



Effect of Acyl Chain Length on the Rate of Phospholipid Flip-Flop and Intermembrane Transfer

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Abstract

The rate at which phospholipids equilibrate between different membranes and between the non-polar environments in biological fluids is of high importance in the understanding of biomembrane diversity, as well as in the development of liposomes for drug delivery. In this work, we characterize the rate of insertion into and desorption from POPC bilayers for a homologous series of amphiphiles with the fluorescent NBD group attached to phosphoethanolamines of different acyl chain lengths, NBD-diC_n-PE with $n = 6, 8, 10,$ and 12 . The rate of translocation between bilayer leaflets was also characterized, providing all the relevant parameters for their interaction with lipid bilayers. The results are complemented with data for NBD-diC₁₄-PE obtained from literature (Abreu et al. *Biophys J* 87:353–365, 2004; Moreno et al. *Biophys J* 91:873–881, 2006). The rate of translocation between the POPC leaflets is not dependent on the length of the acyl chains, while this affects strongly the rate of desorption from the bilayer. Insertion in the POPC bilayer is not diffusion controlled showing a significant dependence on the acyl chain length and on temperature. The results obtained are compared with those previously reported for NBD-LysoC₁₄-PE (Sampaio et al. *Biophys J* 88:4064–4071, 2005), and with the homologous series of single chain amphiphiles NBD-C_n (Cardoso et al. *J Phys Chem B* 114:16337–16346, 2010; *J Phys Chem B* 115:10098–10108, 2011). This allows the establishment of important relations between the rate constants for interaction with the lipid bilayers and the structural properties of the amphiphiles, namely the total surface and the cross-section of their non-polar region.

Keywords Model membranes · Insertion · Desorption · Translocation · Exchange · Stability

Introduction

Biological membranes are mostly formed by lipids and proteins, with small amounts of saccharides covalently attached to them. The lipids are organized in a bilayer, forming a continuous media where the proteins are embedded. The lipid composition of biological membranes depends strongly on the cell type and environment (Cronan 2003; Gerl et al. 2012). Even within a given cell, a distinct lipid composition

is encountered among the various membrane pools (plasma and organelles) and between the two leaflets of the lipid bilayer. This heterogeneity in the lipid composition is first established at the membrane genesis (depending on the location of the enzymes involved in the biosynthesis of the distinct lipids) and is maintained at the expense of ATP due to the action of translocases and transporters (Devaux 1992; van Meer 2011; van Meer et al. 2008). The action of those active transporters is counterbalanced by the basal rate of lipid exchange between the distinct membrane pools due to passive processes that drive the system towards equilibrium. To understand the role and mechanism of the active transporters, it is very important to know the basal rate, due to passive processes.

In addition to its importance in the understanding of cell membrane diversity, the rate of lipid exchange between membrane pools is also of high relevance in the design of lipid-based vehicles for drug delivery (Bozzuto and Molinari 2015; Kohli et al. 2014; Kraft et al. 2014; Pattni et al. 2015)

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as this has a direct impact on their stability in biological fluids.

The exchange of amphiphilic molecules between membrane pools involves de desorption from the donor membrane, diffusion through the aqueous media, insertion in the outer leaflet of the acceptor membrane, and translocation into the inner leaflet. Each step in the overall processes depends on distinct properties of the amphiphile with the size and charge of the polar region influencing mostly the rate of translocation (Colleau et al. 1991; Homan and Pownall 1988), while the rate of desorption is influenced to a larger extent by the length of the non-polar region (Filipe et al. 2014; Pownall et al. 1991; Silvius and Leventis 1993). Due to the presence of the negative charge in the phosphate group of phospholipids, the rate of translocation is usually the rate limiting step in the overall process. Some effect from the non-polar region is however expected because: (i) it affects the size of the amphiphile (a decrease in the rate of diffusion with the increase in the solute size is predicted, namely by the partition–diffusion model for permeation through lipid bilayers); (ii) it may influence the equilibrium location of the polar region in the bilayer; as well as (iii) the local structure and dynamics of the bilayer itself. In a previous work from this group, the effect of having one or two acyl chains attached to the glycerol moiety has been characterized (Moreno et al. 2006). We have observed that at 35 °C, the translocation of the fluorescent amphiphile with two acyl chains (NBD-DMPE) was significantly faster than that of the single chain amphiphile (NBD-LysoMPE). This is in clear contradiction to the partition–diffusion model and points towards a significant effect from the interaction between the amphiphile and the host-bilayer. The bilayer location of several fluorescent amphiphiles containing the NBD group has been characterized by molecular dynamics (MD) simulations and NMR, and it has been found that the position of the NBD group is only slightly affected by the size and shape of the non-polar group (Amaro et al. 2016; Filipe et al. 2011, 2015a, b; Huster et al. 2001, 2003; Loura and Ramalho 2007). Therefore, the distinct rates of translocation encountered should reflect interaction with and local perturbations of the lipid bilayer, in addition to the properties of the polar groups. In this work, we explore the effect of the acyl chain length on the rate of translocation of two acyl chain phospholipids, diC_nPE-NBD.

A strong dependence of the rate of desorption from the bilayer is expected for the homologous series of amphiphiles studied (Cardoso et al. 2011; Ho et al. 2002; Massey et al. 1997; Nichols 1985; Pool and Thompson 1998; Pownall et al. 1991; Sapay et al. 2009; Silvius and Leventis 1993; Simard et al. 2008; Thomas et al. 2002; Zhang et al. 1996). Very little information is available in the literature regarding the rate of insertion of amphiphiles into the bilayer (Cardoso et al. 2011; Cupp et al. 2004; Kleinfeld et al. 1997; Nichols

1985; Pokorny et al. 2000, 2001; Simard et al. 2008). Also, this parameter has not been characterized for typical phospholipids. Nichols et al. evaluated the rate of insertion of phosphatidylcholines labeled with the fluorescent group NBD in one of the acyl chains (NBD-PC), but those amphiphiles behave as lyso-phospholipids due to the preferential location of the polar NBD group in the bilayer/water interface (Loura and Ramalho 2007) and strongly perturb the lipid bilayer (Loura et al. 2008).

The characterization of the rate of all three steps performed in this work (insertion, desorption and translocation) allows the complete characterization of the rate of amphiphile exchange between lipid bilayers. This information is important to understand and predict the rate of passive exchange of phospholipids between distinct biological membranes, as well as the stability of drug delivery vehicles based on lipid bilayers. The amphiphiles characterized in this work (NBD-diC_nPE) behave very similarly to diC_nPC, leading to very small perturbation in PC bilayers (Filipe et al. 2015b) and to rate constants for desorption and translocation similar to those of the corresponding PC (Abreu et al. 2004; Moreno et al. 2006; Wimley and Thompson 1990, 1991). The homologous series characterized ($n=6-14$) leads to the establishment of the parameter dependence on the length of the acyl chain, from which it is possible to estimate the parameters for the biologically and pharmacologically relevant phospholipids whose characterization is not accessible experimentally.

