ACS OMEGA

Article

Cite This: ACS Omega 2017, 2, 6863-6869

http://pubs.acs.org/journal/acsodf

Partition of Amphiphilic Molecules to Lipid Bilayers by ITC: Low-Affinity Solutes

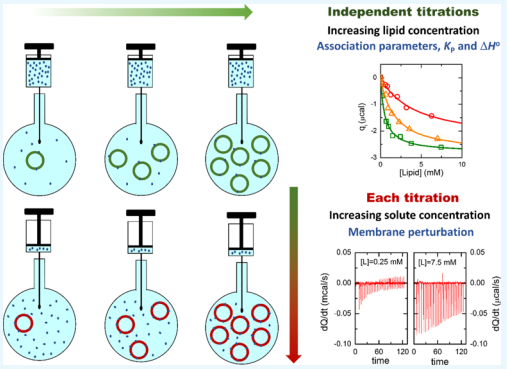
Jaime Samelo,[†] Maria Julia Mora,[‡] Gladys Ester Granero,[‡] and Maria João Moreno*[,†]

[†]Chemistry Department FCTUC, CQC-Biological Chemistry Group, Largo D. Dinis, Rua Larga, 3004-535 Coimbra, Portugal

[‡]Unidad de Investigación y Desarrollo en Tecnología Farmacéutica (UNITEFA), CONICET and Departamento de Ciencias Farmacéuticas, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina

Supporting Information

ABSTRACT: A protocol is developed to allow the accurate characterization of partition to lipid bilayers for solutes with low affinity, using isothermal titration calorimetry. The methodology proposed is suitable for studies using complex membranes, such as intact biomembranes or whole cells. In the method developed, the association is characterized at increasing solute concentrations. This allows the characterization of solute partition into unperturbed membranes, as well as effects induced by high solute concentrations. Most druglike molecules are expected to interact with low-to-moderate affinity with relevant cell membranes. This is due to both the need for a relatively high aqueous solubility of the drug and the poor binding properties of the cell membranes. The methodology is applied to characterize the interaction of antibiotic Rifampicin with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and with lipid bilayers representative of bacterial membranes.



INTRODUCTION

The association of drugs and biological ligands with lipid bilayers is a key step in their permeation through biological membranes, which is determinant for their disposition and availability at the target site.[1,2] There are several methods that may be used to characterize the association of biologically active molecules with, or their permeation through, biomembranes (see ref 3 for a recent review). Among the available approaches, isothermal titration calorimetry (ITC) is particularly important, as it gives information on both the extent of association and the nature of the interactions established.[3−15] The methods available are however limited to ligands with intermediate to high partition coefficients and cannot be used for typical drugs and/or to characterize the association with very ordered lipid bilayers.[4−9] In the usual method used to obtain the association of small molecules with lipid bilayers, the lipid is added to an aqueous solution of the small molecule (solute). After injection *i*, the lipid concentration in the cell increases from that after the previous injection ($[L]^{i-1}$) to $[L]^i$, and this leads to an increase in the number of solute molecules that associate with the lipid bilayer from which heat q_i is evolved, eq 1[6,8,10]

$$q_i = \Delta H^\circ \left(n^i_{S\_L} - n^{i-1}_{S\_L}\left(1 - \frac{V_i}{V_{cell}}\right)\right) + q_{dil} \quad (1)$$

where $n^{i-1}_{S\_L}$ and $n^i_{S\_L}$ are the numbers of moles of solute associated with the lipid bilayer before and after injection *i*, respectively; ΔH° is the molar enthalpy variation associated with the transfer of solute from the aqueous media to the lipid bilayer; V_i and V_{cell} are, respectively, the volume injected in each titration step and the volume of the cell; and q_{dil} is the heat of diluting the concentrated lipid solution. For small solutes at low local concentrations, the association with the lipid bilayer is best described by a partition coefficient from which the amount of solute in the membrane after each lipid injection may be calculated, eq 2

$$n^i_{S\_L} = n_{S\_T}\frac{K_P^{obs}[L^*]^i\overline{V}_L}{1 + K_P^{obs}[L^*]^i\overline{V}_L} \quad (2)$$

where K_P^{obs} is the observed partition coefficient between the aqueous and the lipid phase (hereafter, represented simply by K_P), $[L^*]^i$ is the lipid concentration available for solute association after injection *i*, and $\overline{V}_L$ is the molar volume of the lipid when organized in lipid bilayers.[5,6] The dependence of the heat evolved on the concentration of lipid in the ITC cell is obtained by combining eqs 1 and 2

$$n^i_{S\_L} - n^{i-1}_{S\_L} = \frac{n_{S\_T}K_P\overline{V}_L([L^*]^i - [L^*]^{i-1})}{(1 + K_P[L^*]^i\overline{V}_L)(1 + K_P[L^*]^{i-1}\overline{V}_L)} \quad (3)$$

The amount of lipid added to the cell in each injection is constant throughout the titration, which leads to an approximately linear increase in the concentration of lipid in this compartment (the increase is not strictly linear due to an

Received: August 7, 2017
Accepted: September 25, 2017
Published: October 18, 2017

DOI: 10.1021/acsomega.7b01145
ACS Omega 2017, 2, 6863−6869

increase in the total volume of the solution); also, it is assumed that overflow from the cell occurs faster than equilibration of the injected solution with the content of the cell.

