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Macromolecules in ordered media: 7. Influence of ionic strength and bilayer composition on the association of polyelectrolytes to mixed liposomes

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The effect of both the phospholipid composition of the bilayer and the ionic strength of the medium on the association between poly(2-vinyl pyridine) and mixed liposomes based on dimyristoyl phosphatidic acid and dimyristoyl phosphatidil choline was investigated using fluorescence spectroscopy. Intensity changes upon addition of vesicles have allowed us to estimate the extent of the association and data have been processed as association isotherms. We found that the association is enhanced by increasing both the ionic strength and the anionic phospholipid fraction. However, whereas the negative net charge of the bilayer strongly enhances the interaction, the presence of more and more electrolyte in the aqueous media induces a slight increase of the binding. Moreover, surfaces with at least 50% of charged phospholipid are necessary. Results were consistent with those obtained in preceding reports. © 1997 Elsevier Science Ltd.

(Keywords: polyelectrolyte–liposome interaction; bilayer composition; ionic strength)

INTRODUCTION

The adsorption of polymers, in general, and polyelectrolytes, in particular, at surfaces is the subject of a large number of experimental and theoretical investigations[1–4] because of its wide range of practical applications. Much of the interest lies in the stability that the polymers confer to colloids by generating steric protection against aggregation[5], which depends on the macromolecular characteristics. Therefore, it has been shown that a high polymer concentration induces colloid instability by a depletion phenomenon[6]. Usually, the research deals with the adsorption of a polyelectrolyte at oppositely charged surfaces[1,7–12] or at surfaces with the same charge[1] although the interaction between polyelectrolytes and nonionic surfactants is also of interest[13]. At present, a great variety of surfaces have been studied. In this sense, Dubin and coworkers have focused their investigations on the aggregate formation between polyelectrolytes and micelles[12,14,15] and Hoogeven *et al.* have recently reported the kinetics, the nature of the interaction and the influence of the pH and the ionic strength on the adsorption of polyelectrolytes onto oxide layers[16] as well as the reversibility of the system[17].

On the other hand, aqueous solutions containing polyelectrolytes and bilayer-shaped surfaces are also attracting attention as simple models of complex biological systems. Due to their simplicity and stability the small unilamellar vesicles have been considered useful model membranes, and their use is being extended. Among the most established applications of polyelectrolytes in this field, deserves to be cited the key role of the polymers adsorbed onto the liposome surfaces used as drug delivery vehicles, protecting them from phagocytic cells, and prolonging the liposome carriers' circulation time in the blood[18–21]. Polymer–liposome complexes have also been used to mimic the cellular cytoskeleton[22] and, recently, a wide range of biological activities of cationic polyelectrolytes as bactericidal, antiviral, antitumoral, has been proved[23], being the polyelectrolyte capability to be adsorbed onto the negatively charged surface related to their protection properties.

In recent works we have demonstrated by fluorescence spectroscopy the interactions between poly(2-vinyl pyridine) (P2VPy)[24] or poly(4-vinyl pyridine)[25] and dimyristoyl phosphatidic acid liposomes by means of an increase in fluorescence intensity and a blue shift of the maximum emission wavelength, which indicates the movement of the pyridinium group to a less polar environment when vesicles are present in the aqueous medium. In order to gain insight about the nature of the

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interaction the following contribution was addressed to study the effect of the charge density of the surface on the association. Then the study was extended to poly(2-vinyl pyridine) in the presence of mixed liposomes based on dimyristoyl phosphatidic acid and dimyristoyl phosphatidil choline at different percentages[26]. The results showed that the association was enhanced by the presence of increasing amounts of negative charged phospholipid in the bilayer, denoting the electrostatic nature of the interaction. Furthermore, it was clear that the zwitterionic phospholipid proportion must be lower than the anionic one in order for the association to occur.

To assess the above findings, the purpose of the present study is to explore in more detail the simultaneous influence of the bilayer chemical composition and the solvent ionic strength. As in previous work, the association has been evaluated using the fluorescence intensity as the relevant parameter to obtain the amount of polymer bound to the vesicles. The characterization has been carried out in terms of the binding constant, the stoichiometry and the partition coefficient, and considering the electrostatic effects through equations proposed in a previous report[24].

