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Review

Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods

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Abstract

Optical spectroscopies have been intensively used to determine partition coefficients by a plethora of methodologies. The present review is intended to give detailed and useful information for the determination of partition coefficients and addresses several relevant aspects, namely: (i) definition and calculation of the partition coefficient between aqueous and lipidic phases; (ii) partition coefficients vs. ''binding'' formalisms; (iii) advantages of spectroscopic methodologies over separation techniques; (iv) formalisms for various experimental approaches based on UV–Vis absorption or fluorescence parameters (fluorescence intensity, lifetime, anisotropy and quenching); (v) experimental hints, artifacts and model limitations; and (vi) a brief survey of nonoptical techniques.

Keywords: Partition coefficient; Distribution coefficient; Bilayer; Absorption; Fluorescence; Phase

1. Introduction

The biological world is made of highly heterogeneous media. ''Bulk phase'' chemistry and physics rarely are reasonable approximations to biochemical phenomena. Transport, binding and partition events are ubiquitous. Partition into biomembranes is particularly relevant because many molecules (whether natural or xenobiotics) have target functions directly in biological membranes (e.g., Refs. [1–4]). Moreover, partition always coexists with binding to membrane receptors and transporters. This fact is usually overlooked in studies on biomembrane-associated phenomena, with few exceptions (e.g., Refs. [5,6]).

In the study of the interaction of any compound with model membrane systems, the determination of the partition coefficient should be the first step. After this information is obtained, structural and dynamic studies can then be carried out. The extent of interaction of a solute with a micro-heterogeneous system is evaluated in a quantitative way from its partition coefficient. It is the purpose of this short review to present, in a systematic and critical way, formalisms for its determination and indicate practical hints that should be taken into account. Although the formalisms described are totally general for any kind of partition between two phases, the discussion is centered on their application to model systems of biomembranes (e.g., vesicles). Several works on partition coefficient determination techniques have been published (see later sections), covering problems in toxicology, pharmaceutics, environmental science, food science and structural biochemistry. The octanol/water biphasic system was traditionally used to evaluate partition coefficients, which were then extrapolated to biomembrane/aqueous phase systems, this being an oversimplification [7,8]. To use synthetic phospholipid bilayers instead of biomembranes is not a so strict simplification [9,10].

Regardless of the partition coefficient definition thought as most adequate to describe the partition equilibrium between the two phases, the concentration of the solute in one of the phases (aqueous or lipidic), or both, is aimed. Chromatographic techniques are the only exception to this rule because calculation is based on retention data. Spectroscopic techniques are usually based on the analysis of signals originated from both phases. Thus there is no

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demand for their physical separation, which is a significant advantage [11]; physical separation of phases may be laborious and may result in equilibria perturbation. When spectroscopic techniques are used, the measured parameter is a combination of signals from the ''aqueous'' and ''lipidic'' solute subpopulations, their relative weight depending on the partition coefficient. However, some spectroscopic methods, such as nuclear magnetic resonance (NMR) [12], are not very sensitive and may therefore have to use high solute concentrations. This may lead to saturation phenomena in partition, mainly if independence of the partition coefficient on solute concentration is assumed [13]. Fluorescence techniques, on the other hand, are amongst the most sensitive; low solute concentrations can be used, leading to a small perturbation of the lipidic membranes. It is among our goals to address the role of optical spectroscopies and their contribution to the field, as most of them have simple theoretical backgrounds and are widely used. A brief overview of nonoptical techniques is also provided.

Transcellular (e.g., Ref. [14]) and trans-tissue (e.g., Ref. [15]) drug permeability and diffusion are related to the partition into lipidic biomembranes. These cases will not be addressed. The focus is on the biophysics of partitioning itself and mainly on how to quantify it.

2. Fundamental concepts

On thermodynamic grounds (free energy of transfer of the solute between the two phases), the equilibrium constant described as the mole-fraction partition constant or partition coefficient (partition distribution or ratio is also used, although not frequently) is:

$$K_{p,x} = \frac{\dfrac{n_{S,L}}{n_L + n_{S,L}}}{\dfrac{n_{S,W}}{n_W + n_{S,W}}} \quad (1)$$

where n_W and n_L are the moles of water and lipid, and $n_{S,i}$ are the moles of solute present in each phase (i = W, aqueous phase; i = L, lipid phase). This definition is usually simplified to:

$$K_{p,x} \approx \frac{\dfrac{n_{S,L}}{n_L}}{\dfrac{n_{S,W}}{n_W}} \quad (2)$$

because (i) under most experimental conditions $n_W \gg n_{S,W}$, and (ii) in order to avoid deviations from ideal behavior $n_L \gg n_{S,L}$, i.e., the membrane should not be too overloaded with solute (see Section 4.1).

