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PII: S0032-3861(96)00916-0

Polymer Vol. 38 No. 14, pp. 3553–3560, 1997

0032-3861/97/$17.00 + 0.00

Macromolecules in ordered media: 5. Poly(4-vinyl pyridine)–liposome association induced by electrostatic interactions

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(Revised 25 September 1996)

The association of water-soluble vinyl polymers to dimyristoyl phosphatidic acid unilamellar vesicles as a function of pH, temperature and salt content using steady-state fluorescence and viscometry has been investigated. Poly(4-vinyl pyridine) fluorescence data were converted to association isotherms and discussed in terms of binding and partition models. The results of this report support previous suggestions: (1) in the case of polyions the inclusion of the activity coefficient in both models is essential; (2) the parameters calculated using the two different theoretical approaches can be directly compared by the relating equation proposed for us. Finally, the excellent agreement of steady-state fluorescence and viscometry results has allowed us to assume a model for the polymers approaching and their adsorption onto the surface, where the length of hydrophobic chain as well as the position of the nitrogen atom in the pyridinium ring play an important role. © 1997 Elsevier Science Ltd.

(Keywords: poly(4-vinyl pyridine)–liposomes interaction; partitioning; binding)

INTRODUCTION

Systems of polymer chains at an interface, formed by phospholipid vesicles of spherical topology, have recently received considerable attention. Indeed, such systems have proved useful in many applications because polymers confer stability[1,2], induce the cellular fusion[3,4], provoke changes in the membrane permeability[5–7] and are also of great interest in cell–cell recognition[8,9]. Most theoretical approaches dealing with polymer adsorption at interfaces are based on lattice models[10,11]. However, other treatments including integral equations[12], scaling approaches[13,14] and mean field calculations[15] have also been recently introduced. An overview of theoretical and experimental progress on this area has been reported[16–20].

On the other hand, in studying the biological activity of single molecules (drugs, prodrugs, enzymes, etc.) in the presence of cellular membranes, the association of single probes to biological surfaces has often been characterized by means of the binding[21,22] and partition[23,24] models. Despite the abundant literature on biological membranes, there is only limited theoretical insight into the basic properties of the polymer–bilayer interactions at molecular level. Not many techniques capable of modelling these complex fluid mixtures are available.

We are interested in the physicochemical aspects of the interaction of fluorescent polymer probes with charged unilamellar phospholipid liposomes. Polymer association or adsorption on liquid/solid interfaces is developed in at least two steps: (i) an early and very fast stage where the colloid surface becomes filled by the polymer; and (ii) a slow diffusion process of the polymer through the adsorbed layer. Moreover, the polymer may undergo a conformational change from the bulk domain to the adsorbed state.

At present, our interest has been focused on the association process of poly(4-vinyl pyridine), P4VPy, with phospholipidic vesicles in aqueous media under low pH and ionic strength conditions. In preceding contributions[25,26] we have investigated the association process of poly(2-vinyl pyridine), P2VPy, with liposomes based on dimyristoyl phosphatidic acid under diverse ionic strength, pH and temperature conditions. The methodology used for interpreting the experimental data is based on the combination of fluorescence intensity measurements of polymer–liposome mixtures with the binding and partition models[21–27]. This contribution has been proved to be efficient and useful for analysing, at least semiquantitatively, the association phenomenon of a polyelectrolyte with oppositely charged liposomes. Although P2VPy and P4VPy seem to be similar polymers, we have selected them in order to investigate not only the chain conformation far and close to the liposome surface but also the influence on the adsorption of the placement of the active centre, the nitrogen atom in this case, in the pyridinium ring. In fact, the chemical nature of a chain cannot be ignored if the chains are charged. In these cases physical forces will contribute significantly to the complex formation and its structure. Thus, we think that it is of great interest to analyse the adsorption process onto the liposomes of both *ortho*- and *para*-isomers in the light of the above-mentioned models.

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Notice that the nitrogen on *p*-PVPy is a prime example of a proton acceptor site that protrudes, and the negatively charged oxygen of the phospholipid head on DMPA also protrudes from the bilayer. When the nitrogen on *o*-PVPy is located in the *ortho* position, there is little guidance as how to proceed. One can visualize that the *ortho* site should be less accessible and that it should show up as a reduction in the contact between the charged sites of both PVPy and phospholipid heads.

In the present paper we employ the same method previously reported[25,26] to build up the association isotherms, showing the influence of diverse operational variables on their shape. The essential features of both binding and partition models are compiled. Next, we include a discussion about the role of the electrostatic and non-electrostatic contribution on the values of the basic magnitudes derived from the above-mentioned models, such as the association constant, the number of phospholipids involved in the binding and the partition coefficient. We also give support on the usefulness of new proposed equations[28] obtained from the combination between both models. Lastly, we present an analysis in depth on the comparison between the parameters covering both poly-isomers.