If the term $K_P[L^*]^i\overline{V}_L$ in eq 3 is much smaller than 1, the increment in the number of moles of solute associated with the lipid bilayer after each injection is constant throughout the titration. In this situation, the heat evolved in each injection does not vary significantly as the titration proceeds and is proportional to the enthalpy variation times the partition coefficient, allowing the characterization of only the product of both parameters. To allow obtaining both the partition coefficient and the enthalpy variation, it is necessary to reach lipid concentrations in the cell where the product $K_P[L^*]^i\overline{V}_L$ is close to or higher than unity. In Figure 1, the predicted

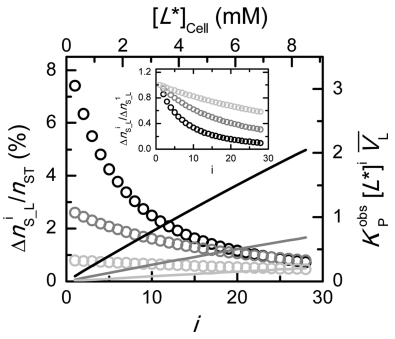


Figure 1. Dependence of the product $K_P[L^*]^i\overline{V}_L$ (lines) and of the increment per injection in the fraction of solute associated with the lipid bilayer (symbols) on the injection number (i) and the concentration of lipid in the cell, for a concentration of lipid in the syringe equal to 50 mM in a typical experiment with 10 μL injections into a 1500 μL cell and for a partition coefficient equal to 300 (open circle black, —), 100 (open circle dark gray, dark gray), or 30 (open circle light gray, light gray). The inset shows the incremental variation of the amount of solute associated with the lipid bilayer normalized by the variation observed in the first injection of lipid.

variation of $K_P[L^*]^i\overline{V}_L$ and the resulting variation in the fraction of solute associated with the lipid bilayers as the titration proceeds are represented, for a concentration of lipid in the syringe equal to 50 mM and distinct values of the partition coefficient. For K_P equal to 300, the product $K_P[L^*]^i\overline{V}_L$ reaches a value of 2 at the end of the titration and there is a clear and nonlinear variation in $n^i_{S\_L} - n^{i-1}_{S\_L}$, allowing the full characterization of the interaction of the solute with the lipid bilayer (K_P and $\Delta H°$). For a partition coefficient equal to 100, $K_P[L^*]^i\overline{V}_L$ reaches only 0.7 at the end of the titration and the variation in $n^i_{S\_L} - n^{i-1}_{S\_L}$ is less accentuated, leading to large uncertainties in the characterization of both K_P and $\Delta H°$ by this method. Lower affinities, exemplified in Figure 1 by K_P = 30, lead to very small variations in $n^i_{S\_L} - n^{i-1}_{S\_L}$, which are due to the nonproportional increase in the lipid concentration in the cell and the dilution of the solute due to the increase in the total volume of the solution that accompanies the addition of lipid aliquots. In this case, it is not possible to obtain K_P or $\Delta H°$, only their product.

The concentration of lipid in the syringe considered in Figure 1 (50 mM) is close to the upper limit that may be achieved with 100 nm large unilamellar vesicles. In fact, for solutes with intermediate polarity, the rate of translocation through the lipid bilayer is expected to be low[4,5] and only the lipid in the outer leaflet of the liposomes is accessible to the solute. Therefore, to achieve a concentration of 50 mM lipid accessible to the solute ([L*]), it is necessary to have at least 100 mM total lipid in the syringe. In spite of this high lipid concentration in the syringe, the maximum concentration in the cell is only 8.5 mM, which makes this method only adequate to characterize the interaction for solutes with intermediate to high affinities for the membrane.

Higher lipid concentrations in the cell could be obtained if the lipid is placed in the cell and titrated with the ligand. This is the usual methodology followed to characterize binding to proteins, the heat evolved being proportional to the fraction of binding sites available in the protein.[14] However, this approach cannot be used to characterize the association of solutes with lipid bilayers due to the inexistence of well-defined binding sites in the bilayers. Variations in the heat evolved as the titration proceeds reflect changes in the affinity of the solute to the lipid bilayer due to perturbation of the membrane by the solute and not to the decrease in empty binding sites.

In this work, we develop a method that uses the advantages of both approaches (lipid in the cell or in the syringe) to allow the characterization of the association parameters for the case of solutes with low affinity for the bilayers. Most drugs are expected to partition to lipid bilayers with a moderate to low affinity, as they must be relatively polar to guarantee a significant solubility in the aqueous media. Also, cell plasma membranes are usually very cohesive, which leads to even lower affinities for druglike solutes.[3,4,16−20] The apical membrane of endothelial and epithelial cells is enriched in cholesterol and sphingomyelin,[21] and bacterial membranes have large amounts of phosphatidylethanolamines (PEs) and phosphatidylglycerol (PG).[22,23] In the method proposed in this work, the lipid is placed in the cell and is titrated with the ligand, which is in the syringe. Several independent titrations with increasing lipid concentrations are performed to allow obtaining the partition coefficient and the enthalpy variation from eqs 1 and 2. For each lipid concentration used, several solute injections are performed until all solution in the syringe is titrated into the lipid in the cell. This allows characterizing the effect of the solute concentration on its interaction with the membranes. In this work, the procedure required for the analysis of the results is developed and made available to the scientific community.

