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[c009] # Negatively cooperative binding of melittin to neutral phospholipid vesicles

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Abstract

The association of basic amphipathic peptides to neutral phospholipid membranes is investigated in terms of binding and partition models. The binding of native and modified melittin to egg-yolk phosphatidylcholine vesicles is studied by steady-state fluorescence spectroscopy. The effect of the ionic strength shows an enhancement of the association as the ionic strength increases. After correction for electrostatic effects by the Gouy–Chapman theory, the melittin binding isotherms could be described by a partition model. In terms of conventional binding mechanisms, which do not take into account electrostatic effects, this would correspond to a negative cooperativity. A plausible way in which the interaction occurs is proposed, based on the calculated Hill coefficient.

Keywords: Peptide–lipid interaction; Binding isotherm; Scatchard plot; Hill plot; Negatively cooperative binding; Partition coefficient; Ionic strength; Salt effect; Melittin

Introduction

The detailed understanding of how macromolecules control the structural and functional characteristics of biomembranes is one of the goals in the interdisciplinary border between chemistry and biology. An approach toward the knowledge of the

exquisite structural and surface-charge features of membranous proteins is based on the use of membrane-active peptides. The mechanism of interaction between charged, natural or synthetic, surface-active peptides and model membranes was the subject of many studies [1-9]. During the binding of a charged peptide to an aqueous–lipid interface, the induced conformational changes and surface-charge modulations yield a peptide–lipid complex having the lowest possible energy state. In order to obtain a quantitative description of the peptide association process, most studies used a membrane–water partition model, which allows the calculation of a partition coefficient for the peptide between the lipid and aqueous phases [10,11]. In the case of small peptides bound to a surface, with or without induced uniform charge density, the Gouy–Chapman theory was generally applied, which proved successful. The method accounts for changes in the bound peptide properties due to the effect of the surface charge distributed over lipid bilayers. The activity coefficient was explained taking into account electrostatic effects and the peptide's effective interfacial charge *v*, and the binding isotherms were fitted using two parameters: the partition coefficient and *v*. However, the determined *v* values are smaller than those expected from the number of ionizable groups in the peptide, *i.e.*, +3, +4, +6 and +6 e.u. for substance P (SP, a peptidic neurotransmitter of pain), mastoparan, dansylcadaverine (DNC)-melittin (cadaverine, 1,5-diaminopentane, is a metabolite derived from decarboxylation of lysine) and melittin, respectively [12]. In order to explain this difference, theoretical treatments were reported [13-15].

Melittin, a 26-residue long antimicrobial amphiphilic polypeptide and major component of the European honey bee (*Apis mellifera*) venom, is one of the best-studied model peptides concerning lipid–protein interactions. In earlier publications, the analysis of binding isotherms obtained upon the interaction of a DNC-melittin derivative, as well as the naturally occurring peptide with zwitterionic phospholipid membranes was performed [16,17]. The approach was extended to the analysis of the binding of the fluorescent probe DNC-SP analogue to neutral phospholipid membranes under various conditions of ionic strength I [18]. The theoretical association isotherms generated from z_p^+ values satisfactorily described the experimental data at low I, and

several parameters included in the equation were varied in order to reproduce better the binding curves obtained at the highest *I*. The partition coefficients derived for both peptides are similar to those deduced according to binding analyses, which are more classical [19]. The formalism allowed determining the values of the molar free energies for the peptide in both aqueous and lipid phases. Such an understanding of the thermodynamic parameters provided information of interest about the energetic of peptide–lipid interactions.

A thermodynamic approach was proposed to quantitatively analyze the binding isotherms of peptides to model membranes as a function of one adjustable parameter, the actual peptide charge in solution z_p^+ [20]. The main features of the approach are: (1) the theoretical expression for the partition coefficient calculated from the molar free energies of the peptide in both aqueous and lipid phases, (2) an equation proposed by Stankowski to evaluate the activity coefficient of the peptide in the lipid phase [15] and (3) the Debye–Hückel equation, which quantifies the activity coefficient of the peptide in the aqueous phase. The interaction of basic amphipathic peptides, *e.g.*, melittin, DNC-melittin and DNC-SP, with neutral phospholipip membranes was studied by spectrofluorimetry. The obtained z_p^+ values were always lower than the electrostatic charge of the peptide and were rationalized by considering that the peptide charged groups are strongly associated with counterions in buffer solution at a given ionic strength. The partition coefficients derived using the z_p^+ values are in agreement with those deduced from the Gouy–Chapman formalism. From the z_p^+ values, the molar free energies for both free and lipid-bound states of the peptides were calculated. The lipid charge density at the bilayer surface modulates the effects of melittin on membranes [21]. Steinem *et al.* showed multilayer formation of melittin on solid-supported phospholipid membranes by shear-wave resonator measurements [22]. The preferential solvation of ternary systems including polymers was studied in this Laboratory by gel permeation chromatography [23]. High-performance size-exclusion chromatography (HPSEC) was used for characterizing the interaction between polyanions and cationic liposomes [24]. In earlier publications, it was studied the binding of vinyl polymers to anionic model membranes [25] and the interaction of polyelectrolytes with oppositely

charged micelles [26] by fluorescence and liquid chromatography. The present report presents a non-linear kinetic approach applied to the lipid–melittin system. Section 2 presents the method. Section 3 discusses the results. Section 4 summarizes our conclusions.

Results and Discussion

Theoretical considerations

The association of peptide molecules to lipid vesicles is generally described by a partition model, in which one considers the membrane as a separate lipid phase in which the peptide can dissolve. The partition model allows the calculation of a partition coefficient K_r of the peptide between the lipid and aqueous phases, defined as the ratio of the activity of the peptide in the lipid phase $a_p{}^L$ to that in the aqueous phase $a_p{}^A$, when secondary effects make the system to deviate from the ideal behaviour:

$$K_r = \frac{a_p^L}{a_p^A} = \frac{c_p^L \gamma_p^L}{c_p^A \gamma_p^A} \qquad (1)$$

where $c_p{}^L$ and $c_p{}^A$ are the concentrations of peptide in the lipid and aqueous phases, respectively, and $\gamma_p{}^L$ and $\gamma_p{}^A$, the activity coefficients in each specific phase, attributed to electrostatic repulsions among the positive peptide molecules in each phase. When the lipid volume is negligible with respect to the solvent volume, the following expression that relates $c_p{}^L/c_p{}^A$ with experimental data can be derived:

$$\frac{c_p^L}{c_p^A} = \frac{\left(\alpha / R_i^*\right)}{(1-\alpha)[P]_T \bar{v}_L} \qquad (2)$$

