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Journal of Chromatography A, 778 (1997) 53–65

JOURNAL OF CHROMATOGRAPHY A

Macromolecules in ordered media VIII. High-performance size-exclusion chromatography as a technique for characterizing the interaction between polyanions and cationic liposomes

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Abstract

The association of polyanions with liposomes based on dimyristoyl phosphatidyl choline and stearylamine at a ratio of 50:50 was investigated in terms of the binding model and, to a lesser extent, of the partition model. Aqueous size-exclusion chromatography has been proposed as a major technique for monitoring the interactions between liposomes and polyelectrolytes and the results were compared with those obtained by fluorescence spectroscopy. Quantitatively, the extent of the association follows the order: Sodium poly(styrene sulfonate) ($K_A = 10\,000\ M^{-1}$), poly(acrylic acid) ($K_A = 2000\ M^{-1}$) and poly(L-glutamic acid) ($K_A = 700\ M^{-1}$). Also, the effect of the ionic strength has been studied, showing an enhancement of the association as the ionic strength increases. Furthermore, a plausible way in which the interaction occurs has been proposed, based on the calculated number of phospholipids involved in the binding and on the polymer's conformational structure. © 1997 Elsevier Science B.V.

Keywords: Liposomes; Binding studies; Mobile phase composition; Polyanions

1. Introduction

Cell membranes are complex organized systems of lipids, proteins and carbohydrates, supported by the cytoskeleton, a two-dimensional network of polyelectrolyte nature, with the function of stabilizing and maintaining membrane integrity [1]. In this field, polymer–liposome complexes with polyelectrolytes attached to the lipid bilayer have been extensively used to model the dynamic motions of the cellular cytoskeleton [2] or to study biological membrane processes [3]. Furthermore, polyelectrolytes have also recently found application in liposome controlled release systems due to their use in both stabilizing liposomes and disrupting liposomes, depending on environmental stimuli such as pH or temperature. As liposome stabilizers, Tirrell et al. have employed ionene [4] and poly(ethylene imine) [5] to stabilize liposomes, Sato et al. [6] have studied maleic acid copolymers and Tsuchida [7] has reported the interaction of poly(N-vinylimidazole), poly(L-glutamic acid) and sodium poly(styrene sulfonate) with dipalmitoyllecithin vesicles or human erythrocytes showing that the interaction of polyanions enhances the membrane's rigidity. Concerning the second application, Tirrell and co-workers [8–

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PII S0021-9673(97)00385-3

10] studied the pH dependence of the interaction of poly(carboxylic acids) and poly(acrylic acids) with vesicles and found that the adsorption decreased upon decreasing the pH, thus modifying the membrane organization from vesicles to micelles. Kono et al. [11] have developed temperature-sensitive liposomes bearing poly(N-isopropyl acrylamide) and have shown their usefulness in drug delivery systems.

On the other hand, polyelectrolytes, either cationic or anionic, have been shown to have a wide range of biomedical applications [12]. For this reason, the study of polyelectrolyte–liposome interactions has received great interest, as these systems can be used to model the answer of the organism cells to such charged polymers. In this regard, Odin et al. [13] have recently shown the adsorption of diallyl polycations with antimutagenic efficiency on soybean phosphatidylcholine membranes. Also, polyanions exhibit important biomedical applications, such as to increase resistance to bacteria and fungi, to enhance immune response, to inhibit adjuvant arthritis and either to depress or stimulate phagocytic activity [14]. Also, the binding between polyelectrolytes and small oppositely charged micelles [15] and studies of polyelectrolyte–protein complex formation have recently been investigated by Dubin's group [16–19]. In this report, we focused on the study of the interaction between small unilamellar cationic liposomes and three polyanions, sodium poly(styrene sulphonate), poly(L-glutamic acid) and poly(acrylic acid). All of them have been used in biomedical applications and have been proved to play a significant role coating liposomal drug carriers [20,21].

Our own work for the last few years has been focused on polycation–anionic vesicle systems and on characterizing the interaction by means of the partition and the binding models [22–27] as well as on the interaction processes between polyelectrolytes and chromatographic gel surfaces [28–30]. The above-mentioned models allow us to determine the association constant and the partition coefficient using results obtained from fluorescence or circular dichroism experiments. Moreover, the partitioning of drugs into a cell membrane has also been measured chromatographically using immobilized, artificial membrane chromatography [31,32].

The main goal of this work is to present high-performance size-exclusion chromatography (HPSEC), with a refractive index detector, as an alternative technique to fluorescence spectroscopy, which is widely used to characterize associations between interacting agents and bilayers [33–35]. The advantages of HPSEC with respect to the experimental drawbacks of the spectroscopic technique are: (1) It avoids the need for the interacting agent to contain a fluorophore group (or the use of probes), (2) the concentration range assayed is widened and (3) the controversial double reciprocal plot is not required, simplifying the method. In order to asses the validity of the HPSEC technique for characterizing the interaction between polymers and vesicles by means of the binding and partition models, the first step consisted of monitoring the interaction of a fluorescent polyelectrolyte, such as sodium poly(styrene sulphonate) (PSS) by fluorescence spectroscopy and afterwards by the chromatographic technique, and comparing equivalent results. In order to contribute to a better and deeper understanding of polyanion–vesicle interactions, the chromatographic experiments have also been extended to other polyanions without spectroscopic characteristics, such as poly(L-glutamic acid) (PGA) and poly(acrylic acid) (PAA). Their association isotherms have been interpreted in terms of the partition model and the binding model, with particular emphasis being placed on the latter. In addition, an analysis of the influence of ionic strength on the interaction has also been conducted, specifically for the systems containing PGA and PAA.

2. Experimental

2.1. Materials

Dimyristoyl phosphatidylcholine (DMPC) and stearylamine (STA) were purchased from Sigma (St. Louis, MO, USA). The polyelectrolytes studied were samples of sodium poly(styrene sulphonate) (PSS), of nominal molar mass, M, 1600 g mol^{-1} from Pressure Chemical (Pittsburgh, PA, USA), poly(L-glutamic acid) (PGA) of $M=13\,600$ g mol^{-1} from Sigma and poly(acrylic acid) (PAA) of $M=5000$ g mol^{-1} from Aldrich (Milwaukee, WI, USA). All

polyelectrolyte samples showed polydispersities lower than 1.1.

