Chain Length Effect on the Binding of Amphiphiles to Serum Albumin and to POPC Bilayers

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · DECEMBER 2010

Impact Factor: 3.3 · DOI: 10.1021/jp105163k · Source: PubMed

6 AUTHORS, INCLUDING:

Hugo Filipe
University of Lisbon
12 PUBLICATIONS **45** CITATIONS
SEE PROFILE

Filipe Gomes
University of Coimbra
13 PUBLICATIONS **110** CITATIONS
SEE PROFILE

Winchil Vaz
New University of Lisbon
106 PUBLICATIONS **4,974** CITATIONS
SEE PROFILE

Maria João Moreno
University of Coimbra
47 PUBLICATIONS **545** CITATIONS
SEE PROFILE

Chain Length Effect on the Binding of Amphiphiles to Serum Albumin and to POPC Bilayers

R. M. S. Cardoso, H. A. L. Filipe, F. Gomes,* N. D. Moreira, W. L. C. Vaz, and M. J. Moreno*

Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Largo D. Dinis, Rua Larga, 3004-535 Coimbra, Portugal

Received: June 5, 2010; Revised Manuscript Received: August 10, 2010

The interaction of small molecules, such as drugs or metabolites, with proteins and biomembranes is of fundamental importance for their bioavailability. The systematic characterization of the binding affinity for structurally related ligands may provide rules that allow its prediction for any other relevant molecule. In this work we have studied a homologous series of fluorescent fatty amines with the fluorescent moiety 7-nitrobenz-2-oxa-1,3-diazol-4-yl covalently bound to the amine group (NBD-C_n; n = 4, 6, 8, 10, 12, 14, and 16) in aqueous solution and associated with BSA or lipid bilayers. We have found a linear dependence with the length of the alkyl chain, up to NBD-C_{10}, for the Gibb's free energy of partition between the aqueous solution and 1-palmitoyl-2-oleoyl phosphatidylcholine bilayers equal to $\Delta\Delta G = -2.5 \pm 0.3$ kJ/mol per methylene group. Additionally, the amphiphiles interacted efficiently with bovine serum albumin, and it was inhibited by fatty acids indicating that binding occurs to the fatty acids highest affinity binding site. The association of the amphiphiles with BSA and POPC bilayers was performed at different temperatures (15−35 °C) allowing for the calculation of the enthalpic and entropic contributions. A value of $\Delta H = -15 \pm 4$ kJ/mol was obtained for all amphiphiles and binding agents. The entropy contribution was always positive and increased with the length of the alkyl chain. The location of the ligand in the biological membrane is also of high relevance, namely because this will determine its effect on biomembrane properties at high ligand concentrations. With this goal, we have measured some photophysical properties of the amphiphiles inserted in POPC bilayers, and we found no significant variation along the series, indicating that the NBD group is located in a region with similar properties regardless of the length of the nonpolar group. An exception was noted for the case of NBD-C_{14} whose parameters were somewhat different from the trend observed.

Introduction

The interaction of amphiphilic molecules with proteins and lipid assemblies is a key property that determines their bioavailability and the rate at which they reach their active location in the cell or organism. The most prevalent models for passive permeation through biological membranes assume that the rate limiting step is the diffusion through the membrane (corresponding to the translocation rate for the case of amphiphilic molecules) and that the partition between the aqueous media and each leaflet of the lipid bilayer is at equilibrium.[1] Within this model, the partition coefficient to the lipid bilayer and the concentration of the ligand in the aqueous media are the most relevant parameters. Therefore, to evaluate the rate of passive permeation through a lipid bilayer the binding affinity between the ligand and both the lipid bilayer and the proteins in the aqueous media must be known.

Several studies have shown that for amphiphilic ligands this simple model based on the equilibrium parameters for the interaction between the ligand and the binding agents fails,[2] and we have observed that in this case the kinetics of the interactions plays a significant role in the rate of overall permeation (Filipe et al., unpublished results). The characterization of the equilibrium parameters is nevertheless of fundamental importance as they may be used for a qualitative screening on potential fast and slow permeating ligands. Additionally, they are very important to optimize the conditions for the experimental studies and analysis models that may conduce the full characterization of the kinetics of the most relevant processes. Some systematic studies on homologous series can be found in the literature[3−13] but the relation between the structure of the amphiphile and its binding affinities is far from being understood, justifying the study of additional series of amphiphiles.

The characterization of structurally related ligands may conduce the establishment of rules between the structure of the ligand and its bioavailability or rate of passive permeation through tight endothelia such as the blood−brain barrier. The systematic study of the association for homologous series of amphiphiles can also provide important information regarding the nature of the interaction and the structure and dynamics of the binding agent. With this goal we have previously characterized the kinetics and thermodynamics of the interaction of NBD-DMPE, NBD-lysoMPE, and DHE with proteins, lipoproteins, and lipid bilayers.[14−19] More recently we have been characterizing the interaction of a homologous series of fluorescent fatty amines, NBD-C_n, that differ only in the length of the alkyl chain. In this work we report on the equilibrium association between the amphiphiles in the NBD-C_n homologous series (n = 4−16) and bovine serum albumin or POPC bilayers as well as on their critical aggregation concentration in aqueous media. Modification of proteins by acylation is a prevalent process in biological systems for the reversible association of proteins and small molecules with biological membranes and proteins.[20,21] The

* Corresponding author.

10.1021/jp105163k
Published on Web 11/19/2010

knowledge of the binding affinity as a function of the length of the hydrophobic chain is of fundamental importance in the understanding of the mechanism and biological role of those associations. The characterization of the homologous series of amphiphiles presented in this study contributes to this goal complementing the work done with fatty acids and acylated peptides.[11,13]

Another important issue in the modification of proteins with hydrophobic moieties is the prevalence of some chain lengths.[20,21] The systematic study of homologous series may provide clues regarding the specificities of the interaction between alkyl chains with a given length and biological membranes or proteins. With this purpose, we have undertaken the systematic characterization of the photophysics of NBD-C_n amphiphiles associated with POPC bilayers so as to obtain information regarding the effects of the alkyl chain length on the location of the polar group in the bilayer.

Materials and Methods

Materials. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA), and bovine serum albumin (BSA) was from Applichem (Darmstadt, Germany). All other reagents and solvents were of analytical grade, or higher purity, from Sigma-Aldrich Química S.A. (Sintra, Portugal).

Equipment. Steady state fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with a thermostatted multicell holder accessory and automatic polarizers. Fluorescence lifetime measurements were done on a home-built TCSPC apparatus with a Horiba-JI-IBH NanoLED at $\lambda_{exc} = 460$ nm, a Jobin-Ivon monochromator, a Philips XP2020Q photomultiplier, and a Canberra instruments TAC and MCA as described previously.[22] UV−vis absorption was performed on a Unicam UV530 spectrophotometer (Cambridge, U.K.). The purity of the amphiphiles synthesized was evaluated by mass spectrometry in the positive and negative mode as described elsewhere,[23,24] by ^{1}H NMR (Bruker spectrometer operating at 400 MHz) and by analytical HPLC with a Diode Array (model G1315D from Agilent, USA) and Fluorescence detector (model G1321A from Agilent, USA).

Synthesis and Purification of NBD-C_n. The method followed is significantly different from that in the literature and is explained below.[9,25−29] The amine was first dissolved in dry chloroform, and excess sodium carbonate was added. NBD-Cl, dissolved in the minimal amount of dry chloroform was added dropwise with constant stirring. The mixture was left in the dark, with stirring, for 2 h at room temperature. A small excess of amine was used and the concentration of amine was ∼20 mg/mL. The reaction mixture was purified using Si60 preparative TLC plates and $CHCl_3$:MeOH:CH_3COOH 80:20:1 as eluent. The product, the most intense yellow band with an $R_f = 0.4-0.8$ (smaller for shorter alkyl chain amphiphiles), was extracted with chloroform and further purified by preparative liquid chromatography using the stationary phase RP18 and acetonitrile:water 75:25 as eluent. The reaction yield was around 20% assuming $\varepsilon_{NBD\text{-}Cn} = 2.1 \times 10^4$ M^{-1} cm^{-1} at the CT band (∼460 nm) in methanol. The purity of the amphiphile was verified by HPLC, ^{1}H NMR, and mass spectrometry, and they were at least 94% pure. See additional materials for details.