where $\bar{v}_L$ is the lipid partial molar volume (0.785 and 0.716L·mol^{-1} for EPC and DPPC, respectively [30]), as well as $(1-\alpha)[P]_T$ is the aqueous free peptide concentration. In Eq. (2) for calculation purposes and, since the peptide is considered to have access only from the vesicle outside [31], α/R_i is corrected by the fraction of lipid in the outer leaflet β, *i.e.*, $(\alpha/R_i^*) = (\alpha/R_i)/\beta$, thus denoting the moles of adsorbed peptide per mole of accessible lipid. For SUVs where *ca.* two thirds of the lipids stay in the outer shell, a value of 0.65 for β can be used. The following expression for describing a real partition equilibrium is obtained by substituting Eq. (2) into (1):

$$\frac{\left(\alpha/R_i^*\right)}{(1-\alpha)[P]_T} = \frac{K_r\bar{v}_L}{\gamma} = \frac{\Gamma}{\gamma} \tag{3}$$

where the activity coefficient γ is equal to ${\gamma_p}^L/{\gamma_p}^A$ and reflects possible unideal peptide–peptide interactions. For small ${c_p}^A$ values, the activity can be replaced by the concentration (*cf.* Eq. 1), ${\gamma_p}^A$ will approach unity, and γ will be equivalent to ${\gamma_p}^L$ as generally assumed in theoretical calculations. Γ is a parameter proportional to the partition coefficient, $\Gamma = K_r\bar{v}_L$, which is a measure of the free energy of the peptide–lipid interactions and independent of peptide concentration:

$$\Gamma = \mathrm{cnt}\,\bar{v}_L \exp\left(\frac{\Delta\overline{G}_p^{o,A} - \Delta\overline{G}_p^{o,L}}{RT}\right) \tag{4}$$

where *cnt* is a constant that depends on the molar masses and densities of both lipid and water, as well as $\Delta\overline{G}_p^{o,A}$ and $\Delta\overline{G}_p^{o,L}$ are the molar free energies of the peptide in the aqueous and lipid phases, respectively. The value of $\Delta\overline{G}_p^{o,A}$ for cationic ion–water interactions is given by [32]

$$\frac{\Delta\overline{G}_p^{o,A}}{RT} = -\frac{N_A\left(z_p^+\right)^2 e^2}{2RT\left(R_p+2R_w\right)4\pi\varepsilon_o}\left(1-\frac{1}{\varepsilon_w}\right) + \frac{\Delta G_{\mathrm{cav}}}{RT} - \frac{4N_A z_p^+ e\mu_w}{RT\left(R_p+R_w\right)^2 4\pi\varepsilon_o} + \frac{4N_A z_p^+ e\theta_w}{2RT\left(R_p+R_w\right)^3 4\pi\varepsilon_o} - \frac{4N_A\left(z_p^+\right)^2 e^2\alpha_w}{2RT\left(R_p+R_w\right)^4 4\pi\varepsilon_o} \tag{5}$$

The free energy of solvation is composed of five terms. The first one refers to the Born charging contributions, *i.e.*, the free-energy change resulting from the transfer of ions from vacuum to a structureless continuum medium, the water solvent with relative permittivity ε_w. A numerical value of 84kJ·mol^{-1} for ΔG_{cav}, the work term, has been used in this study, assuming a tetrahedrally coordinated positive ion as a first approximation. The term involves the work of forming a cavity in the solvent, the work of splitting up the extracted solvent molecules and separating them to infinity, the work to orientate the solvent molecules around the peptide (primary solvation shell), as well as the work of condensing the solvent molecules not used in the solvation of the ion. The three other terms refer to the ion–dipole, ion–quadrupole and ion–induced dipole interactions, respectively. All the parameters included in Eq. (5) are known except R_p (the radius of the peptide ion) and ${z_p}^+$, the latter being the actual peptide-ion charge in solution, which can be different from the physical charge due to partial screening. N_A is the

Avogadro number, $e = 1.602192\text{x}10^{-19}$C, the proton charge, $R = 8.3143\text{J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$, the gas constant, $T = 293$K, the temperature, $R_w = 2.8$Å, the effective radius of solvation of water, $\varepsilon_o = 8.854\text{x}10^{-12}\text{C}^2\cdot\text{N}^{-1}\text{m}^{-2}$ the absolute permittivity of vacuum, $\varepsilon_w = 78.5$, the relative permittivity of water, $\mu_w = 1.86$D, the dipole moment of water, $\theta_w = 3.9\text{x}10^{-26}\text{statC}\cdot\text{cm}^2$, the quadrupole moment of water, and $\alpha_w = 1.65\text{x}10^{-40}\text{C}^2\cdot\text{m}^2\cdot\text{J}^{-1}$, the deformation polarizability, which is a measure of the *distortability* of the water molecule along its permanent dipole axis. A similar expression can be obtained for $\Delta\overline{G}_p^{o,L}$. In order to evaluate the relationship between z_p^+ and ν (the peptide effective interfacial charge for the membrane-bound state of the peptide in the Gouy–Chapman approach), the γ_p^L values were used to determine ν with the expression proposed by Schwarz and Beschiaschvili [10]:

$$\ln\gamma = 2\nu\sinh^{-1}\left[\nu b\left(\alpha/R_i^*\right)\right] \tag{6}$$

Notice that γ in Eq. (6) is equivalent to γ_p^L; the b parameter:

$$b = \frac{e}{\beta A_L\left(8\varepsilon_w\varepsilon_o RTI\right)^{1/2}} \tag{7}$$

is essentially determined by the ionic strength I of the bulk electrolyte, as well as $A_L = 70$ (EPC, DPPC) and 68Å^2 (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol, POPG) are the estimated area of the phospholipid heads [15]. Under our present experimental conditions, $b = 3.10\text{I}^{-1/2}$ for EPC, $b = 2.97\text{I}^{-1/2}$ for DPPC and $b = 3.54\text{I}^{-1/2}$ for dioleoylphosphatidylcholine (DOPC). After correction for electrostatic effects by the Gouy–Chapman theory, the melittin binding isotherms could be described by a partition model. In terms of conventional binding mechanisms, which do not take into account electrostatic effects, this would correspond to a negative cooperativity.

