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Solution properties of polyelectrolytes

VIII[☆]. A comparative study of the elution behaviour on two organic-based packings[☆☆]

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ABSTRACT

Aqueous size-exclusion chromatography was used to analyse the elution behaviour of several standard ionic polymers, including poly(L-glutamic acid), sodium poly(styrene sulphonate) and poly(acrylic acid), different in nature and chain flexibility, as a function of the pH and ionic strength of the eluent. Two organic-based hydrophilic packings, Spherogel TSK PW4000 and Ultrahydrogel 250, were tested in order to select the optimal conditions of macromolecular separation, and the results obtained for each column were compared. A set of calibration graphs for the above polyions as a function of eluent pH and ionic strength were obtained and compared with those obtained for uncharged standards (dextran and polyethylene oxide, PEO) under the same experimental conditions. The divergence between both charged and uncharged plots served to interpret the separation mechanisms for the polyions, other than pure size exclusion. Deviations from ideal elution behaviour have been attributed to ion-exclusion and hydrophobic effects, as a consequence of the repulsive or attractive interactions between the ionizable groups of the polyelectrolyte and the residual surface charge of the support.

INTRODUCTION

In the last decade, size-exclusion chromatography (SEC) has become firmly implanted in the areas of biomedical and life sciences, mainly as a result of the development of high-performance and selective hydrophilic backing columns, allowing the separation and identification of synthetic and natural water-soluble polymers as well as macromolecular assemblies, such as viral particles and liposomes [1–5].

Hydrophilic packings can be classified into two categories, inorganic (mostly silica based) and organic (formed by a three-dimensional polymer network with strategically inserted water-compatible organic functional groups). These functional groups, such as hydroxyl, carboxyl, etc., are dissociated or not depending on the pH of the mobile phase. In some cases, specific interactions between functional groups and certain atoms or ionic groups can distort the macromolecular separation expected from a pure size-exclusion mechanism. In the particular case of elution of a polyelectrolyte through organic- or silica-based packings, specific solute–matrix interactions, such as hydrogen bonding, ion exchange, hydrophobic and ion-exclusion effects, have been thoroughly studied from both experimental and theoretical points of view [6,7].

In this context, we should mention a series of

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papers published by Dubin and co-workers [8–10] on a model based on the reduction in the pore volume accessible to polyions, which predicts ion-exclusion effects. Another model calculates the free energy of partitioning of anionic polyelectrolytes into cylindrical pores of like charge. A theoretical contribution based on the Poisson–Boltzmann equation has recently been reported by Hoagland [11], which examines the interaction of a rod-like polyelectrolyte with nearby surfaces of like charge. Recently, our group [12,13] has evaluated the elution behaviour of sodium poly(styrene sulphonate) (PSS) and proposed an empirical correlation accounting for deviations of this polyion when compared with standard non-ionic polymers.

In this paper, a qualitative analysis of the elution behaviour of PSS, poly(L-glutamic acid) (PGA) and poly(acrylic acid) (PAA) polyions, at different mobile phase compositions and through two different packings, is presented. The studied packings, organic-based ones, were Spherogel TSK PW4000 and Ultrahydrogel 250 (UHG-250). The first has been widely used in the separation of both synthetic and biological macromolecules, however the second has scarcely been tested. Deviations in universal calibration curves, expressed as log $M[\eta]$ *vs.* elution volume, with respect to the uncharged polymer, have been considered in order to evaluate the contribution of non-exclusion effects to the total separation mechanism.

EXPERIMENTAL

Chemical and reagents

Dextran samples purchased from Pharmacia (Uppsala, Sweden) with nominal molecular masses of 10 000, 17 700, 40 000, 66 900, 83 300, 170 000, 500 000 and 2 000 000 g mol^{-1} were used as standards for uncharged polymers. The chromatographic low-molecular-mass range was covered by poly(ethylene oxide) (PEO) standards with molecular masses of 2000 and 4000 g mol^{-1}, from Fluka (Darmstadt, Germany). Studied polyelectrolytes were samples of PGA from Sigma (St. Louis, MO, USA), PSS from Pressure (Pittsburgh, PA, USA) and PAA from Aldrich (Milwaukee, WI, USA) and are listed in Table I. All samples show polydispersities lower than 1.1.

