ELSEVIER

PII: S0032-3861(96)00915-9

Polymer Vol. 38 No. 14, pp. 3545–3552, 1997

0032-3861/97/$17.00 + 0.00

Macromolecules in ordered media: 4. Poly(2-vinyl pyridine)–liposome association induced by electrostatic interactions

Iolanda Porcar, Rosa García, Vicente Soria and Agustín Campos*
Departament de Química Física and Institut de Ciències de Materials (ICMUV), Universitat de València, 46100 Burjassot, València, Spain
(Revised 25 September 1996)

We have investigated the interaction between vesicles based on dimyristoyl phosphatidic acid (DMPA) and a polymer, such as poly(2-vinyl pyridine) (P2VPy), on the basis of both partition and binding models. The resulting association curves are non monotonous being three regions clearly evidenced. The electrostatic and steric origin of the attractive or repulsive interactions are analyzed as a function of pH, ionic strength and temperature using the fluorescence spectroscopy technique. We emphasize the importance of differentiating the ideal definition of the binding constant from the theoretically evaluated including the activity coefficient, γ, to take into account shifts from the ideality of the polymer in both aqueous and lipidic domains. Furthermore, we propose an equation relating both models that predicts fairly well the experimental data.

(Keywords: poly(2-vinyl pyridine)–liposomes interaction; partitioning; binding)

INTRODUCTION

Much of the interest in phospholipid membranes lies with the behaviour of substances associated with the bilayer, such as amphiphilic peptides, membrane proteins and anesthetics as well as drugs, prodrugs and adjuvants. In this context, theoretical and experimental studies provide remarkable views of the complex aqueous liquid–liquid interface in fluid lipid membranes[1–5]. Because of their biophysical and pharmacological importance, some of these systems have been studied at a fundamental level and a wide range of techniques has been used to monitor the array of complex interactions involved.

Several experimental studies of the effect of adding a polymer to a colloid dispersion have been reported[6–11]. These studies mainly concern with the dependence of ordering and association phenomena in the colloid on the type of solvent, polymer length and polymer concentration. The theoretical study of the structural properties of colloid–polymer mixtures was initiated by Vrij who consider the colloid as a hard sphere and the polymers by spheres mutually interpenetrable[12]. In this connection, polyelectrolytes have recently found application in the development of pH sensitive liposomial controlled release systems. This application arises from the fact that polyelectrolytes may be used both to stabilize liposomes and to disrupt liposomes in a pH dependent-manner[13].

On the other hand, organized structures of liposomes and functional synthetic polymers can mimic the dynamic motions of a two-dimensional cellular cytoskeleton attached to a lipid bilayer. In this regard it is worth mentioning the pioneering work of Sunamoto and co-workers[14] who exploited the affinity for liposomes for a certain polysaccharide in order to reinforce the lipid membranes by adsorption of polymers. Moreover, it has recently been reported that liposomes coated with polyethylene glycols have been shown to have prolonged lifetimes *in vitro*, compared to untreated samples[6,15].

This concern prompted us to study the behaviour of charged polymers in the presence of phospholipid-based liposomes of opposite charge in order to promote the adhesion of the polymer onto the outlet surface of the phospholipid bilayers. In this regard, we have recently reported a detailed study based on fluorescence and viscosity measurements of aqueous buffered solutions of poly(4-vinyl pyridine) in the presence of vesicles based on dimyristoyl phosphatidic acid[16,17].

The aim of the present work is to extend this formalism for studying the equilibrium association of a weak polybase such as poly(2-vinyl pyridine) to small unilamellar vesicles based on dimyristoyl phosphatidic acid using fluorescence intensity measurements. In addition, we use two macroscopic models to obtain the association constant, K_A, the number of phospholipids involved in the interaction, N, and the partition coefficient, Γ, as key magnitude representatives of the polymer–lipid association process. Finally, we analyse the level of fulfilment of both models for certain experimental conditions, as well as temperature, ionic strength and pH dependences.

EXPERIMENTAL

Chemicals

Poly(2-vinyl pyridine) (P2VPy) with molar mass 2900 was purchased from Pressure Chemical Co. (Pittsburgh, PA, USA) and dimyristoylphosphatidic acid (DMPA)

* To whom correspondence should be addressed

from Sigma Chem. Co. (St Louis, MO, USA). Both materials were used without further purification.

The experiments were performed using acetate buffered solutions of pH 3.5 and 4.5 and three different ionic strengths: 0.026, 0.049 and 0.102 M. Ethylene diamine tetraacetic acid (EDTA) 1 mM was always present in the solutions to remove all bound divalent cations left on the surface of the membrane vesicles. The P2VPy is always protonated and phospholipid vesicles bear a negative charge for each phospholipid head at the values of pH assayed here.

Preparation of small unilamellar vesicles (SUV)

The stock liposome solution was prepared as follows: DMPA powder was dissolved in a mixture of benzene/methanol (2/1 v/v) and the solution taken to dryness in a round-bottom flask by rotary evaporation. The dried phospholipid film was dispersed to the desired concentration by adding the appropriate volume of the buffer and immersed during 10 min in a heater bath above the transition temperature of the lipid, T_c, for hydration. Then, the phospholipid suspension was vigorously vortexing for 5 min and afterwards sonicated using a microtip sonicator Vibra Cell VC 300 set at 50% duty cycle and level 4. Sonicator tip titanium and non-SUV lipid were removed by ultracentrifugation using a Beckman Microfuge TM at 12 000 rpm × 10 min. In all cases, the temperature during the processes of hydration, vortexing and sonication were kept above the transition temperature of the lipid. For each data point fresh solutions of 1 ml were prepared by dilution with buffer of the appropriate aliquots of the stock solution.