2.2. Conditions

The eluents used were buffers made up of NaH_2PO_4 and Na_2HPO_4, pH 7.0, both reagents being of analytical-reagent grade. The desired ionic strengths, C_s, were adjusted to 0.01 and 0.02 *M*. Water, of chromatographic grade, was obtained from a Milli-Q system from Waters Chromatography Division (Milford, MA, USA) and was tested daily by conductimetry. The solvent used for fluorescence experiments was the phosphate buffer (0.02 *M*). All experiments were performed at room temperature.

2.3. Preparation of liposomes

The stock solution of small unilamellar vesicles of DMPC–STA (50:50, v/v) was prepared by tip ultrasonication followed by ultracentrifugation, as was described in detail elsewhere [25]. Briefly, an appropriate mixture of DMPC and STA was dissolved in chloroform and evaporated in a round-bottomed flask using a rotary evaporator at 45°C. Once formed, the phospholipid film was hydrated by adding the required volume of the buffer solution at 30°C, just above the melting point (23°C) of DMPC, over 10 min and then shaking the mixture gently for 5 min on a vortex-mixer. Afterwards, the solution was sonicated to promote the formation of unilamellar vesicles and it was subsequently ultracentrifuged (10 min, 13 000 *g*) to remove the large vesicles (pellet) from the small unilamellar vesicles.

Fresh samples were prepared by dilution of this stock solution with appropriate aliquots of buffer.

2.4. Fluorescence measurements

Emission fluorescence spectroscopy was conducted on a Perkin-Elmer Model LS-5B luminescence spectrometer that was equipped with a Data Station Model 3700. All spectra were obtained at room temperature with an excitation wavelength of 260 nm, with excitation and emission slits of 5 nm. A battery of independent samples containing a fixed concentration of PSS and increasing concentrations of liposomes were prepared for use in the fluorescence experiments, in order to determine the desired lipid–polymer ratios, R_i. In all cases, the spectra were corrected for background fluorescence as well as for vesicle and solvent light scattering by subtraction of the blank spectrum, which was recorded in the absence of polymer under identical conditions.

2.5. Chromatographic measurements

A Waters Model ALC/GPC 202 liquid chromatograph equipped with an M-45 solvent delivery system, a U6K injector and an R-410 refractive index detector was used. 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, was used as the chromatographic packing material. The interstitial packing volume and the total pore volume were 5.48 and 10.96 ml, as measured with a standard dextran of M_r=2 000 000 g mol^{-1} and ^2H_2O, respectively.

Eluents were degassed and filtered 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, and chromatograms were always obtained at a flow-rate of 1.0 ml min^{-1}.

Two sets of experiments were performed involving binary (polyelectrolyte–buffer) and ternary (polyelectrolyte–liposomes–buffer) systems, using the buffered solution as the mobile phase in all cases. All samples were run twice on the UHG-250 column, and the results shown are the average values obtained.

2.6. Theoretical background

The association of interacting molecules to model vesicles has lately been characterized in terms of two models: The partition and the binding models, both involving association isotherms (the number of bound moles of polymer per mol of accessible phospholipid, α/R_i^*, versus the concentration of free polymer, [P]) in the quantitative interpretation.

Briefly, the binding model describes the polymer–vesicle interaction by an equilibrium between the polymer free in solution, P; the unoccupied membrane sites, S_N, containing *N* phospholipids and the

polymer bound to the liposomes, PS_N, expressed as [36]

$$P + S_N \rightleftarrows PS_N \quad (1)$$

with a characteristic association constant, K_A, whose definition in terms of concentration leads to the following expression [25]

$$\alpha/R_i^* = K_A(1/N - \alpha/R_i^*)[P] \quad (2)$$

which describes an association isotherm by means of K_A and N.

Another equation derived assuming the Langmuir isotherm for the binding process can be used to describe the association isotherm [37],

$$\alpha/R_i^* = K_A x_L^2[(\alpha/R_i^*)^{-2}(1/N) - (\alpha/R_i^*)^{-1}][P]_\infty \quad (3)$$

where x_L is the mole fraction of anionic phospholipid making up the bilayer and $[P]_\infty$ refers to the concentration of free polymer at an infinite distance from the bilayer surface [38]. Since Eq. 3 includes electrostatic effects, it would be more rigorous and would fulfil the experimental results better than Eq. (2).

On the other hand, the partition model gives a quantitative interpretation of the association isotherms on the basis of a membrane–water partition equilibrium of the polymer, yielding the expression [39],

$$\alpha/R_i^* = \frac{\Gamma}{\gamma}[P] \quad (4)$$

where Γ is the pertinent partition coefficient, which depends on the standard Gibbs free energy change per mol as a consequence of the polymer relocation from water to the membrane. γ is the activity coefficient that takes into account the electrostatic repulsions between the charged groups on the polymer.

In addition, as we have previously reported [25], both models can be related, rendering the following relationship between their characteristic parameters (assuming here that $\gamma \approx 1$)

$$\Gamma = K_A\left[\frac{1}{N} - \alpha/R_i^*\right] \quad (5)$$

3. Results and discussion

To validate the idea that HPSEC can be used to measure polymer–liposome interactions, in the first place, we have followed the association of the fluorescent PSS to DMPC–STA liposomes by fluorescence, a technique widely used and which proved very useful for describing such types of interactions. Afterwards, the same system was studied by HPSEC and the results were compared to those obtained by the spectroscopic method.

3.1. PSS–liposomes–buffer system

3.1.1. Fluorescence

Fluorescence spectroscopy has proved to be a powerful tool for monitoring the interaction of small molecules, such as drugs or peptides, [40] as well as of cationic polymers [22–27] with phospholipidic vesicles, because, (1) the fluorescence intensity depends linearly on the concentration of the two possible states of the polymer (associated and aqueous) and (2) the mass of the entire polymer concentration in the system must be conserved.