EXPERIMENTAL

Chemicals

Poly(4-vinyl pyridine) (P4VPy) with molar mass 50 000 was purchased from Polysciences Inc. (Warrington, PA, USA), poly(2-vinyl pyridine) (P2VPy) with molar mass 2900 from Pressure Chemical Co. (Pittsburgh, PA, USA) and dimyristoyl phosphatidic acid (DMPA) from Sigma Chem. Co. (St Louis, MO, USA). All other chemicals were reagent grade and all materials were used without further purification.

Preparation of small unilamellar vesicles (SUV)

Suspension of unilamellar liposomes of DMPA was prepared in acetate buffered solutions with 1 mM EDTA by tip ultrasonication followed by ultracentrifugation as described in detail in the preceding paper[26].

Fluorescence

The association of P4VPy to DMPA vesicles was measured by adding the dispersed vesicles to a fixed amount of polymer and monitoring the change of fluorescence at 375 nm on a Perkin–Elmer Model LS-5B Luminiscence Spectrometer using bandwidths of 5 nm for both the excitation and the emission monochromators. The excitation wavelength was 305 nm.

All experiments were performed in aqueous buffer solutions at pH 3.5 (ionic strengths 0.026 and 0.102 M) and at pH 4.5 (0.065 M). The covered temperature range under the former pH was 5, 20, 37, 56 and 76°C whereas for the latter pH the temperature range was restricted to 5, 20 and 37°C because at highest values the polymer–liposome mixtures exhibit cloudiness and the scattering contribution to the fluorescence emission can grossly contaminate the accuracy of the measurements.

The lipid contribution to the signal was subtracted in the absence of polymer under otherwise identical conditions. Prior to measurements, the samples constituted by polymer and vesicles at different molar ratios were annealed to the desired temperature for 10 min in order to assure equilibrium conditions.

The fluorescence intensity data were analysed in the same manner as previously described[26]. Briefly, the fluorescence of the fully polymer–vesicle association state, I_{max}, was determined using the double-reciprocal plots. Therefore, the fraction of polymer bound or associated to the phospholipid bilayer, α, was obtained from the relationship $\alpha = (I - I_o)/(I_{max} - I_o)$[26,29,30], where I and I_o refer to the polymer fluorescence intensities in the presence and absence of vesicles. Since P4VPy is considered to have access only from the vesicle outside, all the magnitudes including the phospholipid concentration are corrected by the fraction of lipid in the outer leaflet, β, which in the particular case of small unilamellar vesicles is equal to 0.65[31], and is denoted by the superscript (*) throughout the present report.

Viscometry

The viscosity of liposomes in aqueous buffered solvent at pH 3.5 and C_S 0.026 M in the absence and presence of polymer at 2.5 μM concentration has been measured. The measurements were performed in a Ubbelohde capillary viscometer (Model AVS 440, from Schott-Gerate, Germany). At least eight dilutions were automatically obtained by adding the appropriate aliquots of solvent. Under the assayed conditions, the flow time was always above 100 s at 20 ± 0.1°C. Kinetic energy corrections were also included in the calculation of the specific viscosities.

RESULTS AND DISCUSSION

The interaction between P4VPy and small unilamellar vesicles of DMPA has been followed by monitoring the intrinsic fluorescence of the polymer in the presence and in the absence of DMPA liposomes. Emission spectra of 1.25 μM P4VPy alone (dotted line) and in the presence of DMPA vesicles at different phospholipid/polymer molar ratios (continuous line) at 5°C and pH 4.5 are illustrated

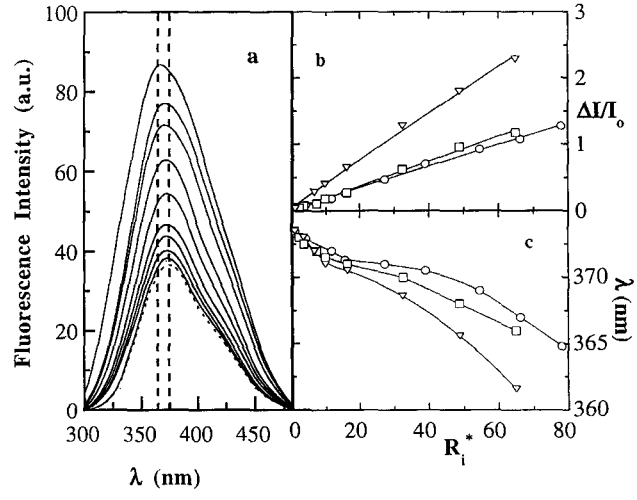

Figure 1 (a) Emission spectra of P4VPy 1.25 μM (dotted line) and in the presence of increasing amounts of DMPA vesicles at 5°C. From bottom to top the accessible lipid to polymer molar ratio are $R_i^* = 0$, 0.8, 3.9, 11.7, 15.6, 27.3, 39.0, 54.6, 66.3 and 78.0. (b) Dependence of the relative intensity of fluorescence upon addition of vesicles at the emission maximum wavelength of the polymer. (c) Dependence of the wavelength of the emission maximum on R_i^*. Experimental conditions: pH 4.5, C_S 0.065 M, temperature: (○) 5, (□) 20 and (▽) 37°C; excitation wavelength 305 nm