Solubility in Aqueous Solutions. The solubility of the monomeric form of NBD-C_n in aqueous solutions at pH 7.4 and 25 °C was assessed via deviations to linearity in the amphiphile absorption and/or fluorescence as a function of the total concentration of amphiphile. The necessary volume of a solution of the amphiphile in methanol was placed in a glass tube, the solvent was evaporated under a stream of nitrogen and the required amount of aqueous solution was added. The solutions were allowed to equilibrate at 25 °C for 2 to 4 h and its absorption and/or fluorescence was measured. For amphiphiles with a small solubility in aqueous solutions and a high tendency to adsorb to the glass tube, $n = 8-12$, the amphiphile in methanol was added directly to the aqueous solution, without preparation of the film. The concentrations of the amphiphile in methanol were varied such that the final concentration of methanol was equal to 0.5%.

Partition Coefficients between the Aqueous Phase and POPC Bilayers. The amphiphile was first dissolved in methanol at the necessary concentration and a small aliquot was squirted into the aqueous buffer (HEPES 10 mM at pH 7.4 with 0.15 M NaCl, 1 mM EDTA and 0.02% NaN_3), while stirring gently with a vortex apparatus, to obtain the required final concentration of NBD-C_n and 1% of methanol. This solution was then mixed with an equal volume of solutions of POPC, in the same buffer, at different lipid concentrations and allowed to equilibrate for 1 to 2 h at 25 °C. Aqueous solutions of NBD-C_{10} are not very stable and this amphiphile was added directly from the methanol solution into the POPC suspensions to avoid the intermediate aqueous solution with a relatively high concentration of the amphiphile. The fluorescence of the samples was measured with a path length such that the maximum optical density (absorption plus scattering) was smaller than 0.1. The POPC was in the form of large unilamelar vesicles (LUV) that were prepared by evaporation of a POPC solution in the azeotropic mixture of chloroform and methanol (87:13, *v*:*v*) followed by hydration of the film with the aqueous buffer and subsequent extrusion (Extrusor from Lipex Biomembranes, Vancouver, British Columbia, Canada) through 100 nm pore size filters (Nucleopore, Whatman, Springfield Hill, U.K.) as described previously.[16] The final concentration of POPC was verified using a modified version of the Bartlett phosphate assay.[30] The total concentration of the fluorescent amphiphiles was equal to, or smaller than, half the value of its CAC in the aqueous solution and the ratio of POPC to bound amphiphile was higher than 50 to guaranty that the properties of the bilayer are not affected by the ligand.[31]

Binding to Bovine Serum Albumin. The equilibrium constant between the amphiphiles and BSA was obtained using the same approach described above for the equilibrium titrations of POPC. The concentration of fluorescent amphiphile was always smaller than half its CAC and much smaller than the lowest BSA concentration used in the titration. To obtain information regarding the binding site of NBD-C_n, the competitive effect of decanoic acid on the binding of NBD-C_{10} was obtained. Albumin aqueous solutions at different concentrations were incubated overnight with decanoic acid, a small aliquot of NBD-C_{10} dissolved in methanol was added, conducing to a final concentration of 40 nM, and the solutions were allowed to equilibrate for 1 to 2 h. The amount of NBD-C_{10} bound to albumin was assessed via the increase in its fluorescence upon binding. As a control, the effect of decanoic acid on NBD-C_{10} fluorescence was also measured. The concentration of decanoic acid was varied from 0 to 20 μM.

Photophysical Characterization of NBD-C_n in Aqueous Solution and in POPC Bilayers. The ionization constant of the amine group of NBD-C_n was measured in aqueous solution for NBD-C_6 at a concentration of 50 μM and for all NBD-C_n ($n = 6-16$) associated with POPC bilayers at a lipid concentration of 0.5 mM and a probe to lipid ratio of 1:100. At alkaline

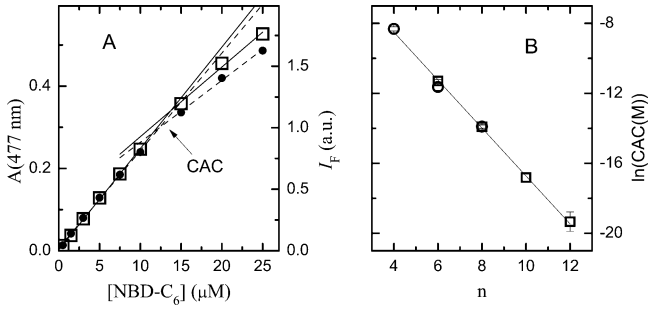

Figure 1. Solubility of the monomeric form of NBD-C_n in aqueous solution, pH 7.4, at 25 °C. Plot A: Typical result for the dependence of NBD-C_6 absorption at the maximum wavelength (□) and fluorescence at 575 nm (●) as a function of its total concentration in aqueous buffer with 0.5% (*v*/*v*) methanol. Plot B: Average and standard deviation of the critical aggregation concentration (CAC) in aqueous solutions with 0 (○) and 0.5% (*v*/*v*) methanol (□), for at least 3 independent experiments. The line is the best fit of ln(CAC) for $n = 6-12$ using the data obtained at 0.5% methanol.

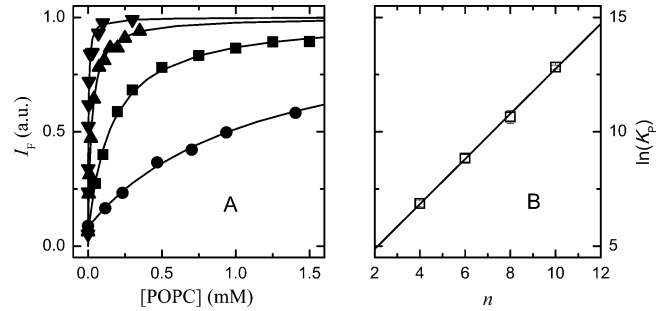

Figure 2. Partition of NBD-C_n between an aqueous solution, pH 7.4, and POPC bilayers at 25 °C. Plot A: Typical results obtained for the titration of NBD-C_4 at 350 nM (●), NBD-C_6 at 170 nM (■), NBD-C_8 at 50 nM (Δ), and NBD-C_{10} at 20 nM (▼). The line is the best fit of eq 1 with $K_P = 1.1 \times 10^3$, 7.7×10^3, 5.0×10^4, and 4.1×10^5 respectively. Plot B: average value of the partition coefficient of all NBD-C_n studied for at least 3 independent experiments, the standard deviation is smaller than the symbols shown. The line is the best fit of a straight line with $\Delta\Delta G = -2.4 \pm 0.2$ kJ/mol.

values of pH, NBD-C_n changes its ionization state and the absorption maximum shifts to smaller wavelengths becoming nonfluorescent. The solutions were prepared in unbuffered aqueous media and the pH was changed by addition of small volumes of NaOH or HCl at the appropriate concentrations. The pH of the solutions was verified using a pH meter and the fluorescence emission spectra was obtained after stabilization of the pH.

The fluorescence lifetime decay, quantum yield and anisotropy were obtained for NBD-C_6 in aqueous solutions, at a concentration of 50 μM, and for all NBD-C_n associated with POPC bilayers at a lipid concentration of 0.5 mM and a probe to lipid ratio of 1:100. The concentration of lipid and fluorescent amphiphile were chosen to guaranty a good signal-to-noise ratio and an optical density at the excitation wavelength (absorption plus dispersion) smaller than 0.1.

Results

Solubility of the Amphiphiles in Aqueous Solutions. Before the characterization of the binding affinities between the ligands and the binding agents (proteins or lipid bilayers) the solubility of the ligand in the aqueous media must be known. This is a very important parameter because if the binding experiments are performed at ligand concentrations where aggregates are present the parameters obtained are apparent and depend on the total concentration of the ligand.[32] The results obtained for the critical aggregation concentration (CAC) of NBD-C_n ($n = 4-12$) are represented in Figure 1. For the amphiphiles with a longer alkyl chain ($n = 6-12$) the experiment was performed in aqueous buffer with 0.5% (*v*/*v*) methanol, and for NBD-C_4 the aggregation was evaluated in the absence of methanol. To ascertain the effect of methanol NBD-C_6 was studied in both solvents, and a small increase in the amphiphile solubility was observed due to the presence of 0.5% methanol (from 9 to 13 μM). The results obtained for a typical experiment with NBD-C_6 in aqueous buffer with 0.5% methanol are shown in Figure 1, plot A. For this amphiphile the CAC could be obtained from the dependence of its absorption or fluorescence with the total concentration, both leading to similar results if the fluorescence was measured at a wavelength and optical path length corresponding to an absorption smaller than 0.1. The CAC of smaller alkyl chains was assessed only from changes in its absorption and for longer ones only from the dependence of its fluorescence quantum yield on the total concentration of amphiphile. From Figure 1, plot A, it can be seen that deviations from linearity are stronger in the fluorescence than in the absorption of the solutions indicating a decrease in the fluorescence quantum yield of the amphiphiles in the aggregated form. For NBD-C_{10} it was also observed a small red shift on the fluorescence spectra upon aggregation. The absorption and/or fluorescence of the solutions with a concentration higher than the indicated CAC were not stable; it always decreased over time but after long intervals the dependence with the total concentration of amphiphile is poorly defined. This indicates that the aggregates are not stable in aqueous solution and slowly precipitate. Additionally, when the solutions were prepared by dissolving a film of the amphiphile, it was not possible to obtain concentrations much higher than the value indicated for the CAC. The critical aggregation concentration indicated is therefore similar to the solubility of the amphiphiles in the aqueous media.