Materials and Methods

Bovine serum albumin (BSA) essentially free of fatty acids was purchased from Applichem (Darmstadt, Germany). The fluorescent phospholipids derivatives NBD-diC_nPE ($n=6, 8, 10$ and 12) were synthesized by addition of NBD-Cl (Fluka) to the required phosphatidylethanolamine [diC_nPE, Avanti Polar Lipids Inc. (Alabaster, Alabama, USA)] in chloroform at 10% molar excess of NBD-Cl in the presence of a slight excess of sodium carbonate. The reaction was allowed to stand for about 12 h at room temperature in the dark, with stirring. The product was isolated and purified by preparative thin layer chromatography on Silica Gel 60 plates (Merck, Portugal) using chloroform/methanol/acetic acid (80/20/1, v/v) as eluent. The phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). All other chemicals were of analytical grade or higher purity from Sigma-Aldrich Química S.A. (Sintra, Portugal).

Absorption spectra were recorded on a Unicam UV530 UV/Vis spectrophotometer and fluorescence measurements were performed on a Cary Eclipse Spectrophotometer (Varian, Victoria, Australia) equipped with a multi-cell holder

accessory and temperature control. Fast kinetics were characterized on a stopped-flow fluorimeter (Hi-Tech model SF-61, Salisbury, UK).

Large unilamellar vesicles (LUVs) were prepared using a water-jacketed extruder (Lipex Biomembranes, Vancouver, BC, Canada). A film of the desired lipid (POPC with or without NBD-diC_nPE) was prepared by evaporation of a pre-equilibrated solution in chloroform/methanol azeotropic mixture (83/17, v/v) as described before (Moreno et al. 2006), the solvent-free residue was hydrated with the aqueous buffer (HEPES buffer 10 mM, pH 7.4, with 150 mM using sodium chloride and 0.02% NaN₃), and the LUVs were prepared with a minimum of 10 steps of extrusion through two stacked polycarbonate filters (Nucleopore) with a pore diameter of 0.1 μm.

The lipid concentration in the final LUV preparation was determined by a modified version of the Bartlett phosphate assay. Concentrations of NBD-diC_nPE and BSA were determined by absorption spectrophotometry, assuming molar extinction coefficients of $2.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 463 nm and $4.39 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 278 nm, respectively.

The method used to characterize the rate of NBD-diC_nPE translocation across the POPC bilayer was based on the reaction of dithionite with the NBD group and consequent loss of fluorescence as reported previously (Moreno et al. 2006). In brief, 10 mM dithionite was added to POPC LUVs containing NBD-diC_nPE at a molar ratio above 1–5000 lipid molecules, and allowed to react for 2 min at room temperature. The reaction was stopped by passing the solution through a Sephadex G75 column, the asymmetrically labeled LUVs (with NBD-diC_nPE only in the inner leaflet) free from dithionite being collected in the column-free volume. The LUVs were incubated at the desired temperature, and at given time intervals small aliquots were collected and analyzed for the fraction of NBD-diC_nPE in the outer leaflet (accessible for reaction with dithionite added to the external aqueous media). The rate constant for translocation (k_f) was obtained from the best fit of Eq. (1) to the concentration of NBD-diC_nPE in the outer leaflet.

$$[\text{NBD}_o]_{(t)} = [\text{NBD}_o]_{(\infty)} - \left\{ [\text{NBD}_o]_{(\infty)} - [\text{NBD}_o]_{(0)} \right\} \exp(-2k_f t), \quad (1)$$

where $[\text{NBD}_o]_{(0)}$, and $[\text{NBD}_o]_{(\infty)}$, are the concentration of NBD-diC_nPE in the outer leaflet of the LUVs at the beginning of the experiment and after equilibration between both leaflets, respectively; $[\text{NBD}_o]_{(t)}$ being its concentration at time t after generation of the asymmetric distribution in the bilayer.

The solubility of NBD-diC₈PE monomeric form in the aqueous buffer was obtained through deviations from linearity in the amphiphile fluorescence as a function of its total concentration as described previously (Cardoso et al. 2010).

The critical aggregation concentration of the other amphiphiles in the homologous series was calculated from the tendency observed with the length of the acyl chain considering the data in literature for diC₁₀ (Martins et al. 2008) and diC₁₄ (Abreu et al. 2004). In the experiments of transfer from the aqueous phase (with or without serum albumin) and LUVs, the amphiphile total concentration was chosen to guarantee that the concentration in the aqueous phase was well below its critical aggregation constant (CAC). The concentrations used were typically close to 20 nM.

The relatively high solubility of NBD-diC₆PE and NBD-diC₈PE in the aqueous phase allows the direct characterization of the kinetics and equilibrium of its association with POPC LUVs. In equilibrium experiments, a small volume of NBD-diC_nPE in methanol (0.5% v/v methanol in the final solution) was added to LUVs solutions of distinct lipid concentrations under gentle stirring and the solutions were allowed to equilibrate for 2–4 h. The partition coefficient between the aqueous phase and the lipid bilayer was obtained from the increase in NBD fluorescence quantum yield upon association with the lipid bilayer, considering a simple partition, Eq. (2), a POPC molar volume (\bar{V}_L) of $0.76 \text{ dm}^3 \text{ mol}^{-1}$ (Greenwood et al. 2006), and that only the lipid in the outer leaflet is accessible to NBD-diC_nPE during the incubation time ($[L]^* = [\text{POPC}]/2$).

$$\text{NBD}_W \xrightleftharpoons{K_p} \text{NBD}_L$$

$$I_F = [\text{NBD}]_T \frac{I_F^{\text{NBD}_W} + I_F^{\text{NBD}_L} K_p \bar{V}_L [L]^*}{1 + K_p \bar{V}_L [L]^*} \quad (2)$$

where $I_F^{\text{NBD}_W}$ and $I_F^{\text{NBD}_L}$ are the fluorescence intensity at unity concentration for NBD-diC_nPE in the aqueous phase or associated with the lipid bilayer, respectively.