Ligand–receptor interaction

Suppose we have a ligand G and receptor R, the reaction can be written

$$\text{G} + \text{R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{GR}$$

where k_1 and k_{-1} are the rate constants of the forward and backward reactions, and so the Law of Mass Action gives us the differential equation of the progress of the reaction

$$\frac{dx}{dt} = k_1 gr - k_{-1} x$$

where r, g and x are the molar concentrations of R, G and the product GR [33]. We are interested in determining the concentration of bound ligand x at equilibrium as a function of the free ligand, g [34]. By setting the derivative equal to zero, we obtain the solution $x = grk_1/k_{-1} = gr/K_D$, where K_D is known as the *dissociation constant* of the reaction [35]. The total amount of the receptor is given by $r_T = r + x$ and, hence, by substituting for r in the above we get

$$x = \frac{1}{K_D} g(r_T - x) \qquad \text{or} \qquad x = \frac{r_T g}{K_D + g}$$

which is more usually written as

$$P_B = \frac{B}{B_{\max}} = \frac{F}{K_D + F} \tag{8}$$

where B is the molar concentration of *bound ligand*, F is the molar concentration of unbound or *free ligand*, $B_{\max}$ is the maximum value of B attainable that occurs when all the receptors are bound to ligand, or density of sites, and K_D is the *dissociation constant* of the binding reaction [36]. Notice that $B_{\max} = r_T$ [37]. A plot that has come to be widely used for visual presentation of the results of ligand–receptor assays is known as the *Scatchard plot* [38]. This plot of B/F *vs.* B is approximately a straight line if the simple model without any complications holds. A major use of the Scatchard plot is as a diagnostic of the type of departure from the simple model. In that case, the straight line is often modified into an upwardly concave curve. Such a curve can be obtained for negatively cooperative binding in which binding at one site makes it less likely that binding will occur at some other sites. On the other hand, a Scatchard plot that exhibits downward concavity could mean the presence of positively cooperative binding. The mathematical model of ligand-receptor interaction results

$$P_B = \frac{B}{B_{\max}} = \frac{F^n}{K_D^n + F^n} \tag{9}$$

where $n < 1$ indicates negative cooperativity and $n > 1$ denotes positive cooperativity. For proteins with n binding sites for ligands, negative cooperativity occurs when

binding at one site interferes with binding at an adjacent site. Eq. (8) can be transformed into a straight-line relationship by first writing

$$\frac{P_B}{1-P_B} = \left(\frac{F}{K_D}\right)^n$$

and then taking logs of both sides

$$\log\left(\frac{P_B}{1-P_B}\right) = h\log F - h\log K_D \qquad (10)$$

where n has been replaced by h for the general case in which cooperativity is not perfect. A plot of log $P_B/(1-P_B)$ *vs.* log$F$, called a *Hill plot*, will be a straight line with slope h [39]. The experimentally determined slope is called the *Hill coefficient* h, which increases with the extent of the cooperativity up to a maximum possible value of the total number of sites n [40]. A value of h less than one could indicate negative cooperativity [41]. At values of P_B below 0.1 and above 0.9, the slopes of Hill plots tend to a value of 1, indicating an absence of cooperativity. This is because at low ligand concentrations there is not enough ligand present to fill more than one site on most protein molecules, regardless of affinity; similarly, at high ligand concentrations, there are extremely few protein molecules present with more than one binding site remaining to be filled. The Hill coefficient is therefore taken to be the slope of the linear, central portion of the graph, where the cooperative effect is expressed to its greatest extent. For systems where cooperativity is complete, the Hill coefficient h is equal to the number of binding sites n. Proteins that exhibit only a partial degree of positive cooperativity may still give a Hill plot with a linear central section, but in such cases h will be less than n, and the linear section is likely to be shorter than that for a system where cooperativity is more near complete.

Melittin and DNC-SP will bind to neutral phospholipid vesicles for the hydrophobic interaction between the nonpolar amino acids and the phospholipid hydrocarbon layer. The binding step is probably accompanied by membrane expansion since the peptide molecules can intercalate between the lipid molecules as evidenced by monolayer experiments. A second consequence of melittin/DNC-SP binding is that the membrane surface becomes positively charged, so that the adsorption of forthcoming

positively charged peptide would be less favoured. Both factors, membrane expansion and electric charge of the membrane, are taken into account in the folowing analyses.

Binding of DNC-melittin to neutral bilayers

The binding isotherms for lipid–melittin and –polyanion model systems have been studied in this Laboratory by steady-state fluorospectroscopy, viscometry, HPSEC, ^{31}P and ^{2}H nuclear magnetic resonance, as well as X-ray diffraction experiments. The binding isotherms, *B vs. F*, for DNC-melittin and melittin to EPC vesicles (fluid phase) at temperature $T = 23°C$ and ionic strength $I = 0.03 mol \cdot L^{-1}$, obtained from spectrofluorimetry, are illustrated in Fig. 1. The plots are far from linear, rejecting the idea that an ideal partition of the peptide into both phases take place. The increased deviation from linearity at high F values suggests a concomitant increase in γ. This is likely due to electrostatic repulsion between the positive charges of neighbouring peptide molecules at the water–lipid interface. The maximum value of B, B_{max}, was extrapolated from a double-reciprocal plot. It results $B_{max} = 0.0699$ and $0.0964 \mu mol \cdot L^{-1}$ for DNC-melittin and melittin. The calculated values of the actual peptide charge in solution, z_p^+, are similar (+1.85 and +1.88 e.u. for DNC-melittin and melittin), as can be predicted for a binding isotherm performed at a given ionic strength I. Notice that the electrostatic charge calculated from the number of ionizable groups in native and modified melittin is +6 e.u. Moreover, an effective charge smaller than that expected from the number of ionizable groups is a well-known phenomenon. The corresponding interpretation is that the actual z_p^+ charge of the cationic polypeptide should be decreased due to the screening effect of counterions in the electrolyte solution, which provides a given I.

The Scatchard (*i.e.*, *B/F vs. B*) plot for the binding of DNC-melittin and melittin to EPC vesicles is shown in Fig. 2. The upwardly concave curve indicates that, for a given B/F value, the corresponding B value is lower than that expected for an ideal simple model. The interpretation suggests a negatively cooperative binding, although it might be also attributed to the heterogeneity of the binding mechanism; the shape of the binding isotherm (Fig. 1) could be represented by a two-site mechanism. The negative

cooperativity is in agreement with the increase in effective interfacial charge, ν, from ν = +1.07 to +1.18 and from +1.09 to +1.17 e.u. for DNC-melittin–EPC and melittin–EPC, respectively, as F increases. However, the melittin results should be taken with care: The data could probably also be fitted by linear regression.

The Hill plots of log $P_B/(1-P_B)$ *vs.* log$F$, at fixed peptide concentrations, for the binding of DNC-melittin and melittin to EPC vesicles are displayed in Fig. 3. For DNC-melittin the fitted line turns out to be:

$$\log\left[P_B/(1-P_B)\right]_{\text{DNC-Mel}} = 8.02 + 1.27\log F \qquad n = 6 \quad r = 0.932 \quad s = 0.276 \quad F = 26.5 \qquad (11)$$

However, if only the first four points are included in the fit the correlation is improved:

$$\log\left[P_B/(1-P_B)\right]_{\text{DNC-Mel}} = 5.56 + 0.888\log F \qquad n = 4 \quad r = 0.992 \quad s = 0.067 \quad F = 122.0 \qquad (12)$$

For melittin, the fitted line results:

$$\log\left[P_B/(1-P_B)\right]_{\text{Mel}} = 5.33 + 0.909\log F \qquad n = 6 \quad r = 0.975 \quad s = 0.077 \quad F = 76.0 \qquad (13)$$

The slopes represent the Hill coefficient h, which results 0.888 and 0.909 for DNC-melittin and melittin, respectively. Both values of h are lesser than one, meaning that the peptide–lipid binding shows negative cooperativity. The intercepts $-h\log K_D = hpK_D$ arise 5.56 and 5.33 for DNC-melittin and melittin, where $K_D{}^h$ is the dissociation constant for the whole peptide. From both slope and intercept, the dissociation constant for each site K_D ensues pK_D = –logK_D values of 6.26 and 5.87 for DNC-melittin and melittin.