The results obtained for NBD-C_n ($n = 4-12$) are shown in Figure 1 plot B. The Gibb's free energy for the equilibrium between the amphiphile dissolved in the aqueous media as monomer and its aggregates changes linearly with the length of the alkyl chain with a $\Delta\Delta G^0 = -1.4$ kJ/mol *per* methylene group. From the results obtained, the predicted solubility of NBD-NH_2 is equal to 50 mM and the solubility of the amphiphile with the longer alkyl chain used in this work, NBD-C_{16}, is 15 pM. The value obtained for NBD-C_8 is similar to that reported in the literature for comparable conditions.[29]

Partition of the Amphiphiles between the Aqueous Solution and POPC Bilayers. Given the solubility of the monomeric form of the amphiphiles, obtained in the previous section, the experimental conditions to obtain the intrinsic parameters for their binding to proteins and lipid bilayers may now be defined. The partition of the amphiphiles between an aqueous solution at pH 7.4 and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) bilayers was measured for those with a solubility of the monomeric form higher than 10 nM which corresponds to the sensibility limit of the method used (NBD-C_n; $n = 4-10$). The method was based on the fluorescence increase that occurs upon partitioning of the amphiphile to the lipid bilayers, typical titration curves are shown in Figure 2 plot A. We have used a simple partition model for the data analysis, where the lipid bilayer is treated as a different phase with a volume given by the molar volume of the lipid, V^-_{POPC}, and its concentration relative to the total volume, eq 1

$$I_F = [\text{NBD-C}_n]_T \frac{\Phi_{\text{NBD-C}_n} + \Phi_{\text{NBD-C}_n\text{\_POPC}} K_P \bar{V}_{\text{POPC}} [\text{POPC}]}{1 + K_P \bar{V}_{\text{POPC}} [\text{POPC}]} \quad (1)$$

The molar volume considered for POPC was 0.795 dm^3/mol,[33] and all lipid was considered (from the outer and inner monolayer of the LUVs) because we have found that the translocation of NBD-C_n occurs within minutes at 25 °C (Cardoso rms et al, unpublished work). The relative fluorescence quantum yields of the amphiphile in water, $\Phi_{\text{NBD-C}_n}$, and inserted in the POPC bilayer, $\Phi_{\text{NBD-C}_n\text{\_POPC}}$, as well as the partition coefficient, K_P, are obtained from the best fit of eq 1 to the experimental results using the solver facility of excel.

The Gibb's free energy for partition between the aqueous solution and the POPC bilayer changed linearly with the length of the alkyl chain up to C_{10}, Figure 2 plot B, with a $\Delta\Delta G^0 = -2.4 \pm 0.2$ kJ/mol. This dependence of the binding energy with the length of the alkyl chain is significantly smaller than that obtained for partition of amphiphiles between water and nonpolar solvents (−3.4 kJ/mol[34]). The results obtained are not easily compared with literature data on partition to lipid bilayers because there are very few studies with homologous series. Exceptions are the partition of primary alcohols (C_nOH; $n = 5-9$) to EggPC bilayers and the interaction of fatty acids and alkylated aminoacids with POPC bilayers that gave $\Delta\Delta G^0 = -2.7 \pm 0.7$ kJ/mol (with $K_P(C_9OH) = 1.4 \times 10^3$),[6] $\Delta\Delta G^0 = -3.4$ kJ/mol (with $K_P(C_{14}OO^-) = 1.8 \times 10^4$ and $K_P(C_{14}OOH) = 5 \times 10^6$),[11] $\Delta\Delta G^0 = -3.1$ kJ/mol (with $K_P(C_{14}OO^-) = 2.4 \times 10^3$)[8] and $\Delta\Delta G^0 = -3.3$ kJ/mol for BPTI-C_n ($n = 12-16$).[12] Those partition coefficients are much smaller than the ones obtained for the NBD-C_n homologous series with the same alkyl chain length (from the linear trend observed we estimate $K_P(\text{NBD-C}_9) = 1.3 \times 10^5$ and $K_P(\text{NBD-C}_{14}) = 1.8 \times 10^7$). The higher partition coefficient obtained in this work for the homologous series NBD-C_n reflects the different relative hydrophobicity of the ligands but it may also be a consequence of the high amphiphile concentrations used in most published work. In this study we have used extremely small ligand concentrations and this guaranty that only the monomeric form of the amphiphile is present in the aqueous solution. At high ligand concentrations aggregation in the aqueous phase cannot be excluded and this would artificially decrease the partition coefficient observed.[32] Additionally, when using large ligand concentrations relative to the total solution volume, the high local concentrations of the ligand attained in the lipid bilayer affect the membrane properties leading to changes in the apparent partition coefficient.[31]

The results shown in Figure 2 have been obtained at 25 °C. The experiments have also been performed at 15 and 35 °C to obtain the thermodynamic parameters for the partition of NBD-C_n between water and the POPC bilayer, the results obtained are shown in Figure 3. The partition coefficient decreased slightly with the increase in temperature for all the amphiphiles in the homologous series. The thermodynamic parameters for the interaction were obtained using the Van't Hoff equation and conduced to an enthalpy of -15 ± 4 kJ/mol independent of the length of the alkyl chain, Figure 3 plot B. The entropy variation upon partition to the POPC bilayer is positive for all amphiphiles studied and its contribution to the partition Gibbs free energy increases with the length of the alkyl chain ($T\Delta S$ varies from 4 to 17 kJ/mol from NBD-C_4 to NBD-C_{10}), Table 1. These results indicate that there is a favorable interaction between the polar NBD group and the POPC bilayer (corresponding to a negative enthalpy) and entropy driven interactions between the nonpolar alkyl chain of the amphiphiles and the POPC bilayer (compatible with the expected thermodynamic parameters for interactions driven by the hydrophobic effect). From the results obtained, the thermodynamic parameters for the partition of other amphiphiles in this homologous series may be calculated and one predicts $\Delta H = -14$ kJ/mol and $T\Delta S = -7$ kJ/mol for the polar NBD group ($n = 0$) and $\Delta H = -16$ kJ/mol and $T\Delta S = 31$ kJ/mol for the amphiphile with the longer alkyl chain studied ($n = 16$).

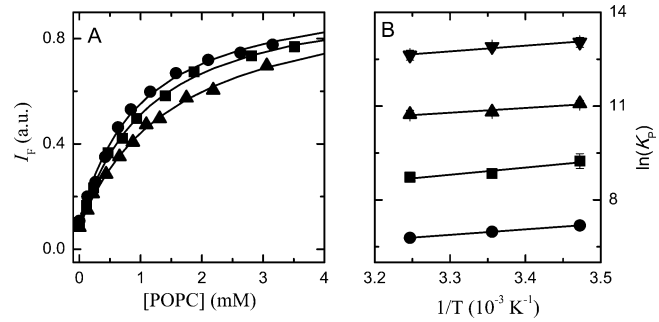

Figure 3. Temperature effect on the partition of NBD-C_n between an aqueous solution, pH 7.4, and POPC bilayers. Plot A: Typical results obtained for the titration of NBD-C_4 at 15 (●), 25 (■), and 35 °C (▲), the line is the best fit of eq 1 with $K_P = 1.3 \times 10^3$, 1.1×10^3, and 8.1×10^2, respectively. Plot B: Van't Hoff plots of the partition coefficient of all NBD-C_n studied, the values shown are the average of at least 3 independent experiments and the standard deviation is smaller than the symbols shown. The line is the best fit of a straight line with $\Delta H = -13$, -17, -14, and -15 kJ/mol for NBD-C_4, C_6, C_8, and C_{10}, respectively.