in *Figure 1a*. It is well-known[32,33] that the different environment of the fluorophore when relocated from water to membrane surface induces a change in the wavelength of the maximum emission to lower values, as well as in the fluorescence intensity, implying that the interaction takes place. In our case, in *Figure 1a* a blue shift is observed from 374 to 364 nm, and a large increase up to 50% of the fluorescence intensity at 374 nm occurs. *Figures 1b, c* show with more detail the relative increases of the fluorescence intensity at 374 nm and the wavelength shifts with the accessible phospholipid/polymer molar ratio, R_i^*, as a function of the assayed temperatures at pH 4.5. Both plots show that, after the addition of 80 mol of phospholipid per mol of polymer, the increase of fluorescence and the decrease of wavelength continued to take place; that is, the P4VPy needs values of R_i^* larger than 80 in order to achieve the plateau observed in the DMPA/P2VPy system (see *Figure 1* in ref. 26).

The fraction of polymer bound to vesicles, α, has been obtained from the experimental data through the so-called doubled-reciprocal plot[29,30,34] (as an example, see *Figure 2*). *Figure 3* shows the initial linear dependence between α and R_i^* for the DMPA/P4VPy system at pH 3.5, ionic strength, C_S, 0.026 M and all the temperatures assayed. From this linear region the number of phospholipid molecules bound per molecule of polymer, N, can be obtained from a particular case, specifically, when α tends to unity, then R_i^* tends to N[27,29,35]. These plots represent the extent of the association between polymer and vesicles and always result in straight lines through the origin of positive slope, at least for low R_i^* values. However, at higher R_i^* values, a saturation is reached suggesting the total binding of polymer to vesicles.

Actually, the experimental results are usually presented through association isotherms, that is, the number of bound polymer moles per mol of accessible phospholipid, α/R_i^*, vs the free polymer concentration $[P]$. The results obtained at pH 3.5 and 4.5 are plotted in *Figures 4* and *5*, respectively, showing the temperature effect exerted on the association isotherms. (Experimental data corresponding to pH 3.5 and C_S 0.102 M are not shown here.) As we observed in similar experiments performed with P2VPy[26], three regions are also evidenced in the isotherms of P4VPy. The first corresponding to the lowest $[P]$ values shows a linear dependence between the number of bound polymer moles per mol of accessible phospholipid and the concentration of free

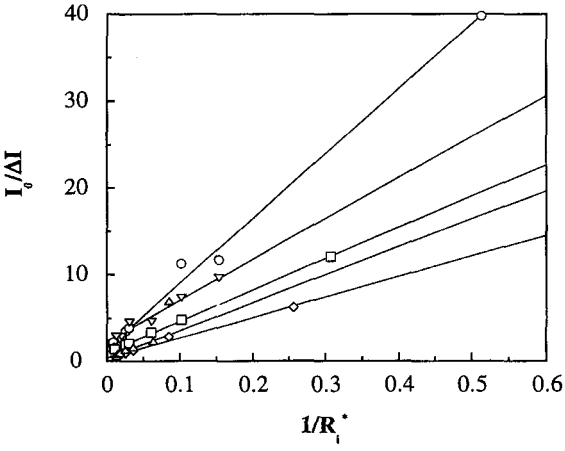

Figure 2 Double-reciprocal plots for the binding of P4VPy to DMPA SUVs at pH 3.5, C_S = 0.026 M and temperature: (○) 5, (□) 20, (▽) 37, (△) 56 and (◇) 76°C

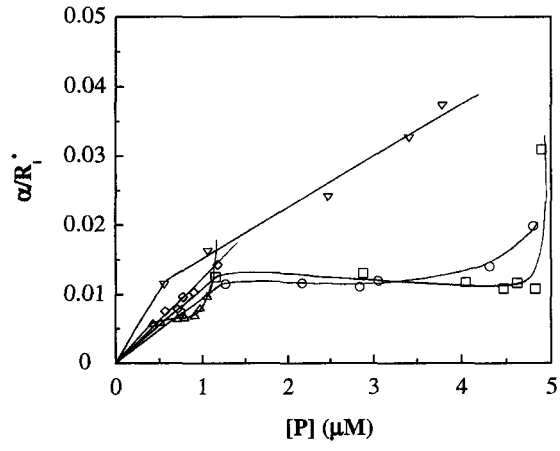

Figure 4 Binding profiles for the association of P4VPy to DMPA unilamellar vesicles at pH 3.5 and C_S = 0.026 M. Symbols for diverse temperatures as in *Figure 2*