The theoretical dissociation constant p($K_D{}^h$) for the whole peptide, in the binding of DNC-melittin and melittin to EPC vesicles (*cf.* Table 1), is calculated for different Hill coefficients h, from the dissociation constant pK_D at each site. When h decreases from one to *ca.* 0.84 (16%), the corresponding theoretical p($K_D{}^h$) value decreases one log unit and, consequently, $K_D{}^h$ increases one order of magnitude for both native and modified melittin.

The partition coefficient Γ is given in Table 2 for the binding of DNC-melittin and melittin to EPC, DPPC, DOPC, POPC and negatively charged POPC/POPG (90/10 mol/mol) vesicles, as well as solid-supported membranes of octanethiol and POPC. The mean average values of $z_p{}^+$ and Γ used for the evaluation of the theoretical binding isotherms are specified, as well as the molar free energies of the peptide in the aqueous

and lipid phases. The ν values obtained for DNC-melittin and melittin in EPC and DPPC are summarized. For a given binding isotherm, the ν values are constant between the experimental error and somewhat smaller than z_p^+, being slightly lower than those reported in the literature [10,12,15,19,22,31,42]. An effective charge smaller than that expected from the number of ionizable groups is a well-known phenomenon, previously described for melittin (+6 e.u.) [10,12,19,22,31,42] and other peptides [2–4]. In the case of melittin, it has been suggested that both arginines (R22 and R24) might be located far from the membrane interface or tightly associated with counterions, and contributing little to the total interfacial charge [12,43,44]. Notice that Γ values, theoretically calculated for both native and modified peptides, are of the same order of magnitude than those derived from the fitting analysis. This means that, for each binding isotherm, the evaluated value of z_p^+ originates a theoretical value of Γ similar to that obtained from the initial slope. However, with the present thermodynamic approach, the determination of Γ and ν parameters is made separately, without any extrapolation from the experimental isotherm. Similar binding constants Γ were derived by Vogel for DMPC vesicles [45]. In general, the greater the electrostatic repulsions (z_p^+) taken into account, the lower the binding constant Γ. Controversial to our findings and those of Steinem *et al.* [22], Okahata *et al.* found a simple Langmuir adsorption isotherm of melittin on 1,2-dipalmitoylphosphatidylethanolamine (DPPE) Langmuir–Blodgett films up to a concentration of 105μmol·L^{-1} [46]. The fact that the authors could neither detect a multilayer adsorption nor a destruction of the membrane at high melittin concentrations, led us to the conclusion that the interaction of melittin with phosphatidylethanolamine monolayers in a crystalline state is significantly different from the interaction with POPC. These different results emphasize the difficulties in comparing different adsorption studies of melittin, since parameters, *e.g.*, membrane fluidity, water content within the membrane, lipid composition and p*H*, considerably influence the adsorption of melittin.

Binding of DNC-substance P to neutral bilayers

The experimental binding curves obtained for DNC-SP were next analyzed using the partition equilibrium model with z_p^+ as adjustable parameter, assuming $\varepsilon_L = 20$, as well as that the peptide was solvated in both aqueous and lipid phases. For a more accurate determination of $\Delta\overline{G}_p^{o,A}$ and γ_p^L, it was considered appropriate evaluating the dielectric permittivity of water ε_w and the inverse Debye length $\kappa = 1/\lambda_D = (2e^2 N_A I/\varepsilon_w \varepsilon_o kT)^{1/2}$, where $k = R/N_A = 1.3807\times10^{-23}\text{J}\cdot\text{K}^{-1}$, as a function of the ionic strength (*cf.* Table 3). In the Gouy–Chapman theory, the λ_D Debye length is a measure of the distance z at which the electric potential $\psi(z)$ decreases by a factor of 1/e (e = Neper number) from its value for a plane, charged surface placed at $z = 0$.

$$\psi(z) = \psi(o)\exp(-\kappa z) \qquad (14)$$

The λ_D length plays a fundamental role in the physics of ionized milieus. In fact, λ_D is nothing but the distance from which two charged objects do not *see* one another in a ionized milieu due to the counterions that they develop in the milieu. The λ_D is, then, a distance such that if $z > \lambda_D$ the potential is practically null, translating thus the screening effect produced by the ions attracted by the charged surface. The order of magnitude is $\lambda_D = 100$Å for an ionic strength $I = 1\text{mmol}\cdot\text{L}^{-1}$ in aqueous solution at room temperature and decreases for incresing I.

As a first approximation, a value of $\Delta G_{cav} = 84\text{kJ}\cdot\text{mol}^{-1}$ and an α-helix conformation for the peptide ($R_p = 6.5$Å) were used. It has been shown by spectroscopy that in the presence of membrane-mimetic solvents and/or surfactants, SP forms a partial α-helix [47,48]. On the other hand, it has been also proposed, based on thermodynamic calculations, that the C-terminal segment of SP could be organized as an α-helix domain in the membrane-bound state [49]. Moreover, CD measurements in our laboratory indicate that in the presence of EPC vesicles, SP and its fluorescent analogue might adopt a partial α-helix conformation.

The partition coefficient Γ for the binding of melittin to dimyristoylphosphatidylcholine (DMPC), DNC-SP to EPC, as well as of peptides mastoparan (14 amino acids) and analogous mastoparan-X to DOPC vesicles is given in Table 4 at different ionic strengths. In particular, for melittin binding to DMPC vesicles, the effective interfacial charge v was only slightly altered at low ionic strength I, but

the partition coefficient Γ decreased. The latter is in contrast to observations with DOPC vesicles [10]. At high I, the partition coefficient Γ remained virtually invariant, whereas the effective interfacial charge ν markedly decreased. An effective interfacial charge of +2 e.u. is small compared to the actual number of +6 e.u. carried by melittin under those conditions. In spite of the electrostatic repulsion of the melittin molecules on the surface, multilayer adsorption takes place [22], which can be explained in the same way as the formation of tetramers in solution. The equilibrium between monomers and tetramers in solution is influenced by two opposite forces, one is the net charge of the peptide, which avoids the formation of tetramers, the other is the hydrophobic exterior of the peptide, which promotes the self-association. Because of the low charge of *ca.* +1.5 e.u. per melittin molecule on the surface [12], the formation of aggregates seems to be reasonable at higher peptide concentrations. From the obtained data, it is not possible to distinguish whether melittin binds as monomer to the surface forming multilayers at higher concentrations, or whether melittin tetramers, which are already formed in solution at higher concentrations, adsorb to the surface as tetramers building multilayers.