The rate constants for NBD-diC₆PE and NBD-diC₈PE association with and dissociation from the POPC LUVs were characterized through the fluorescence increase observed after fast mixture of an aqueous solution of the amphiphile (containing 0.5% methanol) with LUVs at distinct lipid concentrations. The data were well described by a mono-exponential function from which the characteristic rate constant (β) was obtained. The dependence of β on the concentration of LUVs allows the calculation of the rate constants of insertion (k_+) and desorption (k_-), Eq. (3):

$$\text{NBD}_W \xrightleftharpoons[k_-]{k_+ [\text{LUV}]} \text{NBD}_o$$

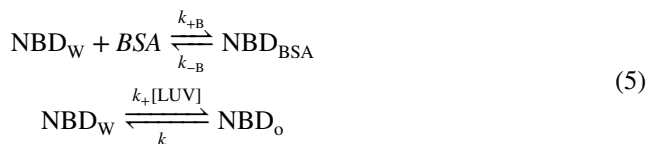
$$\beta = k_- + k_+ [\text{LUV}] \quad (3)$$

The equilibrium association constant may be calculated, from the ratio of the insertion and desorption rate constant, and is related with the partition coefficient by Eq. (4),

$$K_L = \frac{k_+}{k_-} = K_P \bar{V}_L 9.2 \times 10^4 \quad (4)$$

where 9.2×10^4 is the number of POPC molecules per LUV, calculated assuming 100 nm for the LUVs diameter and a POPC area equal to 0.64 nm^2 (Konig et al. 1997; Lantzsch et al. 1996; Smaby et al. 1997).

The aqueous solubility of the amphiphiles with $n > 8$ is below the sensitivity of the method used and therefore the direct association with the LUVs could not be obtained. Those amphiphiles were pre-equilibrated with BSA and the transfer to LUVs was followed through the increase in the fluorescence quantum yield of NBD. The pre-equilibration of NBD-diC_nPE with BSA was done by squirting a small volume of NBD-diC_nPE in methanol (final methanol concentration equal to 0.5% v/v) into a solution of BSA in the aqueous buffer with gently vortex and allowing for equilibration during 2–10 h at 25 °C. The concentration of BSA was selected to ensure that the concentration of the unbound NBD-diC_nPE was well below its CAC. The increase in the fluorescence intensity was well described by a mono-exponential function, which corresponds to the characteristic transfer rate constant (β). From the analytical integration of the differential equations generated from the kinetic scheme describing the transfer between BSA and LUVs,



one obtains Eq. (6) if both steps occurs on similar time scales, which simplifies to Eq. (7) when the association with BSA is in fast equilibrium (rate of dissociation from BSA, k_{-B} , tending towards ∞). The equilibrium association constant with BSA is related with the rate constants of association and dissociation by, $K_B = k_{+B}/k_{-B}$.

$$\beta = \frac{k_{+[\text{LUV}]} + k_{-}(1 + K_B[\text{BSA}])}{\frac{k_{+[\text{LUV}]}{k_{-B}} + 1 + K_B[\text{BSA}]}{k_{-}}} \quad (6)$$

$$\beta = k_{-} + \frac{k_{+[\text{LUV}]}}{1 + K_B[\text{BSA}]} \quad (7)$$

If the dependence of β on the concentration of LUVs follows a straight line, the rate constant of desorption (k_{-}) may be directly obtained from the intercept, Eq. (7). However, to calculate the rate of insertion from the slope it is necessary to know one of the two equilibrium association constants, K_B or K_L . If the variation of β on the concentration of LUVs saturates at high relative concentrations of acceptor LUVs, Eq. (6) must be used which allows obtaining three out of

the four parameters in the equation. Therefore, in exchange experiments, it is always necessary to perform equilibrium studies independently to obtain all the relevant parameters.

The equilibrium association constant (K_B) of NBD-diC_nPE with BSA was obtained following the same methodology as described above for partition into the POPC bilayers, assuming that the amphiphiles associate with the binding site of highest affinity, Eq. (8),

$$I_F = [\text{NBD}]_T \frac{I_F^{\text{NBD}_W} + I_F^{\text{NBD}_{\text{BSA}}} K_B[\text{BSA}]}{1 + K_B[\text{BSA}]} \quad (8)$$

For the long chain amphiphiles, the total concentration used is above their CAC which may influence the equilibrium association constant obtained. The association with BSA is a fast process, occurring within seconds (Abreu et al. 2004; Estronca et al. 2005). A small incubation time was used in this case to prevent amphiphile aggregation (Martins et al. 2008; Santos et al. 2009).

Results

Solubility in the Aqueous Media

The results obtained for the absorption and fluorescence intensity of NBD-diC₈PE as a function of its concentration in the aqueous buffer are shown in Fig. 1.

The molar absorptivity is unchanged within the concentration region evaluated, although there is a small blue shift in the absorption spectra for concentrations above 20 μM indicating a variation in the environment of the NBD group (plot A). This is best seen by the changes in the fluorescence intensity (plot B), with a clear decrease in the fluorescence quantum yield. The value of the critical aggregation concentration was calculated from the intercept between the linear best fit in the regions bellow or above the change in the fluorescence properties, leading to $\text{CAC} = 22 \pm 2 \mu\text{M}$. Together with the CAC of NBD-diC₁₀PE obtained previously (0.7 μM) (Martins et al. 2008), this predicts $\Delta\Delta G = 4.3 \text{ kJ mol}^{-1}$ for each $\Delta n = 1$, and a solubility of the polar group ($n = 0$, glycerol-phosphate-ethanolamine-NBD) of about 20 M. The dependence observed with the number of carbons in each acyl chain is only slightly higher than that obtained for the NBD-C_n series, 3.14 kJ mol^{-1} (Cardoso et al. 2010). This reflects the fact that the two chains in NBD-diC_nPE remain in close contact when the monomer is dissolved in the aqueous solution and therefore, the non-polar surface exposed to the aqueous media is smaller than predicted by the total number of carbons in the acyl chains of amphiphile.