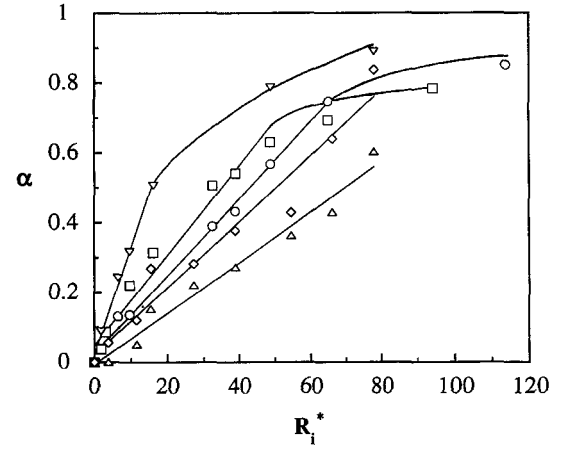

Figure 3 Variation of the fraction of bound P4VPy to DMPA SUVs with the accessible lipid–polymer molar ratio at pH 3.5 and C_S = 0.026 M. Symbols stand for different temperatures as in *Figure 2*

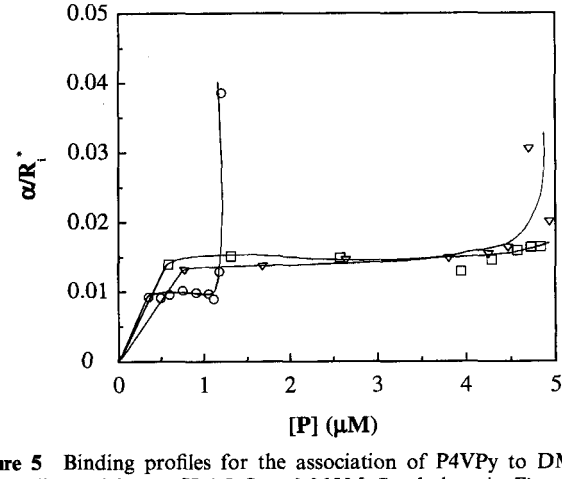

Figure 5 Binding profiles for the association of P4VPy to DMPA unilamellar vesicles at pH 4.5 C_S = 0.065 M. Symbols as in *Figure 1*

polymer, although is not a well-defined region in the present case. In the second zone, the curve bends downwards and reaches similar values of α/R_i^* for any free polymer concentration. The third region is characterized by a sharp increase of α/R_i^* for $[P]$ values close to the initial polymer concentration. It is important to note that this last region has been rarely observed and reported for single molecules much smaller than our macromolecule.

From a qualitative viewpoint the first zone represents the ideality of the interaction whereas the bending in the second one would imply the deviations from the ideality since the presence of residual charges on the P4VPy chain would induce inter- and intrachain electrostatic repulsions and, therefore, the bending of the curve. In addition, the large amount of charges in P4VPy would mean that the electrostatic effects dominate the nature of the interaction, explaining the observed flattening out in practically the whole curve. Finally, the third region can be attributed to the formation of aggregates in the bilayer[27,35–37].

However, for a quantitative discussion we must introduce a basic idea about the nature of the underlying association process. For this purpose, we have chosen two models that, so far, have been applied to account for interactions between liposomes and either small probe molecules[21–24,38] or, more recently, to macromolecules[25,27,35]: partition and binding models. The details of both models have been presented in the preceding paper[26]. Here we will give the background information needed for the interpretation of the results. Briefly, the first model considers the association process in terms of a thermodynamic partitioning equilibrium implying the basic relationship

$$\alpha/R_i^* = \frac{\Gamma}{\gamma}[P] \tag{1}$$

that involves a partitioning coefficient, Γ, and an activity coefficient, γ, which can be mainly attributed to the electrostatic repulsion of the positive charges on neighbouring polymer molecules[39]. The second model assumes a simple binding equilibrium between the free polymer, the free phospholipidic binding sites and the polymer bound to vesicles. This model is characterized by two binding parameters, a binding constant, K_A, and N, and presents two possible approaches depending on the inclusion or not of the non-ideal effects due to repulsions between polycation chains. So the experimental data can fit through the equations

$$\alpha/R_i^* = K_A[1/N - \alpha/R_i^*][P] \tag{2}$$

$$\alpha/R_i^* = \frac{K_A}{\gamma}\left[\frac{1}{N} - \alpha/R_i^*\right][P] \tag{3}$$

where γ takes into account the electrostatic effects (see ref. 26 for details).