TABLE I

NOMINAL MOLECULAR MASSES AND INTRINSIC VISCOSITIES IN PURE WATER AT 25°C OF STUDIED POLYELECTROLYTES

PGA = Poly(L-glutamic acid); PSS = sodium poly(styrene sulphonate); and PAA = poly(acrylic acid)

Samples	M (g mol^{-1})	$[\eta]$ (ml g^{-1})
PGA-1	13 600	181
PGA-2	43 000	985
PGA-3	77 800	2220
PSS-1	1 600	87
PSS-2	16 000	611
PSS-3	31 000	856
PSS-4	88 000	2740
PSS-5	177 000	6080
PAA-1	5 000	44.5
PAA-2	90 000	1430
PAA-3	250 000	5420

Solvents used for viscometric measurements and as eluents in SEC were buffers made up from sodium dihydrogenphosphate and sodium monohydrogenphosphate for pH 7.0 and from sodium acetate and acetic acid for pH 5.0. Desired ionic strengths were adjusted from 0.005 to 0.20 *M*. Reagents used in the preparation of buffers were analytical grade from Merck (Darmstadt, Germany), and the conductivity of the HPLC water used (Merck) was tested daily.

Viscosities

Intrinsic viscosity values, $[\eta]$, for uncharged polymers in pure water at 25.0 ± 0.1°C were evaluated through the viscometric equations $[\eta] = 97.8 \cdot 10^{-3} M^{0.5}$ ml g^{-1} for dextrans [14] and $[\eta] = 2.0 + 0.016 M^{0.76}$ ml g^{-1} for PEO [15], where M = molecular mass. The effects of ionic strength and pH on the viscosity of those non-ionic polymers were ignored [8,12,13].

Viscosity measurements of polyelectrolyte samples at 25.0 ± 0.1°C and pH 5.0 were performed with an automatic Ubbelohde-type AVS 440 capillary viscometer from Schott Geräte (Hofheim, Germany). Original solutions were equilibrated at the working temperature for several hours prior to introduction into the

viscometer. At least five dilutions were obtained by adding the appropriate aliquots of solvent. Kinetic energy corrections were taken into account for the evaluation of $[\eta]$, which was determined by extrapolation to infinite dilution of Füoss plots [16], namely η_{red}^{-1} *vs.* $c^{1/2}$.

Chromatography

The LC equipment consisted of an M-45 solvent-delivery system, a U6K universal injector and an R-401 refractive index detector from Waters (Milford, MA, USA). An Ultrahydrogel 250 (UHG-250) column (30 × 0.78 cm I.D.) packed with hydroxylated poly(methacrylate)-based gel of 250 Å nominal pore size from Waters and a Spherogel TSK PW4000 column (30 × 0.75 cm I.D.) packed with hydroxylated polyether copolymer of 500 Å nominal pore diameter from Beckman Instruments (Galway, Ireland) were used. The chromatograms were recorded by a Yokogawa Electric Works dual-channel recorder (Tokyo, Japan). The exclusion volumes, V_0, and the total column volumes, V_T, were 5.48 and 10.46 ml, respectively, for the UHG-250 column and 5.15 and 10.40 ml, respectively, for the TSK one, as determined with blue dextran ($M = 2\,000\,000$ g mol^{-1}) and ^2H_2O, respectively.

Buffers used as eluents were degassed and filtered through regenerated cellulose 0.45-μm pore diameter filters from Micro Filtration Systems (Dublin, CA, USA). All chromatographic experiments were conducted at room temperature and the column was equilibrated overnight prior to starting any experiment. Chromatograms of polyelectrolytes were obtained at a flow-rate of 1.0 ml min^{-1} by injection of 100 μl of 0.1% (w/v) solute solutions, prepared using the corresponding mobile phase as solvent. Elution volumes of uncharged standards were obtained by extrapolation to zero concentration of peak elution volumes obtained for at least three different injected concentrations. Obtained values were independent of pH and of ionic strength, at least in the range studied here.