EXPERIMENTAL

Chemicals

P2VPy with molar mass 2900 was obtained from Pressure Chemical Co. (Pittsburgh, PA) and dimyristoylphosphatidic acid (DMPA) and dimyristoyl phosphatidyl choline (DMPC) from Sigma Chemical Co. (St Louis, MO). Polymer and phospholipids were used without further purification. The experiments were performed using acetate buffered solutions with 1 mM ethylene diamine tetraacetic acid (EDTA) of pH 3.5 at two different ionic strengths, C_s, 0.050 and 0.102 M. The pK_a values for the different ionic groups are 5.2 for pyridinium, and 3 and 8.5 for the lipid DMPA[27]. Therefore, under the experimental conditions selected the P2VPy is always protonated and the mixed DMPA/DMPC liposomes bear different net negative charges depending on the DMPA molar ratio.

Vesicle preparation

The stock solution of small unilamellar vesicles (SUV) was prepared by tip ultrasonication followed by ultracentrifugation, as previously described in detail[24]. The only difference lies in the fact that in the present case the vesicles were built up starting from DMPA powder mixed with a suitable amount of the neutral phospholipid DMPC in order to achieve the desired DMPA/DMPC molar ratio. For each sample, fresh solutions of 1 ml were prepared by dilution with buffer of the appropriate aliquots of the stock solution.

Measurements

Emission fluorescence spectra were recorded using a Perkin-Elmer Model LS-5B luminescence spectrometer with a thermostated cell holder and equipped with a data station. Throughout the experiments, samples were excited at 262 nm, being the maximum emission wavelength 406 nm, and the excitation and emission slits were both set at 5 nm. A battery of samples containing a fixed concentration of P2VPy (5 μM) and increasing concentrations of vesicles (ranging from 0 to 0.75 mM, in order to achieve the different phospholipid to polymer molar ratios, R_i) was annealed at 20°C to assure equilibrium conditions for 10 min before the measurement was carried out. All emission spectra were corrected for background fluorescence and vesicle and solvent light scattering by subtraction of the appropriate blanks.

The method used takes advantage of two facts: (i) the fluorescence intensity depends linearly on the concentration of the two possible states of the polymer (associated and aqueous); (ii) the mass of the entire polymer concentration in the system must be conserved. As a basic measuring signal for every phospholipid/polymer ratio, we determined the change of the polymer fluorescence intensity at 406 nm upon addition of vesicles ($\Delta I = I - I_0$, I and I_0 being the polymer fluorescence intensities in the presence and absence of liposomes, respectively). However, the relevant variable for knowing the degree of association is the fraction of bound polymer, α, defined as[28,29]

$$\alpha = \Delta I/\Delta I_{max} = (I - I_0)/(I_{max} - I_0) \qquad (1)$$

where I_{max} is the intensity of the polymer totally associated to the liposome. This value cannot always be experimentally obtained but it is possible to evaluate it by using an indirect procedure. Plotting $I_0/\Delta I$ vs $1/R_i$ the experimental data fall on an initial straight line. Keeping the polymer concentration constant, as $1/R_i$ goes to zero, the number of phospholipid moles is so high that one can assume that the polymer has been completely converted into its associated state. If this is so, the intercept will reflect the I_{max} value, and then α can be readily determined according to equation (1).

Since, under these conditions, the polymer only binds to the outer half-layer, and its composition is estimated by about two thirds parts of the total lipid[30], all the magnitudes implying phospholipid moles have been corrected by an accessibility factor of 0.65, denoted by the superscript (*).

However, a more convenient and usual measure of the extent of polymer incorporation is given by α/R_i^*, which accounts for the moles of polymer bound per mole of accessible phospholipid. Then, raw data have been translated into association isotherms, plotting α/R_i^* vs the free polymer concentration [P].

FRAMEWORK

The treatment applied in this paper has been recently[24] published. Here we shall briefly underline its essentials.

Data have been interpreted in terms of two models: (1) a membrane–water partition equilibrium of the polymer; (2) a simple binding equilibrium between the free polymer, the free phospholipid binding sites and the polymer bound to vesicles. The first model gives a quantitative interpretation of the association isotherm through the expression[31,32]

$$\alpha/R_i^* = \frac{\Gamma}{\gamma}[P] \qquad (2)$$

involving a pertinent partition coefficient, Γ, which depends on the standard Gibbs energy change per mole as a consequence of the transition of the polymer from the aqueous to the vesicle media, and an appropriate activity coefficient, γ, reflecting the electrostatic repulsions between the charged groups of the polymer, whether adsorbed onto the surface or free in aqueous solution.