According to both theoretical models, the association isotherms of P4VPy with DMPA vesicles drawn in *Figures 4* and *5* have been characterized in terms of their respective parameters.

Analysis of Γ *(partition model)*

Table 1 shows Γ values for P4VPy–DMPA liposomes mixtures as a function of pH, ionic strength and temperature. Firstly, we will focus our attention on the column labelled 'Experimental' because the remaining data come from equations (5) and (6) that will be introduced later on. Inspection of these experimental data, which have been obtained from the initial slope of the isotherms, shows that pH, ionic strength and temperature affect the Γ values. With regard to the influence of pH and the ionic strength on the association at constant temperature, we can observe that the partition results are enhanced when the ionic strength or pH are raised, as reported for P2VPy[26] and other probe molecules[23,24,39,40]. In this context, it deserves to be noticed that at experimental pH conditions both the liposome and the P4VPy are oppositely charged and the number of charges for each one will be mainly affected by the ionic strength. As concerning this variable influence and disregarding the pH effects, van de Steeg *et al.*[41] have recently introduced the concept of 'screening-enhanced adsorption' and 'screening-reduced adsorption' regimes to account for the adsorption of polyelectrolytes at oppositely charged surfaces by using a mean-field lattice theory. The Γ values seem to follow the first regime which is more typical for highly charged polyelectrolytes, in clear agreement with the behaviour of P4VPy in solution[42]. Consequently, the screening of the electrostatic repulsion between the segments of the P4VPy leads to an increase in the polyion adsorptivity, being the polymer–vesicle interaction strong enough to keep the screened P4VPy adsorbed, although salts also screen the electrostatic attraction between the protonated pyridinium segments and the phospholipid head groups.

On the other hand, phospholipid vesicles can also undergo substantial changes with temperature. Above a certain transition temperature, T_c, the bilayer exists as a two-dimensional liquid and below this temperature behaves as a two-dimensional solid. Furthermore, it has been evidenced that the T_c of DMPA vesicles in the absence of any interacting probe is about 54–56°C depending on the pH experimental conditions[43] but the presence of foreign molecules induces a decrease of phospholipids T_c[43–46]. Regarding *Table 1*, at pH 3.5 (0.026 M) the partition coefficient increases with temperature until 37°C and then, decreases. According to Seelig *et al.*[47] such behaviour in the liquid crystalline

Table 1 Experimental and theoretical partition coefficients for the interaction of P4VPy with DMPA SUVs for all the experimental conditions assayed. Theoretical Γ values have been obtained by coupling partition and binding models through equations (5) and (6)

			$10^{-4}\,\Gamma\,(M^{-1})$					
				Equation (5)			Equation (6)	
pH	C_S (M)	T (°C)	Experimental	A[a]	B[b]	C[c]	B[b]	C[c]
3.5	0.026	5	0.91	0.41	0.25	1.90	0.07	0.87
		20	1.00	0.57	0.26	2.20	0.09	1.12
		37	2.00	0.99	0.58	2.97	0.34	1.78
		56	1.40	0.93	1.00	1.85	0.79	1.38
		76	1.25	1.17	1.24	1.88	0.97	1.25
	0.102	20	5.00	1.48	2.71	6.00	1.83	5.03
4.5	0.065	5	2.80	1.05	2.38	3.50	1.58	2.81
		20	2.50	0.35	0.75	3.40	0.53	2.66
		37	1.60	0.40	0.64	1.93	0.41	1.55

[a] Obtained from double reciprocal and α vs R_i^* plots
[b] From equation (2)
[c] From equation (3)

phase can be attributed to a small and exothermic enthalpy change for the binding. In contrast, at pH 4.5 the increase of the temperature produces the opposite effect in the membrane–water partition coefficient: the binding of P4VPy to the gel state of DMPA decreases with temperature, meaning that when the temperature increases the repulsions between the polymer charges are more enhanced than the attractions between the phospholipidic heads and the polymeric pyridinium groups. This reversal trend exhibited by Γ with pH could be related to a conformational transition of the polyelectrolyte according to that claimed by Thomas and Tirrell[48].

Analysis of K_A *and* N (*binding model*)

With respect to the binding model, the experimental isotherms have been fitted to equations (2) and (3) which involve two adjustable parameters, K_A and N, collected in columns B and C of *Table 2*, respectively, for all assayed conditions. Otherwise, the binding parameters are also achievable by using the α vs R_i^* plot in conjunction with the double-reciprocal plot (*Figures 2* and *3* show an example of these plots), and the results are gathered in columns A in *Table 2*.