Keeping in mind these ideas, changes in the wavelength of maximum emission, $\Delta\lambda$, and in the fluorescence intensity of the polymer, ΔI, occur as more and more moles of liposome are added to the polymer buffered solution, since both variations provide information about the extent of the interaction. Two sets of experiments have been performed at two total polymer concentrations, $[P]_t = 1 \cdot 10^{-4}$ and $1.7 \cdot 10^{-4}$ M. As an example, Fig. 1A shows the fluorescence spectra of PSS ($1 \cdot 10^{-4}$ M) in the absence (upper spectrum) and in the presence of increasing amounts of DMPC–STA vesicles (different R_i^* values). As can be seen, the interaction leads to a small blue shift of the wavelength of maximum emission, λ_{max}, (from 294 to 291 nm) as well as to a relative decrease (by 50%) in the fluorescence intensity at 294 nm when $R_i^* = 8$. The shifting of λ_{max} reveals the relocation of the polymeric sulphonic groups from water to the membrane [41]. Furthermore, the extent of the blue shift gives information about the level of penetration of the fluorescence group into the bilayer [42]. In this case, the small shift (3 nm) allows us to propose that the sulphonic groups of the polyelectrolyte remain on the

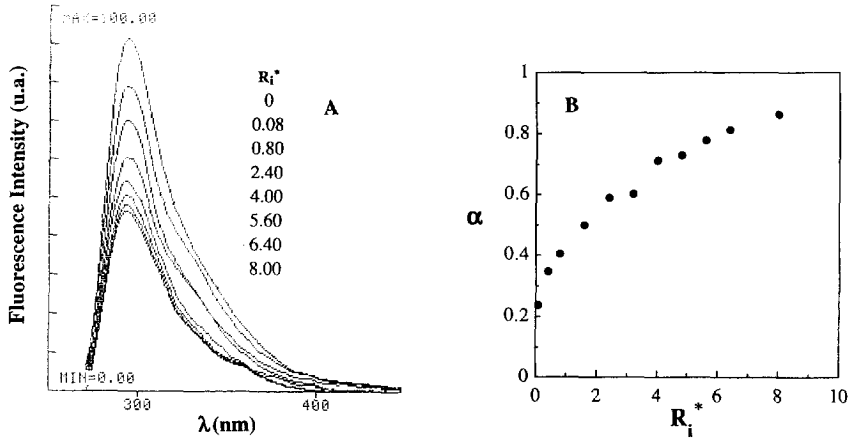

Fig. 1. (A) Emission spectra of PSS in the absence (upper, $R_i^*=0$) and in the presence of increasing amounts of DMPC–STA vesicles. Excitation wavelength, 260 nm. (B) Variation in the fraction of PSS bound to DMPC–STA vesicles with R_i^*.

bilayer surface, forming a monolayer, which will be discussed later.

On the other hand, the fluorescence intensity values at 294 nm, I, for each R_i^* provide us with the necessary experimental raw data to quantify the interaction PSS–DMPC–STA. By means of the well-known double reciprocal plots, that is, $I_o/(I-I_o)$ (with I_o being the intensity of the polymer in the absence of vesicles) versus $1/R_i^*$, the fraction of the polymer bound to the membrane, α, at each value of R_i^*, can be obtained. Specifically, the intercept of the double reciprocal plot $[I_o/(I_m-I_o)]$ gives the intensity of the polymer totally bound to the bilayer, I_m. Then, α is readily calculated through the expression

$$\alpha = (I - I_o)/(I_m - I_o) \quad (6)$$

The α values at each values of R_i^* for the experiment performed at $[PSS]=1\cdot10^{-4}$ M are depicted in Fig. 1B, being $I_o=91.45$ and $I_m=39.15$. As can be seen, α increases progressively with R_i^*, showing a clear tendency to reach a plateau after the initial sharp increase and, at $R_i^*=8$, practically 80% of the macromolecule is bound to the vesicles. Once α is known, the concentration of free polymer in solution is evaluated by using

$$[P] = (1 - \alpha)[P]_t \quad (7)$$

which allows us plot the corresponding association isotherms. These curves show the typical shape that was reported recently [22–27], that is, an initial linear part, denoting ideal association, followed by the bending of the curve, indicating that electrostatic repulsions occur, and a sharp increase of α/R_i^* at values of [P] that are close to $[P]_t$, which indicates the formation of aggregates [25]. Moreover, variation of the polymer concentration has no effect on the isotherm curve, at least in its essential, as will be shown below together with the chromatographic data. Also, isotherms have been plotted as a function of $[P]_\infty$, which can be calculated from [P] using the equation [24],

$$[P]_\infty = (\alpha/R_i^*)^2[P] \quad (8)$$

as we shall see later.

3.1.2. Chromatography

The proposed method for analysing the extent of the interaction by HPSEC is based on the measurement of the height of the polyelectrolyte peak in the elution profile and relies on the assumption that the polymer concentration is linearly correlated with the height of the generated peak. Such an assumption has previously been proved to be reliable, at least throughout the polymer concentration range that was assayed. In this regard, Fig. 2A displays the detector response on injection of diverse buffered PSS solutions, with concentrations ranging from 0.014 to 0.35% (w/v) (chromatograms from bottom to top),

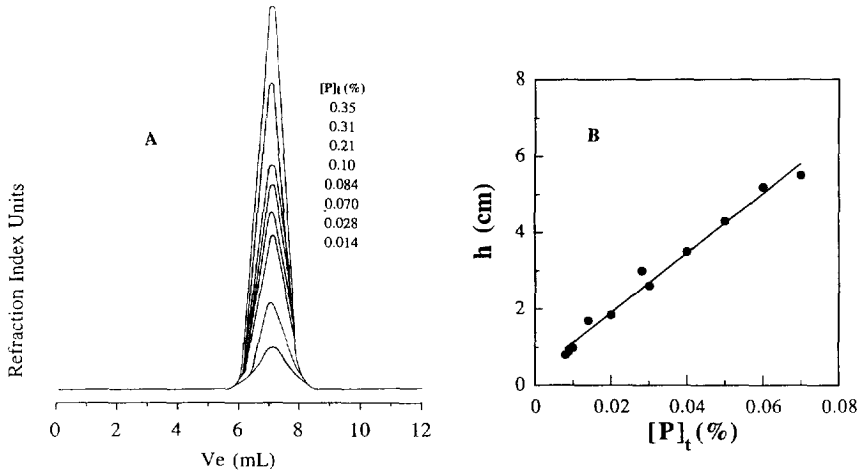

Fig. 2. (A) Elution profiles for 100 μl of PSS at different concentrations on a UHG-250 column. Eluent, phosphate buffer, pH 7.0; ionic strength, 0.02 *M*. (B) Height of the PSS peaks against the injected polymer concentration.

and Fig. 2B depicts the plot of peak height against the concentration of polymer injected, showing a good linear dependence. These kind of plots can be used as calibration graphs for the analysis of chromatograms obtained when the association takes place.

