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Partition of amphiphilic molecules to lipid bilayers by isothermal titration calorimetry

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ABSTRACT

The partition of the amphiphile sodium dodecyl sulfate (SDS) between an aqueous solution and a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayer was followed by isothermal titration calorimetry (ITC) as a function of the total concentration of SDS. It was found that the obtained partition coefficient is strongly affected by the ligand concentration, even after correction for the charge imposed in the bilayer by the bound SDS. The partition coefficient decreased as the total concentration of SDS increased, with this effect being significant for local concentrations of SDS in the lipid bilayer above 5 molar%. At those high local concentrations, the properties of the lipid bilayer are strongly affected, leading to nonideal behavior and concentration-dependent apparent partition coefficients. It is shown that with the modern ITC instruments available, the concentrations of SDS can be drastically reduced while maintaining a good signal-to-noise ratio. The intrinsic parameters of the interaction with unperturbed membranes can be obtained from the asymptotic behavior of the apparent parameters as a function of the ligand concentration for both nonionic and ionic solutes. A detailed analysis is performed, and a spreadsheet is provided to obtain the interaction parameters with and without correction for electrostatics.

During recent decades, the scientific research in biological chemistry has been focused mainly on the study of genes and proteins. Currently, the biological membranes of cells, and in particular their lipid bilayers, is receiving considerable attention, and lipidomics is becoming a hot subject [1]. The current shift in the attention toward the biological membranes is justified by the important role that this class of supramolecular structures plays in the control of cell structure and function working as a semipermeable and dynamic boundary of cells and cell organelles. In particular in the case of organs delimited by tight endothelia (e.g., the blood–brain barrier), the permeation through the membranes of the surrounding cell monolayer is a process that determines the availability of small molecules in the tissue and is a major bottleneck in the development of new drugs. Therefore, the knowledge of the partition of small molecules between aqueous media and the lipid bilayer is of extreme importance. Calorimetry is a well-established technique in ligand-binding studies between small molecules (ligands) and proteins [2] but has been less often used in the characterization of the interaction with lipid bilayers. This is due both to the small heats expected for an interaction that in many cases is driven mostly by the hydrophobic effect and to the fact that the data analysis procedures are less well established and not commercially available. Recent developments in isothermal titration calorimetry (ITC)[1] have resulted in instruments with very high sensitivity, allowing the use of this technique in the characterization of the interaction of some nonpolar ligands with lipid bilayers [3]. It has also been found that the interaction of a large variety of ligands with lipid bilayers in the liquid-disordered state is accompanied by relatively large and negative molar enthalpies [4–10]. Membranes are binding agents with a high plasticity, and without well-defined binding sites, that change their structure significantly (and in some cases irreversibly) due to the presence of high concentrations of ligands [11–15]. Therefore, the usual protocols followed for the characterization of binding to proteins, both in experimental design and in data analysis, are not adequate. In the absence of specific interactions between the ligand and the lipid assemblies, the local concentration of ligand in biological systems is usually small and the relevant parameter is the intrinsic partition coefficient between the aqueous phase and unperturbed lipid bilayers. This

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[1] *Abbreviations used:* ITC, isothermal titration calorimetry; SDS, sodium dodecyl sulfate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid.

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parameter is not easily obtained for hydrophobic or amphiphilic ligands due to their tendency to aggregate in the aqueous phase [16]. In addition, for ligands with a strong affinity for the lipid phase, the experiment must be conduced at low lipid concentrations and high local concentrations of ligand in the lipid bilayer are attained, inducing strong deviations from ideal behavior.

Several methods have been proposed for the measurement of partition coefficients between aqueous solutions and lipid bilayers as well as different expressions for the partition coefficient (see, e.g., Refs. [15,17–19]). It is not the purpose of the current article to review the different approaches. In this work, we explore the effect of the total ligand concentration used and show that, for some ligands such as sodium dodecyl sulfate (SDS), the ligand concentration may be reduced to values where the membrane properties are not affected by the ligand and, consequently, the intrinsic parameters for the interaction may be obtained directly from the titration experiment.

For this purpose, we titrated aqueous solutions of SDS with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers at different total concentrations of ligand. The amphiphile SDS was chosen because its solubility in aqueous media is well characterized and also because some work has already been done on the characterization of its interaction with lipid bilayers by ITC [7,12,20,21].

Materials and methods

POPC was obtained from Avanti Polar Lipids (Alabaster, AL, USA), and all other reagents and solvents were of the highest commercially available purity and obtained from Sigma–Aldrich Química (Sintra, Portugal).

Aqueous suspensions of lipids were prepared by evaporating a solution of the lipid in chloroform/methanol (87:13, v/v) by blowing dry nitrogen over the heated (blowing hot air over the external surface of the tube) solution and then leaving the residue in a vacuum desiccator for at least 8 h at 23 °C. The solvent-free residue was then hydrated with Hepes buffer (10 mM, pH 7.4) with 0.15 M sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.02% NaN_3 (aqueous solution). The hydrated lipid was subjected to several cycles of vortex/incubation at the specified temperature for at least 1 h to produce a suspension of multilamellar vesicles that was then extruded, using a minimum of 10 passes, through two stacked polycarbonate filters (Nucleopore) with a pore diameter of 0.1 μm [22]. The final phospholipid concentration was determined using a modified version of the Bartlett phosphate assay [23].