The method is applied to the characterization of the interaction of antibiotic Rifampicin (Rif) with lipid bilayers prepared from pure 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and from a mixture 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE)/POPC/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) at a molar ratio of 5:3:2, which model, respectively, intracellular membranes of eukaryotic cells and bacterial membranes.[21−23] In aqueous solution at neutral pH values, Rifampicin has a negatively charged phenolic group (pK_a = 1.7) and a positively charged piperazine nitrogen (pK_a = 7.9),[24] presenting a global charge equal to −0.24 at the pH used in this work (7.4). Given the size, polarity, and charge of Rifampicin, a slow rate of translocation through the hydrophobic core of the lipid bilayer would be expected. However, the rate of permeation of Rifampicin through POPC membranes has been characterized using the pH variation approach and permeation is reported to occur in less than 1 s.[25,26] Accordingly, all the lipid was considered accessible to the ligand ([L*] = [L]).

RESULTS AND DISCUSSION

The heat evolved due to titration of Rifampicin (RIF) with POPC and with the ternary mixture POPE/POPC/POPG

DOI: 10.1021/acsomega.7b01145
ACS Omega 2017, 2, 6863−6869

(5:3:2) following the usual protocol (addition of lipid to the solute) is shown in Figure 2.

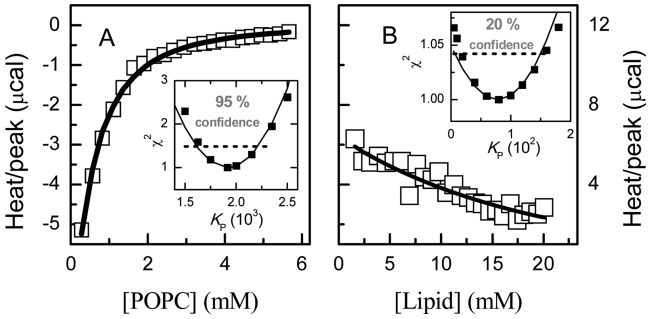


Figure 2. Titration of Rifampicin following the usual protocol, with lipid added to the solute. Plot (A) shows the heat evolved due to the addition of 10 μL aliquots of POPC, 20 mM, into a buffer solution containing RIF at 10 μM, whereas in plot (B), aliquots of the ternary mixture POPE/POPC/POPG (5:3:2) at 30 mM are added to 10 μM RIF. The lines are the best fit of eqs 1 and 2, with parameters $K_P = 1.9 \times 10^3$ and 8×10^1 for plots (A) and (B), respectively. The insets show the dependence of the quality of the best fit on the value of the partition coefficient to highlight the large uncertainty associated with this parameter for the data in plot (B), the black solid lines are the best fit of a parabola, and the dashed lines correspond to the confidence intervals at the percentage indicated (95% for plot (A) and 20% for plot (B)).

RIF associates efficiently with the POPC membranes with a high partition coefficient ($K_P = 1.9 \times 10^3$) and a negative enthalpy variation ($\Delta H° = -10.5$ kJ/mol). On the other hand, the affinity of RIF toward the ternary lipid mixture is low ($K_P \cong 8 \times 10^1$) and shows a positive enthalpy variation. In this case, the heat evolved changes almost linearly with the lipid concentration, which prevents a rigorous characterization of the association of RIF with this membrane. The values obtained for the partition coefficient compare well with previously published data using derivative spectrophotometry, where K_P was equal to 1.2×10^3 and 1.2×10^2 for dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol bilayers, respectively.[27] Note that the definitions of K_P in the present work and in the cited reference are different; the values shown in the text have been converted for the definition used in this work (eq 2).

The insets in Figure 2 show the dependence of the quality of the best fit (χ^2) on the value of K_P.[28] The association of RIF with the POPC membrane is well-characterized, $1.6 \times 10^3 < K_P < 2.2 \times 10^3$ with 95% confidence. However, for the ternary mixture, there is only 20% confidence that the value of K_P is included in the interval $[7, 1.5 \times 10^2]$, the upper limit going up to 2.9×10^2 for 95% confidence. Given the large uncertainty associated with the parameters for the partition of RIF toward the POPE/POPC/POPG (5:3:2) membrane, we have performed new titrations with the addition of RIF to lipid solutions in the cell, allowing the use of higher lipid concentrations. The results obtained are shown in Figures 3 and 4.

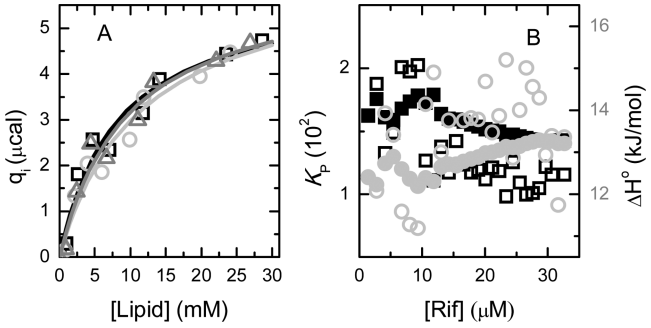


Figure 4. Plot (A): heat evolved in the 1st (□), 10th (dark gray), and 28th (light gray) addition of RIF to liposomes prepared from the ternary mixture POPE/POPC/POPG (5:3:2) as a function of the lipid concentration in the cell; the lines are the best fit of eqs 1 and 2. The dependence of K_P (■, □) and $\Delta H°$ (symbols in light gray) on the concentration of RIF in the cell (extent of titration) is shown in plot (B) considering the heat evolved due to each RIF addition (open symbols) or the cumulative heat from the first injection to that being considered (filled symbols).