Let us analyse in depth the data shown in *Table 2*, by first discussing the parameters obtained using the more simplified method (column A). The results show that at pH 3.5 the association constant increases with temperature from 5 to 56°C, then decreases, whereas at pH 4.5 K_A does not seem to follow any trend with temperature. On the other hand, K_A increases with ionic strength. As to the number of phospholipids involved in the binding, N increases with ionic strength but does not show any rule either by changing temperature or pH. Hence, only the variation of ionic strength seems to have a clear effect on the N value.

With regard to the values of K_A and N collected in column B from *Table 2*, the association constant increases with pH and ionic strength but does not show a clear tendency with the temperature for both pH values. As for the number of phospholipids involved in the binding, N decreases when ionic strength increases but it is very difficult to establish a trend with temperature or pH. In any case, this method for obtaining the binding parameters does not take into account the deviation from the ideality due to the secondary effects caused by the large number of polymer charges. Columns C compile the parameters K_A and N obtained by fitting the experimental data to equation (3), that is, taking into account the activity coefficient. We can see that an increase in whichever of the experimental variables, such as temperature, ionic strength or pH, results in a decrease of the value of the association constant. It must also be recalled that an increase in pH goes parallel with an increase in ionic strength, that is, a decrease of the number of polymer charges and a screening by counter-ions of the salt together. So, it is not surprising that at 20°C when the pH of the solution changes from 3.5 to 4.5, the diminution of K_A is more acute than that happening when only the ionic strength is increased at pH 3.5 from 0.026 to 0.102 M.

Table 2 Equilibrium binding parameters for the interaction of P4VPy with DMPA SUVs using different methods under all the assayed conditions

pH	C_S (M)	T (°C)	$10^{-5} K_A$ (M^{-1})			N		
			A[a]	B[b]	C[c]	A[a]	B[a]	C[c]
3.5	0.026	5	3.56	1.7	9.1	86	69	48
		20	4.51	1.5	8.8	79	58	40
		37	5.56	1.4	8.6	56	24	29
		56	12.49	2.8	6.3	140	28	34
		76	11.92	2.6	6.0	101	21	32
	0.102	20	13.57	7.6	7.8	92	28	13
4.5	0.065	5	11.20	8.1	7.0	107	34	20
		20	2.42	1.5	5.1	70	20	15
		37	2.99	1.6	2.7	74	25	14

[a] Obtained from double reciprocal and α vs R_i^* plots
[b] From equation (2)
[c] From equation (3)

By comparison of the association constants of P4VPy with those obtained for P2VPy at the same conditions (see *Table 1* from ref. 26), we can see that P4VPy presents K_A values slightly higher than P2VPy. This fact is consistent with the values of the Gibbs free energy of the association process calculated with both polymer and phospholipid charges and radii (not published yet).

On the other hand, N diminishes with temperature from 5 to 37°C at both pH values and then increases at pH 3.5. N also diminishes with ionic strength and pH at a given temperature. Moreover, the comparison of N values for both polymers suggests that this magnitude gets very small values, in general, providing that the polymer has a lot of pyridinium groups, and hence a large number of possible charges to interact with the bilayer. However, P2VPy, with a lesser quantity of pyridinium groups in its chain than P4VPy (27 against 492, respectively), presents values of N slightly smaller than P4VPy. It might be speculated that PVPy exhibits different conformations and orientations when adsorbing onto the bilayer depending on the location of its functional group, that is *o*-PVPy or *p*-PVPy. Both, the molecule size and the different position of the nitrogen in the pyridinium group with respect to the backbone chain would lead to different macromolecular conformation when approaching the vesicle surface. On one hand, the large size of P4VPy, close to 500 units, would allow it to adopt a random coil structure, being a long flexible chain depending on the pH and ionic strength conditions. However, the 27 units of P2VPy would limit its conformational structure behaving as a wormlike chain, partially flexible and more solid than P4VPy. On the other hand, the nitrogen atom of the pyridinium group placed in the *ortho* position would be very close to the backbone chain. Such spatial hindrance would obligate the macromolecule to approach the surface in a parallel plane, carrying with it the remaining units in the same arrangement and allowing the whole chain to lay out onto the surface. On the contrary, in the case of P4VPy the macromolecule presents more motional freedom allowing it to approximate the bilayer in any plane and inducing the formation of loops in the polymeric backbone. *Figure 6* depicts a schematic illustration of the approach to the outlet part and the arrangement covering a patch of the bilayer of both polymer chains. As can be seen, for P4VPy only a few segments of the macromolecule would be in close contact with the membrane, forming the remaining segments loops and tails and, consequently, only a small number of phospholipids would be involved in the interaction. In contrast, a short chain such as P2VPy is forced to adopt a more restricted conformation being totally extended on