Fluorescence

Steady-state fluorescence measurements were performed on a Perkin–Elmer Model LS-5B Luminescence Spectrometer with a thermostated cuvette compartment and equipped with a Data Station. The excitation wavelength was 262 nm, with both excitation and emission slit windows set at 5 nm. Measurements were carried out at five temperatures: 5, 20, 37, 56 and 76°C in order to cover a wide range of values below, about and above T_c, 55 and 54°C at pH 3.5 and 4.5, respectively, for DMPA[18].

In all cases, the emission spectra were corrected for background fluorescence and vesicle and solvent light scattering by subtraction of the blank spectrum. It must be mentioned that liposomes as membrane models have the advantage of minimizing the scattered light when using fluorescence techniques compared with other bilayer formats.

In order to cover a composition range from 0 to 200 for lipid/polymer molar ratio, a battery of samples containing a fixed concentration of P2VPy and increasing concentrations of phospholipid vesicles was annealed at the desired temperature for 10 min to assure equilibrium conditions, before any measurement was carried out.

The relative intensity of fluorescence is expressed as $\Delta I/I_o = (I - I_o)/I_o$, where I and I_o are the polymer fluorescence intensities in the presence and absence of vesicles, respectively. Therefore, the fraction of bound polymer, α, can be calculated as[19,20]

$$\alpha = \frac{\Delta I}{\Delta I_{max}} = \frac{I - I_o}{I_{max} - I_o} \quad (1)$$

I_{max} being the intensity value when the polymer is fully associated to the liposome. However, usually I_{max} cannot be directly obtained from experimental measurements. In order to circumvent this drawback, several authors[19–21] have used the so-called double-reciprocal plot given by

$$\frac{I_o}{I - I_o} = \frac{I_o}{\Delta I} = \left(\frac{I_o}{I_{max} - I_o}\right)\left(1 + \frac{N}{K_A[P]_t R_i^*}\right) \quad (2)$$

where K_A denotes the polymer–lipid association constant, $[P]_t$ the total polymer concentration, and R_i^* the accessible lipid-to-probe molar ratio defined as $R_i^* = \beta \times R_i = \beta \times$ (moles of phospholipid)/(moles of probe). β stands for the accessibility factor equal to 0.65 for small unilamellar vesicles[22]. Throughout the paper, magnitudes with superscript (*) will be affected by the factor β. A linear least-squares fit of equation (2) will provide I_{max} from the intercept, allowing the α calculation through equation (1).

THEORY

The association of probe molecules to phospholipid vesicles can be described by either a partition model[23–25] or by a binding model[26,27]. The former considers a water-membrane partition equilibrium modulated by electrostatic charges and implies that the probe becomes dissolved in the phospholipid bilayer due to favourable solvation effects exerted by the lipid. In contrast, the latter model proposes a simple binding equilibrium between the free probe, P, the unoccupied membrane sites containing N phospholipids in each one, S_N, and the probe bound to lipid sites, PS_N, assuming the binding sites are independent and equivalent.

Next, we shall briefly describe both models as well as a modification of one of the binding models proposed here to account for the association of a polycation to model membranes.

Partition model (modulated by charges)

Basically, the partition model considers the lipid and the aqueous solutions as separate phases. It is assumed a partition equilibrium that relates the molar amount of bound probe per mole of accessible lipid, α/R_i^*, with the concentration of the free probe, $[P]$, as follows[23–25]

$$\alpha/R_i^* = \frac{\Gamma}{\gamma}[P] \quad (3)$$

where Γ is a partition coefficient determined by the difference in the free-energy of the probe between lipidic and aqueous media, and γ is its activity coefficient representing the deviation from the ideality. Equation (3) predicts the α/R_i^* vs $[P]$ plot, so-called as association isotherm, to be a straight line through the origin though the linear functionality is gradually lost decreasing the slope. This bending of the curves is due to non-ideal probe–probe repulsion interactions denoting their electrostatic nature[25] and represented by γ. In particular, a strict thermodynamic analysis of the adsorption equilibrium defines γ as the ratio of probe activity coefficients in the lipid and the aqueous phases, that is, $\gamma = \gamma_P^L/\gamma_P^A$.

Binding model

This model assumes a simple binding equilibrium between P, S_N and PS_N expressed as

$$P + S_N \rightleftarrows PS_N$$

characterized by an association constant, K_A, given in terms of molar concentration by

$$K_A = \frac{[PS_N]}{[P][S_N]} \quad (4)$$

In this context, the fraction of probe bound to vesicles, α, is defined by $\alpha = [PS_N]/[P]_t$ and the total polymer concentration as $[P]_t = [PS_N] + [P]$. On the other hand, the total concentration of binding sites is given by $[S]_t = [PS_N] + [S_N]$. Recalling that every site consists of N lipids, it can also be expressed as $[S]_t = [L]_t^*/N$, where $[L]_t^*$ is the total concentration of accessible lipid. Then, the concentration of unoccupied sites at the equilibrium will be

$$[S_N] = [S]_t - [PS_N] = [S]_t - \alpha[P]_t = \frac{[L]_t^*}{N} - \alpha[P]_t \quad (5)$$

Equation (5) can be rewritten by introducing the accessible lipid-to-probe molar ratio as $R_i^* = [L]_t^*/[P]_t$. Taking into account the above expressions, the association constant given in equation (4) can be written as

$$K_A = \frac{\alpha[P]_t}{[P]\left[\frac{[L]_t^*}{N} - \alpha[P]_t\right]} = \frac{\alpha/R_i^*}{[P][1/N - \alpha/R_i^*]} \quad (6)$$