The heat evolved when RIF is added to the buffer solution is due to its dilution from 200 μM (concentration in the syringe) to concentrations in the range from 1.4 to 33 μM. The observation that this heat is small and essentially unchanged throughout the titration indicates that there is no change in the aggregation state in this range of concentrations (1.4−200 μM) and was interpreted as RIF being in the monomeric state. As the concentration of lipid in the cell increases, the heat evolved due to the addition of each 10 μL of the RIF solution becomes more positive, indicating that a higher fraction of solute has partitioned into the lipid bilayer and that the partition is endothermic. These experiments have been performed at additional lipid concentrations, and the heat evolved in the first,

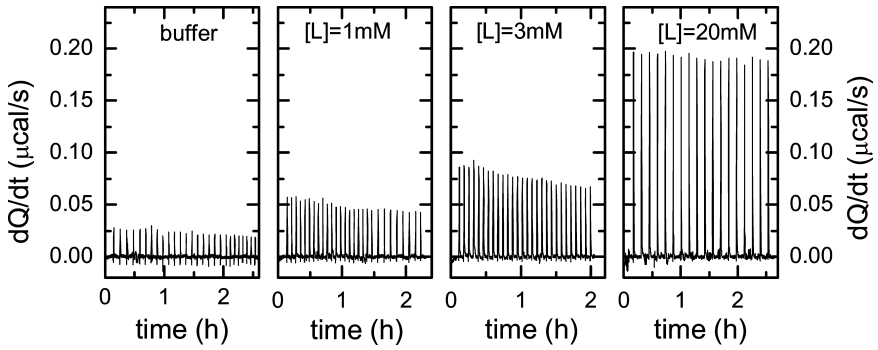


Figure 3. Titration of RIF into different concentrations of POPE/POPC/POPG (5:3:2; indicated in the plot): the left panel corresponds to the heat of dilution of RIF into the buffer solution. The concentration of RIF in the syringe is equal to 200 μM in all titrations, leading to concentrations of 1.4 and 33 μM after the first and last injections of 10 μL, respectively.

DOI: 10.1021/acsomega.7b01145
ACS Omega 2017, 2, 6863−6869

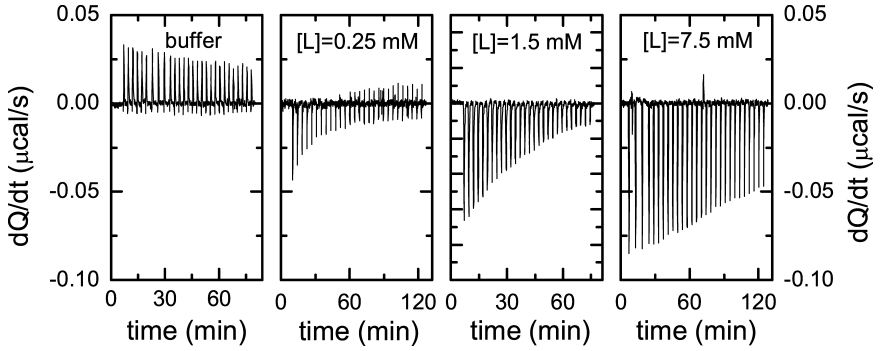


Figure 5. Titration of RIF into different concentrations of POPC (indicated in the plot): the left panel corresponds to the heat of dilution of RIF into the buffer solution. The concentration of RIF in the syringe is equal to 200 μM in all titrations, leading to concentrations of 1.4 and 33 μM after the first and last injections of 10 μL, respectively.

last, and at injection 10 is represented in Figure 4 as a function of the lipid concentration in the cell. This data was analyzed with eqs 1 and 2 to obtain the partition coefficient and the interaction enthalpy variation (Microsoft Excel spreadsheet provided in the Supporting Information). The parameters resulting from the heat evolved due to each ligand addition are represented in Figure 4B by open symbols. There is a significant variation in the values obtained for K_P and ΔH° throughout the titration, which is essentially random, with perhaps a small decrease in K_P as the concentration of ligand increases. The similarity of the heats per injection obtained as the titration proceeds is also visible in plot A. The filled symbols in plot B correspond to the parameters obtained from the cumulative heat evolved (from injection 1 to injection i). The increased sampling reduces the uncertainty associated with the parameters without a strong dependence on the concentration of ligand. The average values obtained from the heat evolved in each titration are $K_P = 1.3(\pm 0.3) \times 10^2$ and ΔH° 13 $\pm$ 1 kJ/mol, which compare well with the results obtained from the cumulative heats at the end of the titration, $K_P = 1.4 \times 10^1$ and ΔH° = 13 kJ/mol.

Under the conditions of these experiments, the local concentration of Rifampicin in the lipid bilayer is always very small (always less than 0.3%, which is the concentration attained in the last injection into the smaller lipid concentration and corresponds to a ratio of accessible lipid to bound RIF equal to 375). These small local concentrations are not expected to perturb significantly the lipid bilayer and substantiate the results obtained, association parameters independent of the concentration of ligand.