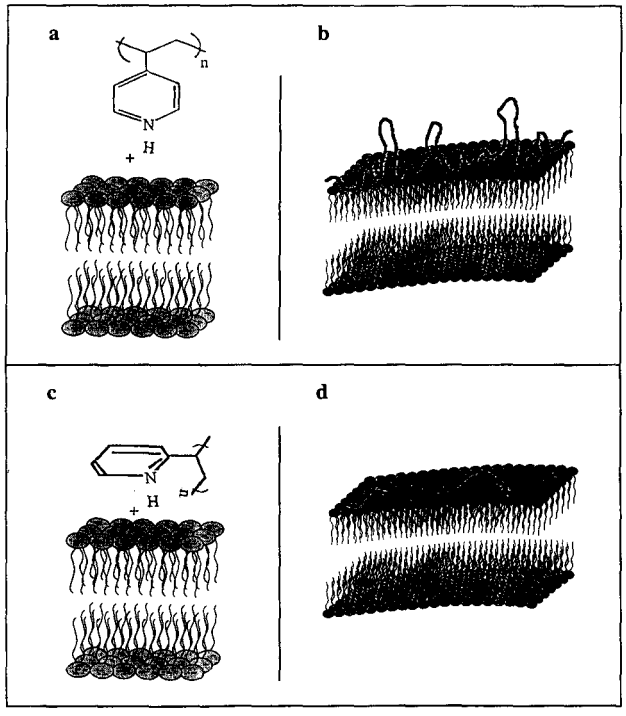

Figure 6 Schematic drawing of the approach of a P4VPy chain (a) and its arrangement (b) onto a DMPA vesicle surface, as well as the approach of a P2VPy chain (c) and its arrangement (d) on the vesicle surface

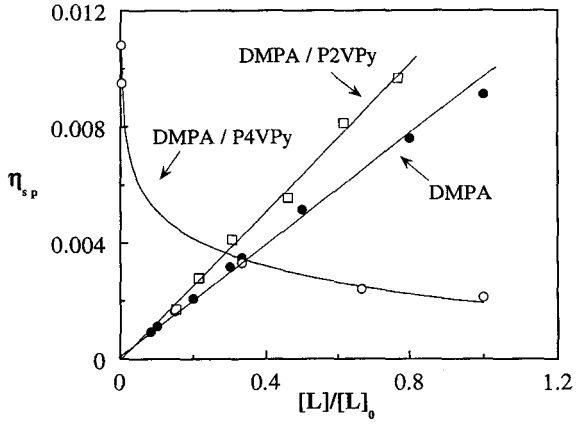

Figure 7 Specific viscosity vs reduced liposome concentration in the absence (●) and in the presence of P2VPy (○) and P4VPy (□) at a 2.50 μM concentration

the bilayer and showing a major number of binding points.

In fact, such an idea is supported by the conclusions drawn from the viscometry technique. The linear dependence of the specific viscosity η_{sp} on liposome concentration is well demonstrated in accordance with the Einstein–Simha equation[49]

$$\eta_{sp} = 2.5 V_h n_o ([L]/[L]_o) \quad (4)$$

where V_h is the hydrodynamic particle volume, n_o the number of liposomes per millilitre of initial sample and $[L]/[L]_o$ the reduced liposomal concentration, and considering the liposomes as spheres. *Figure 7* shows viscosity measurements on the liposome assay in the absence and presence of both polymers. As can be seen, the linear functionality between the specific viscosity and the reduced liposome concentration predicted by equation (4) is observed for both DMPA and P2VPy/DMPA systems. However, the presence of P4VPy in the vesicle dispersion not only loses the linear dependence but also produces the opposite effect: a sharp increase on the η_{sp} at infinite dilution similar to the so-called polyeletrolyte effect. These features would imply that the P2VPy–DMPA vesicle complex continues being a sphere and it would demonstrate that P2VPy interacts with the bilayer by sticking onto the surface. On the contrary, the P4VPy–liposome complex does not adopt a spherical shape in agreement with the proposal that the polymer interacts with the bilayer by forming loops and, therefore, involving a minor number of phospholipids in the binding than P2VPy.

With respect to the changes in the behaviour of Γ, K_A or N with temperature in the range 37–56°C we ought to pay attention to the fact that this temperature range includes the DMPA liposome solution T_c and one may think that the fluidity degree of the bilayer will also moderate the interaction.

Analysis of binding parameters by coupling models

From a quantitative viewpoint and recalling the direction followed in a preceding paper, we have proceeded next to relate both binding and partitioning models and to check the degree of coincidence between them. To this end, the partition coefficient for each experimental condition has been calculated using the different pairs of K_A and N values through the equations[26]

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

and

$$\Gamma = \frac{K_A}{N} \quad (6)$$

with the following features: equation (6) has been obtained considering the number of occupied membrane sites negligible to the total sites. Equation (5) does not contain such an approach. In any case both equations have been obtained taking into account the activity coefficient in both models. (For more details, see the preceding paper[26].)