Therefore, the elution profile of the binary system will show a single peak due to the polymer sample, whereas column elution of the ternary system (at the same polymer concentration) will give two peaks, corresponding to the free polyelectrolyte and the polymer–vesicle complex or associated polymer, provided that the complex is stabilized by strong coulombic forces and that it is not in dynamic equilibrium with the free polymer during the chromatographic process. By relating the heights of the peaks with the polymer concentration, one can estimate the extent of the association. In other words, with h_t being the peak height in the binary system and with $h_t \equiv [P]_t$; h_f being the peak height of free polymer in the ternary system and $h_f \equiv [P]$, the difference will correspond to the amount of polymer that is associated or bound to vesicles, e.g., $h_a = (h_t - h_f)$ and $h_a \equiv \alpha[P]_t$.

Finally, we must relate the chromatographic data with the association isotherms since these plots are used to describe the interaction process, together with the theoretical models outlined above. If this is so, interpolation of h_a and h_f data in the calibration graph (see Fig. 2B) will yield the associated and free polymer concentrations, respectively, with the former value being easily converted into α/R_i^* by means of the relationship

$$\frac{\alpha}{R_i^*} = \frac{[P]_{\text{associated}}/[P]_t}{[L]_t^*/[P]_t} = \frac{[P]_{\text{assoc}}}{[L]_t^*} \tag{9}$$

where the accessible phospholipid concentration, $[L]_t^*$, is known. Therefore, as many isotherm points can be obtained as there are polymer peaks in every experiment, with and without liposomes.

Fig. 3 shows a typical pair of PSS elution profiles

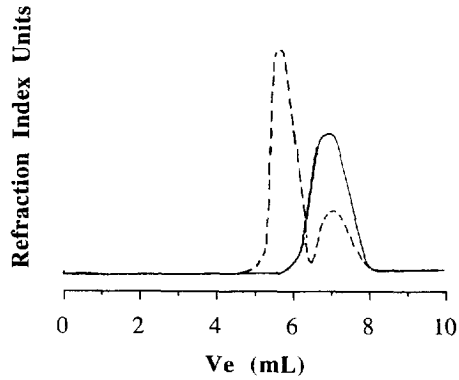

Fig. 3. Elution profiles for 100 μl of PSS (0.05%) with (dashed line, $R_i^* = 3.24$) and without (continuous line) liposomes. Eluent as in Fig. 2.

at a constant concentration (0.05%, w/v) in the absence (continuous line) and in the presence (dashed line) of DMPC–STA vesicles ($R_i^* = 3.24$). As can be seen, the binary system generates a peak at 7.1±0.1 ml, which is associated with PSS, whereas two peaks appear in the elution profile of the ternary system. The first one occurs at a retention volume of 5.5±0.1 ml and is due to the elution of the polymer–liposome complex, which permeates into the least number of pores because of its higher size, leading to it being eluted first. The second peak, with a retention volume of 7.1±0.1 ml, can be identified as the free polymer peak by comparison with the binary system (see Fig. 2A). In addition, a difference can be seen in the heights of the peaks at 7.1 ml, which clearly indicates the diminution of the free polymer in solution at the expense of its incorporation into the liposomes, since the total concentration is the same in both binary and ternary systems. Moreover, isolated measurements using a UV detector at 270 nm, for which the complex does not absorb, have shown a single peak corresponding to the free PSS at the same elution volume.

As explained before, data from different pairs of runs (at diverse liposome and PSS concentrations) have been converted into association isotherms, as in Fig. 4, and analyzed in the light of the partition and the binding models. The best fit of data through Eq. (2) gives values of $K_A = 14\,000\ M^{-1}$ and $N = 2$.

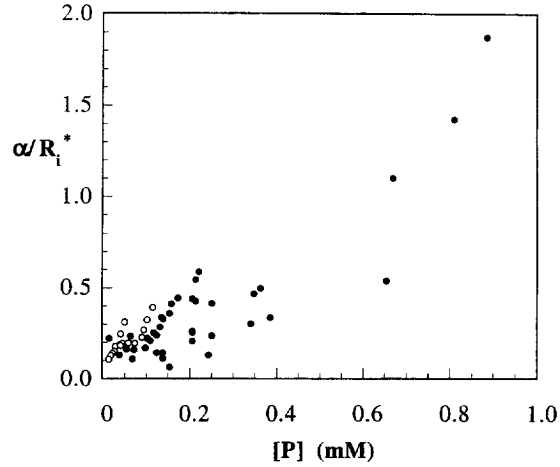

Fig. 4. Association isotherm of PSS with DMPC–STA vesicles plotted by using chromatographic (●) and fluorescence (○) experimental data.

However, since the cationic stearylamine could be not homogeneously distributed between the inner and the outer sides of the bilayer and the polymer is supposed to interact exclusively with the outer side, it seems more appropriate to use Eq. (3), which takes into account the fraction of charged molecules in the bilayer, x_L. In such cases, the best fit of experimental data has been obtained for $x_L = 0.4$, giving binding parameters equal to $K_A = 10\,000\ M^{-1}$ and $N = 2$. On the other hand, the partition coefficient is evaluated from the slope of the initial part of the isotherm, which, for this system, gives $\Gamma_{exp} = 2000$. Furthermore, the two models have been related through Eq. (5) from the pairs of K_A and N values, obtaining the following calculated partition coefficients: $\Gamma_{cal} = 2130$ (with $K_A = 14\,000\ M^{-1}$ and $N = 2$) and 1900 (with $K_A = 10000\ M^{-1}$ and $N = 2$). These values are very close to the value of 2000, which was determined graphically from Fig. 4, and suggest that the content of stearylamine on the inner side would be slightly higher than on the outer side of the bilayer.