A proper rearrangement of equation (6) leads to the relationship between α/R_i^* and $[P]$

$$\alpha/R_i^* = K_A[1/N - \alpha/R_i^*][P] \quad (7)$$

Attempts have been made to obtain a useful expression, mainly to evaluate K_A from fluorescence data. According to that postulated by some authors[19,20] for low molecular weight probes at very dilute concentration, the number of occupied membrane sites with respect to those unoccupied can be neglected, so that $[PS_N] \ll [S_N]$. The extension of this argument to equations (5) and (6) shows that $[S_N] \approx [S]_t$ and consequently $[L]_t^*/N \gg \alpha[P]_t$ and $\alpha/R_i^* \ll 1/N$. The above approximations reduce equation (7) to

$$\alpha/R_i^* = \frac{K_A}{N}[P] \quad (8)$$

An alternative approach to evaluate K_A and N can be done using simultaneously the so-called double-reciprocal (see equation (2)) and α vs R_i^* plots. Thus, from the first plot the ratio N/K_A can be extracted as a whole, whereas from the linear part of the second one it can easily be observed that R_i^* tends to N when α tends to unity[16,17,19].

Proposed extension of the binding model

The binding model or more precisely, equation (7) does not take into account so far the non-ideal interactions in the binding constant definition. Therefore, we believe that a more accurate description of the binding process would require to define K_A in terms of activities instead of molar concentrations of the components. Thus

$$K_A = \frac{a_{(PS_N)}}{a_{(P)}a_{(S_N)}} = \frac{[PS_N]\gamma_{(PS_N)}}{[P]\gamma_{(P)}[S_N]} \quad (9)$$

$a_{(PS_N)}$, $a_{(P)}$ and $a_{(S_N)}$ being the activities of probe–membrane complexes, free probe and unoccupied membrane sites, and $\gamma_{(PS_N)}$ and $\gamma_{(P)}$ the activity coefficients of the probe bound to vesicle and of the free probe, respectively. At this point, we assume that $a_{(S_N)} = [S_N]$ and the activity coefficients $\gamma_{(PS_N)}$ and $\gamma_{(P)}$ are the same values than those corresponding to γ_P^L and γ_P^A coming from the partition model. In the light of this argument, equations (7) and (8) can be rewritten in the following manner:

$$\alpha/R_i^* = \frac{K_A}{\gamma}\left[\frac{1}{N} - \alpha/R_i^*\right][P] \quad (10)$$

$$\alpha/R_i^* = \frac{K_A}{\gamma N}[P] \quad (11)$$

Relation between models

At present, three treatments have been explained in order to describe the association isotherms: (i) the partition model given in equation (3); (ii) the binding model through equations (7) and (8); and (iii) the extension of the latter described by equations (10) and (11) proposed here. Recently, Zouni *et al.*[28] have demonstrated that under certain experimental conditions, the traditional partition and binding models predict the same experimental behaviour. Our next purpose has been to find valid relationships between all the characteristic binding parameters, K_A, N and Γ, for any experimental conditions. For this reason, we have coupled equation (3) with equations (10) or (11) yielding this relationship as

$$\Gamma = K_A\left[\frac{1}{N} - \alpha/R_i^*\right] \quad (12)$$

and

$$\Gamma = \frac{K_A}{N} \quad (13)$$

respectively.

It is worthwhile emphasizing that for the first time, equations (12) and (13) have been derived by taking into account γ of the fluorescent probe, in contrast with the expressions given by other authors where this coefficient is absent. In this regard, equation (13) was previously reported by Zouni *et al.*[28] considering γ equal to 1. Furthermore, as we shall demonstrate in the Results section, equation (12) leads to better predictions of the experimental data and, hence, validating the assumptions made to describe the lipid–polymer association process more rigorously than other formalisms reported up-to-date.

RESULTS AND DISCUSSION

The binding of P2VPy to DMPA small unilamellar vesicles, above and below the main phase transition temperature, has been determined by fluorescence spectroscopy. P2VPy of molar mass 2900 has twenty-seven pyridinium groups that dominate its fluorescence emission spectrum, with the excitation maximum of 262 nm and the emission maximum at 402–410 nm depending on the temperature and other experimental conditions, such as pH and ionic strength. *Figure 1a* shows the emission spectra of P2VPy in the absence (bottom) and presence of increasing amounts of phospholipids at 37°C, pH 4.5 and $C_s = 0.026$ M. As it can be seen, the progressive addition of DMPA-SUV modifies the original P2VPy spectrum not only by increasing the fluorescence emission intensity but also by shifting the