The value predicted for the CAC of NBD-diC₁₄PE is significantly smaller than that reported previously (Abreu et al. 2004), highlighting the difficulty in the direct

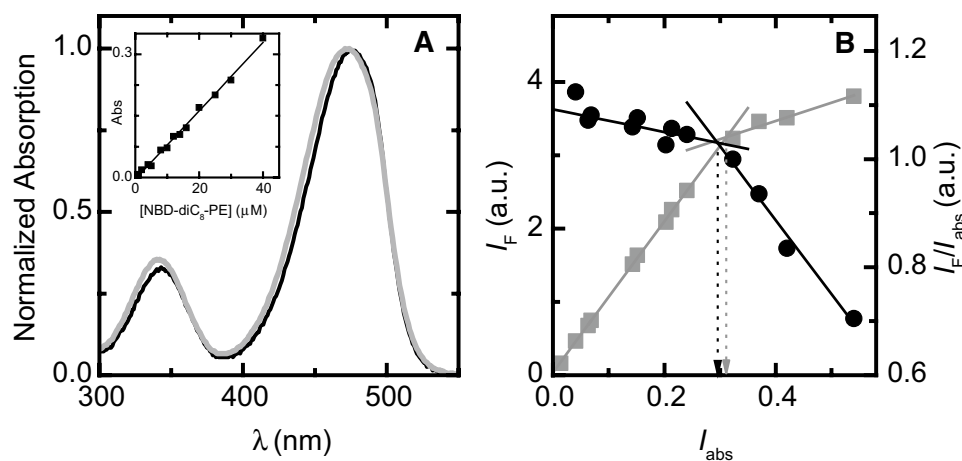


Fig. 1 Characterization of the critical aggregation concentration (CAC) of NBD-diC₈-PE. **A** Normalized absorption spectra at a concentration of 20 (thick black line) and 40 (thick gray line) μM, and dependence of the molar absorptivity at the maximum with the total concentration (insert). **B** Fluorescence intensity when excited at 475 nm (filled gray square) and ratio of the fluorescence intensity and

the intensity of light absorbed (filled black circle), the solid lines are the linear best fit to the regions below or above the CAC, their intercept corresponds to the CAC and is represented by the vertical dot lines ($I_{\text{abs}} = 0.312$ and 0.295 , respectively, corresponding to concentrations equal to 22.9 and 21.5 μM)

characterization of the solubility of amphiphiles with very small values of CAC.

Rate of Translocation Through the POPC Bilayer

The rate constant of translocation (k_t) of the amphiphiles from the inner to the outer leaflet of the POPC bilayer was characterized via the irreversible quenching of NBD fluorescence by dithionite. Typical results obtained for NBD-diC₈PE are shown in Fig. 2. Immediately after preparation of the asymmetric membranes, the fraction of NBD in the outer leaflet of the LUVs is small, upper curve in plot A. As the asymmetric LUVs are allowed to equilibrate, the fraction of NBD accessible to dithionite increases, reaching 0.4 after about 24 h (plot B). For symmetric LUVs, one would

expect a fraction of lipid (and therefore NBD-diC₈PE) equal to 0.5. The smaller value encountered in this work indicates that at the high lipid concentration used (up to 20 mM at the LUV preparation step), a significant fraction of the lipid is in internal bilayers (Cardoso et al. 2011; Martins and Moreno 2016; Martins et al. 2012).

The translocation rate constant for NBD-diC₆PE and NBD-diC₁₀PE was also studied and the results obtained are shown in Fig. 3, together with those previously reported for NBD-diC₁₄PE (NBD-DMPE) (Moreno et al. 2006). No significant effect of the acyl chain length is observed at 35 °C. At the temperature extremes characterized, 15 and 55 °C, small differences may be observed, with larger temperature dependence for the shorter acyl chain amphiphiles. This leads to a more positive enthalpy

Fig. 2 Characterization of the rate of translocation NBD-diC₈-PE. **A** Decrease in the normalized fluorescence intensity due to reaction with dithionite. The black lines are the best fit of a mono-exponential function. **B** Fractional fluorescence intensity decrease due to reaction with dithionite during 30 s, which corresponds to the fraction of NBD-diC₈-PE in the outer leaflet of LUVs, as a function of the time of equilibration at 35 °C. The line is the best fit of Eq. (1), leading to $k_t = 7.7 \times 10^{-5} \text{ s}^{-1}$

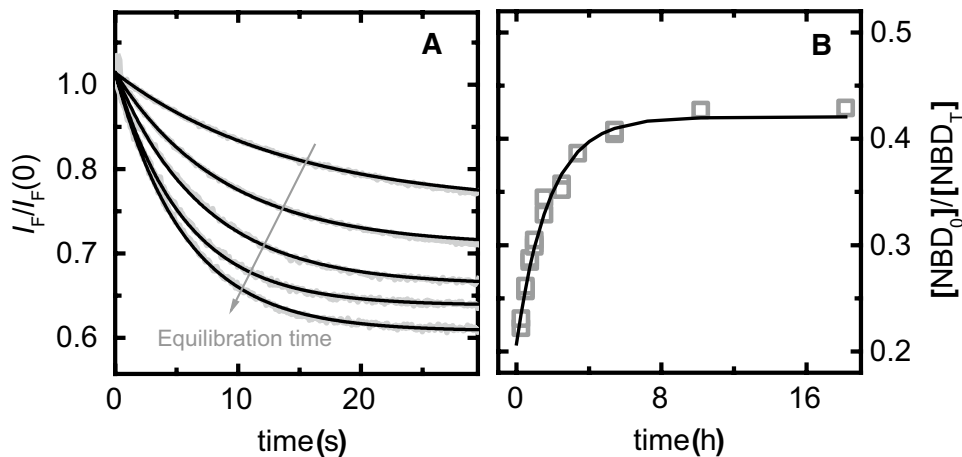
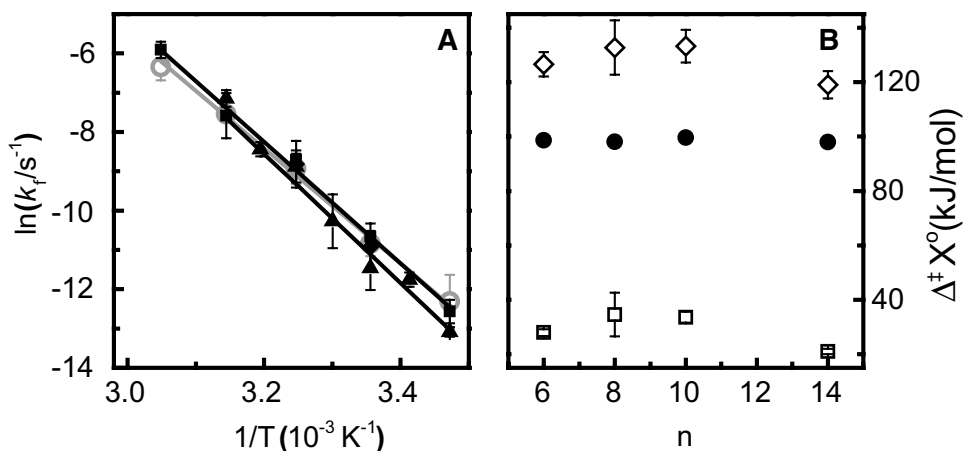