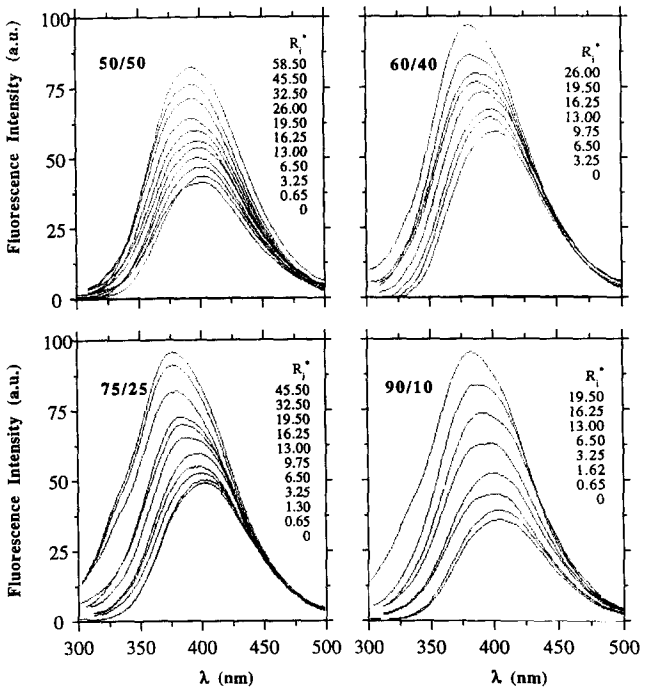

Figure 1 Emission spectra of P2VPy in the absence (bottom, $R_i^* = 0$) and in the presence of increasing amounts of vesicles of different DMPA/DMPC compositions at diverse ionic strengths. Excitation wavelength 262 nm

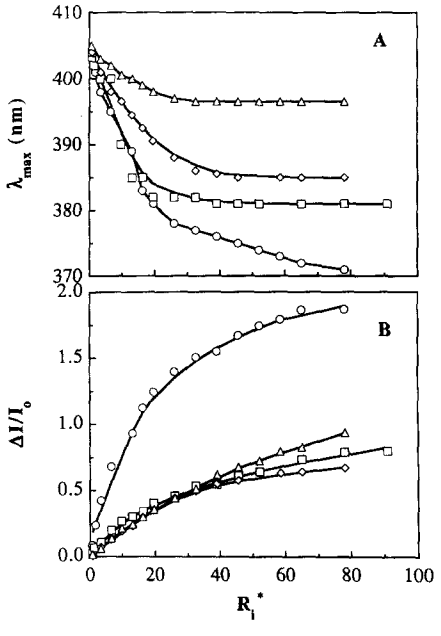

Figure 2 Changes in the wavelength of the emission maximum (A) and in the fluorescence intensity at 406 nm (B) vs R_i^* at diverse DMPA/DMPC compositions and ionic strengths: (○) 90/10, 0.102 M; (□) 75/25, 0.050 M; (◇) 60/40, 0.102 M; (△) 50/50, 0.050 M

The second model is derived on the basis of an equilibrium between the polymer free in solution, P, the unoccupied membrane sites containing N phospholipids, S_N, and the polymer bound to the liposomes PS_N, expressed as[33,34]

$$P + S_N \overset{K_A}{\rightleftharpoons} PS_N$$

with a characteristic association constant, K_A.

Table 1 Effect of the vesicles addition on the P2VPy fluorescence spectrum at different ionic strengths

	DMPA/DMPC composition (mol/mol)				
Δλ (nm) in spectra	40/60	50/50	60/40	75/25	90/10
0.026 M[a]	0	8	14	19	26
0.050 M	0	10	18	24	30
0.102 M	0	13	20	29	35

[a] Data from ref. 26

As we have already reported[24], K_A can be conventionally described in terms of concentrations, leading to the expression

$$\alpha/R_i^* = K_A[1/N - \alpha/R_i^*][P] \quad (3)$$

or, more accurately, in terms of activities, as

$$\alpha/R_i^* = \frac{K_A}{\gamma}\left[\frac{1}{N} - \alpha/R_i^*\right][P] \quad (4)$$

involving an activity coefficient that takes into account the electrostatic effects, as in the preceding model. Equation (4) has been proved to be very useful for fitting the association isotherms corresponding to systems formed by DMPA liposomes and vinyl pyridine-based polymers of molar mass 2900 and 50 000. Undoubtedly equation (4) will become more accurate than equation (3) as the molar mass of the macromolecule increases, since the electrostatic repulsions between polymer chains also increase.