Table 1 also compiles the theoretical values of Γ calculated for all experimental conditions. Comparison of theoretical and experimental data point out that the best agreement is obtained through equation (5) and using the binding parameters, K_A and N, from equation (3). Specifically, when using equation (6) for relating both models, deviations between experimental and theoretical Γ values are of about 50%, independent of the method used (columns A, B or C). However, when the relationship is given by equation (5) and the binding parameters are obtained by fitting the association isotherms to equation (3), see column C, the mean deviation is less than 4.5% which, at least from a quantitative point of view, is successful providing all the assumptions are made.

CONCLUSIONS

In keeping with previous results on the interactions between liposomes and probe molecules[29,32,33], the

emission fluorescence spectra of P4VPy bound to DMPA vesicles are indicative of a lipidic environment. In fact, in the presence of liposomes based on DMPA (see *Figure 1*) the emission maximum experienced a slight blue shift (up to 10 nm). An increase of the fluorescence intensity by a factor of 2.5 was also observed. In contrast to that reported for P2VPy[26], P4VPy shows a linear dependence between the variations of the fluorescence intensity (see *Figure 1b*) and of the maximum wavelength (see *Figure 1c*) with R_i^* in the whole range studied here. This trend denotes that the total association of P4VPy to vesicles has not already been achieved, so that a higher liposomal concentration would be required in order to reach the saturation. Unfortunately, the performance of experiments with molar ratios above is not possible because the stability of liposomes is questioned.

The results are described in terms of association isotherms of polymer to surfactants. The first stage of the isotherm involves binding of individual polymer molecules to the vesicles, and the last stage occurring when the free polymer concentration approaches the total concentration is a strong binding related to the self-association of the P4VPy. In the intermediate stage, the deviation from the linearity is more pronounced and significant than that shown by small molecules[23,24,37,40,50,51] and even by another polyion such as P2VPy[26]. As $[P]$ increases, γ reaches increasing values higher than unity, which indicates that the real behaviour of the macromolecule–liposome system is mainly dominated by the repulsions between adsorbed–adsorbed or adsorbed–free polycations. In contrast with P2VPy, the P4VPy chain is larger, carrying a high number of positive charges and therefore, intense repulsions and large deviations from ideality are expected.

For a better understanding of the association process, the isotherms have been discussed assuming the partition and the binding models both include the electrostatic effect by means of the activity coefficient. The main goal of this work has been to state that, as was previously concluded for P2VPy, the Γ data calculated through our proposed equation (5) (see ref. 26) using K_A and N obtained by fitting the experimental isotherms with equation (3) show the best agreement with the experimental data. In fact, the deviation is less than 4.5% in contrast with the value of 50% obtained using any of the other methods.

With regard to the effect of the different experimental variables, such as temperature, pH or ionic strength, on the characteristic parameters, the following trends have been noticed: (i) the partition coefficient increases with the ionic strength and pH at constant temperature, whereas at constant ionic strength and pH 3.5, Γ enhances with the temperature in the gel state of the phospholipid but decreases in the liquid crystalline state; (ii) the binding parameters, K_A and N involved in the association obtained with the proposed equation (3), diminish when the three experimental solution variables increase, with the only exception at pH 3.5 in the liquid phase of phospholipids for which N increases.

It also deserves to be mentioned that the feature about the different conformational approach of the polymers to the vesicles arises from the comparison of their number of phospholipids involved in the binding process. Thus, P2VPy would approach the vesicle surface rod-like being fully adsorbed onto the liposome membrane offering all its pyridinium groups to the liposome, whereas P4VPy in solution would adopt a random coil structure and the binding might occur by forming loops and therefore involving only few pyridinium groups. This speculation is based on the fact that the chain length in solution and the position of the nitrogen in the pyridinium group are decisive in the interaction phenomenon and it has gained further verification by the viscometric results.

Lastly, it should be stressed that polymer–polymer interactions cannot be ignored because, as expected, such interactions will strongly affect the polymer size and shape near to the liposome interface. In a first approach, we have introduced in the present study an overall activity coefficient, γ, in order to account for the above-mentioned effect. In a next contribution the meaning of γ will be analysed in depth in the framework of the polymer–liposome interaction, including the quantitative evaluation close to and away from the phospholipid bilayer.

ACKNOWLEDGEMENTS

This work was partially supported by grants (PM95-0149) and (PB95-1109) from the Comisión Interministerial de Ciencia y Tecnología (Spain). One of us, I. Porcar, is indebted to the Ministerio de Educación y Ciencia (Spain) for a fellowship grant.

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