Fig. 3 Effect of temperature and acyl chain length on the rate of NBD-diC_n-PE translocation. **A** Temperature dependence of the translocation rate constant, for diC₆ (filled black squares), diC₈ (filled black diamond), diC₁₀ (filled black triangle), and diC₁₄ (open gray circle). **B** Effect of the acyl chain length on the thermodynamic parameters for translocation at 35 °C, $\Delta^\ddagger G^\circ$ (filled black circle), $\Delta^\ddagger H^\circ$ (open black diamond), and $T\Delta^\ddagger S^\circ$ (open black square)



variation associated with the formation of the transition state ($\Delta^\ddagger H^\circ$) for the case of the shorter acyl chains, which is compensated by a more positive entropy variation ($T\Delta^\ddagger S^\circ$). This effect being more significant for intermediate acyl chain lengths, as observed previously for the NBD-C_n homologous series (Cardoso et al. 2011). Larger enthalpy and entropy variations associated with the formation of the transition state in translocation have also been obtained for NBD-LysoMPE when compared with NBD-DMPE. This suggests the occurrence of particularly strong interactions (favored by enthalpy) between intermediate acyl chain amphiphiles and may result from packing constraints in the POPC bilayer due to the presence of the cis double bond at carbon 9.

Rate of Transfer Between the Aqueous Phase and POPC LUVs

The aqueous solubility of the amphiphiles with the shorter acyl chain lengths (diC₆ and diC₈) allows the direct characterization of their transfer between the aqueous phase and the POPC LUVs, taking advantage of the large increase in the fluorescence quantum yield when associated with the POPC bilayer. Typical results obtained for NBD-diC₆PE at 14 °C are shown in Fig. 4.

The fluorescence increase was well described by a mono-exponential function, from which the rate constant of transfer (β) is directly obtained. The rate of equilibration between the aqueous phase and the outer leaflet of POPC LUVs was fast, with a strong dependence on the concentration of

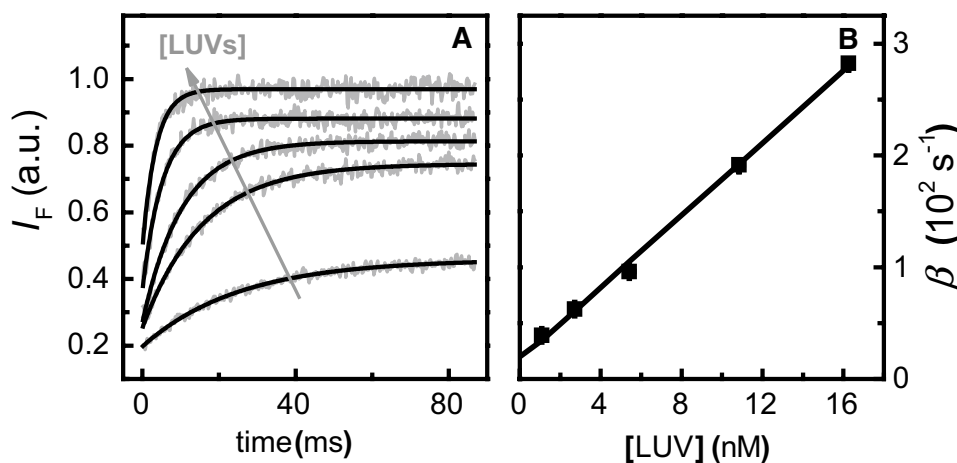


Fig. 4 Transfer of NBD-diC₆-PE between the aqueous phase and POPC LUVs. **A** Fluorescence increase due to the addition of various concentrations of POPC LUVs to an aqueous solution of 50 nM NBD-diC₆-PE at 14 °C (gray line), the black lines are the best fit of a mono-exponential function. **B** Characteristic rate constant of trans-

fer (β) as a function of LUV concentration. The symbols (filled black squares) are the average of at least three independent experiments (standard deviation smaller than the size of the symbols) and the line is the best fit of Eq. (3), with $k_+ = 1.4 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ and $k_- = 2.0 \times 10^1 \text{ s}^{-1}$

LUVs. From this dependence, the rate constant of insertion and desorption may be calculated, Eq. (3).

The experiments were only performed in the temperature range from 12 to 20 °C, because at higher temperatures it was too fast to be measured accurately. The temperature dependence is shown in Fig. 7 and the extrapolation to 35 °C leads to $k_+ = 7.3 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ and $k_- = 1.2 \times 10^2 \text{ s}^{-1}$. Those rate constants are several orders of magnitude larger than found for NBD-diC₁₄PE (Abreu et al. 2004), and closer to those obtained for NBD-LysoMPE (Sampaio et al. 2005).

To evaluate the effect of the acyl chain length, the equilibration of NBD-diC₈PE between the aqueous phase and POPC LUVs was also characterized. The results obtained are shown in Fig. 5A.

Both the rate of insertion and desorption were smaller than observed for NBD-diC₆-PE allowing the characterization up to higher temperatures. The values of the rate constants will be discussed below, in comparison with those obtained for the other amphiphiles in the homologous series.

Rate of Exchange Between BSA and POPC LUVs

The aqueous solubility of the amphiphiles with longer acyl chain length [diC₁₀ and diC₁₂ studied in this work, and diC₁₄ (Abreu et al. 2004)] was too small to allow the direct characterization of their interaction with POPC LUVs. To avoid aggregation in the aqueous phase, they were first equilibrated with BSA and the parameters for their interaction with the POPC LUVs were obtained from the rate of exchange between BSA and the LUVs. NBD-diC₈-PE was characterized following both methods to allow a critical comparison.

The results obtained for diC₈ are shown in Fig. 5B. The characteristic rate constant for exchange between BSA and the LUVs was not well described by a straight line and the parameters obtained when analyzing the results assuming fast equilibrium for the interaction of the amphiphile with BSA, Eq. (7), were not in agreement with those obtained directly by transfer from the aqueous phase to the LUVs. However, when considering a steady state for the exchange between BSA and the LUVs, Equations (5) and (6), good agreement was obtained for the rate constants of insertion and desorption obtained by the two methods.

To obtain the rate constants for interaction with the LUVs, it is necessary to know the equilibrium association constant with BSA. This parameter was characterized for diC₈, diC₁₀, and diC₁₂ and showed very little dependence with the length of the acyl chain, being between 8×10^5 and $2 \times 10^6 \text{ M}^{-1}$ at 35 °C. The temperature dependence was also very small, and within the experimental uncertainty. In addition, this parameter was similar to that measured previously for diC₁₄ (Abreu et al. 2004; Estronca et al. 2005). Uncertainty in the binding affinity to BSA influences proportionally the rate constant of insertion calculated from the exchange experiment, while the rate constant of desorption from the LUVs and dissociation from BSA are not altered. To avoid introducing noise in the kinetic parameters measured, we have opted to fix the equilibrium binding constant at 10^6 M^{-1} for all amphiphiles and temperatures studied.