Finally, as seen in Fig. 4, results from both techniques agree fairly well, showing that the SEC method offers a wider range of concentrations that can be studied and, therefore, can be used in a wider range of applications than the fluorescence measurements.

3.2. PGA–liposomes–buffer system

In order to study the effect of electrolyte content in the milieu, experiments were performed at two ionic strengths. Fig. 5 shows the detector response on injection of diverse polymer concentrations in buffered solutions (binary systems) at ionic strengths of 0.01 *M* (part A) and 0.02 *M* (part B) as well as the corresponding calibration graphs (parts C and D, respectively). As we can see from Fig. 5A–B, there is an increase in the retention volume of PGA from 7.80±0.05 ml to 8.50±0.05 ml as the ionic strength of the mobile phase increases, which has also been reported in a previous work [43]. This shifting of elution volumes to higher values was attributed to the screening of charges by counter-ions in both the polyelectrolyte and the chromatographic support. The final result is a decrease in the electrostatic repulsions between the polyanion chain and the

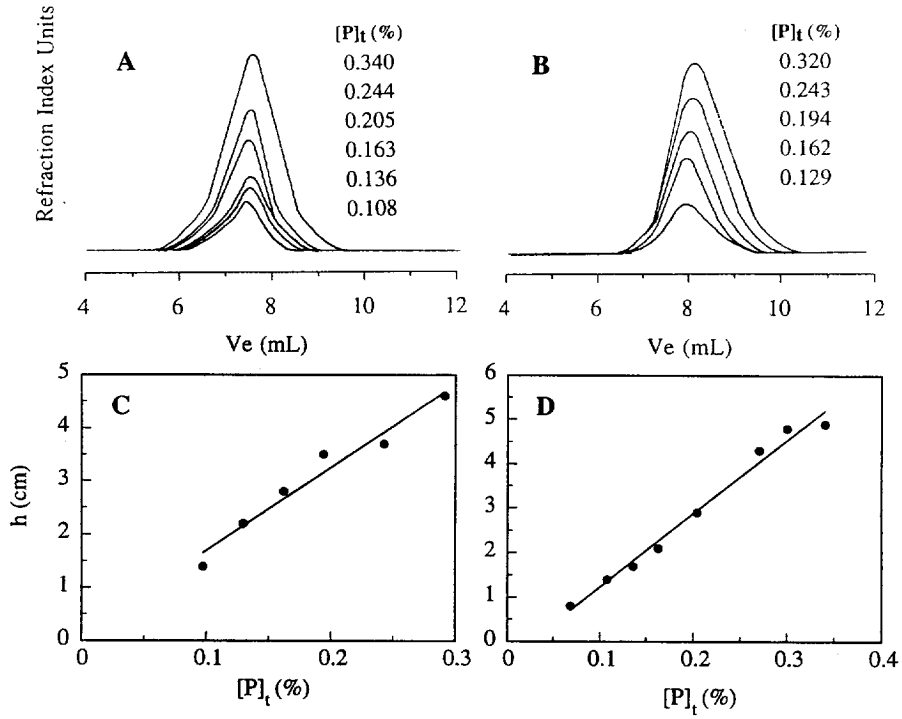

Fig. 5. Elution profiles for PGA at different concentrations on a UHG-250 column and with different ionic strengths: (A) 0.01 *M* and (B) 0.02 *M*. (C) and (D) Height of the PGA peaks versus the injected polymer concentration at C_s 0.01 and 0.02 *M*, respectively.

negative charges on the gel surface and, consequently, retarded polymer elution.

Again, a good linear correlation was found between the height of the polymer peak and its concentration, as can be seen in Fig. 5C–D. By following the same experimental procedure as explained in the preceding section, several pairs of binary and ternary systems were run on the column at different $[P]_t$ and R_i^* values. As an example, Fig. 6 shows the chromatograms obtained with 0.244% PGA in the absence and in the presence of liposomes at $C_s = 0.01$ *M* (part A) and $C_s = 0.02$ *M* (part B), with $R_i^* = 5.27$ and 5.74, respectively. In contrast to the PSS elution profiles, an important difference arises for PGA, that is, the lack of a polymer–liposome complex peak in the elution of ternary systems. However, the height of the PGA peak (or its area) is smaller than that in the binary system, denoting a diminution in the concentration of free polymer in solution. Would such behaviour signify that the interaction does not occur? It can be argued that the association does take place since the decrease in the concentration of free polymer suggests that it has been incorporated in or associated with the vesicle. The lack of the complex peak could be attributed to its electrostatic adsorption onto the ionic groups of the chromatographic packing, which is negatively charged, due to the existence of residual positive charges on the bilayer surface of the polymer–liposome complex. This argument would be supported by two facts: (1) The small extent of the interaction involves a low number of cationic stearylamine molecules in the association (see the small values of the association constants that are given below) and (2) as previously reported [26], the molar mass of the polymer and, therefore, the polymer structure in solution plays an important role in the association with liposomes. Hence, the more flexible PGA structure, because of its molar mass (ca. ten times higher than that of PSS), implies that it folds over itself when approaching the bilayer, thus exhibiting a fewer number of anionic groups on interaction, with many more cationic charges remaining on the complex surface. Both effects would lead