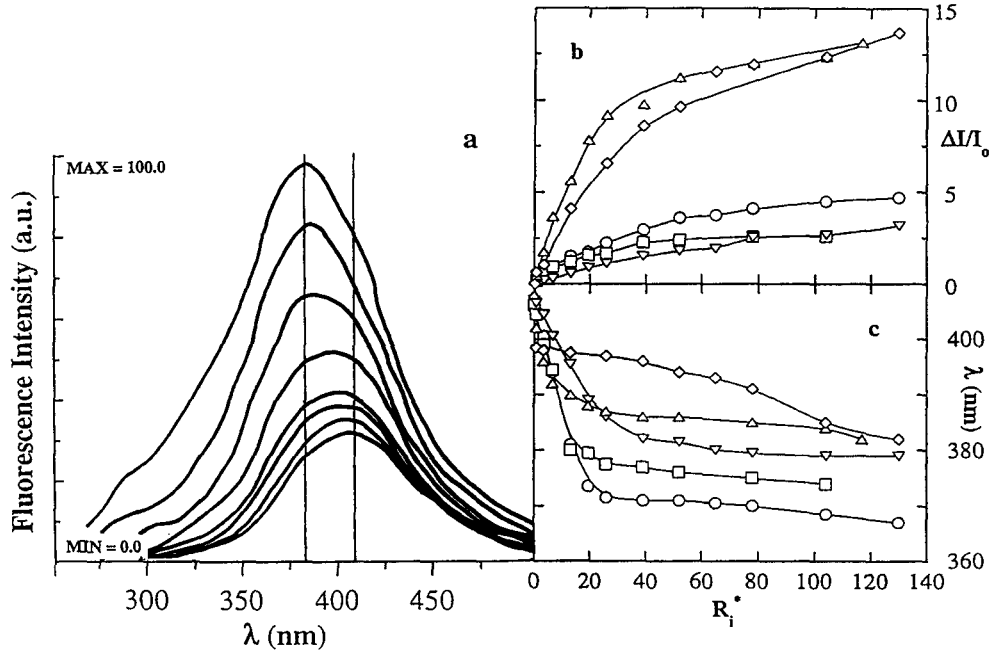

Figure 1 (a) Emission spectra of P2VPy 3.75 μM (bottom) and in the presence of increasing amounts of DMPA vesicles at 37°C. From bottom to top the accessible lipid to polymer molar ratio are $R_i^* = 0$, 0.65, 3.25, 6.5, 13, 19.5, 26 and 39. (b) Dependence of the relative intensity of fluorescence upon 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 0.026 M; temperature: (○) 5, (□) 20, (▽) 37, (△) 56 and (◇) 76°C; excitation wavelength 262 nm

emission wavelength to lower values, specifically from 407 to 380 nm. This spectral blue shifting is induced by fluorochrome relocation from water to a more hydrophobic solvent environment due to the phospholipid bilayer. This behaviour is in agreement with the observed blue shifting of a fluorophore upon membrane binding, which is thought to involve relocation to an environment with a lower dielectric constant such as the phospholipidic membrane[19,29,30].

Moreover, the parallel increase in fluorescence intensity observed upon association with the membrane could be attributed to a conformational change of the polymeric chains into a more ordered structure as a consequence of binding. In order to clarify the figure only spectra for R_i^* values up to 39 have been shown. Other R_i^* values corresponding to 52, 65, 78, 104 and 130 were measured and registered but they have not been pictured for the sake of simplicity.

Figures 1b and *c* clearly show the changes in both relative intensity and wavelength of the emission maximum after the addition of increasing amounts of SUV, respectively, at the same pH and C_s values and five temperatures.

By using the double-reciprocal plot, i.e. $I_o/\Delta I$ vs $1/R_i^*$, the change in intensity for P2VPy totally bound can be extrapolated making possible the calculation of the fraction of bound polymer at each molar ratio. As an example, *Figure 2* shows these plots for the association of P2VPy with DMPA based liposomes at pH 3.5, $C_s = 0.026$ M, and different temperatures. As expected a linear relationship given by equation (2) is obtained, which allow us to evaluate the I_{max} from the intercept, and α through equation (1) at each phospholipid composition. Similar plots have been built up for the remaining experimental conditions (not shown here). The values of polymer bound to SUV have been plotted in *Figure 3* against R_i^* for temperatures varying in the

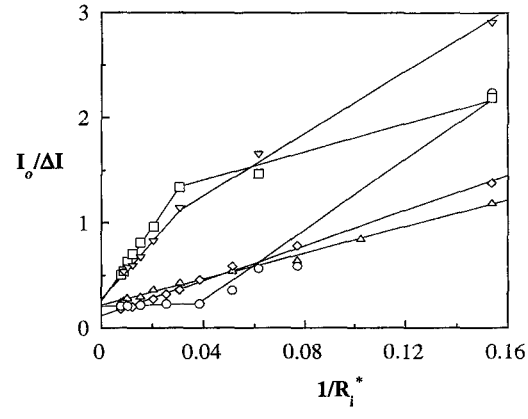

Figure 2 Double-reciprocal plots for the binding of P2VPy to DMPA SUVs at pH 3.5 and $C_s = 0.026$ M. Symbols stand for different temperatures as in *Figure 1*

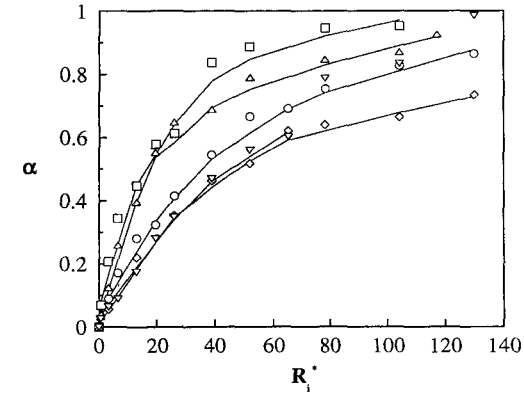

Figure 3 Variation of the fraction of bound P2VPy to DMPA SUVs with the accessible lipid-polymer molar ratio at pH 4.5 and $C_s = 0.026$ M. Symbols as in *Figure 1*