RESULTS AND DISCUSSION

The approach most commonly followed to analyse secondary effects in aqueous SEC of polyelectrolytes compares the calibration graphs obtained under the same experimental conditions both for the polyion under study and for some uncharged polymer used as a reference. In this context, some controversy arises about the physical magnitude that best defines the hydrodynamic volume of polyions. Thus, whereas several authors [17–19] believe that elution volumes, V_e, in aqueous SEC of polyelectrolytes are determined by the hydrodynamic volume, $M[\eta]$, only at ionic strengths that are sufficiently high to allow electric double-layer effects to be considered negligible, Potschka [20] shows that any macromolecule, regardless of its shape (solid sphere, expanded coil or more or less flexible rod), elutes at any condition according to a universal parameter, namely the viscosity radius, R_η, directly related to $M[\eta]$. Therefore, in what follows the product $M[\eta]_I$ will be used as the representative parameter of polyelectrolyte size at any external salt concentration, I.

Whereas pH causes negligible changes or no changes at all in the intrinsic viscosity of flexible polyelectrolytes, as experimentally ascertained for the polymers studied here and in agreement with previous reports [8,12], added salts do indeed cause large changes [21]. It is generally admitted that [22,23]:

$$[\eta]_I = [\eta]_\infty + SI^{-1/2} \quad (1)$$

where $[\eta]_I$ and $[\eta]_\infty$ stand for intrinsic viscosities at the ionic strength I and at $I \rightarrow \infty$, respectively, and S is related to the stiffness of the macromolecule according to Odijk [24]. In what follows units for $[\eta]$ and I will be ml g^{-1} and mol l^{-1}, respectively.

Eqn. 1 plots are shown in Fig. 1 for the three polyelectrolytes under study. As seen in the figure good linear correlations hold in the range of ionic strength so far studied (0.005–0.20 M), allowing evaluation of $[\eta]_\infty$ and S values through least-square fits. As seen, the higher the molar mass of the polyanion the larger the slope values of Fig. 1, as expected from the greater contribution to $[\eta]_I$ of the electrostatic persistence length, L_e, of the macromolecular chains. Moreover, good linear correlations of $\log[\eta]_I$ and $\log[\eta]_\infty$ *vs.* $\log M$ (not shown) allowed us to obtain Mark–