Considering the mass balance $[S]_t=[S]_W+[S]_L$ and the simplified formulation of partition constant (Eq. (2)), the membrane-bound solute mole fraction, x_L, is derived as a function of the phospholipid concentration, [L]:

$$x_L = \frac{K_{p,x}[L]}{[W] + K_{p,x}[L]} \quad (3)$$

where [W] is the molar concentration of water (approximately 55.5 M at 25 °C and 55.3 M at 37 °C).

It is also common in the literature to present the partition constant in a similar way to Eq. (2), but with the lipid and water amounts represented by their volumes, V_i:

$$K_p = \frac{\dfrac{n_{S,L}}{V_L}}{\dfrac{n_{S,W}}{V_W}} \quad (4)$$

K_p, as defined in Eq. (4), is sometimes referred to as the Nernst partition coefficient, evoking the pioneering work of W. Nernst on solute distribution between immiscible liquids [16].

The relationship between the two formulations of partition constants (Eqs. (2) and (4)) is simply:

$$K_p = K_{p,x}\frac{\gamma_W}{\gamma_L} \quad (5)$$

where γ_i is the molar volume of water, i = W, or lipid, i = L. Although trivial, it is not uncommon that this relevant detail is overlooked, and different partition constants are intercompared. The handbook of Marsh [17] is a good source for γ_L data, and the online database LIPIDAT [18] can be used as a source for studies on membranes, namely for lipid mixtures. Regarding these last ones, in systems including cholesterol, the condensation effect of this lipid should be taken into account [19].

Before proceeding to the discussion of the methodologies, several subjects related to the partition coefficient will be commented. The first one is related to indirect methods of estimating the membrane partition coefficient, the appreciation of the octanol/water partition coefficient (K_{ow}), or the solute water solubility being used as criteria. The octanol/water partition coefficient has been the subject of extensive literature (as described e.g., in Ref. [20]), and its determination has aimed at obtaining predictive relationships [21]. However, octanol is not a good membrane mimic [7,8]. Membranes are complex entities with specific interface interactions and a hydrocarbon like interior and in addition lateral heterogeneities [22]. Presently, biphasic systems consisting of mixtures of neutral and charged phospholipids are used as better membrane models. In this situation, electrostatic interactions are the ruling factor in case of charged solutes (e.g., Ref. [23]). The relevant theories that rationalize interaction with charged interfaces [24,25] are out of the scope of this work; it should be stressed that the global partition constant as described by

Eq. (2) or Eq. (4) includes contributions of hydrophobic and electrostatic interactions. An alternative but related approach is to use water solubility, which can give some insight on the magnitude of K_p. Examples for peptides and their limitations are given by White et al. [26].

A different way to describe the interaction of a solute with a membrane is by considering a binding mechanism. In this case, a chemical equilibrium between the solute and one or more lipid molecules is considered, and a conventional binding isotherm is obtained (e.g., Refs. [27,28]). In this case two parameters describe the process, a dissociation constant K_d and the number of lipid molecules that are associated with a solute molecule, i.e., that constitute a binding site. We consider that the interaction of a solute should be quantified by the partition formalism described above, in agreement with others [26]. In fact, among other reasons, there is no molecular counterpart for describing "lipidic binding sites", as if some kind of receptors for the solute would exist; the membrane is a supramolecular system and the solute interaction is controlled by the lipidic ensemble. No direct comparison can be made of published K_d and K_p values, and the best procedure would be to analyse the raw binding data (if available) on the framework of the partition formalism.

As can be concluded from the definition of partition coefficient, the determination of K_p is dependent only on the determination on the amount of solute in interaction with the membrane (or in the aqueous phase), since the total amount of solute is usually known. For this purpose two types of methodologies can be used.