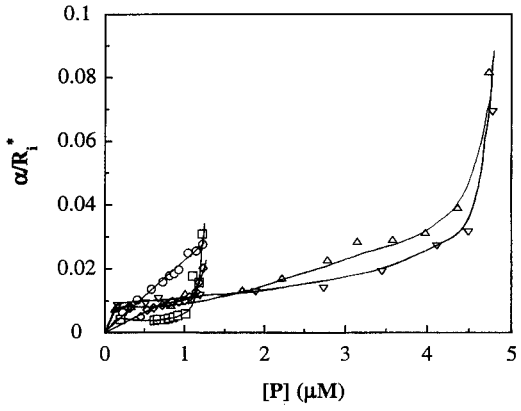

Figure 4 Binding profiles for the association of P2VPy to DMPA unilamellar vesicles at pH 3.5 and $C_s = 0.026$ M. Symbols for diverse temperatures as in *Figure 1*

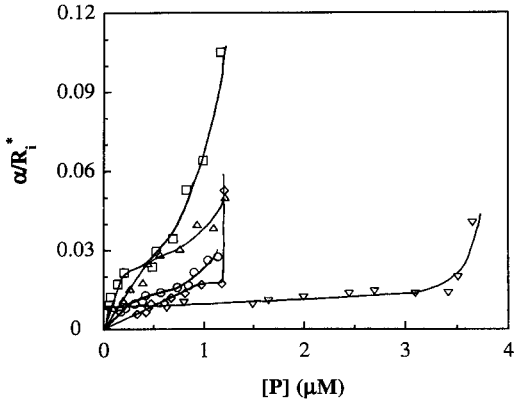

Figure 5 Binding profiles for the association of P2VPy to DMPA unilamellar vesicles at pH 4.5 $C_s = 0.026$ M. Symbols as in *Figure 1*

range 5–76°C, at pH 4.5 and C_s 0.026 M. These plots represent the extent of the association between the polymer and the vesicles, and two trends can be clearly distinguished in this figure. At low R_i^* values a linear dependence of positive slope is observed, whereas for higher R_i^* values ($R_i^* \approx 50$ in this case) a 'plateau' is reached meaning that the saturation or maximum binding of the polymer is achieved.

Nevertheless, the models described in the Theory section to analyse the association are based on equations relating to the ratio of associated polymer to accessible lipid, α/R_i^*, with the free polymer concentration. Therefore, it seems more reasonable to use the association isotherms, α/R_i^* vs $[P]$, to interpret the experimental data. In this regard, *Figures 4–6* show the association isotherms for the P2VPy bound to DMPA model membranes as a function of the three variables assayed: temperature, ionic strength and pH. Specifically, *Figures 4* and *5* illustrate the temperature effect exerted on the association isotherms at pH 3.5 and 4.5, respectively; and *Figure 6* the ionic strength effect at fixed pH and temperature. At first sight, all the isotherms show as a general trend, three zones well differenced independently of the experimental conditions. The first region corresponds to low values of free polymer where the dependence of bound polymer on concentration is linear. The second one, shows that the degree of binding is gradually deviating from the linearity causing a continuous flattening out of the association isotherm.

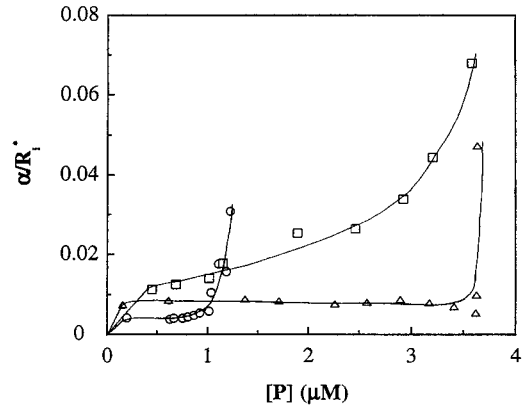

Figure 6 Binding profiles for the association of P2VPy to DMPA unilamellar vesicles at pH 3.5 and 20°C and different ionic strengths: (○) 0.026; (□) 0.050; and (△) 0.102 M

This behaviour, evidenced here for a short chain of a polyelectrolyte, seems to be in agreement with studies previously reported by other authors on melittin[19,24], mastoparan[31] or alamethicin[32] interacting with vesicles, and it clearly denotes that electrostatic interactions play an important role on the P2VPy–DMPA association. Finally, at very high $[P]$ values close to the initial polymer concentration, a sharp increase in the α/R_i^* values is observed. This dramatic change in ellipticity is not usual when small probe molecules are used but it can be considered typical in the case of polyelectrolytes[16,17], as the P2VPy studied here. Such behaviour could also indicate aggregate formation in the bilayer phase in a similar way as it has been reported for the peptides alamethicin[32] and succinylated melittin[33].

In order to analyse the data in terms of the models previously described, the characteristic association parameters, K_A, N and Γ, have been evaluated from the binding isotherms (*Figures 4–6*).

Binding model

The values of K_A and N have been obtained according to:

(a) a direct method through equation (1) and α vs R_i^* plot;
(b) the binding model by means of equation (7); and
(c) a modification of the binding model proposed in this paper and given by equation (10), which takes into account the activity coefficient, γ.

Table 1 summarizes the K_A and N values under the temperature, pH and C_s conditions assayed. Some comments about these values deserve to be made in order to check the goodness of the different ways suggested. First, the K_A and N values seems to be insensitive with the C_s or pH changes (column A from *Table 1*). We assume a 1/1 stoichiometric interaction between the pyridinium and the phospholipid charged groups. Moreover, for a homopolymer, N_{max} agrees with the polymerization degree, being 27 for P2VPy sample studied here. As can be seen, the N values obtained are always greater than N_{max}, so that method A seems to be unsuitable to describe the association process. Second, the K_A values gained with either method B [equation (7)] or C [equation (10)] show a decrease when increasing temperature (except at 5°C) and C_s and an increase with pH, as a general trend. In addition, we consider these N