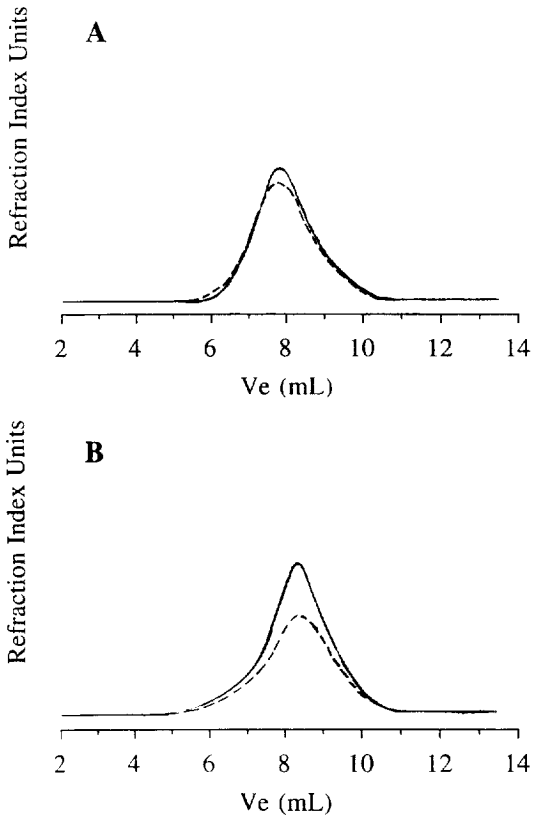

Fig. 6. Elution profiles for 100 μl of PGA (0.244%) with (dashed line) and without (continuous line) liposomes at $C_s = 0.01$ *M*, $R_i^* = 5.27$ (A), and 0.02 *M*, $R_i^* = 5.74$ (B).

to a positively charged complex that would be adsorbed onto the anionic gel surface by electrostatic attraction. Furthermore, the existence of positively charged patches on the surface vesicle is perfectly plausible, given that the liposome has an average radius of 800 Å and each phospholipid head occupies ca. 70 $Å^2$, leading to approximately $5 \cdot 10^4$ cationic groups on the surface, whereas a PGA chain bears 136 anionic groups, from which, up to two are involved in the binding (see Table 1).

Fig. 7 depicts the association isotherms obtained at the two assayed ionic strengths, 0.01 *M* (part A) and 0.02 *M* (part B). Data were interpreted by means of the two models and the data are shown in Table 1. Three fits using different x_L values have been performed according to Eq. (3), and these are also drawn in Fig. 7. At $C_s = 0.01$ *M*, the best fit comes from using $x_L = 0.4$, as can be seen in Fig. 7A. In

Table 1
Binding parameters, K_A and *N*, obtained from the association isotherm data by using the binding model (Eq. (3)) at different ionic strengths and x_L values for the PGA–DMPC–STA system

C_s (*M*)	x_L	K_A (M^{-1})	*N*	Γ_{cal}	Γ_{exp}
0.01	0.3	500	0.5	975	1200
	0.4	600	0.5	1170	
	0.5	500	0.5	975	
0.02	0.4	700	2	248	208
	0.5	800	2	364	
	0.7	600	2.5	212	

Experimental and calculated (using Eq. (5)) partition coefficients are also shown.

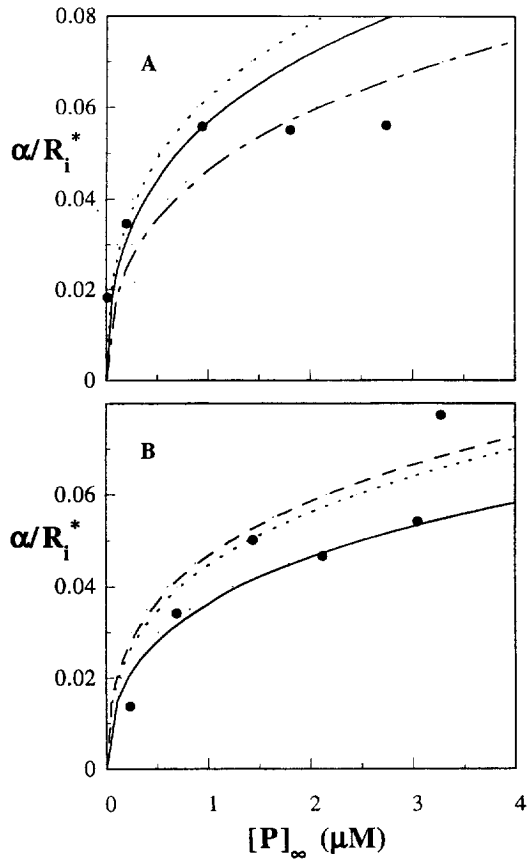

Fig. 7. Association isotherms of PGA with DMPC–STA vesicles at different ionic strengths: (A) 0.01 *M* and (B) 0.02 *M*. Curves correspond to the fits of experimental data (●) calculated using Eq. (3) at different x_L values: $x_L = 0.3$ (– · –); $x_L = 0.4$ (———); $x_L = 0.5$ (· · ·) and $x_L = 0.7$ (– – –).

addition, for this x_L value, the value of the calculated partition coefficient obtained from these binding parameters is also the closest to the experimental one (graphically evaluated). On the other hand, at C_s = 0.02 *M*, as Fig. 7B shows, the best fit is reached with $x_L = 0.4$, although the Γ_{exp} value seems to be better predicted using $x_L = 0.7$ ($\Gamma_{cal} = 212$ and $\Gamma_{exp} = 208$).

Concerning the effect of ionic strength on the interaction, it should be stressed that both K_A and N values increase with increasing ionic strength. In particular, whereas K_A increases slightly, the number of phospholipids involved in the binding quadruples. Such behaviour implies that the presence of electrolytes in solution enhances the association, probably due to a diminution of electrostatic repulsions as a consequence of the screening of the polymer charges, as has also be found for systems formed by polycations and anionic liposomes [25,26]. On the other hand, it seems that at both ionic strengths, the cationic probe would be distributed more in the inner layer than in the outer part of the bilayer.