The non-linear dependence of β on the concentration of LUVs in the exchange of NBD-diC₈PE, indicates that dissociation from BSA and desorption from the POPC bilayer occur on similar time scales. In this case, the best fit of

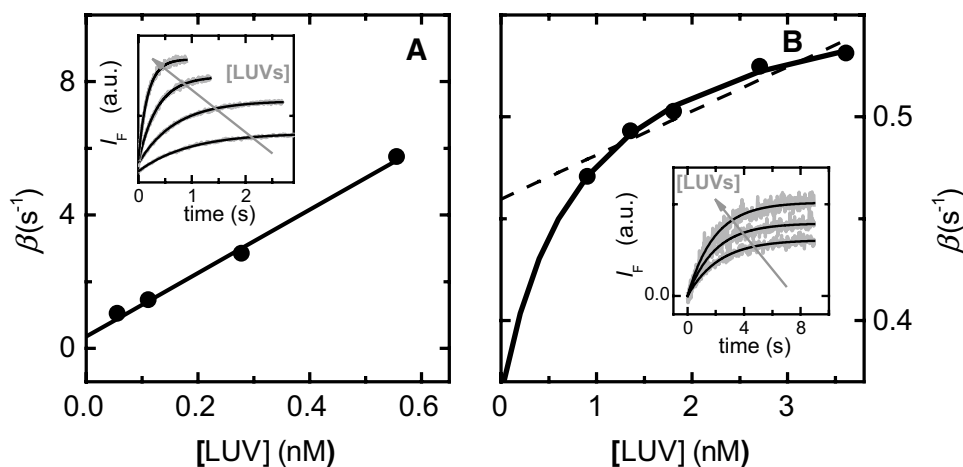


Fig. 5 Transfer of NBD-diC₈-PE between the aqueous phase (A) or associated with BSA (B) and POPC LUVs, at 20 °C. **A** Dependence of the characteristic transfer rate constant (β) on the concentration of LUVs (filled black circle) and best fit of Eq. (3) (black line) with $k_+ = 9.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ and $k_- = 3.6 \times 10^{-1} \text{ s}^{-1}$. The inset shows the fluorescence variation from which β was obtained. **B** Dependence of the

characteristic transfer rate constant (β) on the concentration of LUVs (filled black circle) and best fit of Eq. (7) (dashed black line), with $k_+ = 2.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $k_- = 4.6 \times 10^{-1} \text{ s}^{-1}$. The solid black line (continuous black line) is the best fit of Eq. (6), with $k_+ = 7.3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, $k_- = 3.6 \times 10^{-1} \text{ s}^{-1}$, $K_B = 1.0 \times 10^6$, and $k_{-B} = 5.7 \times 10^{-1} \text{ s}^{-1}$. The inset shows the fluorescence variation from which β was obtained

Eq. (6) allows obtaining both rate constants. At small concentrations of lipid, the exchange is limited by desorption from the LUVs, while as the lipid concentration increases the characteristic exchange rate constant tends towards the rate of dissociation from the BSA. The results obtained at 20 °C show that at this temperature dissociation from BSA (k_{-B}) is faster than desorption from the bilayer (k_{-}). However, the dependence of k_{-} with temperature is more accentuated and at 30 °C or above, the rate constant for dissociation from the BSA becomes the slower step.

Typical results obtained for diC₁₀ and diC₁₂ are shown in Fig. 6. The slower kinetics observed for those amphiphiles allows its characterization up to 35 °C. At this temperature, desorption of NBD-diC₁₀PE from the POPC bilayer occurs in the same time scale as dissociation from BSA, with the characteristic rate constant of transfer increasing with the concentration of lipid non-linearly tending towards the rate constant of dissociation from BSA at high LUVs concentrations. The rate of desorption from the POPC bilayer is more affected by temperature and at 25 °C or below, a linear dependence of β on [LUV] is observed indicating that equilibration of NBD-diC₁₀PE between the aqueous phase and BSA occurs faster than the interaction with the POPC bilayer.

The increased hydrophobicity of NBD-diC₁₂PE decreases the rate of desorption from the POPC bilayer to a larger extent than the rate of dissociation from BSA. As a consequence, association with BSA may be considered at fast equilibrium, and the characteristic rate constant of exchange increases linearly with the concentration of LUVs. In this case, the rate of exchange between BSA and the outer leaflet

of LUVs occurs in the same time scale of translocation into the inner leaflet, and the time dependence of the fluorescence increase is not well described by a mono-exponential function. The rate constants of insertion and desorption into/from the outer leaflet of the POPC bilayer were obtained through the global best fit of the results obtained at the different lipid concentrations, with the numerical integration of the differential equations obtained from the kinetic scheme (5) assuming fast equilibrium for the association with BSA. The dash lines in Fig. 6B correspond to the fluorescence increase due to interaction with the outer leaflet of the POPC LUVs, highlighting the contribution from translocation into the inner leaflet.

The temperature dependence of the rate constants is represented in Fig. 7 for all the amphiphiles studied in this work.

The rate of desorption from the lipid bilayer is strongly temperature dependent for all amphiphiles studied in this work, with $\Delta^\ddagger H^\circ$ varying from 80 to 100 kJ mol⁻¹ with no systematic dependence on the acyl chain length. A similar result was obtained previously for phospholipids (Wimley and Thompson 1990), and for pyrene-labeled phospholipids (Massey et al. 1982), with somewhat smaller enthalpy variations observed for lyso-phospholipids with NBD in the head group (Sampaio et al. 2005) and for NBD-PC (Nichols 1985; Nichols and Pagano 1981). The Gibbs free energy variation upon formation of the transition state in desorption is however strongly affected by the length of the acyl chain, with $\Delta\Delta^\ddagger G^\circ = 6.1$ kJ mol⁻¹ per methylene group in each acyl chain at 35 °C. This is slightly above 3/2 of the value encountered for the single chain amphiphiles NBD-Cn