Binding of the Amphiphiles by Bovine Serum Albumin. Another relevant parameter that determines the bioavailability of the ligands is their binding to proteins present in biological fluids. Albumin is the most abundant protein in serum and is generally used as a model protein to mimic the binding ability of proteins in biological fluids. The equilibrium constant for binding of the amphiphiles to BSA at 25 °C is shown in Figure 4. The experiments were done at a large excess of the binding agent and therefore the equilibrium constants presented correspond to the highest affinity binding site. The binding constants were obtained from the best fit of eqs 2 and 3

$$[\text{NBD-C}_n\text{\_BSA}] = \frac{[\text{NBD-C}_n]_T K_B [\text{BSA}]}{1 + K_B[\text{BSA}]} \cong \frac{[\text{NBD-C}_n]_T K_B [\text{BSA}]_T}{1 + K_B[\text{BSA}]_T} \quad (2)$$

$$I_F = \Phi_{\text{NBD-C}_n} [\text{NBD-C}_n] + \Phi_{\text{NBD-C}_n\text{\_BSA}} [\text{NBD-C}_n\text{\_BSA}] \quad (3)$$

Typical titration curves obtained for all amphiphiles studied (NBD-C_n: $n = 4-10$) are shown in plot A and the dependence of the binding constant with the length of the alkyl chain is shown in plot B together with literature values for the binding of fatty acids to serum albumins. The best fit of a straight line to the energetics of binding of the NBD-C_n homologous series is also shown in Figure 4 plot B corresponding to $\Delta\Delta G^0 = -2.6 \pm 0.9$ kJ/mol. The average value is similar to that found for partition between water and the POPC bilayer, but a clear deviation from a linear behavior is evident indicating a sigmoid dependence with the length of the alkyl chain. This behavior was also observed for the binding of fatty acids, and the

TABLE 1: Equilibrium Parameters for the Aqueous Solubility of NBD-C_n in the Monomeric Form and Its Interaction with POPC Bilayers and with BSA, at 25 °C

		partition to POPC			binding to BSA		
NBD-C_n	CAC (M)	K_P	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	K_B (M^{-1})	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)
$n = 4$	$2.5 \pm 0.3 \times 10^{-4}$	$1.1 \pm 0.1 \times 10^{3}$	-13 ± 4	4 ± 4	$1.7 \pm 0.1 \times 10^{4}$	-15 ± 2	9 ± 2
$n = 6$	$1.1 \pm 0.1 \times 10^{-5}$	$6.9 \pm 1.0 \times 10^{3}$	-17 ± 4	5 ± 4	$6.3 \pm 0.3 \times 10^{4}$		
$n = 8$	$1.3 \pm 0.1 \times 10^{-7}$	$4.8 \pm 0.3 \times 10^{4}$	-14 ± 4	13 ± 4	$5.2 \pm 0.3 \times 10^{4}$		
$n = 10$	$5.0 \pm 1.3 \times 10^{-8}$	$4.0 \pm 0.4 \times 10^{5}$	-15 ± 4	17 ± 4	$6.3 \pm 1.3 \times 10^{6}$	-17 ± 5	21 ± 5

relatively higher affinity of the amphiphiles with an intermediate length of the alkyl chain was taken as evidence for a somewhat well-defined length of the albumin binding site. The data shown in Figure 4 corresponds to the binding of fatty acids by bovine[7] and human[3−5,10] serum albumin at 37 °C. Results for the binding of all fatty acids at 25 °C are not available in the literature, but there is evidence for a small increase in the binding constant when the temperature is decreased to 25 °C.[10,35] The comparison of the results reported for HSA and BSA indicates a stronger binding affinity between the fatty acids and bovine serum albumin.[5,36] Our results are therefore comparable to the binding constants of fatty acids to the higher affinity binding site of BSA.

The binding of NBD derivatives to BSA as been previously reported by other authors[9] and a sigmoidal dependence on the length of the alkyl chain was also observed. The binding constants reported are substantially different from those obtained in this work and a quantitative comparison is not possible. The discrepancy may be due to the different experimental methodologies followed, where higher amphiphiles concentrations conduced to binding at BSA saturation conditions.

The temperature dependence of the binding constant of NBD-C_4 and NBD-C_{10} was also studied and the results obtained are shown in Figure 5. The enthalpy contribution to the interaction is similar for both amphiphiles (−15 and −17 kJ/mol for n = 4 and 10 respectively) and similar to that obtained for the partition of those amphiphiles to POPC bilayers. The entropy contribution to the Gibb's free energy of binding was always favorable and increased with the length of the alkyl chain, in agreement with that predicted by the hydrophobic effect. The values obtained in this work for the enthalpy of the interaction of NBD-C_n with BSA are significantly less negative than those reported for the binding of laurate and myristate to the highest affinity binding site of HSA[10] but similar to those reported for the binding of decanoate to HSA[35] and for the binding of short and medium chain fatty acids to BSA.[7]

In addition to the binding affinity it is very important to know the binding site in the protein because competition between different ligands in the biological media will influence the amount of amphiphile bound. To evaluate the identity of the binding site, we have titrated NBD-C_{10} with BSA in the presence of different concentrations of the fatty acid with the same alkyl chain length (FA-C_{10}). The results obtained for a total concentration of NBD-C_{10} equal to 40 nM are shown in Figure 6.

The experimental results were analyzed with the equilibrium equations assuming a single binding site in BSA and competitive binding of NBD-C_{10} and decanoic acid (FA-C_{10}) to the same binding site, kinetic Scheme 1 and eq 4. The conversion between

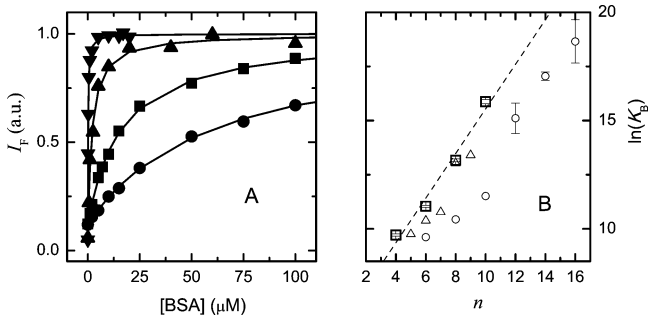

Figure 4. Binding to bovine serum albumin, pH 7.4, at 25 °C. Plot A: Typical results obtained for the titration of NBD-C_4 at 100 nM (●), NBD-C_6 at 50 nM (■), NBD-C_8 at 50 nM (▲), and NBD-C_{10} at 25 nM (▼). The line is the best fit of eqs 2 and 3 with $K_B = 1.7 \times 10^4$, 6.2×10^4, 5.2×10^5, and 7.4×10^6 M^{-1}, respectively. Plot B: Average and standard deviation of the logarithm of the binding constant of each NBD-C_n for at least 3 independent experiments (□). The average and standard deviation for the literature values of the binding of fatty acids to the highest affinity binding site of bovine (Δ)[7] and human (○)[3−5,10] serum albumin at 37 °C is also shown. The line is the best fit of a straight line to the results obtained in this work for NBD-C_n with $\Delta\Delta G = -2.6 \pm 0.9$ kJ/mol.

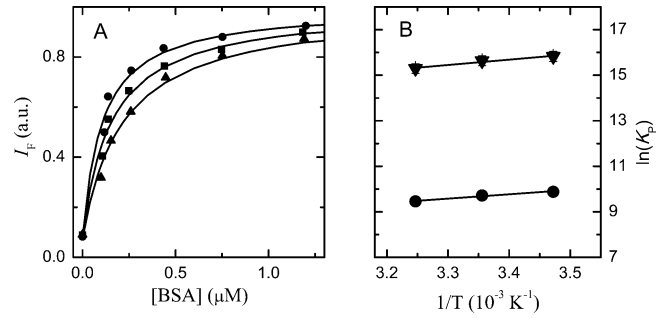

Figure 5. Temperature effect on the binding of NBD-C_n between an aqueous solution, pH 7.4, and BSA. Plot A: Typical results obtained for the titration of NBD-C_{10} at 15 (●), 25 (■), and 35 °C (▲), the line is the best fit of eqs 2 and 3 with $K_B = 9.4 \times 10^6$, 6.4×10^6, and 4.6×10^6 M^{-1}, respectively. Plot B: Van't Hoff plots of the binding constant for NBD-C_4 and C_{10}, the values shown are the average of at least three independent experiments and the standard deviation is smaller than the symbols shown. The line is the best fit of a straight line with $\Delta H = -15$ and −17 kJ/mol for NBD-C_4 and C_{10}, respectively.