Table 1 Equilibrium binding parameters for the interaction of P2VPy 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[b]	C[c]
3.5	0.026	5	11.7	3.8	9.6	52	13	26
		20	13.8	1.3	7.5	243	21	23
		37	5.5	1.0	7.3	80	12	12
		56	3.3	0.9	6.0	38	8	16
		76	11.8	0.8	3.7	108	6	18
	0.050	20	4.4	1.1	5.9	33	8	12
	0.102	20	2.9	0.8	4.2	122	18	8
4.5	0.026	5	10.9	2.0	5.5	48	7	14
		20	19.1	1.8	9.8	30	3	9
		37	4.0	1.3	8.9	77	12	6
		56	13.2	1.1	8.2	32	2	8
		76	14.0	0.8	4.4	81	5	12

[a] Obtained from equation (2) and α vs R_i^* plot
[b] From equation (7)
[c] From equation (10)

values as more reliable since they are always lower than N_{max}. The explanation of the influence of each experimental variable on K_A and N will be discussed below. We shall focus the analysis on the results obtained by using the method C proposed here that takes into account the non-ideal behaviour of the polymer–polymer interactions, through the γ coefficient.

Influence of the experimental conditions on K_A

Data inspection on column C from *Table 1* reveals that binding constant decreases as temperature increases under fixed pH and C_s values (see column C in *Table 1*). This trend clearly agrees with that observed in similar studies of interaction between Daunomycin (an anthracycline antibiotic) and plasma membranes[21] and could be due to the thermodynamic nature of the interaction, since the binding of P2VPy to DMPA vesicles is an exothermic process. Indeed, according to the van't Hoff equation, negative values of the reaction enthalpy predict a decrease in K_A with temperature. This statement has sense, since, on one hand, the polymer–vesicle association process has a negative entropy increment due to the polymer molecules move from the bulk solvent to a more ordered structure as the liposome bilayer; and on the other, it is a spontaneous process (thermodynamic calculations not published yet on the free energy gave negative values). Obviously, according to the van't Hoff equation, the association constant K_A tends to decrease when the temperature increases, in clear agreement with an exothermic transformation.

In regard to the ionic strength influence on K_A at fixed temperature and pH, it is clearly seen in *Table 1* that as C_s is increased the binding constant decreases, showing that $\log K_A$ is linear with $\log C_s$, in agreement with that reported by other authors[19,34]. In general, the effect of added salts always causes a decrease in the intensity of the electrostatic interaction, and in particular for polyelectrolytes, like P2VPy, implies a decrease in the effective charge density as a consequence of the screening by counter-ions. Therefore, the observed behaviour is expected for a process in which mainly charge–charge interactions are involved[35], as in the present report.

From the comparison of association constant values shown in *Table 1* at the same ionic strength and temperature, we can notice that increasing pH causes surprisingly an opposite effect to that observed with C_s, i.e. at pH 4.5 the association constants reach higher values than at pH 3.5. Such a behaviour was not expected since it is well-known that both adding salts and increasing pH imply a loss of effective charge on polycations. Therefore, the observed anomalous behaviour should be attributed to a balance between the polymer–liquid electrostatic attractions and the polymer–polymer (intra or intermolecular) repulsions. Obviously, it can be concluded that attractive interactions are more intense than repulsive ones as the pH is raised, as corroborated from the above K_A values closely related with the extent of the polymer–lipid association process.

Influence of the experimental conditions on N

As it can be seen in column C from *Table 1*, the number of phospholipid heads involved in the binding, N, diminishes as the temperature is raised until a value of 37–56°C and then increases. Likely, it could be invoked that the change in the physical state, gel or liquid crystal, of phospholipids is responsible for the observed trend, since it has been reported that the presence of a probe induces transition temperatures lower than that of the pure lipid[18,36]. This fact has been explained by a disordering influence of the polymer chain on the head group packing[37].

On the other hand, comparison of N values at constant temperature shows that the number of phospholipids per polymer molecule involved in the binding is markedly attenuated by increasing either the ionic strength or the pH. For instance, when pH is raised one unit or C_s is doubled, N decreases to its half, whereas a four-fold increase of C_s induces a reduction of the initial value of N to its third. For polycations, as P2VPy, an increase in pH or C_s values leads to a screening of positive charges on the protonated pyridinium ring and so, to a weaker interaction with less number of phospholipid per binding site, as observed from the results compiled in *Table 1*.

Partition model: analysis of Γ

Data falling into the first zone (low R_i^*) from the adsorption isotherms plotted in *Figures 4–6*, were also fitted with equation (3), which allows us to obtain the Γ values compiled in *Table 2*. At a fixed temperature, Γ increases as both pH or C_s do. Although under these conditions there are less effective charges, due to a decrease of the Debye screening length on both negative liposome and polycation, the association process is enhanced. Such behaviour could indicate that the charges on P2VPy chains interact more favourably with their ionic atmospheres when located close to the bilayer than they do in a purer aqueous surrounding[31]. In other words, the electrostatic repulsions between like-charges (polymer–polymer or lipid–lipid) are less favoured compared to the electrostatic polymer–lipid attractions, where this trend is in fair agreement with similar studies reported by other authors[31].