For DNC-SP binding to EPC, the average z_p^+ values are obtained at different ionic strengths (Table 4). The z_p^+ slightly decreases as the ionic strength increases, as usually observed for polyelectrolytes [50,51], due to partial screening of the counterions in the electrolyte solution. This diminution is in agreement with a Hill coefficient $h < 1$ (Table 1) or negative cooperativity. The obtained z_p^+ values are lower than the electrostatic charge of SP (+3 e.u.) [52] (Table 4). Once z_p^+ is known, the free molar energies of DNC-SP between the water $\Delta\overline{G}_p^{o,A}$ and lipid phases $\Delta\overline{G}_p^{o,L}$ have been estimated as a function of the ionic strength as well as Γ values. Both $\Delta\overline{G}_p^{o,A}$ and $\Delta\overline{G}_p^{o,L}$ display negative values, as well as their absolute values decrease as the ionic strength increases. As a first glance, this fact reveals that the thermodynamic process of dissolving the peptide in both phases is spontaneous, as observed for DNC-melittin and melittin (Table 2). The differences ($\Delta\overline{G}_p^{o,A} - \Delta\overline{G}_p^{o,L}$) are positive values and slightly increase with the ionic strength, denoting a more favourable thermodynamic process of the peptide to be dissolved in the lipid phase. Notice that, for a given ionic strength, the values of Γ

(Table 4) are similar to those deduced from the Gouy–Chapman/partition equilibrium approach [3].

For the binding of mastoparan to DOPC, the calculated Γ values increase with the ionic strength I and are similar to those previously obtained by Schwarz *et al.* [3], by using the Gouy–Chapman formalism (Table 4). Notice also that the calculated ν values are smaller than the actual z_p^+ charge and similar to those previously reported [3]. Looking at the data, we see a distinct increase of the effective charge when I is raised. This suggests that the underlying conformational changes imply a displacement of charges closer to the interface. The considerations above are, of course, generally applicable to similar peptide–lipid systems, particularly mastoparan– and mastoparan-X–DOPC. When, instead of mastoparan, the analogous peptide mastoparan-X interacted with DOPC within the I range of 0.0–0.4mol·L^{-1}, the absolute magnitudes of Γ became slightly larger, whereas effect of I was practically the same as far as the relative change is concerned [3,53]. However, the variation in I does not change the effective interfacial charge, suggesting largely alike conformations in both cases. Of course, this does still allow changes of Γ owing to the ionic-atmosphere interactions. The Γ values for native and modified melittin are affected in one or two orders of magnitude by I. As $K_D{}^h$ is inversely proportional to Γ, the quotients between pairs of $K_D{}^h$ values at two different I values have been calculated from the corresponding quotient of Γ values as $K_D^{h-h_o} = K_D{}^h / K_D^{h_o} = \Gamma_o/\Gamma$. Here, h and h_o are the Hill coefficients at ionic strengths I and I_o, respectively.

From data in Table 4, the plot of the relative theoretical $K_D^{h-h_o}$ *vs.* I (*cf.* Fig. 4) shows that the first point of the four curves is superposed. In particular for mastoparan-X in the power curve $K_D^{h-h_o} = aI^b$, the exponent is $b = -0.132$, indicating that theoretical $K_D^{h-h_o}$ is less sensitive to I, and an increase in I of 1mol·L^{-1} decreases $K_D^{h-h_o}$ by 0.5. However, for mastoparan in the power curve $b = -0.536$, pointing to that theoretical $K_D^{h-h_o}$ is more sensitive to I, and an increase in I of 1mol·L^{-1} decreases $K_D^{h-h_o}$ by 0.9. Melittin and DNC-SP with exponents $b = -0.218$ and -0.317, respectively, present an intermediate behaviour, and an increase in I of 1mol·L^{-1} decreases $K_D^{h-h_o}$ by 0.7. As no dependence of K_D on I is considered, the corresponding decrease in h should be in

the range 0.3–1.2. The results may cause an enhancement of the negatively cooperative character of the four peptide–vesicle bindings, which is in agreement with the increase in ν from +1.07 to +1.18 and from +1.09 to +1.17 e.u. for DNC-melittin–EPC and melittin–EPC, as F increases.

From the preceding results the following conclusions can be drawn.

1. Fluorescence spectroscopy measurements likely suggest a similar lipid-bound state for both native and modified melittin.

2. In the thermodynamic approach, both Gouy–Chapman and Debye–Hückel formalisms apply the Poisson–Boltzmann equation to calculate the influence either of a plane charged surface or between two charged spheres over the structure of the adjacent ionized liquid. In both cases, the *Debye length* is the magnitude of the medium with physical meaning.

3. Melittin, a positively charged amphipathic peptide of 26 amino acid residues, strongly associates with zwitterionic lipid vesicles, taking different structural states depending on the ionic strength in the buffer solvent. The partition coefficient (measuring the affinity of binding in the limit of high dilution) increases with the ionic strength, indicating that the peptide charges interact more favourably with their ionic atmospheres when located close to the bilayer than they can do in purely aqueous surroundings. The behaviour is similar to that observed previously for mastoparan [3].

4. The interaction of melittin with EPC is significantly different from the interaction with DPPC vesicles. These different results emphasize the difficulties in comparing different adsorption studies of melittin since parameters, *e.g.*, membrane fluidity, water content within the membrane, lipid composition and p*H*, considerably influence the adsorption of melittin. The behaviour is similar to that observed previously with DPPE and POPC [22].

5. Melittin association to neutral phospholipid vesicles can be described by a simple model incorporating a water–membrane partition equilibrium, modulated by electrostatic charging of the membrane, as the basic peptide accumulates at the interface. The surface potential induced in this way counteracts the association of further peptide. It turns out that this effect can be satisfactorily treated by using a

Gouy–Chapman approach. Further melittin binding is difficult because the repulsion of like charges becomes the dominant mechanism. In terms of conventional binding mechanisms, which do not take into account electrostatic effects, this would correspond to a negative cooperativity. The dissociation constant pK_D^h for the whole peptide, in the binding of native and modified melittin to EPC vesicles, is dependent on the Hill coefficient h. A decrease in h of 16% causes an increase in K_D^h of one order of magnitude.

Work is in progress on testing the utility of high-performance size-exclussion chromatography to confirm the results presented in this paper, *i.e.*, binding of melittin to lipid.