This method has also been applied to characterize the association of RIF with liposomes prepared from pure POPC, to allow a comparison between the results obtained with the usual and modified methods. The results are shown in Figures 5 and 6. In contrast to what was observed for the ternary mixture, the heat evolved decreases significantly as the titration proceeds, especially for small concentrations of lipid, Figure 5. The dependence of the heat evolved in each solute injection on the lipid concentration is shown in Figure 6A, together with the best fit with eqs 1 and 2, and the values obtained for K_P and ΔH° are shown in plot B as a function of the concentration of RIF at the end of the respective injection. A strong decrease is observed in the partition coefficient as the titration proceeds. At the smallest concentration of RIF (first injection, leading to a total concentration of RIF in the cell equal to 1.4 μM), the association with the POPC liposomes is very efficient ($K_P = 2.5 \times 10^3$). Under these conditions, the ratio of lipid to RIF in the

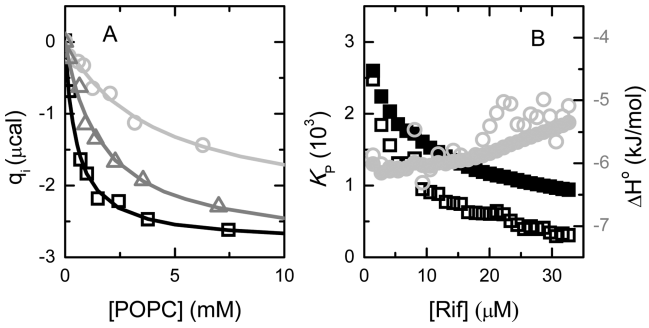


Figure 6. Plot (A): heat evolved in the 1st (□), 10th (dark gray), and 28th (light gray) addition of RIF to liposomes prepared from POPC as a function of the lipid concentration in the cell; the lines are the best fit of eqs 1 and 2. The dependence of K_P (■, □) and ΔH° (symbols in light gray) on the concentration of RIF in the cell (extent of titration) is shown in plot (B) considering the heat evolved due to each RIF addition (open symbols) or the cumulative heat from the first injection to that being considered (filled symbols).

membrane (POPC/RIF_M) is 529 at the lower lipid concentration, being even higher at the other lipid concentrations. As more RIF is added to the liposomes, the local concentration in the membrane increases, and this hinders the association of additional solute molecules, leading to a decrease in the observed association affinity. The addition of RIF to a POPC solution already containing 10 μM RIF (injection 9) leads to $K_P = 0.8 \times 10^3$, and for the highest concentration of RIF (33 μM, injection 28), $K_P = 0.3 \times 10^3$. At this high total solute concentration, POPC/RIF_M = 135 at the smallest lipid concentration and increases to 322 at the highest. The corresponding local concentrations of RIF are not very high (0.7 and 0.3%, respectively), but these results indicate a strong perturbation of the membrane, which decreases the affinity of RIF toward the membrane. In previous studies, we have characterized the effect of the concentration of other solutes (sodium dodecyl sulfate,[6] chlorpromazine,[5] and bile salts[4]) and found that below 4% these small molecules do not affect significantly their association with the POPC bilayer. These small solutes are charged at the pH value used (single negative charge for sodium dodecyl sulfate and bile salts and a single positive charge for chlorpromazine), but at the high ionic strength used, the electrostatic potential in the membrane introduced by the ligands at those small concentrations is negligible.[5] Rifampicin is a zwitterion and therefore the stronger perturbation observed cannot be due to the generation of a large electrostatic potential in the membrane. The results

DOI: 10.1021/acsomega.7b01145
ACS Omega 2017, 2, 6863−6869

obtained in this work may be due to the larger molecular weight of Rifampicin, which interacts with a higher number of lipids in the membrane.

The enthalpy variation associated with the partition of RIF from the aqueous media to the POPC bilayer does not change significantly with the concentration of RIF in the range studied, with only a small decrease (from −6 kJ/mol at concentrations below 10 μM to −5.1 kJ/mol at the highest concentration considered).

The values obtained for the partition coefficient and enthalpy variation using the new method are not in quantitative agreement with those obtained by the usual method, Figure 2. It is however important to stress that the two methods lead to nonequivalent conditions, which may affect the results obtained. The titration shown in Figure 2 was obtained with a total concentration of RIF in the cell equal to 10 μM and a total concentration of POPC in the syringe equal to 20 mM with 10 μL injections. After the first injection, the concentration of POPC in the cell is equal to 140 μM, all being considered accessible to the solute (γ = 1). The calculated local concentration of RIF in the membrane is equal to 1% (POPC/RIF_M = 95) after the first lipid addition and decreases to 0.2 (POPC/RIF_M = 484) at the end of the titration. The different parameters obtained from the two methods used may be due to a significant perturbation of the POPC bilayer at the beginning of the titration when following the most common method. It is important to stress that when using that method membrane perturbation is most significant at the beginning of the titration and will affect the results obtained throughout the titration. In contrast, when following the method proposed in this work, membrane perturbation is most likely to occur at the end of the titration, when higher solute concentrations are attained.