With regard to the temperature effect, at fixed pH and C_s, two tendencies are observed. Γ increases as T does until it reaches a value in the range 37–56°C, and then diminishes. Again, it is clear that the partition of the P2VPy is controlled by the physical state of the phospholipids involved in the bilayer. This Γ-dependence on T exhibits an opposite trend to that followed by the N values in the binding model, where

Table 2 Experimental and theoretical partition coefficients for the interaction of P2VPy with DMPA SUVs for all the experimental conditions assayed. Theoretical Γ values have been obtained by coupling partition and binding models through equations (12) and (13)

			10^{-4} Γ (M^{-1})					
				Equation (13)			Equation (12)	
pH	C_s (M)	T (°C)	Experimental	A[a]	B[b]	C[c]	B[b]	C[c]
3.5	0.026	5	2.2	2.3	2.9	3.7	2.3	2.1
		20	2.5	0.6	0.6	3.3	0.5	2.9
		37	4.0	0.7	0.8	6.1	0.7	4.9
		56	3.0	0.9	1.2	3.8	1.0	2.8
		76	1.5	1.1	1.3	2.1	1.2	1.7
	0.050	20	3.5	1.2	1.6	5.4	1.3	3.8
	0.102	20	5.0	0.2	0.4	5.3	0.4	4.9
4.5	0.026	5	4.0	2.3	2.9	3.9	2.5	3.1
		20	10.0	6.3	6.0	10.9	5.5	8.8
		37	12.5	0.5	1.1	14.8	0.9	13.6
		56	7.5	4.1	5.5	10.0	5.2	8.2
		76	2.0	1.7	1.7	3.7	1.5	2.9

[a] Obtained from equation (2) and α vs R_i^* plot
[b] From equation (7)
[c] from equation (10)

the latter values show a more reasonable trend as we shall discuss further.

Analysis of binding parameters by coupling models

Finally, it seems interesting to discuss the values of the different association parameters by combining the two models through equations (12) and (13) and by comparison of three methods of calculating K_A and N (options A, B and C). In addition to the experimental partition coefficient data, *Table 2* also compiles the theoretical predictions of Γ obtained as previously mentioned for any assayed conditions. As can be seen, the predicted Γ using the approximated equation (13) and the direct method (A) are far away from the corresponding experimental data. When calculating Γ with the values from method B the predictions improve but still deviations of about 48% are found. However, the best agreement between experimental and theoretical Γ values was found using the K_A and N values coming from method C and equation (12). In this case, the comparison between data on the last column in *Table 2* with the experimental Γ values gives an average deviation lower than 8% which is satisfactory enough recalling the complexity of the calculations.

CONCLUSIONS

The interaction of P2VPy with DMPA unilamellar vesicles has been investigated by measuring the fluorescence intensity of the polymer in the presence of different concentrations of lipid vesicles, above and below T_c, at diverse pH and ionic strength values. The binding is experimentally evidenced by an increase in fluorescence intensity and a blue shift observed upon association of the polymer with the model membranes. The maximum wavelength shifts were up to 39 nm at a lipid to polymer ratio of 130 under the specific conditions of 5°C and pH 4.5 (as shown in *Figure 1c*), and an increase in intensity of 13 units at other conditions (see *Figure 1b*). Such large spectral shifts are indicative of a change in the dielectric constant of the medium surrounding the fluorophore and have also been described for small molecules[38–40].

The association isotherms of P2VPy with DMPA vesicles yield a more pronounced flattening than that observed for small molecules as melittin or mastoparan, which indicates a great contribution of the electrostatic repulsions between pyridinium groups at the expense of polymer–lipid attractions. Furthermore, a drastic increase in α/R_i^* is observed at free polymer concentrations close to total $[P]_t$. Such change, not usual in small probe molecules, would be the typical polyelectrolytic behaviour[16,17,40] and it could be attributed to aggregate formation.

In order to gain insight in the association process, two models have been applied to analyse the experimental data: the partition and the binding models. The latter has been extended by including the electrostatic secondary effects through the activity coefficient, γ, since for large molecules highly charged as the polycation P2VPy the interaction is better characterized by using activities instead of concentrations.

As a general trend, the association constant K_A, representing the binding process, is greatly enhanced by increasing either temperature or pH and by decreasing the ionic strength of the solvent (see *Table 1*).

The number of phospholipids involved in a binding site, N, is markedly attenuated by the presence of salts or by increasing the pH due to the screening of effective charges on both the polymer and the liposome (bilayer). In contrast, the temperature dependence of N seems to be highly influenced by the physical state of the phospholipids. As is well-known, phospholipids in a bilayer can undergo a temperature-dependent phase transition from gel state at $T < T_c$ to a liquid-crystalline state at $T > T_c$. In the gel state the lipid chains are in a stretched conformation and packed tightly. So their motional freedom is restricted compared to the high mobility that they exhibit in the liquid-crystalline or fluid state. In the light of these arguments, it is expected that N decreases with temperature in the liquid state more than one phospholipid head would be bound to a pyridinium

group. This behaviour has been corroborated in our results compiled in *Table 1*.

With regard to the values of Γ given by the partition model, it can be concluded that its dependence on the experimental variables, pH, C_s and T, is the opposite to that followed by N. This trend is plausible in the light of equations (12) and (13). Provided that Γ is a measure of the affinity between P2VPy and DMPA vesicles, the data reveal that the physical state, gel or liquid, of the lipid has no effect on the polymer binding as in the case of melittin with negatively-charged phospholipids[18], and in contrast with results reported for other probe-vesicle systems[5,28,42].

Finally, the theoretical values of Γ calculated through equation (12) proposed here, including γ on the binding model, show the best agreement with the experimental data. Therefore, to take into account the activity coefficient in the binding model is essential for an accurate description of the association process, at least when highly charged polyions are involved.

Furthermore, the formalisms applied to P2VPy have been subsequently extended to P4VPy in the accompanying paper[43].

ACKNOWLEDGEMENTS

This work was partially supported by the 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.