Experimental Procedures

Materials

Melittin, SP and monodansylcadaverine [*N*-(5'-aminopentyl)-5-dimethylamino-1-naphthalensulphonamide, DNC] were purchased from Serva (Heidelberg, Germany). Dipalmitoyl–L–α-phosphatidylcholine (DPPC) and guinea-pig liver tranglutaminase (protein-glutamine:amine γ-glutamyl transferase, EC 2.3.2.13) were from Sigma (St. Louis, MO). Egg-yolk phosphatidylcholine (EPC) was purchased from Merck (Darmstadt, Germany) and purified according to Singleton *et al.* [27]. Salts, buffers and reagents were of the highest purity available.

Transglutaminase-mediated chemical modification of melittin and substance P

The procedure for the transglutaminase-mediated incorporation of DNC into Q25 of melittin (DNC-melittin) was similar to that described in Refs. [16,17]. Experimental conditions for the selective labelling of SP at Q5 with a DNC-SP probe were reported [18]. Both fluorescent analogues have been shown to retain the biological activity of the native sequences. Thus, DNC-SP was functionally active on guinea-pig trachea [18], and DNC-melittin had the same haemolytic effect than native melittin on red blood cells.

Preparation of small unilamellar vesicles (SUV)

EPC or DPPC were dispersed in Mops–NaOH buffer, pH 7.0, at a given NaCl concentration in the 0–1mol·L^{-1} range and vortexing for 10 min. The dispersion was next sonicated for 20 min on ice (EPC) or at a temperature above the phase-transition of the phospholipid (DPPC), by using an ultrasonic generator with a microtip probe (Vibra Cell, Sonics and Materials, Inc., Daubury, CT) at a power setting 4 and 50% duty cycle. The samples were then centrifuged for 15 min at 35 000×g to remove probe particles and the remaining multilamellar aggregates. The lipid content in the resulting SUV preparations was determined by a phosphorous assay [28]. The integrity of SUV preparations was controlled by negative-stain electron microscopy [29].

Fluorescence spectroscopy

Steady-state fluorescence measurements were recorded using a Perkin Elmer LS–50 fluorescence spectrophotometer, with 1.0×1.0cm quartz cuvette. The excitation and emission bandwidths were 5nm. Spectra were corrected compared to quinine sulphate, as well as blanks, subtracted to remove the Raman line, light scattering and any residual fluorescence from non-peptide components. The excitation wavelength was set at 280nm for melittin or DNC-melittin, and 330nm for DNC-SP. In lipid–peptide mixtures, the changes in the emission fluorescence intensity at $\lambda = 330$nm (melittin, DNC-melittin) and $\lambda = 510$nm (DNC-SP), I^{λ}, were analyzed as a function of R_i (lipid–peptide molar ratio) and, from the fluorescence intensity increase, the fraction of bound peptide α defined by $\alpha = (I^{\lambda}–I^{\lambda}_{\text{free}})/(I^{\lambda}_{\text{bound}}–I^{\lambda}_{\text{free}})$ was estimated. The $I^{\lambda}_{\text{bound}}$ value was extrapolated from a double-reciprocal plot. All the measures were performed in triplicate.

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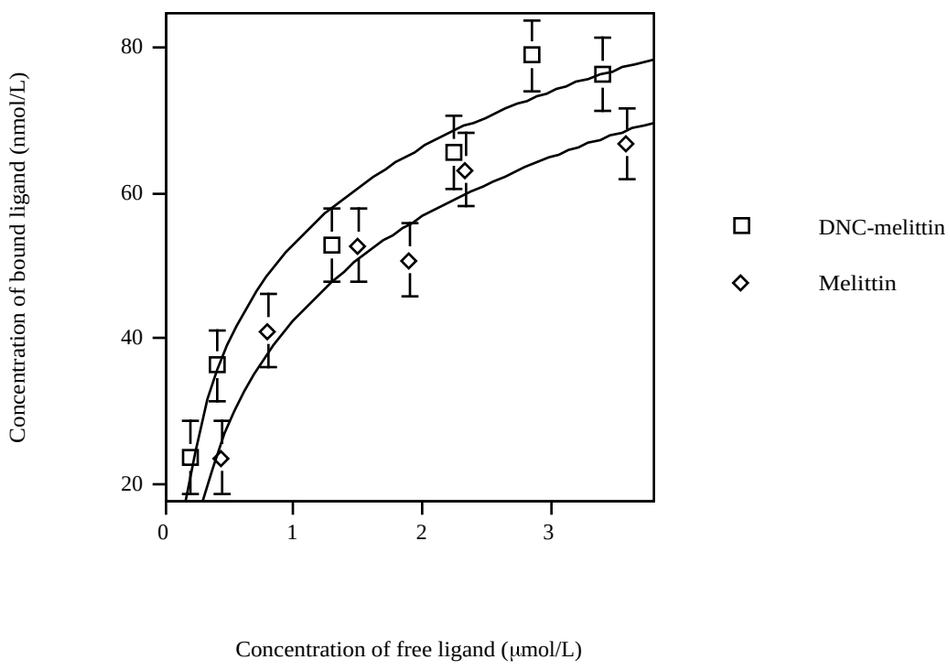
Figure captions

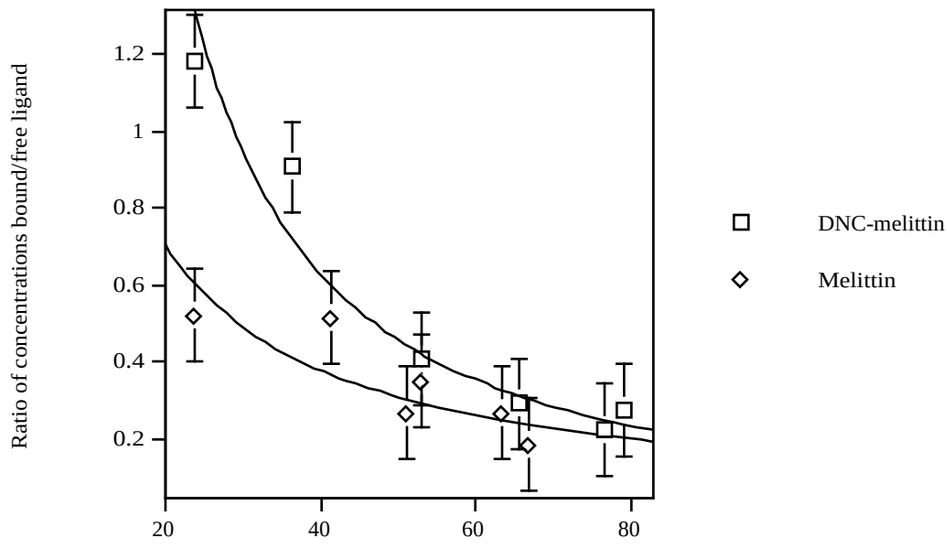
Fig. 1. Binding isotherms (*B vs. F*) of DNC-melittin– and melittin–EPC at $T = 23°C$ and $I = 0.03 mol \cdot L^{-1}$ in buffered solution containing $50 mmol \cdot L^{-1}$ Mops–NaOH, pH 7.0; 1mM EDTA.