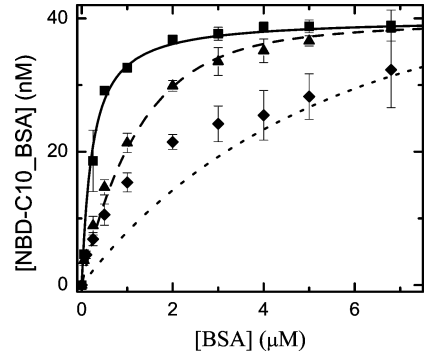

Figure 6. Binding of NBD-C_{10} to bovine serum albumin, pH 7.4, at 25 °C in the presence of different concentrations of decanoic acid: 0 (■), 2.5 (▲), or 10 μM (♦). The lines correspond to the global best fit of eq 4 to the titrations performed in the presence of decanoic acid at concentrations ≤2.5 μM leading to $K_B = 4.8 \times 10^6$ M^{-1} and $K_I = 2.0 \times 10^6$ M^{-1}.

SCHEME 1

$$\text{NBD-C}_{10} + \text{BSA} \xleftrightarrow{K_B} \text{NBD-C}_{10}\_\text{BSA}$$

$$\text{FA-C}_{10} + \text{BSA} \xleftrightarrow{K_I} \text{FA-C}_{10}\_\text{BSA}$$

the fluorescence intensity observed and the amount of amphiphile bound was performed according to eq 3.

$$[\text{NBD-C}_{10}\_\text{BSA}] = \frac{[\text{NBD-C}_{10}]_T K_B[\text{BSA}]}{1 + K_B[\text{BSA}]}$$

$$[\text{FA-C}_{10}\_\text{BSA}] = \frac{[\text{FA-C}_{10}]_T K_I[\text{BSA}]}{1 + K_I[\text{BSA}]}$$

$$[\text{BSA}] = [\text{BSA}]_T - [\text{FA-C}_{10}\_\text{BSA}] - [\text{NBD-C}_{10}\_\text{BSA}] \quad (4)$$

For concentrations of fatty acid equal to 2.5 μM or smaller, the model describes the experimental results very well leading to the parameters: $K_B = 4.8 \times 10^6$ M^{-1} and $K_I = 2.0 \times 10^6$ M^{-1}. At 5 μM of FA-C$_{10}$ deviations from the behavior predicted by eq 4 are visible and at higher concentrations the misfit is evident, see titration at 10 μM of FA-C$_{10}$ in Figure 6. At those high concentrations of FA-C$_{10}$, the fraction of NBD-C$_{10}$ bound to BSA (observed fluorescence) is higher than the amount predicted. The deviations from the predicted behavior are only significant for small concentrations of BSA, where the ratio of fatty acid to protein is high ($[\text{FA} - \text{C}_{10}]_T/[\text{BSA}]_T > 1$). Those results indicate that the binding of the second fatty acid affects the ability of albumin to bind NBD-C$_{10}$ increasing the binding affinity. Allosterism in the binding of ligands by BSA has been observed before.[35]

The value obtained for the binding constant of decanoic acid to BSA is significantly higher than that usually considered, the reference value for binding to the highest affinity site on HSA is $K_B = 1 \times 10^5$ M^{-1} at 37 °C,[3] but similar to some of the binding affinities reported in the literature, $K_B = 1 \times 10^6$ M^{-1} at 25 °C.[35] As discussed above, the difference may be due to the effect of temperature ($K_B = 3.5 \times 10^6$ M^{-1} at 4 °C[35]), or due to different processes of protein purification.[35] Direct measurements of binding of decanoic acid to BSA are not available but the results for shorter alkyl chain fatty acids indicate that the binding affinity of BSA is stronger than that of HSA.[5,36] The equilibrium constant obtained in this work for the binding of decanoic acid to BSA is therefore in agreement with the expected value and allow us to conclude that NBD-C$_{10}$ binds to the highest affinity site of FA-C$_{10}$ on BSA.

Photophysical Characterization of NBD-C$_n$ Inserted in POPC Bilayers. After knowing the binding affinities between the ligands and the typical binding agents found in biological media it is important to know the location of the bound amphiphile. In the previous section we have obtained information regarding the binding site on BSA from the competitive binding with fatty acids. Lipid bilayers do not possess well-defined binding sites and the relevant parameter is the depth position of the amphiphile. In this section we will obtain information regarding this parameter from the photophysical properties of the amphiphiles inserted in the POPC bilayer and their dependence on the length of the alkyl chain.

The results obtained for the time-resolved and steady state fluorescence emission of NBD-C$_n$ inserted in POPC bilayers are shown in Figures 7−10.

The fluorescence lifetime of the amphiphiles in methanol was 5.2 ± 0.1 ns independently of the length of the alkyl chain. The behavior in aqueous solution was also studied for NBD-C$_6$ and it was well described by a monoexponential with a lifetime of 0.9 ns. In contrast, the amphiphiles inserted in POPC bilayers showed a complex fluorescence decay that, at the experimental conditions reported, was well described by a biexponential, Figure 7. The short lifetime is not due to amphiphile in the aqueous phase; at the lipid concentration used, 0.5 mM, more than 95% is inserted in the lipid bilayer for all the amphiphiles studied. The complex decay is therefore originated from the amphiphile inserted in the membrane. Both lifetimes, and respective amplitudes, are relatively independent of the alkyl chain length except for a small monotonic decrease in the lifetime of the long-lived component. There is also a significant increase in the weight of the small lifetime component for NBD-C$_{14}$.

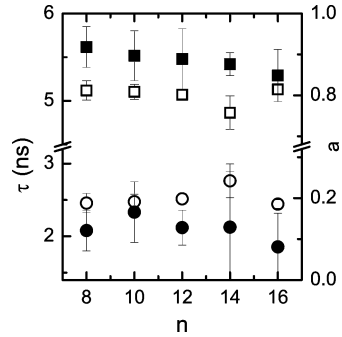

Figure 7. Time resolved fluorescence emission of NBD-C$_n$ inserted in POPC bilayers at a probe to lipid ratio of 1:100 for $\lambda_{exc} = 460$ and $\lambda_{em} = 530$ nm and 25 °C. The values shown are the average and standard deviation of the fluorescence lifetimes (■ and ●) and their respective amplitudes (□ and ○) obtained from 3 to 4 independent experiments.

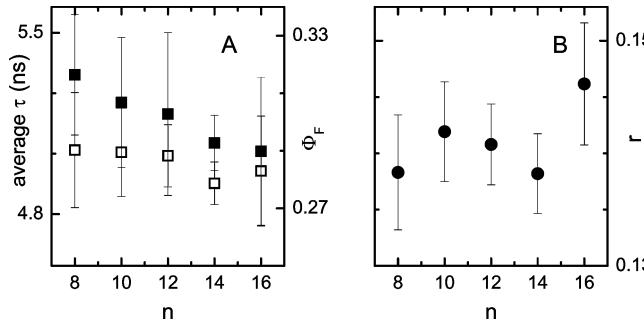

Figure 8. Fluorescence emission parameters for NBD-C$_n$ inserted in POPC bilayers at a probe to lipid ratio of 1:100 and 25 °C. Plot A: average lifetime (■) and fluorescence quantum yield (□); the values shown correspond to the average and standard deviation of 2 (Φ_F) or 3 ($\bar{\tau}$) independent experiments. Plot B: Average value of the fluorescence anisotropy in the range $\lambda_{em} = 525-560$ nm for $\lambda_{exc} = 475$ nm, (●). The values shown correspond to the average and standard deviation of four independent experiments.