The affinity of Rifampicin for liposomes (representative of bacterial membranes; enriched in PE and PG,[22,23] POPE/POPC/POPG 5:3:2) is much lower than that for pure POPC membranes. This is evident in both the smaller partition coefficient and the unfavorable enthalpy variation obtained for the ternary lipid mixture. These results are partially due to repulsion between the negatively charged RIF (−0.24) and the negative POPE/POPC/POPG membrane. However, this repulsion is expected to be small due to the high ionic strength used. The lower affinity for the ternary lipid mixture may also be due to the higher lateral density and order of these membranes due to the presence of large amounts of phosphatidylethanolamine and phosphatidylglycerol.[22] These lipids interact with each other by hydrogen bonds, in contrast with phosphatidylcholines, which only possess acceptor groups and are therefore unable to interact with each other by H-bonds. In fact, the main transition temperature (from gel to fluid) of POPC is equal to −3 °C,[29] whereas that of POPE is 26 °C.[30] Although POPG lipids may interact with each other by hydrogen bonds, this is counterbalanced by electrostatic repulsion between the negatively charged head groups, and the phase transition temperature of pure POPG is 1 °C.[31] When POPG is mixed with uncharged lipids, the charge repulsion is smaller and the more packed gel phase is stabilized, this being particularly relevant for mixtures with phosphatidylethanolamine lipids.[32] The phase diagram for this ternary mixture is not known, but at the temperature used in this work (25 °C), this membrane is expected to be in the fluid phase, although more ordered and densely packed than the pure POPC membrane.

CONCLUSIONS

In this work, a methodology is proposed in which the association of solutes and lipid bilayers may be characterized from the titration of the lipid with the solute. One advantage of this approach is the possibility of achieving higher lipid concentrations, permitting the characterization of the association of solutes with low affinity toward the membrane. Another important advantage is that each lipid concentration corresponds to an independent titration, avoiding, in this way, artifacts from eventual membrane perturbation by the solute. In the usual protocol, the local concentration of solute in the membrane is higher at the beginning of the titration, and if the solute affects the properties of the membrane, this will propagate to all titration points with unpredictable effects on the heat profile obtained. In the methodology proposed in this work, the concentration of solute is progressively increased in each titration, which allows evaluating the effect of the solute concentration on its affinity for the membrane. This effect may also be characterized through the use of distinct solute concentrations following the usual protocol.[5,6] A major advantage of this method is that smaller local concentrations of solute are characterized first, in contrast with the usual protocol where the local concentration of the solute is higher at the beginning of the titrations.

An important characteristic of the method proposed in this work is that the membrane is not significantly diluted during each titration experiment. In contrast, when following the usual protocol, the membranes are at a very high concentration in the syringe and are diluted into the ITC cell. If the membrane contains molecules with intermediate affinity toward the lipid bilayer (such as the products of lipid hydrolysis), the fraction associated with the membrane varies significantly as the lipid concentration is changed. In fact, the usual protocol may only be used with very pure synthetic lipids as otherwise a high and complex (not constant throughout the titration) heat of dilution is obtained, which masks the heat evolved due to the titration of the relevant solute. In contrast, the method proposed in this work is compatible with the use of very complex systems, such as cell membranes or even whole cells.

MATERIALS AND METHODS

POPC, POPG, and POPE were from Avanti Polar Lipids, Inc., Alabaster, Alabama, and all other reagents and solvents were of highest commercially available purity from Sigma-Aldrich Química S.A., Sintra, Portugal. Rifampicin 99.95% was from Parafarm, Argentina.

Aqueous suspensions of lipids were prepared by evaporating a solution of the lipid in chloroform/methanol (87:13, v/v) by blowing dry nitrogen over the heated (blowing hot air over the external surface of the tube) solution and then leaving the residue in a vacuum desiccator for at least 8 h at 23 °C. The solvent-free residue was then hydrated with Tris buffer, 10 mM, pH = 7.4 with 0.15 M sodium chloride, 1 mM ethylenediaminetetraacetic acid, and 0.02% NaN_3 (aqueous solution). The hydrated lipid was subjected to several cycles of vortex/incubation at the specified temperature for at least 1 h to produce a suspension of multilamellar vesicles that were subjected to three cycles of freeze and thaw (with an extrusion step between each cycle) as further extruded with a minimum of 10 passes, through two stacked polycarbonate filters (Nucleopore) with a pore diameter of 0.1 μm.[33] The final

DOI: 10.1021/acsomega.7b01145
ACS Omega 2017, 2, 6863−6869

phospholipid concentration was determined using a modified version of the Bartlett phosphate assay.[34]

Titrations were performed on a VP-ITC from MicroCal (Northampton, MA) at 25 °C, with injection speed 0.5 μL/s, stirring speed 459 rpm, and reference power 10 μcal/s. The titration was performed with additions of 10 μL/step. Depending on the method followed, the lipid/solute was either in the syringe or in the cell. All solutions were previously degassed for 10 min. All titrations have been performed at 25 °C.

The obtained thermogram was integrated using data analysis software Origin 7.0 as modified by Microcal to deal with ITC experiments, and eqs 1 and 2 were fitted to the resulting differential titration curve using Microsoft Excel and Solver. The concentrations in the cell were calculated taken into account the volume that overflows the cell due to the addition of solution from the syringe, considering that overflow is faster than mixing. The predicted heat evolved in the titration step i is calculated by eq 1 to 3. The molar volume considered for the lipids was 0.756 dm^3/mol.[35] It was considered that only the lipid in the outer leaflet of the liposomes is accessible to Rifampicin during the time scale of the experiments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01145.

Excel spreadsheet with the procedure to obtain the best fit of ITC data according to the proposed methodology (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mmoreno@ci.uc.pt. Tel: 351-239-854481.