Fig. 2. Scatchard (*B/F vs. B*) plot of the binding of DNC-melittin– and melittin–EPC.

Fig. 3. Hill plot of DNC-melittin– and melittin–EPC with $B_{max} = 82.4$ and $96.4 nmol \cdot L^{-1}$, respectively.

Fig. 4. Plot of relative theoretical $K_D^{h-h_o}$ *vs.* I ($mol \cdot L^{-1}$) for DNC-SP–EPC and mastoparan–DOPC.

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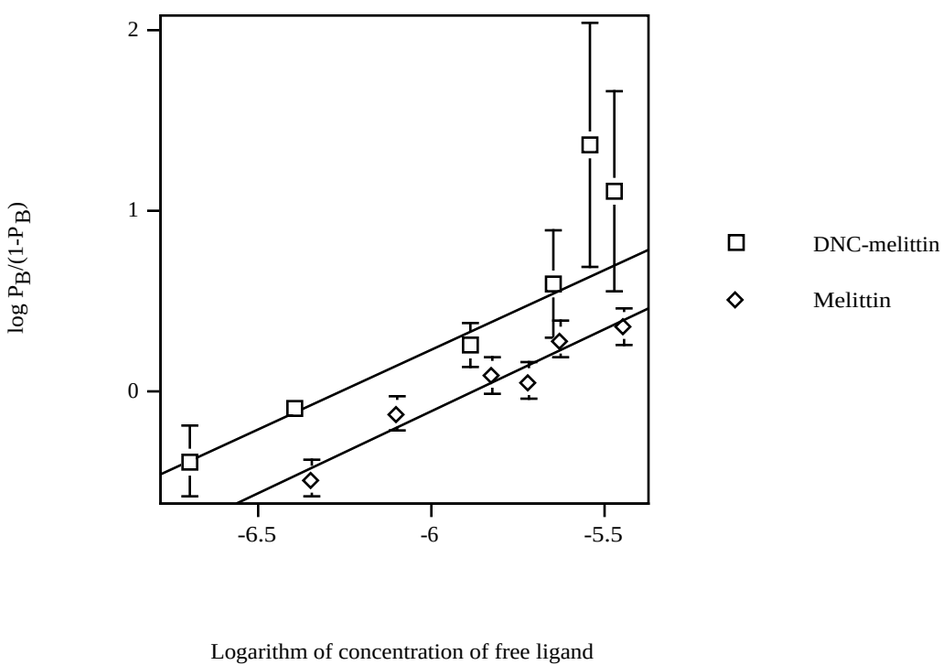
1
0.8
0.6
0.4
0.2
20
40
60
80
DNC-melittin
Melittin
Concentration of bound ligand (nmol/L)


log $P_B/(1-P_B)$
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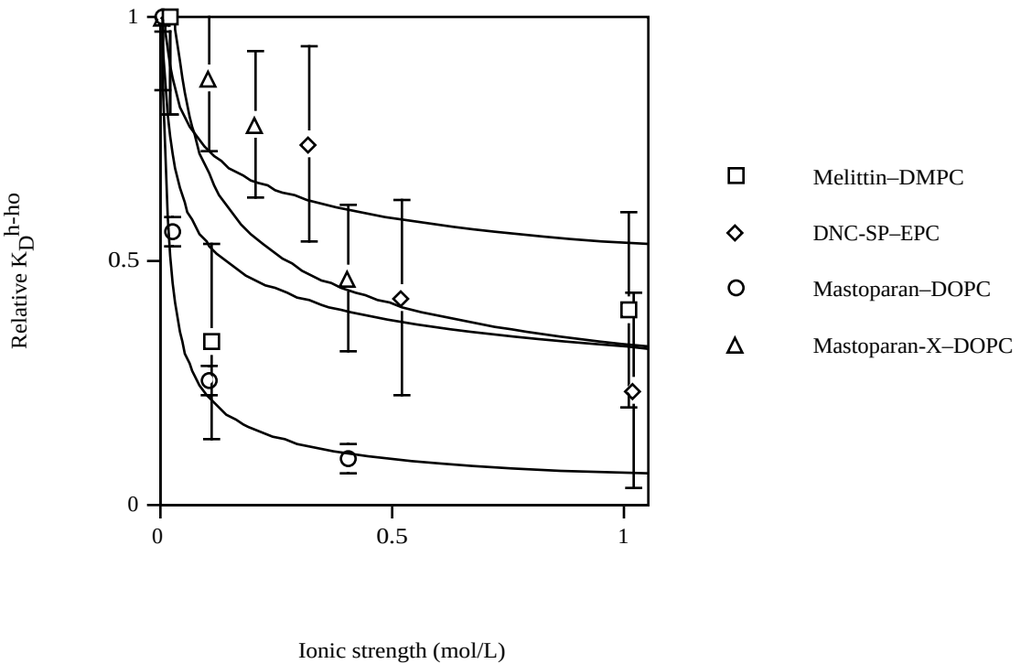
1
0
-6.5
-6
-5.5
DNC-melittin
Melittin
Logarithm of concentration of free ligand



SEE TABLES IN THE FOLLOWING PAGES

Table 1

Theoretical dissociation constant p(K_D^h) for the peptide in the binding of melittins to EPC vesicles for different Hill coefficients

Peptide	Hill coefficient, h												
	0.800	0.820	0.840	0.860	0.880	0.888	0.900	0.909	0.920	0.940	0.960	0.980	1.000
DNC-melittin	5.01	5.13	5.26	5.38	5.51	5.56	5.63	5.69	5.76	5.88	6.01	6.13	6.26
Melittin	4.70	4.81	4.93	5.05	5.16	5.21	5.28	5.33	5.40	5.52	5.63	5.75	5.87

Table 2

Actual peptide charge in solution z_p^+, molar free energies of the peptide in the aqueous $\Delta\overline{G}_p^{o,A}$ and lipid $\Delta\overline{G}_p^{o,L}$ phases, partition coefficient Γ and effective interfacial charge ν for DNC-melittin and melittin in EPC, DPPC, DMPC, DOPC, POPC and POPC/POPG vesicles, and solid-supported membranes of octanethiol and POPC[a]