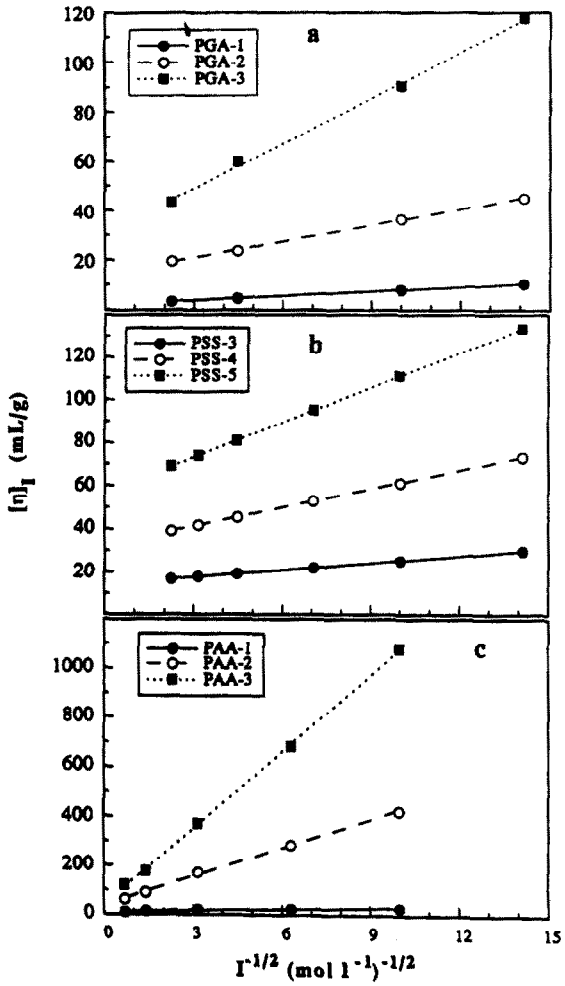

Fig. 1. Intrinsic viscosities of poly(L-glutamic acid), PGA (a); sodium poly(styrene sulphonate), PSS (b) and poly(acrylic acid), PAA (c) in acetate buffer (pH 5.0) as a function of the inverse square root of ionic strength.

Houwink constants K and a ($[\eta] = KM^a$) at the diverse I values studied.

Chromatograms of PGA-1, PSS-2 and PAA-1, as examples, on TSK PW4000 and UHG-250 columns in pure water (curves a) and in phosphate buffer (pH 7.0) at various ionic strengths (curves b–g) are shown in Fig. 2. In all cases the peaks are Gaussian and become broader with increasing ionic strength. In contrast to the SEC behaviour of dextran or any uncharged polymer, where the effects of I on elution volumes are negligible, it can be observed in Fig. 2 that the retention volumes of all polyanions significantly

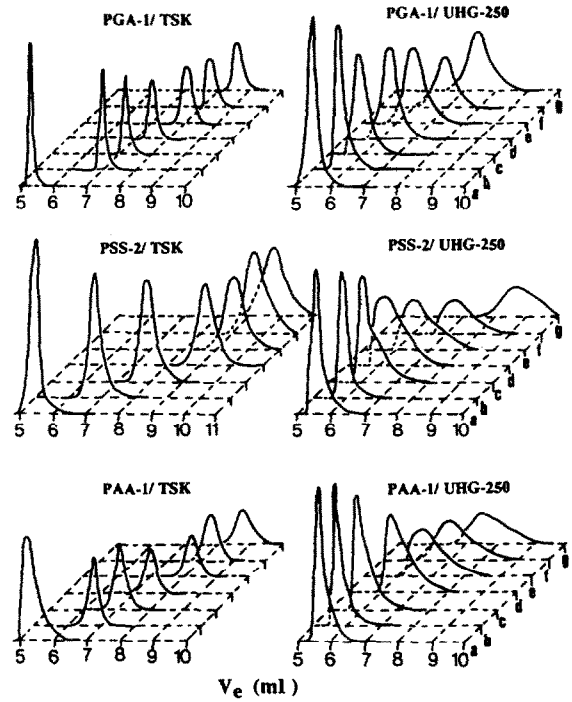

Fig. 2. Comparison of the elution profiles of poly(L-glutamic acid) (PGA-1), sodium poly(styrene sulphonate) (PSS-2) and poly(acrylic acid) (PAA-1) on TSK PW4000 and on UHG-250 columns at pH 7.0 and various ionic strengths: (a) pure water; (b) 0.005 M; (c) 0.01 M; (d) 0.02 M; (e) 0.05 M; (f) 0.10 M; and (g) 0.02 M.

increase with increasing ionic strength of the mobile phase. Both TSK PW4000 [25,26] and UHG-250 [4,13,27] are hydroxylated polymer-based gels containing residual carboxyl groups. At low ionic strength and pH > pK_a(–COOH), these groups become dissociated and the gels will then exhibit negative residual charges on their surfaces. Consequently, the elution volumes of polyanions shift towards lower values, denoting polymer–gel electrostatic repulsion. This effect can be suppressed at high ionic strength, but not always [9]. At pH 7.0 all ionic groups of both packing and polyelectrolyte sample should be negatively ionized. Therefore, in pure water (chromatograms a) or in dilute buffer solution at $I = 0.005$ M (chromatograms b), solutes cannot enter into pores because of electrostatic repulsions and elute near the exclusion volume, V_0, and their peak shapes are sharp regardless of their molar masses. With increasing eluent ionic

strength, the screening of charges implies a decrease of electrostatic repulsion and, as a result, solute elution profiles become broader according to the increasing amount of pore volume accessible to the polyelectrolyte. Note that the TSK PW column has wider pores than the UHG-250 column, so solutes can enter into a larger pore volume, as shown by the higher elution volumes at the same ionic strength on TSK PW than on UHG-250 (compare profiles b for the same sample in both columns). As a consequence, at moderate I values the predominant separation mechanism in TSK PW gel seems to be size exclusion, whereas in UHG-250, at the same I values, it is ion exclusion.