RESULTS AND DISCUSSION

The experiments presented in this report were carried out in order to investigate the effect of both the aqueous salt content and the surface charge density of the vesicles on the association of the polymer with mixed vesicles. Thus, in a set of experiments we have measured the change in P2VPy fluorescence intensity upon the addition of different lipid vesicles concentrations. In different sets we also varied the phospholipidic composition DMPA/DMPC: 90/10, 75/25, 60/40, 50/50, 40/60, 25/75 and 0/100 (mol/mol), being in all cases the ionic strength of the buffered solution 0.050 M. A second series of experiments were performed repeating the sets at a higher ionic strength, 0.102 M.

As an example of raw data, *Figure 1* illustrates the spectra of P2VPy in the absence and in the presence of increasing amounts of DMPA/DMPC vesicles, either 50/50, 75/25 at C_s 0.050 M and 60/40, 90/10 at C_s 0.102 M. It can be seen that in the presence of lipids, the P2VPy fluorescence intensity increases and concomitantly the wavelength of the emission maximum shifts towards lower values. Both changes in the emission spectral properties are determined by the microenvironment polarity, that is, the polymer relocates from the polar aqueous solution to another environment with a lower dielectric constant[35] (the lipid bilayer), suggesting the association. Qualitatively, similar spectral shiftings were observed for the experiments carried out with vesicles DMPA/DMPC 90/10, 75/25, 60/40 and 50/50 at both ionic strengths. Compositions 40/60, 25/75 and 0/100 were also assayed but no changes in fluorescence intensity were observed under these conditions. Such results indicate that the interaction between P2VPy and DMPA/DMPC vesicles does not take place when the DMPC content is over the DMPA one.

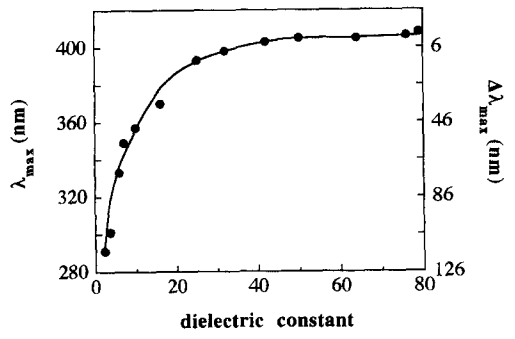

Figure 3 Dependence of P2VPy fluorescence wavelength on solvent dielectric constant

Table 2 Fitting parameters for the analysis of association isotherm data by the partition model for several phospholipidic compositions and diverse ionic strengths

	$10^{-3}\Gamma$ (M^{-1})		
DMPA/DMPC (mol/mol)	0.026 M[a]	0.050 M	0.102 M
50/50	1.75	2.54	3.47
60/40	2.65	4.69	5.64
75/25	4.94	6.05	8.34
90/10	10.17	12.26	23.01

[a] Data from ref. 26

To facilitate the reading of the spectral variations upon the addition of vesicles shown in *Figure 1*, the changes in both the wavelength of the emission maximum and in the fluorescence intensity at 406 nm as a function of the accessible lipid-to-polymer ratio are represented in *Figure 2*. As can be seen in *Figure 2A* the shift towards lower wavelengths is clear in the four cases. In addition, regardless of the ionic strength, the shifting is more pronounced as the anionic phospholipid content increases. *Table 1* compiles the maxima changes of λ_{max} observed for all conditions assayed. As can be seen, concerning both effects studied, the blue shifting increases with the DMPA in the bilayer and/or the salt content. On the other hand, the variation of fluorescence intensity plotted in *Figure 2B* shows a dramatic increase of the optima association conditions, i.e. 90/10 and 0.102 M, in comparison with the other three compositions assayed, which only show slight differences between their values.