The average lifetime, weighted by the fluorescence intensity,[37] is shown in Figure 8, plot A, and a small, but significant, decrease with the increase in the alkyl chain length is observed. The fluorescence quantum yield was also measured and its absolute value was calculated with respect to that of NBD-DMPE, $\Phi_F = 0.4$.[38] The fluorescence quantum yield obtained for all amphiphiles in the NBD-C$_n$ homologous series, inserted in the POPC bilayers, is significantly smaller than that of NBD-DMPE and does not show a strong dependence on the length of the alkyl chain. There is only a small decrease from NBD-C$_{10}$ to NBD-C$_{16}$, in agreement with the decrease observed in the average lifetime, and the fluorescence quantum yield obtained for NBD-C$_{14}$ is somewhat below the overall behavior. The fluorescence emission spectrum was the same for all

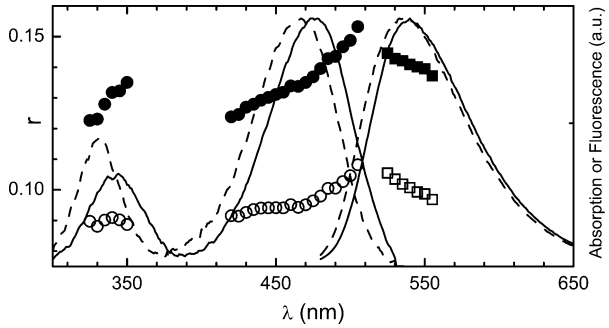

Figure 9. Absorption and fluorescence spectra of NBD-C_{16} (—) and NBD-DMPE (– –) inserted in POPC bilayers at a probe to lipid ratio of 1:100 and 25 °C. The fluorescence anisotropy as a function of the excitation wavelength for $\lambda_{em} = 540$ nm (●, ○), and as a function of the emission wavelength for $\lambda_{exc} = 475$ nm (■, □), is also shown.

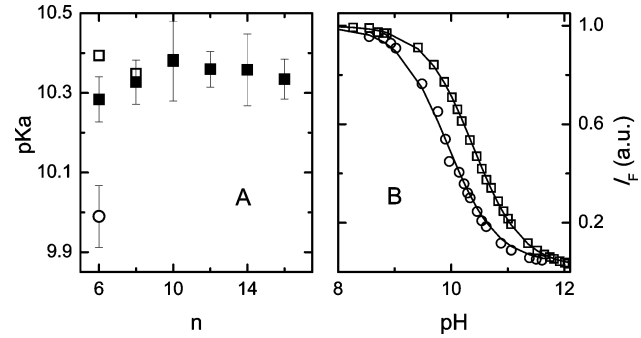

Figure 10. Ionization properties of NBD-C_n in the alkaline region of pH for a lipid concentration of 0.5 mM and a probe to lipid ratio of 1:100, at 25 °C. Plot A: Ionization constant of NBD-C_6 in aqueous solution (○) and NBD-C_n inserted in POPC bilayers (■). The pK_a corrected for the fraction of amphiphile in the aqueous phase is also shown (□). Plot B: Typical titration curve obtained for NBD-C_6 in aqueous solution (○) and for NBD-C_{16} inserted in the lipid bilayer (□).

amphiphiles in the series except for NBD-C_{14} that was somewhat blue-shifted with an intermediate behavior between the remaining NBD-C_n and NBD-DMPE (results not shown). [The small blue shift in the emission spectra of NBD-C_{14} was observed for excitation in the red edge. This effect was however only present in some preparations of the amphiphile in POPC bilayers.] It should be noted that the fluorescence quantum yield shown in Figure 8 was calculated from the total fluorescence emission, and not from the intensity at a given wavelength, it is therefore not directly affected by shifts in the fluorescence spectra.

The steady state anisotropy (r) in the emission range 525−560 nm, for excitation at 475 nm, is shown in Figure 8, plot B. The variation observed in the average lifetime predicts a small increase in the anisotropy with the length of the alkyl chain and this trend is in fact observed. However, the behavior observed for NBD-C_{14} is again distinct from that of the other amphiphiles in the homologous series and the anisotropy recovered is somewhat smaller. The anisotropy obtained for all NBD-C_n amphiphiles is significantly higher than that of NBD-DMPE inserted in DOPC bilayers and excited at 465 nm, $r = 0.093$,[39] or inserted in POPC bilayers for $\lambda_{exc} = 475$ nm ($r = 0.10$, Figure 9). This difference is in agreement with the longer lifetime observed for NBD-DMPE ($\bar{\tau} = 7.8$ nm[39]), allowing a more efficient depolarization during the excited state, but is much higher than predicted by the Perrin equation for the dependence of r on the excited state lifetime.[37]

The anisotropy of NBD amphiphiles inserted in micelles or lipid bilayers has been reported to depend on the excitation (and emission) wavelength.[39,40] The behavior found for the NBD-C_{16} and NBD-DMPE in POPC bilayers is shown in Figure 9. The results obtained for all NBD-C_n amphiphiles were very similar with a small shift in the anisotropy values, Figure 8 plot B, but the same dependence with the excitation (emission) wavelength.

The results obtained for NBD-DMPE are very similar to those reported for this amphiphile inserted in DOPC bilayers with a small increase in the value of the anisotropy as the excitation is shifted toward the red and a significant increase in the red edge of the spectra.[39] For excitation at longer wavelengths than those shown in Figure 9, the anisotropy increases even more sharply, but it is strongly affected by the scattering from the lipid vesicles. The anisotropy obtained for the NBD-C_n amphiphiles is always higher than that of NBD-DMPE and shows a stronger dependence on the excitation wavelength outside the red edge of the excitation spectra. The normalized absorption and emission spectra of NBD-C_{16} and NBD-DMPE, inserted in the POPC bilayers, are also shown in Figure 9. There is a 10 nm shift in the absorption maximum, both for the $\pi\pi^*$ and CT absorption bands,[27] and this is reduced to 4 nm in the fluorescence emission spectra.

The ionization constant of NBD-C_n is an additional property that may give some insight regarding the properties of the environment around of the NBD group. Two ionization constants have been reported for NBD covalently bound to aliphatic amines, one at very low values of pH and the second at slightly alkaline pH.[25] The NMR spectra of water-soluble NBD derivatives[25] and the zeta potential of NBD-DMPE inserted in DOPC vesicles[41] indicates that at neutral pH the most abundant species is globally neutral with the fatty amine being un-ionized. In alkaline solutions the NBD plus amine becomes negative and nonfluorescent but the structure of the anionic form is still not well established.[41] We have followed the decrease in the fluorescence intensity as a function of the increase in the pH and the ionization constants for NBD-C_6 in aqueous solution and NBD-C_n inserted in POPC bilayers were obtained, the results are shown in Figure 10. The pK_a obtained for NBD-C_6 in aqueous solutions, 9.99 ± 0.08, is in good agreement with that found previously for other amine linked NBD amphiphiles.[25] Insertion of NBD-C_6 in the POPC bilayer increases the value of its pK_a by 0.3 units, to 10.28 ± 0.06. The ionization constant does not depend strongly on the length of the alkyl chain although there a significant variation with a higher value of pK_a for the amphiphiles with intermediate chain length. The decrease in pK_a for the shorter amphiphiles may be due to a significant fraction in the aqueous phase and the predicted values after this correction are also shown in Figure 10. In fact, after this correction, the results indicate that the value of pK_a of the amphiphiles inserted in the POPC bilayer decreases slightly with the increase in the alkyl chain length.

The values found in this work for the ionization constant of NBD-C_n inserted in POPC bilayers are much smaller than the reported values for NBD-PE in DOPC bilayers, p$K_a = 11.5$.[41] This difference is not due to the presence of the phosphate group in the case of NBD-PE because the ionization constant of deacylated NBD-PE in aqueous solution is similar to the value found in this work for NBD-C_6, p$K_a \cong 10$.[41] The lower pK_a values obtained for NBD-C_n must therefore reflect the different environment around the NBD group when inserted in the lipid bilayer.

Discussion

The thermodynamic parameters obtained for the interaction of the amphiphiles with POPC bilayers and with BSA indicated the existence of favorable interactions between the polar portion of the amphiphile (NBD group) and the lipid bilayer as well as with the protein. Additionally, the nonpolar portion of the amphiphile (alkyl chain) increased the binding affinity via an increase in the entropy of the bound state which changed fairly linearly with the length of the alkyl chain. This conduced to more negative values of the Gibbs free energy of amphiphile association with POPC bilayers and BSA as the length of the alkyl chain increased with similar increments for association with both binding systems, −2.4 and −2.6 kJ/mol per methylene group, respectively. The incremental variation obtained for the aggregation Gibb's free energy was however significantly smaller, −1.4 kJ/mol. This difference indicates that the hydrophobic portion of the amphiphile that is hidden from water upon aggregation is much smaller than that removed from water contact due to association with POPC bilayers or BSA being compatible with a dimerization of the amphiphiles at concentrations immediately above the CAC reported.

The nonlinear increment on the Gibb's free energy along the homologous series for association with BSA indicates the existence of an optimal length of the alkyl chain that conduces to a relatively stronger interaction, and this was expected due to limited size of the hydrophobic pocket in the protein binding site. The structure of HSA associated with palmitic acid has been characterized[42] and the results show that the 16 carbon alkyl chain is not in its stretched conformation indicating a smaller optimal length for the ligand hydrophobic chain.