This complex behaviour is better illustrated through universal calibration plots. Thus, log $M[\eta]_I$ *vs.* V_e graphs are depicted in Fig. 3 for PGA and dextran on TSK PW4000 gel at different ionic strengths and at pH 5.0 (acetate buffer, Fig. 3a) and at pH 7.0 (phosphate buffer, Fig. 3b). As mentioned above, elution curves of PGA

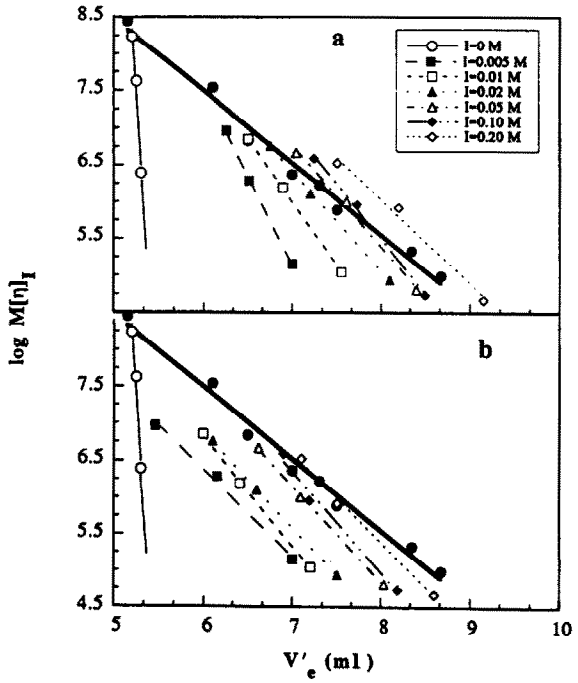

Fig. 3. Universal calibration plots for dextran (●) in pure water and poly(L-glutamic acid) (PGA) on a TSK PW4000 column at different ionic strengths and pH values: (a) pH 5.0 and (b) pH 7.0.

are far from the reference one at low ionic strength, approach the dextran curve with increasing saline content and cross it at pH 5.0 and high ionic strengths ($I = 0.10$ and 0.20 M). Although the influence of pH is less pronounced than that of ionic strength, dextran yields a single curve at any pH value while PGA plots deviate most from the reference curve at high pH values. This result is a consequence of the variation in the surface charge density provoked by the dissociation of carboxyl groups of both the PGA polymer and the support. At the lowest pH and highest I values, the elution volumes of polyelectrolyte (Fig. 3a) shift towards values higher than would be expected from ideal behaviour, probably because of the appearance of salt-induced matrix–solute hydrophobic interactions. In this regard, Mori [28] has reported hydrophobic retention of PSS on different supports at relatively high ionic strengths.

Because this general trend is also followed by the other studied polyanions (PSS and PAA) on both TSK PW and UHG-250 columns, a detailed analysis on the above deviations deserves to be undertaken. Since hydrodynamic volumes of solutes are not affected by pH, as mentioned above, changes in elution volumes with pH taking place at fixed ionic strength can be attributed to repulsive ionic effects between solute and gel surfaces. Effectively, PGA and matrix contain carboxyl groups, with pK_as in both cases about 4.25 and, whereas at pH 5.0 the degree of dissociation is about 50%, at pH 7.0 all functional groups are completely dissociated, greatly increasing the electrostatic repulsion. Of course, pH values lower than pK_a would diminish electrostatic effects but could also cause precipitation of polyanion and instability of supports. Note that for chromatographic purposes it is important to distinguish between the pH of eluent and the pH of the injected PGA solution. Whereas the former is constant, the latter depends on polymer concentration, this dependence being more pronounced as ionic strength decreases and molar mass increases [12]. In order to minimize this drawback all polyelectrolyte samples were injected at a constant concentration, namely 0.1% (1 g l^{-1}, dilute solution). In spite of this, changes in the pH of