ORCID

Maria João Moreno: 0000-0003-3076-9905

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was partially supported by the Portuguese "Fundação para a Ciência e a Tecnologia" (FCT) through projects 007630 UID/QUI/00313/2013 and PT2020_PTDC_DTP-FTO_2784_2014, cofunded by COMPETE2020-UE. M.J.M. thanks the ERASMUS MUNDUS Action 2—PRECIoSA: Programe of Exchange & Cooperation for International Studies between Europe and South America Grant Agreement No 2012-246/001-001-EMA2 for the postdoctoral fellowship that allowed the development of this work.

■ REFERENCES

(1) Smith, D.; Artursson, P.; Avdeef, A.; Di, L.; Ecker, G. F.; Faller, B.; Houston, J. B.; Kansy, M.; Kerns, E. H.; Kramer, S. D.; Lennernas, H.; van de Waterbeemd, H.; Sugano, K.; Testa, B. Passive Lipoidal Diffusion and Carrier-Mediated Cell Uptake Are Both Important Mechanisms of Membrane Permeation in Drug Disposition. *Mol. Pharmaceutics* **2014**, *11*, 1727−1738.

(2) Testa, B.; Waterbeemb, G. F.; Guy, R. *Pharmacokinetic Optimization in Drug Research*; Wiley-VCH: Weinheim, 2001.

(3) Deleu, M.; Crowet, J. M.; Nasir, M. N.; Lins, L. Complementary biophysical tools to investigate lipid specificity in the interaction between bioactive molecules and the plasma membrane: A review. *Biochim. Biophys. Acta, Biomembr.* **2014**, *1838*, 3171−3190.

(4) Coreta-Gomes, F. M.; Martins, P. A. T.; Velazquez-Campoy, A.; Vaz, W. L. C.; Geraldes, C. F. G.; Moreno, M. J. Interaction of Bile Salts with Model Membranes Mimicking the Gastrointestinal Epithelium: A Study by Isothermal Titration Calorimetry. *Langmuir* **2015**, *31*, 9097−9104.

(5) Martins, P. T.; Velazquez-Campoy, A.; Vaz, W. L. C.; Cardoso, R. M. S.; Valerio, J.; Moreno, M. J. Kinetics and Thermodynamics of Chlorpromazine Interaction with Lipid Bilayers: Effect of Charge and Cholesterol. *J. Am. Chem. Soc.* **2012**, *134*, 4184−4195.

(6) Moreno, M. J.; Bastos, M.; Velazquez-Campoy, A. Partition of amphiphilic molecules to lipid bilayers by isothermal titration calorimetry. *Anal. Biochem.* **2010**, *399*, 44−47.

(7) Heerklotz, H. Interactions of surfactants with lipid membranes. *Q. Rev. Biophys.* **2008**, *41*, 205−264.

(8) Tsamaloukas, A. D.; Keller, S.; Heerklotz, H. Uptake and Release Protocol for Assessing Membrane Binding and Permeation by way of Isothermal Titration Calorimetry. *Nat. Protoc.* **2007**, *2*, 695−704.

(9) Filipe, H. A. L.; Coreta-Gomes, F. M.; Velazquez-Campoy, A.; Almeida, A. R.; Peixoto, A. F.; Pereira, M. M.; Vaz, W. L. C.; Moreno, M. J. Synthesis and Characterization of a Lipidic Alpha Amino Acid: Solubility and Interaction with Serum Albumin and Lipid Bilayers. *J. Phys. Chem. B* **2013**, *117*, 3439−3448.

(10) Velazquez-Campoy, A.; Freire, E. Isothermal Titration Calorimetry to Determine Association Constants for High-Affinity Ligands. *Nat. Protoc.* **2006**, *1*, 186−191.

(11) Claveria-Gimeno, R.; Vega, S.; Abian, O.; Velazquez-Campoy, A. A look at ligand binding thermodynamics in drug discovery. *Expert Opin. Drug Discovery* **2017**, *12*, 363−377.

(12) Simões, C. J. V.; Almeida, Z. L.; Costa, D.; Jesus, C. S. H.; Cardoso, A. L.; Almeida, M. R.; Saraiva, M. J.; Pinho e Melo, T. M. V. D.; Brito, R. M. M. A novel bis-furan scaffold for transthyretin stabilization and amyloid inhibition. *Eur. J. Med. Chem.* **2016**, *121*, 823−840.

(13) Bastos, M.; Bai, G.; Gomes, P.; Andreu, D.; Goormaghtigh, E.; Prieto, M. Energetics and partition of two cecropin-melittin hybrid peptides to model membranes of different composition. *Biophys. J.* **2008**, *94*, 2128−2141.

(14) Velazquez Campoy, A.; Freire, E. ITC in the Post-Genomic era... ? Priceless. *Biophys. Chem.* **2005**, *115*, 115−124.

(15) Domingues, M. M.; Bianconi, M. L.; Barbosa, L. R. S.; Santiago, P. S.; Tabak, M.; Castanho, M.; Itri, R.; Santos, N. C. rBPI(21) interacts with negative membranes endothermically promoting the formation of rigid multilamellar structures. *Biochim. Biophys. Acta, Biomembr.* **2013**, *1828*, 2419−2427.

(16) Estronca, L. M. B. B.; Moreno, M. J.; Vaz, W. L. C. Kinetics and thermodynamics of the association of dehydroergosterol with lipid bilayer membranes. *Biophys. J.* **2007**, *93*, 4244−4253.

(17) Estronca, L. M. B. B.; Moreno, M. J.; Laranjinha, J. A. N.; Almeida, L. M.; Vaz, W. L. C. Kinetics and thermodynamics of lipid amphiphile exchange between lipoproteins and albumin in serum. *Biophys. J.* **2005**, *88*, 557−565.