Length dependent values for the $\Delta\Delta G$ could also be originated from a non linear dependence of the ligand energy in the aqueous phase (due to folding of the hydrocarbon chain or amphiphile aggregation)[34] as this behavior was also observed for partition of long chain fatty acids between aqueous and nonpolar organic phases.[43] It is generally assumed that folding of the hydrocarbon chain is only significant for lengths equal to or higher than 16 carbons[34] and only the monomer is present in the studies performed in this work. Therefore, length dependent values of $\Delta\Delta G$ obtained in this work should reflect distinct energetics for the solvation of the additional hydrocarbon segments in the binding agent.

Lipid bilayers are highly structured in the direction perpendicular to the bilayer surface with four regions being clearly identified, two of which in the hydrophobic region.[44] As the length of the alkyl chain is increased, the amphiphile is expected to interact with additional regions in the lipid bilayer and their distinct solvation properties may conduce to non linear increments in the Gibb's free energy of the interaction. The center of the bilayer behaves as decane and extends up to about 6 Å from the center of the lipid bilayer (from C_{16} to $\sim C_{10}$ of a DPPC bilayer) followed by a region with properties of a soft polymer that extends up to 20 Å (from $\sim C_9$ to carbonyl).[44,45] In this work we report on the energies of the interaction of NBD-C_n with POPC bilayers for $n = 4-10$, and we have obtained a linear dependence of the partition Gibb's free energy with the length of the alkyl chain. The value obtained for $\Delta\Delta G$ was also independent of temperature (from 15 to 35 °C) reflecting the same enthalpic contribution to the partition of all amphiphiles studied (C_4-C_{10}). This behavior can result from similar solvation properties of both regions in the lipid bilayer or may be an indication that the alkyl chain of NBD-C_{10} is only in the soft polymer region of the lipid bilayer. Unfortunately the two possible behaviors cannot be distinguished because the partition of longer amphiphiles in this series cannot be measured accurately due to aggregation in the aqueous phase. We are currently performing Molecular Dynamics simulations to obtain information regarding the location of the amphiphile alkyl group in the POPC bilayer.

On the basis of the competitive binding of NBD-C_{10} and FA-C_{10} to BSA, we conclude that the highest affinity binding site is the same for both ligands. The crystallographic structure of fatty acids bound to HSA shows the stabilization of the bound ligand by formation of hydrogen bonds and proximity of positive charges.[42] The binding affinity obtained for NBD-C_n is actually stronger than that of FA-C_{10} (4.8 vs 2 μM^{-1}), and at first, it is difficult to reconcile the involvement of electrostatic interactions in the stabilization of the complex and binding to the same site in the protein. It should however be noticed that, at pH 7.4, BSA has nearly 200 charged aminoacids with a net charge of −17.[46] The lost of the electrostatic interaction with the carboxylate may easily be compensated by favorable interactions between NBD (with a large dipole moment[27]) and nearby charged groups on BSA.

We have undertaken an extensive characterization of the photophysics of the homologous series of amphiphiles (NBD-C_n) when inserted in POPC bilayers, to gain insight regarding the location of the NBD group and the effect of the alkyl chain length. The parameters obtained do not change strongly along the series indicating a relatively similar position for the NBD group. An exception is noted for NBD-C_{14} that systematically shows deviations from the trend observed for the other amphiphiles: larger amplitude for the short fluorescence lifetime component, smaller fluorescence quantum yield, small blue shift in the emission spectra, and lower steady state fluorescence anisotropy. The interpretation of those results in terms of the location of the NBD group is however very difficult because the photophysical parameters of NBD amphiphiles associated with lipid bilayers are still not well understood. The multi exponential fluorescence decay is not observed in homogeneous liquids with a low viscosity but has been observed for some solvent mixtures and viscous solvents[26,27] being usually found for NBD amphiphiles inserted in micelles or lipid bilayers.[38−40] Several possible explanations have been indicated such as quenching due to hydrogen bonding with the solvent,[26] self-quenching,[38,47] solvent relaxation,[39] internal conversion to nonemissive excited states,[27] or distinct NBD populations,[28,39] but the reconciliation of all the different parameters and distinct media has not been done. Nevertheless, the relative weight of the two lifetimes is similar for all NBD-C_n and comparable to that observed for NBD-PE under equivalent conditions.[38,39,47] The lifetime of both components is significantly smaller for NBD-C_n and from this result one could deduce that the NBD group is in a more polar environment[27] than for the case of NBD-DMPE. This interpretation is corroborated by the lower energy of the absorption and fluorescence bands of NBD-C_n and their smaller fluorescence quantum yields and could justify the smaller change in pK_a observed for NBD-C_n upon insertion in the lipid bilayer. Being in a more polar environment, it is tempting to conclude that the NBD group of NBD-C_n is more exposed to the aqueous solution, at a larger distance from the center of the bilayer, than in the case of NBD-DMPE. When covalently bound to the headgroup of PE phospholipids, the NBD group was found to lay at a distance between 20 and 31 Å from the bilayer midplane, at or above the lipid phosphate group.[48] It is therefore difficult to convey a larger distance for the case of NBD-C_n. Additionally, the significantly higher anisotropy of NBD-C_n ($\cong$0.14 as compared with 0.10) indicates

that the group is in a region with a higher viscosity (the difference cannot be explained only on the basis of the smaller average lifetime using the Perrin equation[37]). Another property that has been used as indication of restricted mobility and slow solvent relaxation is the red edge excitation shift (REES). The fluorescence emission maxima of NBD-C_n inserted in POPC bilayers is at 540 nm for excitation at 460 nm and is shifted to 543 nm for $\lambda_{exc} = 500$ nm. At those conditions we have observed a red shift of 4 nm for NBD-DMPE, from 536 to 540 nm, similar to that observed in DOPC bilayers[39] although the spectra obtained by us in POPC bilayers are significantly red-shifted. The magnitude of the REES indicates that the NBD group of NBD-C_n is in a region similar to that of NBD-DMPE although with a mobility somewhat less restricted. This result is in agreement with the relative values obtained for the fluorescence lifetimes and quantum yields as well as variation of pK_a upon insertion, but it is incompatible with the higher values obtained for the fluorescence anisotropy of NBD-C_n.

The photophysical parameters of NBD-C_n can also be compared with those of NBD-PC (NBD covalently bound to the acyl chain of phosphatidyl cholines). The photophysical parameters obtained for NBD-C_n are in fact very similar to those observed for NBD-PC: higher steady-state anisotropy, smaller average lifetime, and emission at lower energies when compared with NBD-PE.[49] Using photophysical methods and molecular dynamic simulations, it has been proposed that the NBD group of NBD-PC is localized in the glycerol-phosphate region when inserted in PC bilayers,[49−53] at a similar position as NBD from NBD-PE, allowing to conclude that the position of the NBD group in the lipid bilayer is not significantly constrained by the linkage to the acyl chain of the PC molecule or to the headgroup of PE. The similar location proposed for the NBD group from both NBD-PE and NBD-PC, despite the relatively distinct photophysical properties of both fluorophores when inserted in the lipid bilayer, indicates that the relation between the photophysical parameters of NBD and its location in the lipid bilayer is not straightforward being dependent on specific interactions with the solvent and not simply on the average properties of the solvent at a given depth in the lipid bilayer.[54]

We cannot therefore interpret the distinct behavior observed for NBD-C_{14} as compared with the other amphiphiles along the homologous series. However, the non monotonic dependence on the length of the alkyl chain indicates that the interactions between the alkyl chain and the lipid bilayer are relevant. Modification of proteins by acylation is a prevalent mechanism for their reversible interaction with biological membranes and other proteins, and myristoylation is most commonly observed.[21,55] It is therefore tempting to relate the distinct behavior observed for NBD-C_{14} with a specific role for this length of the hydrophobic tail. The singularity may be associated with the depth dependent properties of the lipid bilayers that change from a soft polymer to a liquid like solvent at the center of each monolayer, corresponding approximately to the position of the POPC double bond. We are currently performing molecular dynamic simulations on the homologous series of NBD-C_n inserted in POPC bilayers to gain a better understanding on the interactions of the amphiphile with the POPC bilayer and on their effects on the location of the NBD and methyl groups.