2.1. Physical separation of free and "membrane-bound" molecules is involved

This allows direct determination of the partition coefficient and a plethora of approaches can be used such as centrifugation, equilibrium dialysis, membrane filtration, and chromatography (see Section 5). These methods are unavoidable in case that no physical signal (usually spectroscopic) can be directly related to one of the species, such as described below; one of the few exceptions is an indirect fluorescence approach that will be described later in Section 3.2. The main disadvantages are related to the possible incomplete separation of the phases (vesicle centrifugation is not an easy process and remaining lipid can exist in the supernatant, or lipid can be adsorbed, e.g., at membranes used for filtration); in addition, equilibrium perturbation can occur. This class of methods will be briefly addressed in Section 5.

2.2. No physical separation of free and "membrane-bound" molecules is involved

In this case the global system signal response is a combination of the free and bound molecules signals. Most of the techniques used in this case are spectroscopic, with a few exceptions (e.g., titration calorimetry [29] and ζ potential determination [30]). It implies that measurements should be carried out at several lipid concentrations, which is easily achieved by the addition of a lipid stock suspension. As will be described later, the parameter to be monitored follows a hyperbolic-like dependence on lipid concentration.

The partition coefficient does not contain, in principle, any kind of topographical information about the solute location in the membrane, which can be either adsorbed at the membrane interface or, in the case of a nonpolar species, internalized. If this structural information is aimed, it can be obtained via other spectroscopic methodologies such as differential fluorescence quenching [31–33] or energy transfer [34].

For the purpose of partition coefficient determination one must specify the lipidic concentration. If the compound is internalized in the membrane, all the lipidic bilayer volume is available for incorporation, otherwise when the solute is restricted essentially to the interface region (slow translocation as compared to the partition equilibrium), one-half should be considered. For small unilamellar vesicles (SUV), which are considered bad bilayer models due to all the consequences introduced by their small radius of curvature, about 60% of the lipid is assumed to be in the outer hemilayer [35].

3. Optical spectroscopies involved in methodologies without physical phase separation

3.1. UV–Vis absorption spectrophotometry

This class of methodologies is based on the change of an absorption parameter upon incorporation of molecules into membranes. Using direct UV–Vis absorption measurements, Kaminoh et al. [36] determined partition coefficient values from Eq. (6)[1] (Table 1).

$$A = \frac{A_W + K_p \gamma_L [L] A_L}{1 + K_p \gamma_L [L]} \quad (6)$$

As the total concentration of the partitioning molecule and its absorbance in aqueous solution, A_W, are known, the limit absorption in the lipidic environment, A_L, can be calculated from an A vs. [L] plot. Furthermore, the method was extended to the determination of the partition coefficient of the protonated, HA, and ionized, A^-, forms of a weak acid with an ionization constant, K_a, based on the measurement of the molar absorption coefficient, ε, for different

[1] All the equations presented were adapted to the notations used in the present work. The subscripts W and L stand for the value of the parameter in aqueous phase and the value that would be obtained if all the partitioning molecules were in the lipid phase, respectively.

Table 1
The most used optical spectroscopy methodologies for the calculation of the partition coefficient of a fluorescent molecule between lipid and aqueous phases

Parameter	Equation	Requirements	Reference
Absorption (A)	$A = \frac{A_W + K_p\gamma_L[L]A_L}{1 + K_p\gamma_L[L]}$	Different absorptivities, ε, in lipid and aqueous phases.	[127] (related equations appear in Ref. [36])
Fluorescence intensity (I)	$I = \frac{I_W + K_p\gamma_L[L]I_L}{1 + K_p\gamma_L[L]}$	Different quantum yields, ϕ, in lipid and aqueous phases.	[53,72] (related equations appear in Refs. [70,127–130])
Fluorescence steady-state anisotropy (r)	$r = \frac{r_W((\gamma_L[L])^{-1} - 1) + r_L K_p \varepsilon_L \phi_L/(\varepsilon_W \phi_W)}{(\gamma_L[L])^{-1} - 1 + K_p \varepsilon_L \phi_L/(\varepsilon_W \phi_W)}$	Different anisotropy in lipid and aqueous phases. The fluorescence emission intensity from both phases must be comparable.	[2]
Fluorescence lifetime weighted quantum yield ($\bar{\tau}$)	$\bar{\tau} = \frac{\bar{\tau}_W + K_p\gamma_L[L]\bar{\tau}_L}{1 + K_p\gamma_L[L]}$	Different fluorescence lifetimes in lipid and aqueous phases. The fluorescence emission intensity from both phases must be comparable.	[53]