3.3. PAA–liposomes–buffer system

The same experimental procedure has been followed for the present system. First of all, injection of the binary system PAA/buffer at different PAA concentrations and at two C_s values and, secondly, runs of the ternary system, PAA–liposomes–buffer under the same experimental conditions, varying the R_i^* ratio. Fig. 8A–B depicts the PAA chromatograms obtained for the two assayed C_s values, with the elution volumes of the maximum of the peaks being found at 5.90±0.05 and 6.20±0.05 ml for C_s 0.01 and 0.02 *M*, respectively, and again emphasizing the higher PAA retention volume at $C_s = 0.02$ M, as found for PGA. In the same way, Fig. 8C–D reveal a good linear dependence of the peak height on the polymer concentration, as required in order to apply the proposed SEC method. Fig. 9 shows, as an example, the comparison of the elution profiles of 0.23% PAA in the absence and in the presence of liposomes at $C_s = 0.01$ *M* and with $R_i^* = 3.13$ (part A) and at $C_s = 0.02$ *M* and with $R_i^* = 2.06$ (part B). In all cases, a decrease in the height of the peak (and its area), corresponding to the free polymer in the ternary system, was noticed, compared with the binary system, which provides evidence of the

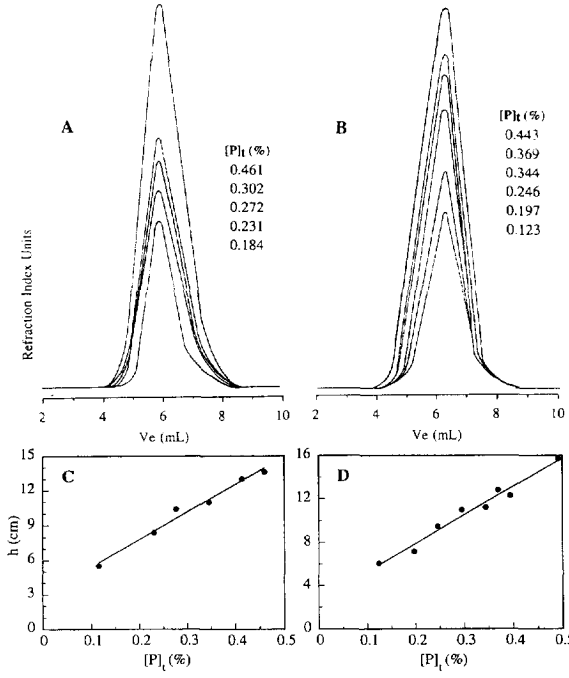

Fig. 8. Elution profiles for PAA at different concentrations on a UHG-250 column and at different ionic strengths: (A) 0.01 *M* and (B) 0.02 *M*. (C) and (D) Height of the PGA peaks against the injected polymer concentration at $C_s = 0.01$ and 0.02 *M*, respectively.

formation of the PAA–vesicle complex, even though the peak for this complex does not appear. The same arguments as those presented for the preceding system can be given for the lack of the complex peak in this case, although an additional reason can be argued. As is well known, polyacrylic acids are

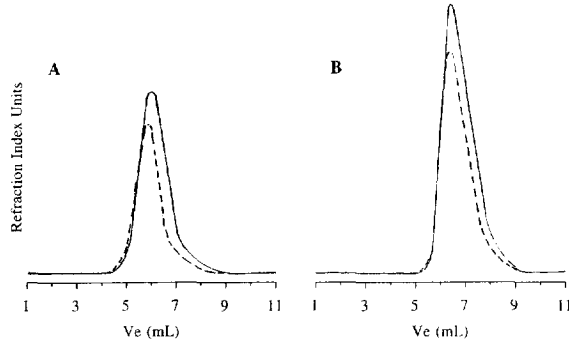

Fig. 9. Elution profiles for 100 μl of PAA (0.23%) with (dashed line) and without (continuous line) liposomes at $C_s = 0.01$ *M*, $R_i^* = 3.13$ (A) and at $C_s = 0.02$ *M*, $R_i^* = 2.06$ (B).

Table 2
Binding parameters, K_A and N, obtained from the association isotherm data by using the binding model (Eq. (3)) at different ionic strengths and x_L values for the PAA–DMPC–STA system

C_s (M)	x_L	K_A (M^{-1})	N	Γ_{cal}	Γ_{exp}
0.01	0.4	500	0.3	1600	1550
	0.5	800	0.5	1550	
	0.7	300	0.32	900	
0.02	0.3	2000	1	1500	1500
	0.4	1000	1	226	
	0.5	1700	2	774	
	0.7	1000	2	275	

Experimental and calculated (using Eq. (5)) partition coefficients are also shown.

usually prepared by hydrolysis of narrow polyacrylates, with such procedures often being incomplete, resulting in non-polar moieties remaining on the PAA chain, which causes an increase in the positive net charge on the complex and, consequently, a strong attractive interaction with the chromatographic support. This behaviour is reflected in the lower values of K_A obtained for the PAA complexes (see Table 2) when compared with those for PSS.

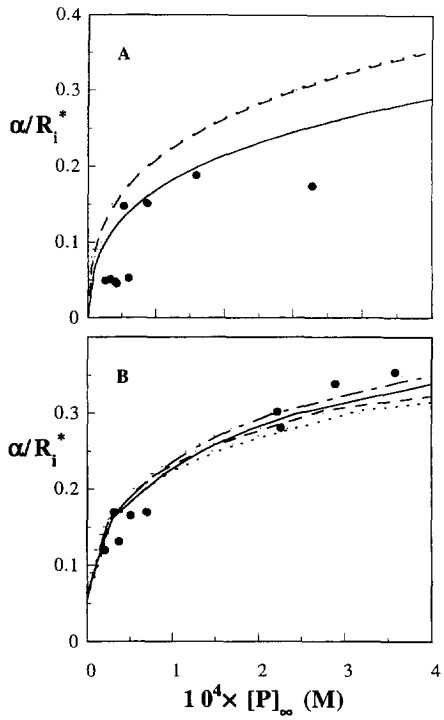

Fig. 10. Association isotherms of PAA with DMPC–STA vesicles at different ionic strengths: (A) 0.01 M and (B) 0.02 M. Curves correspond to the fits of experimental data (●) calculated using Eq. (3) at different x_L values: $x_L=0.3$ (– · –); $x_L=0.4$ (———); $x_L=0.5$ (· · ·) and $x_L=0.7$ (– – –).

In Fig. 10, plots of the association isotherms for this system at $C_s=0.01$ and 0.02 M are shown, parts A and B, respectively, together with the theoretical fittings built up with different x_L values according to Eq. (3). Inspection of this figure reveals that the best fit of the experimental data seems to correspond to values of $x_L=0.4$ and 0.3 for $C_s=0.01$ and 0.02 M, respectively. The values of the quantitative binding parameters, K_A and N (from Eq. (3)), as well as the calculated (using Eq. (5)) and experimental (graphically determined) partition coefficients are gathered in Table 2 for different x_L values and for each ionic strength. As can be seen, Γ_{cal} correctly predicts the Γ_{exp} value when using $x_L=0.4$ and 0.3 for each set of data, in accordance with the fittings plotted in Fig. 10. Furthermore, the effect of ionic strength on the association of PAA to liposomes is similar to that found for PGA, that is, both K_A and N increase as C_s does.

