographic behaviour of NaPSS (analysed through eqn. 9) on U-250 and I-250 using 0.2 *M* acetate buffer (pH 4.0) as eluent. A good fit was observed for the different c_p values applied. Note that under these conditions the behaviour on the polymer-based support was quasi-ideal, a slight

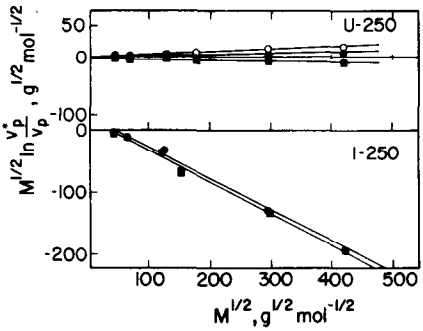

Fig. 3. Comparison of the plots of eqn. 9 obtained for NaPSS at different concentrations on U-250 and I-250 using 0.2 *M* acetate buffer (pH 4.0) as eluent. Symbols as in Fig. 2.

concentration effect being apparent. It must be pointed out that although this plot plausibly offers a more precise way to analyse deviations from ideal SEC under any given experimental conditions than conventional log $M[\eta]$ *vs.* elution volume plots, in the region of positive slopes it does not allow an assignment of the specific contributions of operating individual secondary effects, and for this reason it is difficult to establish unambiguously a trend for the variation of the slope with c_p at this ionic strength. On the other hand, it must be noted that under these experimental conditions significant ion exclusion still remains when using I-250, which corroborates a surface residual charge density markedly higher than that for U-250.

Concerning the meaning of the variation of V_p^* (or its equivalent K_p) with the polyion molecular weight, the results in Figs. 2 and 3 show that as a general trend throughout the plot, the lower is M the closer is the value of K_p to unity (ideal SEC). This is reflected in the negative slope zone by a decrease in V_p^* (or K_p) as M increases, *i.e.*, the ion-exclusion effect is largest for the largest polyelectrolytes. In contrast, in the positive slope region V_p^* (or K_p) increases when M increases, *i.e.*, the secondary effects (hydrophobic and other interactions) are more pronounced for the largest molecules. It is worth mentioning in this regard that a similar qualitative dependence of K_p on M for synthetic polymers in organic media has been reported for $K_p > 1$ for polystyrene [36] and for $K_p < 1$ for poly(N-vinyl-3,6-dibromocarbazole) [37], both with tetrahydrofuran as eluent.

The results presented so far have been obtained for NaPSS, conventionally used as a model chain-like polyelectrolyte. It has been shown that for this polyion the expression proposed is valid when using two kinds of supports under very different experimental conditions. However, the interest of most chromatographers using aqueous SEC is increasingly focusing on biopolymers, particularly peptides and proteins. In order to test the general applicability of eqn. 9 for the analysis of secondary effects in the elution behaviour of these biomolecules, we selected from the literature the set of chromatographic data reported by Irvine [23]. That study can be considered as a suitable framework to check eqn. 9, because a wide variety of peptides and proteins covering a molecular weight range from 574 to 66 000 g mol^{-1} were eluted with mobile phases of widely varying composition. Briefly, the author reported on the elution of the biopolymers on a TSK G2000SW column with eluents of low pH containing different concentrations of phosphoric, trifluoroacetic or heptafluorobutyric acid as ion-pairing agents.

In order to test eqn. 9, two approximations were made. First, log M instead of log $M[\eta]$ was used in the calculation of V_p^*, obtained in a similar way to that mentioned above. Second, as absolute uncharged standards (*e.g.*, dextrans, PEO) were not employed in Irvine's work, we were obliged to select one calibration graph (among the reported) as a relative reference system, namely, 0.1 M phosphoric acid as mobile phase (see Fig. 2C in ref. 23). This eluent composition presumably permitted a minimization of secondary effects because of both an effective charge screening of the biopolymer (owing to the moderately high ionic strength) and the low hydrophobicity of this ion-pairing agent relative to the other acids used.

Fig. 4. depicts the fitting of the data from Irvine's paper (see Fig. 2 in ref. 23) taking the above assumptions into account. Inspection of Fig. 4 reveals that as both the ionic strength and hydrophobicity of the ion-pairing acid increase, the slope shifts from negative towards positive values. Some considerations can be made in relation

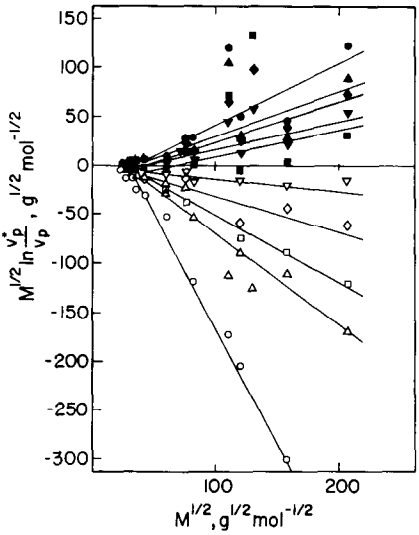

Fig. 4. Plot of eqn. 9 for a series of different peptides and proteins eluted on TSK G2000SW with eluents of low pH containing different concentrations of phosphoric acid (○ = 0.005 *M*; △ = 0.02 *M*), trifluoroacetic acid (□ = 0.005 *M*; ▽ = 0.01 *M*; ▼ = 0.015 *M*; ◆ = 0.02 *M*) or heptafluorobutyric acid (◇ = 0.005 *M*; ■ = 0.01 *M*; ▲ = 0.015 *M*; ● = 0.02 *M*) as ion-pairing agents. A mobile phase consisting of 0.1 *M* phosphoric acid was taken as a reference corresponding to the horizontal line in the plot. Primary chromatographic data were taken from ref. 23.

to the secondary effects taking place under each particular chromatographic condition. First, the fits are in general good in the negative slope zone, where the predominant interaction induced by the very low pH of the mobile phase is likely to be an electrostatic repulsion between the positively charged silica-based support (as suggested by the author) and the peptides and proteins exhibiting net positive charge. Second, in the positive slope zone hydrophobic interaction is likely to be the main secondary effect taking place. However, the fits in this instance are in general not as good. A possible explanation for this behaviour could be that the biopolymers under these conditions exhibit at least two kinds of hydrophobic sites on the surface: on the one hand the non-polar chains of the ion-paired acid molecules, and on the other specific surface hydrophobic patches which can vary widely both in nature and topological distribution from one protein or peptide to another. It can be easily understood that the physico-chemical processes underlying surface recognition and interaction of different ion-paired proteins with the chromatographic matrix are complex and that deviations from linearity due to intrinsic biopolymer heterogeneities are not surprising. Anyway, a general trend is clearly observed in the plot in the positive slope region, so that an increase in ion-pairing acid concentration and replacement of trifluoroacetic by heptafluorobutyric acid give rise to an increase in the slope.

Finally, it is worth mentioning that a functionality similar to that in eqn. 9 has been reported [38] for the elution behaviour on a size-exclusion support of another type of biopolymer, namely double-stranded DNA restriction fragments, of great interest for biotechnologists and molecular biologists. In particular, for large DNA fragments, an anomalous retention behaviour of unknown mechanism was observed

where the retention parameter increased as a function of $L^{1/2}$, L being the chain length of the DNA fragment. Again, it is apparent that the interpretation of non-ideal SEC elution behaviour of biopolymers, including peptides, proteins and polynucleotides, is particularly complicated when the mobile phase composition strongly favours compatibility between the support and the solute and that more sophisticated treatments are needed to quantitatively account for the different interactions involved in deviations from pure size exclusion.

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