Acknowledgment. The authors acknowledge Júlio Lopes Sampaio for the execution and analysis of the mass spectrometry measurements as well as João Sérgio Melo and João Pina for the help with the execution and analysis of the time-resolved fluorescence measurement. NMR data was obtained at the Nuclear Magnetic Resonance Laboratory of the Coimbra Chemistry Centre, Universidade de Coimbra, supported in part by Grant REEQ/481/QUI/2006 from FCT, POCI-2010 and FEDER, Portugal. This work was supported by research grants from the Portuguese Ministry for Science and Higher Education through FCT, via the PTDC program cofinanced by the European Union.

Supporting Information Available: Characterization of the purity of the fluorescent amphiphiles used in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Andreas, M.; Peter, P. *ChemPhysChem* **2009**, *10*, 1405.
(2) Sawada, G. A.; Barsuhn, C. L.; Lutzke, B. S.; Houghton, M. E.; Padbury, G. E.; Ho, N. F. H.; Raub, T. J. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 1317.
(3) Ashbrook, J. D.; Fletcher, J. E.; Spector, A. A. *J. Biol. Chem.* **1972**, *247*, 7038.
(4) Ashbrook, J. D.; Spector, A. A.; Santos, E. C.; Fletcher, J. E. *J. Biol. Chem.* **1975**, *250*, 2333.
(5) Spector, A. A. *J. Lipid Res.* **1975**, *16*, 165.
(6) Jain, M. K.; Wray, L. V. *Biochem. Pharmacol.* **1978**, *27*, 1294.
(7) Brown, N. A.; Wilson, A. G. E.; Bridges, J. W. *Biochem. Pharmacol.* **1982**, *31*, 4019.
(8) Froud, R. J.; East, J. M.; Rooney, E. K.; Lee, A. G. *Biochemistry* **1986**, *25*, 7535.
(9) Matsushita, Y.; Takahashi, M.; Moriguchi, I. *Chem. Pharm. Bull.* **1986**, *34*, 333.
(10) Pedersen, A. O.; Honore, B.; Brodersen, R. *Eur. J. Biochem.* **1990**, *190*, 497.
(11) Peitzsch, R. M.; McLaughlin, S. *Biochemistry* **1993**, *32*, 10436.
(12) Pool, C. T.; Thompson, T. E. *Biochemistry* **1998**, *37*, 10246.
(13) Murray, D.; Matsumoto, L. H.; Buser, C. A.; Tsang, J.; Sigal, C. T.; Ben-Tal, N.; Honig, B.; Resh, M. D.; McLaughlin, S. *Biochemistry* **1998**, *37*, 2145.
(14) Martins, P. A.; Gomes, F.; Vaz, W. L. C.; Moreno, M. J. *Biochim. Biophys. Acta* **2008**, *1778*, 1308.
(15) Estronca, L. M. B. B.; Moreno, M. J.; Vaz, W. L. C. *Biophys. J.* **2007**, *93*, 4244.
(16) Moreno, M. J.; Estronca, L. M. B. B.; Vaz, W. L. C. *Biophys. J.* **2006**, *91*, 873.
(17) Sampaio, J. L.; Moreno, M. J.; Vaz, W. L. C. *Biophys. J.* **2005**, *88*, 4064.
(18) Estronca, L. M. B. B.; Moreno, M. J.; Laranjinha, J. A. N.; Almeida, L. M.; Vaz, W. L. C. *Biophys. J.* **2005**, *88*, 557.
(19) Abreu, M. S. C.; Moreno, M. J.; Vaz, W. L. C. *Biophys. J.* **2004**, *87*, 353.
(20) Nadolski, M. J.; Linder, M. E. *FEBS J.* **2007**, *274*, 5202.
(21) Resh, M. D. *Biochim. Biophys. Acta-Mol. Cell Res.* **1999**, *1451*, 1.
(22) de Melo, J. S.; Fernandes, P. F. *J. Mol. Struct.* **2001**, *565*, 69.
(23) Kalvodova, L.; Sampaio, J. L.; Cordo, S.; Ejsing, C. S.; Shevchenko, A.; Simons, K. *J. Virol.* **2009**, *83*, 7996.
(24) Schwudke, D.; Hannich, J. T.; Surendranath, V.; Grimard, V.; Moehring, T.; Burton, L.; Kurzchalia, T.; Shevchenko, A. *Anal. Chem.* **2007**, *79*, 4083.
(25) Meyers, H. W.; Jurss, R.; Brenner, H. R.; Fels, G.; Prinz, H.; Watzke, H.; Maelicke, A. *Eur. J. Biochem.* **1983**, *137*, 399.
(26) Lin, S.; Struve, W. S. *Photochem. Photobiol.* **1991**, *54*, 361.
(27) Feryforgues, S.; Fayet, J. P.; Lopez, A. *J. Photochem. Photobiol. A-Chem.* **1993**, *70*, 229.
(28) Galinier, F.; Bertorelle, F.; Fery-Forgues, S. *C. R. Acad. Sci. Ser. II Fascicule C-Chim.* **2001**, *4*, 941.
(29) Bertorelle, F.; Lavabre, D.; Fery-Forgues, S. *J. Am. Chem. Soc.* **2003**, *125*, 6244.
(30) Bartlett, G. R. *J. Biol. Chem.* **1959**, *234*, 466.
(31) Moreno, M. J.; Bastos, M.; Velazquez-Campoy, A. *Anal. Biochem.* **2010**, *399*, 44.
(32) Santos, A.; Rodrigues, A. M.; Sobral, A.; Monsanto, P. V.; Vaz, W. L. C.; Moreno, M. J. *Photochem. Photobiol.* **2009**, *85*, 1409.
(33) Wiener, M. C.; White, S. H. *Biophys. J.* **1992**, *61*, 428.
(34) Tanford, C. *J. Mol. Biol.* **1972**, *67*, 59.
(35) Lee, I. Y.; McMenamy, R. H. *J. Biol. Chem.* **1980**, *255*, 6121.
(36) Meisner, H.; Neet, K. *Mol. Pharmacol.* **1978**, *14*, 337.
(37) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Kluwer Academic/Plenum Publishers: New York, 1999.
(38) Brown, R. S.; Brennan, J. D.; Krull, U. J. *J. Chem. Phys.* **1994**, *100*, 6019.
(39) Chattopadhyay, A.; Mukherjee, S. *Biochemistry* **1993**, *32*, 3804.

(40) Rawat, S. S.; Mukherjee, S.; Chattopadhyay, A. *J. Phys. Chem. B* **1997**, *101*, 1922.
(41) Chattopadhyay, A.; London, E. *Biochim. Biophys. Acta* **1988**, *938*, 24.
(42) Simard, J. R.; Zunszain, P. A.; Ha, C. E.; Yang, J. S.; Bhagavan, N. V.; Petitpas, I.; Curry, S.; Hamilton, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 17958.
(43) Goodman, D. S. *J. Am. Chem. Soc.* **1958**, *80*, 3887.
(44) Tieleman, D. P.; Marrink, S. J.; Berendsen, H. J. C. *Biochim. Biophys. Acta* **1997**, *1331*, 235.
(45) Bemporad, D.; Essex, J. W.; Luttmann, C. *J. Phys. Chem. B* **2004**, *108*, 4875.
(46) Peters, T. *All about Albumin. Biochemistry, Genetics, and Medical Applications*; Academic Press: New York, 1996.
(47) Arvinte, T.; Cudd, A.; Hildenbrand, K. *Biochim. Biophys. Acta* **1986**, *860*, 215.
(48) Wolf, D. E.; Winiski, A. P.; Ting, A. E.; Bocian, K. M.; Pagano, R. E. *Biochemistry* **1992**, *31*, 2865.
(49) Mukherjee, S.; Raghuraman, H.; Dasgupta, S.; Chattopadhyay, A. *Chem. Phys. Lipids* **2004**, *127*, 91.
(50) Abrams, F. S.; London, E. *Biochemistry* **1993**, *32*, 10826.
(51) Huster, D.; Muller, P.; Arnold, K.; Herrmann, A. *Biophys. J.* **2001**, *80*, 822.
(52) Huster, D.; Muller, P.; Arnold, K.; Herrmann, A. *Eur. Biophys. J. Biophys. Lett.* **2003**, *32*, 47.
(53) Loura, L. M. S.; Ramalho, J. P. P. *Biochim. Biophys. Acta-Biomembr.* **2007**, *1768*, 467.
(54) Greenough, K. P.; Blanchard, G. J. *J. Phys. Chem. A* **2007**, *111*, 558.
(55) Boutin, J. A. *Cell. Signall.* **1997**, *9*, 15.

JP105163K