In order to reassure the assumption made about the correlation of the blue shifting in the maximum wavelength with the change of the environment polarity of the pyridinium groups (and then the dielectric constant of the medium), we have measured the fluorescence spectrum of P2VPy in a series of different dioxane/water mixtures of known dielectric constant[36]. The λ_{max} corresponding to each spectrum has been plotted against the dielectric constant in *Figure 3*. Such a curve has allowed us to convert the maxima blue shifts of P2VPy at the different experimental conditions assayed (compiled in *Table 2*) to an estimate dielectric constant, and then to suggest the depth of penetration of P2VPy into the bilayer. It is well known that the dielectric constant of the hydrophobic part of the bilayer and the aqueous phase are about 2 and 78, respectively[30]. Furthermore, Epand and Leon[37], in a study about phospholipid bilayers with fluorescent probes, reported that for a dielectric constant of 10 the

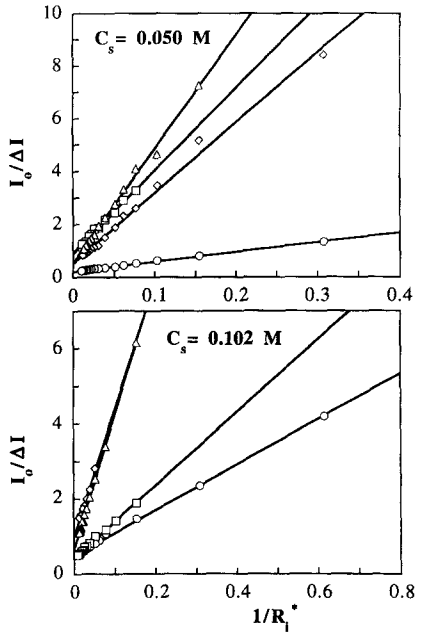

Figure 4 Double-reciprocal plots for the binding of P2VPy to DMPA/DMPC small unilamellar vesicles (SUVs) at both ionic strengths assayed. Symbols stand for different DMPA/DMPC compositions, as in *Figure 2*

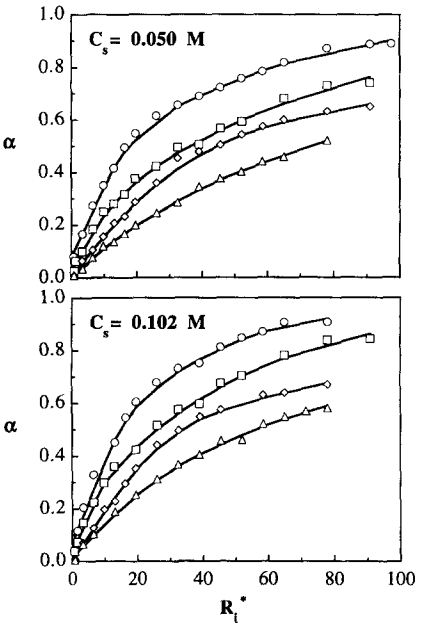

Figure 5 Variation of the fraction of P2VPy bound to DMPA/DMPC SUVs with the accessible lipid to polymer molar ratio at both ionic strengths assayed. Symbols for DMPA/DMPC compositions as in *Figure 2*. Solid lines are to guide the eye

chromophore was located in the polar part of the lipid although very close to the hydrocarbonated chain. Thus, inspection of *Figure 3* shows that the highest shift of λ_{max} observed (35 at DMPA/DMPC 90/10, ionic strength 0.102 M) would correspond to a dielectric constant value of 18, suggesting a slight penetration of the pyridinium group into the membrane located between the polar groups of the phospholipids. This hypothesis would be totally in accordance with that reported by us for P2VPy/DMPA systems[25] and deduced from viscosity measurements. Besides, from *Figure 3* and *Table 2* we can conclude that the lower the