REFERENCES

1. Napper, D. H., *Polymeric Stabilization of Colloidal Dispersions*. Academic Press, London, 1983.
2. Ringsdorf, H., Simon, J. and Winnik, F. M., in *Colloid–Polymer Interactions, Particulate, Amphiphilic and Biological Surfaces*, ed. P. Dubin and P. Tong, ACS Symposium Series 532, American Chemical Society, Washington, DC, p. 216.
3. Forster, S., *Adv. Polym. Sci.*, 1995, **120**, 53.
4. Scranton, A. B., Rangarajan, B. and Klier, J., *Adv. Polym. Sci.*, 1995, **122**, 3.
5. Barghouthi, S. A., Puri, R. K. and Eftink, M. R., *Biophys. Chem.*, 1993, **46**, 1.
6. Hristova, K. and Needham, D., *Macromolecules*, 1995, **28**, 991.
7. TeGrotenhuis, W. E., Radke, C. J. and Denn, M. M., *AIChE Journal*, 1994, **40**, 283.
8. Meijer, E. J. and Frenkel, D., *J. Chem. Phys.*, 1994, **100**, 6873.
9. Ringsdorf, H., Sackmann, E., Simon, J. and Winnik, F. M., *Biochim. Biophys. Acta*, 1993, **1153**, 335.
10. Buján-Núñez, M. and Dickinson, E., *Molec. Phys.*, 1993, **80**, 431.
11. Podgornik, R., and Jönsson, B., *Europhys. Lett.*, 1993, **24**, 501.
12. Vrij, A., *Pure Appl. Chem.*, 1976, **48**, 471.
13. Thomas, J. L. and Tirrell, D. A., *Acc. Chem. Res.*, 1992, **25**, 336.
14. Sunamoto, J., Iwamoto, K., Takeda, M. and Yuzuriha, T., in *Polymers in Medicine*, ed. E. Chielling and P. Giwit. Plenum, New York, 1983, p. 157.
15. Pincus, S. P. and Adelman, D., *Science*, 1990, **248**, 354.
16. Porcar, I., Gómez, C. M., Pérez-Payá, E., Soria, V. and Campos, A., *Polymer*, 1994, **35**, 4627.
17. Porcar, I., Gómez, C. M., Codoñer, A., Soria, V. and Campos, A., *Macromol. Symp.*, 1995, **94**, 171.
18. Bernard, E., Faucon, J. F. and Dufourcq, J., *Biochim. Biophys. Acta*, 1982, **688**, 152.
19. Bonmatin, J. M., Ph.D. thesis, Universite de Bordeaux I, France, 1987.
20. Pérez-Payá, E., Ph.D. thesis, Universitat de València, Spain, 1992.
21. Escriba, P. V., Ferrer-Montiel, A. V., Ferragut, J. A. and González-Ros, J. M., *Biochemistry*, 1990, **29**, 7275.
22. Stankowski, S., *Biophys. J.*, 1991, **60**, 341.
23. Stankowski, S. and Schwarz, G., *Biochim. Biophys. Acta*, 1990, **1025**, 164.
24. Kuchinka, E. and Seelig, J., *Biochemistry*, 1989, **28**, 4216.
25. Schwarz, G. and Beschiaschvili, G., *Biochim. Biophys. Acta*, 1989, **979**, 82.
26. Vogel, H., *FEBS Lett.*, 1981, **134**, 37.
27. Thiaudière, E., Siffert, O., Talbot, J. C., Bolard, J., Alouf, J. E. and Dufourcq, J., *Eur. J. Biochem.*, 1991, **195**, 203.
28. Zouni, A., Clarke, R. J., Visser, A. J. W. G., Visser, N. V. and Holzwarth, J. F., *Biochim. Biophys. Acta*, 1993, **1153**, 203.
29. Alouf, J. E., Dufourcq, J., Siffert, O., Thiaudiere, E. and Geoffroy, C., *Eur. J. Biochem.*, 1989, **183**, 381.
30. Mi, Z. and Burke, T. G., *Biochemistry*, 1994, **33**, 10325.
31. Schwarz, G. and Blochmann, U., *FEBS*, 1993, **318**, 172.
32. Rizzo, V., Stankowski, S. and Schwarz, G., *Biochemistry*, 1987, **26**, 2751.
33. Stankowski, S., Pawlak, M., Kaisheva, E., Robert, C. H. and Schwarz, G., *Biochim. Biophys. Acta*, 1991, **1069**, 77.
34. Zhang, F. and Rowe, E. S., *Biochim. Biophys. Acta*, 1994, **1193**, 219.
35. Antonietti, M., Kaul, A. and Thünemann, A., *Langmuir*, 1995, **11**, 2633.
36. Bader, H., Dorn, K., Hupfer, B. and Ringsdorf, H., *Adv. Polym. Sci.*, 1985, **18**, 1512.
37. Record, M. T. Sr, Anderson, C. F. and Lohman, T. M., *Q. Rev. Biophys.*, 1978, **11**, 103.
38. Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*. Plenum Press, New York, 1983.
39. Burke, T. G., Mishra, A. K., Wani, M. C. and Wall, M. E., *Biochemistry*, 1993, **32**, 5352.
40. Pérez, J. A., Haro, I., Martín, I., Alsina, M. A. and Reig, F., *Analytica Chimica Acta*, 1995, **303**, 65.
41. Porcar, I., García, R., Gómez, C. M., Soria, V. and Campos, A., *Progr. Colloid Polym. Sci.*, 1996, **100**, 356.
42. Dufourcq, J. and Faucon, J. F., *Biochim. Biophys. Acta*, 1977, **467**, 1.
43. Porcar, I., García, R., Soria, V. and Campos, A., *Polymer*, 1997, **38**, 3553.