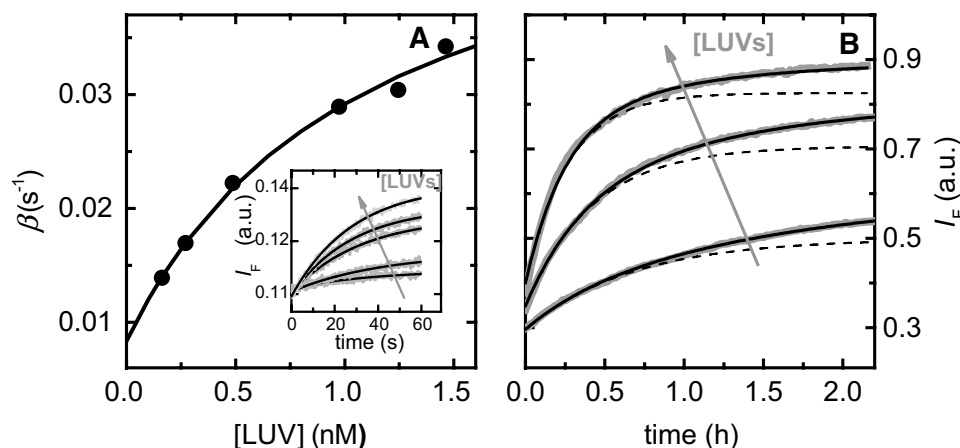


Fig. 6 Exchange of NBD-diC₁₀-PE (plot A) or NBD-diC₁₂-PE (plot B) from BSA and POPC LUVs, at 35 °C. **A** Dependence of the characteristic transfer rate constant (β) on the concentration of LUVs (filled black circle) and best fit of Eq. (6) (continuous black line) with $k_{+} = 1.8 \times 10^9$ M⁻¹ s⁻¹, $k_{-} = 8.3 \times 10^{-3}$ s⁻¹, $K_B = 1.0 \times 10^6$ M⁻¹, and $k_{-B} = 5.2 \times 10^{-2}$ s⁻¹. The inset shows the fluorescence variation from which β was obtained. **B** Experimental results obtained for

the fluorescence intensity of NBDdiC₁₂-PE (continuous gray line) at three different lipid concentrations, global best fit of the numerical integration of the differential equations obtained from the kinetic scheme (5) (continuous black line) with $k_{+} = 9.3 \times 10^6$ M⁻¹ s⁻¹, $k_{-} = 2.7 \times 10^{-4}$ s⁻¹, $K_B = 1.0 \times 10^6$, and $k_f = 1.6 \times 10^{-4}$ s⁻¹, and corresponding mono-exponential assuming negligible translocation during the exchange (dashed black line)

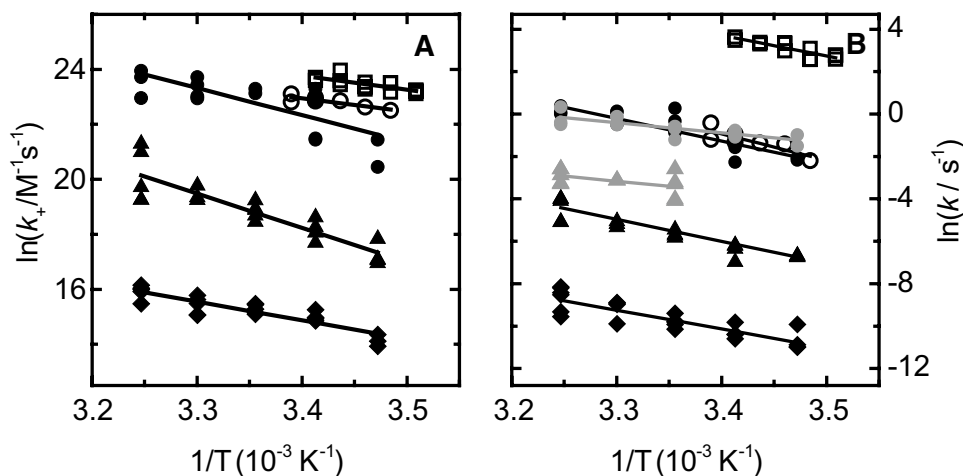


Fig. 7 Temperature dependence of the rate of insertion into (plot A) and the rate of desorption from the POPC bilayer (black symbols) or dissociation from BSA (gray symbols) (plot B), for all amphiphiles studied in this work: NBD-diC₆-PE (open black squares), NBD-diC₈-PE (open black circles, filled black circles), NBD-diC₁₀-PE (filled

black triangles), and NBD-diC₁₂-PE (filled diamond). The association with the POPC LUVs was characterized directly by transfer from the aqueous phase (open symbols) or from the amphiphile equilibrated with BSA (closed symbols)

($\Delta\Delta^\ddagger G^\circ = 3.5 \text{ kJ mol}^{-1}$), as expected. The entropy variation associated with the formation of the transition state in desorption is consequently strongly dependent on the length of the acyl chain, changing from $17 \pm 6 \text{ kJ mol}^{-1}$ for diC₆ to $-26 \pm 4 \text{ kJ mol}^{-1}$ for diC₁₂. This variation reflects the larger hydrophobic surface exposed to the aqueous phase as the acyl chain increases. In this respect, it is interesting to note that the rate constants of desorption of NBD-diC_n-PE are similar to those observed for the NBD-C_n with the same non-polar surface area (for example, $k_-(\text{diC}_8) \cong k_-(\text{C}_{12})$).

The rate of insertion in the POPC bilayer is only weakly dependent on temperature for the case of the amphiphile with the shorter acyl chain (diC₆) leading to an enthalpy variation upon formation of the transition state, $\Delta^\ddagger H^\circ = 40 \pm 5 \text{ kJ mol}^{-1}$. The most hydrophobic amphiphile in the homologous series (diC₁₄) showed $\Delta^\ddagger H^\circ = 85 \pm 5 \text{ kJ mol}^{-1}$ (Abreu et al. 2004), and the higher enthalpy variation was observed for NBD-diC₁₀-PE, $\Delta^\ddagger H^\circ = 101 \pm 12 \text{ kJ mol}^{-1}$. Stronger temperature dependence of the insertion rate constant for amphiphiles with intermediate acyl chain lengths

was previously observed for the NBD-C_n homologous series (Cardoso et al. 2011), and may be related with the location of the amphiphile methyl groups relative to the POPC double bond (Filipe et al. 2011).

The values obtained for the rate constant of insertion at 35 °C are shown in Table 1. There is a strong dependence on the length of the acyl chains, with k_+ being close to diffusion control for diC₆ and diC₈, decreasing to $8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for diC₁₂ being even smaller diC₁₄ (Abreu et al. 2004). The rate of insertion obtained for this homologous series is much slower than previously reported for phospholipids with a single acyl chain (Sampaio et al. 2005) or when the NBD group is located in one of the acyl chains (Nichols 1985). This suggests a direct relation between this parameter and the aqueous solubility of the amphiphile, possibly its solubility in the ordered water at the surface of the lipid bilayer.

On the other hand, the comparison between the dependence of the insertion rate constant on n for this homologous series and that of the single chain amphiphiles NBD-C_n highlights the importance of the non-polar cross-section.