the actual injected sample with respect to the pH of eluent could affect the extent of repulsive polymer–substrate interactions, and these contributions, though small, could add to the general trend followed by calibrations with pH.

Fig. 4 depicts calibration curves for PGA, PSS and PAA on both TSK PW4000 (Fig. 4a–c) and UHG-250 (Fig. 4d–f) columns at pH 5.0 and different ionic strengths. As can be seen, different sets of elution curves depending on I are obtained for each polyion/gel system. Comparison of Fig. 4a–c should illustrate the different elution behaviour of polyelectrolytes on the same TSK PW4000 column and eluents. Thus, the predominant mechanism for the PGA/TSK system, at low and moderate I values, is ion exclusion; ideal SEC is obtained at $I = 0.10$ M, and at higher I values adsorptions or attractive polymer–gel interactions are observed. It must be kept in mind, as commented above, that pH 5.0 is the pH value most suitable for elution because about 50% of ionizable groups of both polyanion (PGA) and gel remain protonated. Regarding the PSS/TSK system (Fig. 4b), at $I < 0.01$ M electrostatic repulsion effects predominate and pure SEC is achieved at $I = 0.01$ M. At higher I values the calibration curves shift towards higher elution volumes than those of the reference one, and they become vertical at the highest studied I. In fact, under these conditions elution volumes seem to be independent of solute molar mass, and they reach the total volume of column whatever the polyelectrolyte molar mass. In the system PAA/TSK (Fig. 4c), size exclusion is the predominant separation mechanism in the range $0.01 \leq I \leq 0.05$ M. Under these conditions repulsion and adsorption secondary effects would appear to cancel each