4. Conclusions

The chromatographic results of the three polyanions as a whole at a fixed ionic strength (0.02 M) show, in first place, that the elution volume is not influenced by the polyanion concentration, undoubtedly due to the low molar masses chosen [44,45]. Secondly, the order found in the elution volumes on the UHG-250 column was PAA<PSS<PGA, in accordance with their respective hydrodynamic volumes ($V_h \equiv M[\eta]$, with $[\eta]$ being the intrinsic viscosity), as previously reported [43,46]. Thirdly, the essential requirement of the method has been fulfilled in all cases, that is, a good linear correlation exists between the height of the polymer peak and the injected concentration. Lastly, chromatograms of the ternary system (polyelectrolyte–liposomes–buffer) have shown a second peak corresponding to the complex only in the PSS elution profiles. The lack of that peak in the other systems has been attributed to electrostatic adsorption of the complexes, PAA–DMPC–STA and PGA–DMPC–STA, onto the col-

umn because of the remaining positive charges on the bilayers once the interaction has occurred.

In order to perform a global analysis of the extent of the interaction, we may draw our attention to the fitting parameters of the obtained association isotherms. Firstly, it should be mentioned that throughout the paper the partition model has only been used for checking the fitness of the different binding parameters obtained. As explained in Section 2.6, this model involves two parameters, Γ and γ, although the latter has been neglected in order to simplify the discussion. Therefore, analysis of the interaction as well as of the ionic strength effect has been reduced to the results obtained by applying the binding model. A more detailed discussion through the partition model would require both Γ and γ to be taken into account, which we shall do in future work.

Concerning the optimal binding parameters, that is, those that best reproduce the experimental partition coefficients, it can be concluded that PSS interacts more with the cationic vesicles ($K_A = 10\,000\ M^{-1}$) than PAA does ($K_A = 2000\ M^{-1}$) and that PAA interacts more than PGA ($K_A = 700\ M^{-1}$), as already suggested by the chromatographic behaviour. With regard to the influence of ionic strength on the interaction, it has been observed that the association constant increases with increasing ionic strength (see Tables 1 and 2). It is well known that an increase in the electrolyte concentration causes a diminution in the degree of ionization, both on the polyelectrolyte chains and on the cationic vesicles, so that the attractive polymer–liposome interactions as well as polymer–polymer repulsions will be reduced. Thus, a plausible explanation for the observed trend should lie in the fact that the intensity of the coulombic repulsions diminish more dramatically than the polymer–liposome attractive ones, resulting in the overall association being enhanced. A similar behaviour was also found for systems formed by a polycation interacting with negative liposomes of dimyristoyl phosphatidic acid [24–26].

In addition, in the light of the N values obtained for the three polyanions and taking into account their respective number of subunits, we can speculate about how the interaction takes place in each case. As is well known, the charges of a polyelectrolyte chain play an important role in its structure in solution and, therefore, in the interaction. Thus, the short chain of PSS (eight monomers) forces it to adopt a rod-like conformation, although with slight motional freedom due to the relatively high ionic strength (0.02 M), so that one molecule would have two contact points ($N=2$) with the bilayer, as shown in Fig. 11. With respect to PGA, its conformational structure would be different, depending on the ionic strength, and probably on its method of interaction. So, at 0.01 M, about 90 units would offer a worm-like structure and the interaction would take place by the extreme of the polymer chain involving one phospholipid per two PGA molecules ($N=0.5$). However, at 0.02 M, the higher amount of electrolyte screens an important fraction of the polyelectrolyte charge and the PGA would adopt a random coil conformation, showing two binding sites (see Fig. 11). The seventy monomers of the PAA chain reflect a conformation that is similar to that of PGA, although with only one site being involved in the binding at C_s 0.02 M ($N=1$), probably due to the incomplete ionization of this polyelectrolyte, as previously outlined.

However, as mentioned above, the fraction of charged molecules making up the bilayer has been varied throughout the different fits performed because of the possible heterogeneous distribution of stearylamine among the two layers. The results obtained ($x_L < 0.5$) allow us to deduce that the cationic probe has been slightly distributed in a

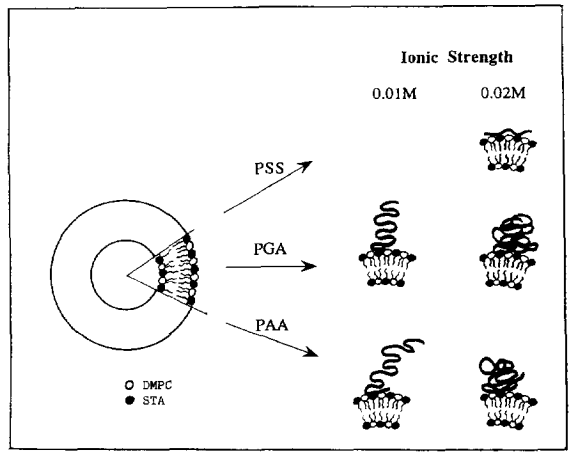

Fig. 11. Schematic representation of the interaction of sodium poly(styrene sulphonate), poly(L-glutamic acid) and poly(acrylic acid) with DMPC–STA (50:50, v/v) liposomes.

minor proportion in the outer layer than in the inner part of the vesicle.

The main conclusion of this report is that HPSEC has been proved to be a very useful technique for quantifying the association of polyelectrolytes with liposomes. In addition, the proposed method has the advantage of simplicity, overcomes the need to have a fluorophore group on the adsorbate and avoids the inaccuracy of the spectroscopic technique, especially when the R_i range is small and the respective intensities barely differ. Finally, it deserves to be mentioned that a calibration graph using areas (with the help of a computer program) instead of peak height would be a more correct procedure and progress towards this end is currently being made.

Acknowledgments

Financial support from the Comisión Interministerial de Ciencia y Tecnología (project No. PB95-1109) is gratefully acknowledged.

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