Table 1 Parameters for the interaction of NBD-diC_n-PE with POPC LUVs, measured at or extrapolated to 35 °C

n	Kinetics			Equilibrium
	Translocation k_t (s^{-1})	Insertion k_+ ($\text{M}^{-1} \text{s}^{-1}$)	Desorption k_- (s^{-1})	Partition coefficient K_p ^b
6	$(1.2 \pm 0.3) \times 10^{-4}$	$(4.6 \pm 1.3) \times 10^{10}$	$(2.4 \pm 0.6) \times 10^2$	1.9×10^3
8	$(1.5 \pm 0.6) \times 10^{-4}$	$(1.9 \pm 0.4) \times 10^{10}$	3.6 ± 1.5	1.7×10^4
10	$(0.8 \pm 0.4) \times 10^{-4}$	$(5.6 \pm 2.6) \times 10^8$	$(1.4 \pm 0.3) \times 10^{-2}$	5.6×10^5
12	–	$(8.1 \pm 2.7) \times 10^6$	$(2.1 \pm 0.9) \times 10^{-4}$	4.7×10^5
14 ^a	$(1.1 \pm 0.4) \times 10^{-4}$	$(2.3 \pm 0.1) \times 10^6$	$(2.8 \pm 1.2) \times 10^{-5}$	1.1×10^6

^aFrom Abreu et al. (2004)

^bCalculated from the ratio of the insertion and desorption rate constants, K_L

For NBD-diC_n-PE, $\Delta^\ddagger G^0$ increases linearly from $n=8$ to $n=12$ with $\Delta\Delta^\ddagger G^0 = 5 \text{ kJ mol}^{-1}$, showing a smaller dependence for smaller and larger acyl chains. The dependence of $\Delta\Delta^\ddagger G^0$ with n for the single chain homologous series is maximal between $n=10$ and 14 , with $\Delta\Delta^\ddagger G^0 = 2 \text{ kJ mol}^{-1}$. Also, the rate constant of insertion obtained for the double chain amphiphile is smaller than anticipated from the NBD-C_n with the same non-polar surface, or aqueous solubility (Cardoso et al. 2010). This indicates that insertion is also influenced by the cross-section of the non-polar region of the amphiphile, supporting the interpretation that insertion into lipid bilayers is not diffusion limited. To occur the insertion of the amphiphile in the membrane, it is necessary to have a free area in the membrane surface with an adequate size.

For NBD-diC₈-PE and NBD-diC₁₀-PE, it was possible to characterize the rate of dissociation from BSA (gray symbols in Fig. 7B). At 35 °C, the rate constant was equal to 1.2 and $7.3 \times 10^{-2} \text{ s}^{-1}$ for diC₈ and diC₁₀, respectively. This process occurs faster than previously observed for DHE [$2 \times 10^{-2} \text{ s}^{-1}$ (Estronca et al. 2014)], despite the higher binding affinity ($K_B \cong 1 \times 10^6$ and $4 \times 10^4 \text{ M}^{-1}$, for NBD-diC_n-PE and DHE, respectively). The temperature dependence observed was much weaker than for desorption from the lipid bilayer, with $\Delta^\ddagger H^0 = 37 \pm 7 \text{ kJ mol}^{-1}$ for diC₈.

Equilibrium Association with the POPC LUVs

The equilibrium association from the aqueous phase into the POPC LUVs was characterized directly for the amphiphiles with a relatively high solubility in the aqueous phase, diC₆ (between 15 and 35 °C) and diC₈ (at 25 °C), and calculated from the ratio of the insertion and desorption rate constants (K_L) for all amphiphiles in the homologous series. A fair agreement was observed for the results obtained by both methods, Table 1. As expected, the extent of association with the POPC bilayer increases as the hydrophobicity of the amphiphile increases. The Gibbs free energy variation decreases linearly up to $n=10$, with $\Delta\Delta^\ddagger G^0 \cong -4 \text{ kJ mol}^{-1}$, but it levels off for longer acyl chains.

Conclusion

The results presented in this work clearly show that crossing the lipid bilayer center cannot be described by random diffusion for strongly amphiphilic molecules with sizes comparable to those of the lipids that forms the bilayer. In contrast, crossing the bilayer center occurs by translocation, with the rate of this process depending on the energy difference between the equilibrium position of the amphiphile in each bilayer leaflet and at the highest energy state. The observation that the rate of translocation is the same for all amphiphiles in the homologous series indicates that

the solubility of the polar groups in the non-polar center of the bilayer is the limiting step in the process. Also, the observation that the rate of translocation is not dependent on the mismatch between the length of the NBD-diC_n-PE and the bilayer constituent lipid (POPC), does not support the formation of stable or transient pores in the bilayer as has been observed by MD simulations (Tieleman and Marrink 2006), at least for the small concentrations of solute used.

For NBD-diC_n-PE with $n \leq 10$, the rate of translocation is much smaller than the rate of desorption from the lipid bilayer. This leads to a relatively fast equilibration of the lipid between the outer leaflets of lipid vesicles, with little variation in the composition of the inner leaflets. This property may be of relevance to prepare asymmetric liposomes. In contrast, for $n \geq 14$, the rate of desorption from the lipid bilayer is significantly slower than the rate of translocation between leaflets. For liposomes prepared from those phospholipids, populations with different lipid compositions may coexist in the same solution permitting studies of amphiphile exchange.

The results provided by this work are also of relevance in the preparation of liposomes from lipid mixtures. To guarantee homogeneity in the lipid composition of the liposomes at all lipid concentrations, the sample must be allowed to equilibrate for at least 3 times the characteristic time for the slowest step (leading to less than 5% deviation from equilibrium). For the case of NBD-diC₁₄-PE (similar to DMPC), the equilibration time at 35 °C must be at least 30 h, this increasing to 8 days if equilibration is performed at 25 °C. This highlights the importance of guaranteeing a homogeneous distribution of the lipids before preparation of the liposomes.

The comparison between the rate constants of insertion into and dissociation from the POPC bilayer for this homologous series and the single chain amphiphiles NBD-C_n, gives important information regarding the contribution of non-polar regions. While the rate of desorption is mostly influenced by the total non-polar surface exposed to the aqueous media, the rate of insertion is also affected by the cross-section of the non-polar region of the amphiphile. The different effects of both properties may be used to design amphiphilic molecules with optimized affinity for lipid membranes, with a high potential in the early steps of drug design.

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Compliance with Ethical Standards

Conflict of interest Filipe M Coreta-Gomes, Winchil L. C. Vaz, Maria João Moreno declare that they have no conflict of interest.

Research Involving with Human and Animal Rights This article does not contain any studies with human participants or animals performed by any of the authors.

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