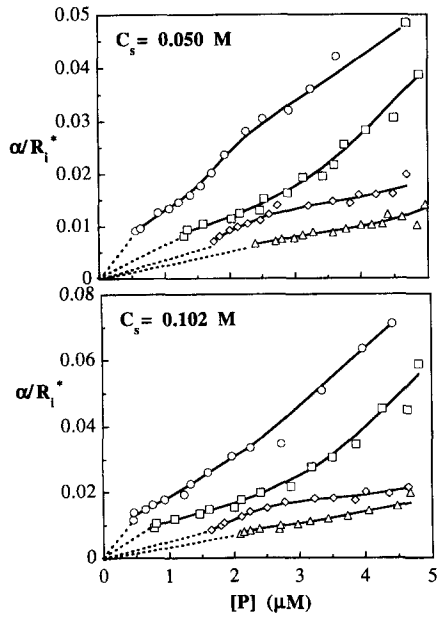

Figure 6 Phospholipidic composition effect on association isotherms for P2VPy/(DMPA/DMPC) system at both ionic strengths assayed. Symbols for DMPA/DMPC compositions as in *Figure 2*. Solid and dashed lines are to guide the eye

blue shift the higher the dielectric constant and, consequently, the pyridinium group will be located closer to the surface. Moreover, when the anionic phospholipid content is lower than 50% no change in λ_{max} is observed, denoting that the P2VPy is not associated to the vesicle but free in the aqueous phase.

Coming back to the intensity data, they have been used to construct the association isotherms, as we will explain later, and to characterize the polymer–liposome interaction. Moreover, the effects of both ionic strength and phospholipidic composition will be evaluated and discussed in terms of the characteristic parameters of the applied models. The I_{max} values determined by means of plots $I_o/\Delta I$ vs $1/R_i^*$ (see *Figure 4* as an example) were used to calculate the fraction of polymer bound to vesicles at each R_i^* for all sets of experiments. In this regard, *Figure 5* shows the dependence of the fraction α on the lipid/polymer ratio in all the assayed conditions. Inspection of this figure reveals an initial linear relationship, which is lost at higher R_i^* values. In addition, at a given R_i^* and ionic strength values, the higher DMPA content in the bilayer the higher the fraction of bound polymer. A final comparison of both parts of *Figure 5* indicates that the association seems to be enhanced when increasing the ionic strength in all cases.

Figure 6 shows the respective association isotherms built up from the α/R_i^* data obtained as explained before. The figure depicts how the change in the charge density of the bilayer affects the binding of the cationic polymer at the ionic strengths 0.050 M (top) and 0.102 M (bottom). Some comments deserve to be made: (i) the shape of the curves is similar to that observed for other polyelectrolyte/liposome systems[24–26], as well as for small molecules in the presence of vesicles[38–40]; (ii) in both panels, for a given free polymer concentration, the molar amount of polymer bound per mole of accessible lipid is decreasing as the percentage of the zwitterionic phospholipid making up the bilayer increases. Similar behaviour has already been reported for the systems constituted by melittin and mixed vesicles made up of palmitoyl oleoyl phosphatidyl choline/palmitoyl oleoyl phosphatidyl glycerol[41], as well as those formed by dibucaine and dimyristoyl phosphatidyl choline/dimyristoyl phosphatidyl glycerol vesicles[22].

These isotherm curves have been theoretically analysed applying the two models described above: the partition and the binding models, and consequently, the partition coefficient as well as the association constant and the number of phospholipids involved in the binding, which characterize the interaction, have been obtained by using equations (2), (3) and (4). First we will pay attention to the partition model.

Partition model

In order to attempt a fit of isotherm data, equation (2) involves two parameters, Γ and γ. The activity coefficient, γ, can be defined using either the Gouy–Chapman approach for charged surfaces[32] or the virial approach[30] which takes into account the finite size of the adsorbing molecules, being therefore more appropriate for macromolecules such as P2VPy. However, in the present work we are only interested in the punctual value of γ, and not in its characterization or theoretical treatment through any model what will be attempted in a future report, currently in progress. Regarding the partition coefficient Γ, according to equation (2) it corresponds with the initial slope of the association isotherm shown in *Figure 6*, the values being compiled in *Table 2*, together with those obtained at C_s 0.026 M and reported in a previous paper[26] for comparison, and in order to attempt a richer discussion. It should be mentioned that despite the lack of points at the lowest [P] values in some cases, the similarity of the curve shape with other systems previously reported[24–26,38–40], enables us to speculate about an initial linear zone in the isotherm for what the partition coefficient could be considered as hypothetical (dashed line). In the light of data from *Table 2*, the partition coefficient of P2VPy onto the membrane is enhanced upon the increase of DMPA content in the bilayer and the electrolyte added to the buffered solution. Such behaviour can be interpreted recalling the ionic nature of the system, that is, polycationic chains and oppositely charged bilayers. Thus, an increase of the salt content in the solution promotes the association due to the decrease of intra- and inter-chain polymer repulsions, because of the screening of charges, being then the polymer–liposome attractions enhanced. This salt dependence was also observed for P2VPy/DMPA systems[24], as well as for mastoparan/dioleoylphosphatidylcholine (DOPC) vesicles[39].