Peptide–vesicle	expected z_p^+ (e.u.)	z_p^+ (e.u.)	$\Delta\overline{G}_p^{o,A}$ (kJ·mol^{-1})	$\Delta\overline{G}_p^{o,L}$ (kJ·mol^{-1})	Γ (L·mol^{-1})	ν (e.u.)
DNC-melittin–EPC[b]	6.00	1.64	-105.0	-146.7	405000	1.26
Melittin–EPC[b]	6.00	1.68	-113.4	-153.9	253000	1.26
DNC-melittin–DPPC[c]	6.00	1.57	-91.0	-134.4	209000	1.27
Melittin–DPPC[c]	6.00	1.52	-81.0	-126.0	356000	1.26
Melittin–DMPC[d]	6.00	0.00[k]	–	–	400000	0.00[k]
Melittin–DMPC[e]	6.00	0.00[k]	–	–	320000	0.00[k]
Melittin–DOPC[f]	6.00	1.80	–	–	30000	–
Melittin–POPC[g]	6.00	2.20	–	–	2100	–
Melittin–POPC/POPG (90/10 mol/mol)[h]	6.00	1.90	–	–	45600	–
Melittin–supported POPC[i]	6.00	–	–	–	–	1.50
Melittin–POPC[j]	6.00	–	–	–	9000	1.20

[a] In all calculations ε_L was 20 and it was considered that the peptide is solvated in both aqueous and lipid phases.
[b] I = 0.03mol·L^{-1}, T = 23ºC.
[c] I = 0.03mol·L^{-1}, T = 50ºC.
[d] From Ref. 45 at I = 0.1005mol·L^{-1}, T = 15ºC.
[e] From Ref. 45 at I = 0.1005mol·L^{-1}, T = 26ºC.
[f] From Ref. 10 at I = 0.11mol·L^{-1}.
[g] From Ref. 31 at I = 0.02mol·L^{-1}, T = 25ºC.
[h] From Ref. 19 at I = 0.105mol·L^{-1}, T = 25ºC.
[i] From Ref. 22 at I = 0.125–0.225mol·L^{-1}, T = 21ºC.
[j] From Ref. 42 at I = 0.11mol·L^{-1}, T = 20ºC.
[k] Assumed.

Table 3

Values for the dielectric permittivity of the aqueous medium ε_w, inverse Debye length $\kappa = 1/\lambda_D$ and b parameter at different ionic strengths I

Parameter	Ionic strength I (mol·L^{-1})				
	0.00	0.02	0.32	0.52	1.02
ε_w	78.355	77.0	73.5	70.0	64.0
κ (Å^{-1})	0.000	0.047	0.194	0.252	0.371
λ_D (Å)	∞	21.28	5.15	3.97	2.70
b (EPC and DPPC)	∞	21.770	5.528	4.443	3.317
b (DOC)	∞	25.032	6.258	4.909	3.505

Table 4

Actual peptide charge in solution z_p^+, molar free energies of the peptide in the aqueous $\Delta\overline{G}_p^{o,A}$ and lipid $\Delta\overline{G}_p^{o,L}$ phases, partition coefficient Γ and effective interfacial charge ν for melittin in DMPC, DNC-SP in EPC, and mastoparan and mastoparan-X in DOPC and POPC vesicles at different ionic strengths I[b]

Peptide–vesicle	Property	Ionic strength I (mol·L^{-1})										
		0.005	0.020	0.025	0.105	0.110	0.205	0.320	0.405	0.520	1.010	1.020
Melittin–DMPC	expected z_p^+ (e.u.)	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
	Γ (L·mol^{-1})[b]	–	60000	–	–	180000	–	–	–	–	150000	–
	relative $K_D^{h-h_o}$[c]	–	1.000	–	–	0.333	–	–	–	–	0.400	–
	ν (e.u.)[b]	–	1.90	–	–	1.90	–	–	–	–	0.30	–
DNC-SP–EPC	expected z_p^+ (e.u.)	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
	z_p^+ (e.u.)	–	1.62	–	–	–	–	1.94	–	1.90	–	1.86
	$\Delta\overline{G}_p^{o,A}$ (kJ·mol^{-1})	–	-100.9	–	–	–	–	-172.3	–	-162.6	–	-153.0
	$\Delta\overline{G}_p^{o,L}$ (kJ·mol^{-1})	–	-143.1	–	–	–	–	-205.3	–	-196.9	–	-188.7
	Γ (L·mol^{-1})	–	9742	–	–	–	–	13203	–	22923	–	41680
	relative $K_D^{h-h_o}$[c]	–	1.000	–	–	–	–	0.738	–	0.425	–	0.234
Mastoparan–DOPC	expected z_p^+ (e.u.)	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
	z_p^+ (e.u.)	2.08	–	2.06	1.96	–	–	–	1.88	–	–	–
	Γ (L·mol^{-1})	2418	–	4325	9525	–	–	–	25741	–	–	–
	Γ (L·mol^{-1})[d]	1650	–	3100	7000	–	–	–	17000	–	–	–
	relative $K_D^{h-h_o}$[c]	1.000	–	0.559	0.254	–	–	–	0.094	–	–	–
	ν (e.u.)	1.20	–	1.36	1.24	–	–	–	1.58	–	–	–
	ν (e.u.)[d]	0.95	–	1.50	1.40	–	–	–	1.75	–	–	–

Table 4 (…/…)

Table 4 (.../...)

Peptide–vesicle	Property	Ionic strength I (mol·L^{-1})										
Mastoparan-X–DOPC	expected z_p^+ (e.u.)	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
	Γ (L·mol^{-1})[d]	–	–	–	13000	–	–	–	27000	–	–	–
	Γ (L·mol^{-1})[e]	14000	–	–	16000	–	18000	–	30000	–	–	–
	relative $K_D^{h-h_o}$ [c,d]	–	–	–	1.000	–	–	–	0.481	–	–	–
	relative $K_D^{h-h_o}$ [c,e]	1.000	–	–	0.875	–	0.778	–	0.467	–	–	–
	ν (e.u.)[d]	–	–	–	1.20	–	–	–	1.20	–	–	–
	ν (e.u.)[e]	1.40	–	–	1.80	–	2.00	–	1.80	–	–	–
Mastoparan-X–POPC	expected z_p^+ (e.u.)	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
	Γ (L·mol^{-1})[e]	36000	–	–	22000	–	18000	–	19000	–	–	–
	relative $K_D^{h-h_o}$ [c,e]	1.000	–	–	1.636	–	2.000	–	1.895	–	–	–
	ν (e.u.)[e]	1.90	–	–	2.50	–	2.30	–	2.10	–	–	–

[a] In all calculations ε_L was 20 and it was considered that the peptide is solvated in both aqueous and lipid phases.
[b] From Ref. 12.
[c] K_D is the dissociation constant at each site; h is the Hill coefficient at ionic strength I; h_o is the Hill coefficient for the minimal ionic strength: I_o = 0.020mol·L^{-1} (DNC-SP–EPC) and 0.005mol·L^{-1} (mastoparan–DOPC).
[d] From Ref. 3.
[e] From Ref. 53.