Titrations were performed on a VP-ITC instrument from MicroCal (Northampton, MA, USA) at 25 °C, injection speed 0.5 μl s^{-1}, stirring speed 459 rpm, and reference power 10 μcal s^{-1}. As recommended by the manufacturer, a first injection of 4 μl was performed before the experiment was considered to start to account for diffusion from/into the syringe tip during the equilibration period, but the injected amount was taken into consideration in the calculations. The titration proceeded with additions of 10 μl per step. Due to the small concentrations of ligand used in this work, binding to some equipment parts, in particular to the filling syringe, may be significant, reducing the amount of ligand available. To overcome this difficulty, after cleaning thoroughly with water, the equipment (cell or syringe, whichever was to contain the ligand) was rinsed with a solution with the same composition as the solution to be used before filling it with the required titration solution. All solutions were previously degassed for 15 min.

The obtained thermogram was integrated using the data analysis software Origin 7.0 as modified by Microcal to deal with ITC experiments, and the resulting differential titration curve was fitted with the appropriate equations using Microsoft Excel and Solver. The concentrations in the cell were calculated, taking into account the volume that overflows the cell due to the addition of solution from the syringe considering that overflow is faster than mixing, meaning that the composition of the solution leaving the cell is the equilibrium composition before the addition [2]. The predicted heat evolved in titration step i is calculated by Eq. (1), and the best fit of the model to the experimental values was performed through minimization of the square deviations between the experimental and predicted heat per injection for all of the titration steps:

$$q_i = \Delta H\left(n^i_{\mathrm{SDS\_Lipid}} - n^{i-1}_{\mathrm{SDS\_Lipid}}\left(1 - \frac{V_i}{V_{\mathrm{Cell}}}\right)\right) + q_{\mathrm{dil}}, \tag{1}$$

where V_i is the volume injected, V_{Cell} is the cell volume, ΔH is the molar partition enthalpy, $n^i_{\mathrm{SDS\_Lipid}}$ is the number of moles of SDS bound to the lipid after injection i, and q_{dil} is the dilution heat. The amount of SDS that partitions into the lipid bilayer is calculated from Eq. (2), which describes the relative amount of ligand in the aqueous and bilayer phases according to a simple partition model:

$$K_{\mathrm{P}}^{\mathrm{obs}} = \frac{n_{\mathrm{SDS\_Lipid}}/V_{\mathrm{Lipid}}}{n_{\mathrm{SDS\_Water}}/V_{\mathrm{Water}}} = \frac{n_{\mathrm{SDS\_Lipid}}/([\mathrm{POPC}]\bar{V}_{\mathrm{POPC}}V_{\mathrm{T}})}{n_{\mathrm{SDS\_Water}}/V_{\mathrm{T}}}, \tag{2}$$

where it is considered that $V_{\mathrm{T}} \approx V_{\mathrm{Water}}$. The molar volume, $\bar{V}_{\mathrm{POPC}}$, was 0.795 dm^3/mol [24]. Other partition models have been proposed, and their relative advantages and inconveniences were discussed in a recent review [17]. In dilute systems, the different models conduce to equivalent descriptions of the partition process and the respective partition coefficients are easily interconverted. Only the POPC in the outer monolayer was considered due to the slow translocation rate for SDS at 25 °C [20]. The partition coefficient obtained directly from the ratio of the concentration in both phases, $K_{\mathrm{P}}^{\mathrm{obs}}$, is dependent on the total concentration of ligand and is related to the intrinsic partition coefficient (to an uncharged bilayer), K_{p}, via the potential at the bilayer surface, Ψ_0:

$$K_{\mathrm{P}}^{\mathrm{obs}} = K_{\mathrm{P}}e^{-\frac{zF\Psi_0}{RT}}. \tag{3}$$

The surface potential is due to the charge imposed in the bilayer (surface charge density), σ, by the negatively charged SDS molecule. This parameter was calculated using the Gouy–Chapman theory (see Refs. [7,12] and references cited therein) using a value of 68 $Å^2$ for the surface area of POPC, A_{L}, and 30 $Å^2$ for SDS, A_{SDS} [7]:

$$\begin{aligned}\sigma^2 &= 2000\varepsilon RT\sum_i C_i\left(e^{-z_iF\Psi_0/RT} - 1\right)\\ \sigma &= \frac{e_0}{A_{\mathrm{L}}}\frac{n_{\mathrm{SDS\_Lipid}}/n_{\mathrm{Lipid}}}{1 + (n_{\mathrm{SDS\_Lipid}}/n_{\mathrm{Lipid}})(A_{\mathrm{L}}/A_{\mathrm{SDS}})}\end{aligned} \tag{4}$$

For the fitting procedure, values for the partition coefficient and surface potential were initially guessed and the corresponding charge density was calculated according to Eq. (4). The two independent calculations of the charge density (the first line is the charge density calculated from the surface potential, and the second line is the charge density calculated from the partition coefficient) were compared, and the sum of their square deviation was minimized through Solver in an iterative procedure, where different values for the surface potential and partition coefficient were generated until the charge density calculated from both expressions was the same.

Results and discussion

The results obtained when the standard protocol for protein–ligand binding experiments is followed [2,25] are given in the Supplementary material together with several technical aspects of the experimental procedures and data analysis. This protocol is not