(18) Abreu, M. S. C.; Moreno, M. J.; Vaz, W. L. C. Kinetics and Thermodynamics of Association of a Phospholipid Derivative with Lipid Bilayers in Liquid-Disordered and Liquid-Ordered Phases. *Biophys. J.* **2004**, *87*, 353−365.

(19) Slater, S. J.; Ho, C.; Taddeo, F. J.; Kelly, M. B.; Stubbs, C. D. Contribution of Hydrogen-Bonding to Lipid−Lipid Interactions in Membranes and the Role of Lipid Order - Effects of Cholesterol, Increased Phospholipid Unsaturation, and Ethanol. *Biochemistry* **1993**, *32*, 3714−3721.

(20) Boggs, J. M. Lipid Intermolecular Hydrogen-Bonding - Influence on Structural Organization and Membrane-Function. *Biochim. Biophys. Acta, Rev. Biomembr.* **1987**, *906*, 353−404.

(21) Gerl, M. J.; Sampaio, J. L.; Urban, S.; Kalvodova, L.; Verbavatz, J. M.; Binnington, B.; Lindemann, D.; Lingwood, C. A.; Shevchenko, A.; Schroeder, C.; Simons, K. Quantitative analysis of the lipidomes of

DOI: 10.1021/acsomega.7b01145
ACS Omega 2017, 2, 6863−6869

the influenza virus envelope and MDCK cell apical membrane. *J. Cell Biol.* **2012**, *196*, 213−221.
(22) Oursel, D.; Loutelier-Bourhis, C.; Orange, N.; Chevalier, S.; Norris, V.; Lange, C. M. Lipid composition of membranes of Escherichia coli by liquid chromatography/tandem mass spectrometry using negative electrospray ionization. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 1721−1728.
(23) Cronan, J. E. Bacterial membrane lipids: Where do we stand? *Annu. Rev. Microbiol.* **2003**, *57*, 203−224.
(24) Newton, D. W.; Kluza, R. B. pKa Values of Medicinal Compounds in Pharmacy Practice. *Drug Intell. Clin. Pharm.* **1978**, *12*, 546−554.
(25) Hermann, K. F.; Neuhaus, C. S.; Micallef, V.; Wagner, B.; Hatibovic, M.; Aschmann, H. E.; Paech, F.; Alvarez-Sanchez, R.; Kramer, S. D.; Belli, S. Kinetics of lipid bilayer permeation of a series of ionisable drugs and their correlation with human transporter-independent intestinal permeability. *Eur. J. Pharm. Sci.* **2017**, *104*, 150−161.
(26) Krämer, S. D.; Aschmann, H. E.; Hatibovic, M.; Hermann, K. F.; Neuhaus, C. S.; Brunner, C.; Belli, S. When barriers ignore the "rule-of-five". *Adv. Drug Delivery Rev.* **2016**, *101*, 62−74.
(27) Rodrigues, C.; Gameiro, P.; Reis, S.; Lima, J.; de Castro, B. Derivative spectrophotometry as a tool for the determination of drug partition coefficients in water/dimyristoyl-L-alpha-phosphatidylglycerol (DMPG) liposomes. *Biophys. Chem.* **2001**, *94*, 97−106.
(28) Kemmer, G.; Keller, S. Nonlinear least-squares data fitting in Excel spreadsheets. *Nat. Protoc.* **2010**, *5*, 267−281.
(29) Curatolo, W.; Sears, B.; Neuringer, L. J. A Calorimetry and Deuterium NMR-Study of Mixed Model Membranes of 1-Palmitoyl-2-Oleylphosphatidylcholine and Saturated Phosphatidylcholines. *Biochim. Biophys. Acta, Biomembr.* **1985**, *817*, 261−270.
(30) Wang, Z.-q.; Lin, H.-n.; Li, S. S.; Huang, C.-h. Calorimetric Studies and Molecular Mechanics Simulations of Monounsaturated Phosphatidylethanolamine Bilayers. *J. Biol. Chem.* **1994**, *269*, 23491−23499.
(31) Wiedmann, T.; Salmon, A.; Wong, V. Phase-Behavior of Mixtures of DPPC and POPG. *Biochim. Biophys. Acta, Lipids Lipid Metab.* **1993**, *1167*, 114−120.
(32) Garidel, P.; Blume, A. Miscibility of phosphatidylethanolamine-phosphatidylglycerol mixtures as a function of pH and acyl chain length. *Eur. Biophys. J.* **2000**, *28*, 629−638.
(33) Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. Production of Large Unilamellar Vesicles by a Rapid Extrusion Procedure - Characterization of Size Distribution, Trapped Volume and Ability to Maintain a Membrane-Potential. *Biochim. Biophys. Acta, Biomembr.* **1985**, *812*, 55−65.
(34) Bartlett, G. R. Phosphorus Assay in Column Chromatography. *J. Biol. Chem.* **1959**, *234*, 466−468.
(35) Wiener, M. C.; White, S. H. Structure of a Fluid Dioleoylphosphatidylcholine Bilayer Determined by Joint Refinement of X-ray and Neutron-Diffraction Data. 2. Distribution and Packing of Terminal Methyl-Groups. *Biophys. J.* **1992**, *61*, 428−433.

DOI: 10.1021/acsomega.7b01145
ACS Omega 2017, 2, 6863−6869