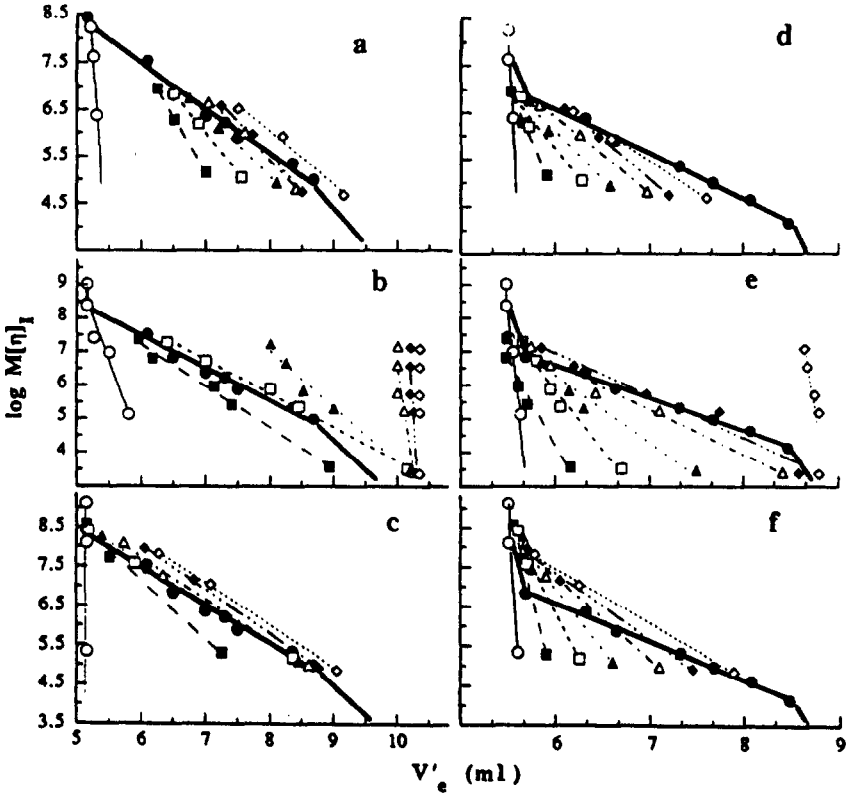

Fig. 4. Universal calibration plots for dextran (●) in pure water and poly(L-glutamic acid) (PGA) (a), sodium poly(styrene sulphonate) (PSS) (b) and poly(acrylic acid) (PAA) (c) on a TSK PW4000 column. Parts d, e and f refer to calibration of the same polyions on a UHG-250 column. In all cases acetate buffer solution at pH 5.0 has been used. The meaning of the symbols is the same as in Fig. 3.

other out. At higher I values, adsorption of polymer by gel takes place. In summary, under fixed I and pH conditions, elution volumes in TSK columns are in the order PGA < PAA < PSS. This order is in agreement with the non-polar character of solutes, that is with their expected hydrophobic interactions. In fact, in this gel hydrophobic interactions seem to play a more important role than electrostatic ones, since if electrostatic interactions were predominant the order of elution volumes would be the inverse of that above [electrostatic repulsions should be in the order PSS > PAA = PGA as expected from their pK_as, namely pK_a(PSS) < pK_a(PAA) = pK_a(PGA)].

Calibrations in UHG-250 (Fig. 4d–f) seem to follow similar trends to the above ones. In this gel, however, electrostatic effects seem to be the most important secondary effects, since, except for PSS at very high ionic strength ($I = 0.20$ M), adsorption effects seem to be negligible. This behaviour sounds reasonable, because on the one hand UHG-250 gel displays less apolar zones than TSK gel and, on the other, it has a higher percentage of ionizable groups per area unit, that is a larger surface charge density. Ideal SEC, of course, can only be obtained at higher I values in this gel. It is worth remarking that the usual order in elution volumes PGA < PAA < PSS is not obeyed by PAA samples with the largest molecular masses, which cross the reference calibration curve. This anomalous behaviour at the highest molecular masses can be explained by assuming additional specific polymer–gel interactions, via hydrogen bonds between the carbonyl ester group of methacrylate, the monomer base of UHG gel and the –COOH lateral groups of PAA (or PGA). These cooperative additional adsorptions, the larger the higher the molecular mass of solute, are in agreement with the increase in alcohol preferential sorption with polymer molecular mass reported by Horta and Katime [29] for poly(methyl methacrylate)–benzene–butanol systems, in which specific interactions between polymer and alcohol take place, and are also in line with the studies of Molyneaux and Vekavakayanondha [30] on specific interactions between phenols and poly(vinyl pyrrolidone). In these systems, a decrease in phenol–polymer interactions with decreasing polymer molar mass is also observed, as ascertained by the proportion of active sites on the polymeric chain occupied by the phenol.

From the complex behaviour mentioned above, it is deduced that the chemical nature of the polyelectrolyte is the main factor governing its elution behaviour, since secondary effects depend on the one hand on the ionizable group density (pK_a) and, on the other, on the extension of non-polar zones. Likewise, from the comparison of elution behaviours of a given polyanion on diverse columns, the following consequences can be pointed out:

(a) Regarding the electrostatic repulsion effects, modern gels based on cross-linked hydrophilic polymers seem to be more convenient for aqueous size-exclusion chromatography studies than the traditional silica-based ones.

(b) For the two gels studied here, the antagonistic secondary effects under the usual working conditions seem to be better equilibrated in UHG-250 than in TSK PW4000. In the latter electrostatic repulsions are weaker but undesirable adsorptions are present, at least for the solutes studied here.

Finally, it must be pointed out that the best experimental conditions (pH and I of mobile phase) in SEC of synthetic or biological polyelectrolytes depend on the solute/support system under study. Each specific problem demands an optimum selection of column and a rigorous search for the most suitable eluent.

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