Concerning the effect of the bilayer composition on the association, we can also argue that the polymer–vesicle attractions will dominate when the anionic phospholipid fraction constituent of the bilayer increases, since the negative charge density on the surface also does.

Binding model

Experimental data have also been analysed in the light of the binding model. Therefore, equations (3) and (4) have been fitted to the association isotherms displayed in *Figure 6*, yielding sets of the characteristic parameters K_A and N, which have also been compiled in *Table 3*. The inspection of data reveals that K_A and N increase with the anionic phospholipid content, whereas an increase of

Table 3 Fitting parameters for the analysis of association isotherm data by the binding model for several phospholipidic compositions and diverse ionic strengths through equations (3) (a) and (4) (b)

DMPA/DMPC (mol/mol)	10^{-4} K_A (M^{-1})			N		
	0.026 M[a]	0.050 M	0.102 M	0.026 M[a]	0.050 M	0.102 M
50/50[(a)]	0.91	1.09	1.48	5	4	4
60/40[(a)]	3.08	4.50	5.24	11	9	8
75/25[(a)]	7.77	8.22	9.95	13	11	9
90/10[(a)]	17.30	19.00	23.30	15	12	10
50/50[(b)]	0.91	1.05	1.08	5	4	3
60/40[(b)]	3.24	4.87	5.05	11	9	8
75/25[(b)]	7.77	8.02	8.40	13	10	8
90/10[(b)]	18.50	20.50	27.3	14	12	10

[a] Data from ref. 26

the electrolyte content in the solution yields an increase of K_A, but a diminution of the number of phospholipids involved in the binding. It is noteworthy that the influence of the bilayer composition on K_A and N is more significant than the ionic strength of the solvent. Otherwise, both parameters reach similar values either by applying the conventional binding model through equation (3), or by taking into account the non-ideal behaviour due to repulsions between polycation chains, with equation (4). Such trends show again that at the highest ionic strength, the screening of the charges on the pyridinium groups weakens the intra- and inter-chain repulsions and, consequently, enhances the association of the polymer to the vesicles. In addition, the parallel slight diminution of N can be attributed to the folding of the polymer over itself (from a stiff rod to a more flexible conformation), having then access to the liposomal surface with a smaller radius and, consequently, involving a less number of anionic phospholipidic heads. Concerning the drastic increase of K_A with the charge density of the liposome surface, it is clear evidence of the electrostatic nature of the interaction: attractions result favoured and the number of phospholipids involved in the binding increases, as a result of the higher number of them making up the surface.

Finally, the comparison between the two series of parameters calculated either with equation (3) or equation (4) points out that the results scarcely differ due to the fact that the curves do not deviate so much from linearity, in contrast to other systems[24–26,32,37,38], probably because the presence of high amounts of counterions in the solvent (0.05 and 0.102 M) diminishes the repulsions. Consequently, γ reaches values very close to unity, showing the interaction to be an apparent ideal behaviour.

CONCLUSIONS

The present findings, together with those reported in a previous work[26] clearly reveal a predominant electrostatic nature of the overall interaction between P2VPy and DMPA/DMPC small unilamellar vesicles. Furthermore, the need has been demonstrated for the bilayer to be formed by at least 50% phospholipids, to carry a net negative charge in order for the polymer–liposome association to take place.

In short, both the association constant and the partition coefficient increase with the ionic strength, denoting an enhancement of the polymer association. In contrast, the number of phospholipids involved in the binding diminishes, probably due to the folding of the polymer chain over itself. Concerning the effect of the bilayer composition on the association, whatever the ionic strength was, all the characteristic parameters, Γ, K_A and N, increase with the DMPA molar fraction. In addition, changes in the emission maximum wavelength of P2VPy dissolved in solvents of different dielectric constants lead us to propose that the association of the polymer to the vesicle takes place through a partial insertion of the pyridinium group into the polar part of the bilayer.

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