All the equations were adapted to the notations used in the present work. [L] is the total lipid concentration and γ_L is its molar volume. The subscripts W and L stand for the aqueous and the lipid phase, respectively. All equations can be simplified to a single generic formulation (Eq. (17) in the text).

lipid concentrations, [L]. Keeping the concentration of the partitioning molecule constant, $K_{p,x}$ can be obtained using the following general equation,

$$\varepsilon = \frac{\varepsilon_{W,A^-} + \varepsilon_{L,A^-}K_{p,x,A^-}[L]/[W] + (\varepsilon_{W,HA} + \varepsilon_{L,HA}K_{p,x,HA}[L]/[W])10^{pK_a - pH}}{1 + K_{p,x,A^-}[L]/[W] + (1 + K_{p,x,HA}[L]/[W])10^{pK_a - pH}} \quad (7)$$

When either pH is sufficiently higher or sufficiently lower than pK_a, Eq. (7) can be simplified to Eq. (8) or Eq. (9), respectively:

$$\varepsilon = \frac{\varepsilon_{W,A^-} + \varepsilon_{L,A^-}K_{p,x,A^-}[L]/[W]}{1 + K_{p,x,A^-}[L]/[W]} \quad (8)$$

$$\varepsilon = \frac{\varepsilon_{W,HA} + \varepsilon_{L,HA}K_{p,x,HA}[L]/[W]}{1 + K_{p,x,HA}[L]/[W]} \quad (9)$$

Kaminoh et al. [36] applied the last two equations to their data after some rearrangements to obtain fits with linear equations. Nevertheless, linearization should be avoided (see footnote 3).

Despite the overall simplicity of Eq. (6), its practical application is usually limited to systems with low light scattering background signals, such as micellar solutions [37]. When the direct application of this spectrophotometric method is prevented by high background signals, caused by the presence of liposomes [38–43] or cells [44], the problem can be minimized by the use of second derivative spectrophotometry (with respect to the wavelength, λ), based on an equation similar to Eq. (6):

$$D = \frac{D_W + K_p\gamma_L[L]D_L}{1 + K_p\gamma_L[L]} \quad (10)$$

where,

$$D = \frac{\partial^2 A}{\partial \lambda^2} \quad (11)$$

In a recent study, Rodrigues et al. [43] indicated that similar results can be obtained using the first or third derivatives of the absorption spectra, instead of the second derivative.

UV–Vis spectrophotometry can also be used to indirectly obtain partition coefficients. Vermeir et al. [45] used absorption measurements as a standard method to determine the apparent Michaelis constant of an enzyme, K_M^{app}, at different lipid volume fractions, $\alpha = V_L/V_W$ (proportional to [L]). These data were used to calculate the K_p value of the enzyme substrate, by analyzing the K_M^{app} vs. α plot.

Circular dichroism techniques can also be potentially used to determine partition coefficients. The parameters calculated by Schwarz and Beschiaschvili [46], for instance, show that this technique can be used to obtain K_p values, although these authors did not effectively calculated a partition coefficient. Other optical absorption techniques, such as infra-red and Raman scattering spectroscopies, although powerful to solve problems related to structure, are not commonly used for partition coefficient determination.

Instead of using single wavelength measurements, the whole absorption spectrum at different lipidic concentrations can be used in a multi-parametric analysis. Although this procedure leads, in principle, to a better statistical analysis, it is not common in the literature and it will not be described in detail.

3.2. Fluorescence spectroscopy

The simplest fluorescence spectroscopy methodologies used to calculate partition coefficients consist in the use of a

hydrophilic fluorescent indicator to quantify the concentration of the partitioning molecule in the aqueous phase [47,48], allowing a direct calculation of the partition coefficient. However, these methodologies are restricted to a few practical situations. For most of the cases, the partition coefficient of a molecule between a lipid and an aqueous phase can be evaluated by fluorescence spectroscopy as long as: (i) there is a difference in a fluorescence parameter of the partitioning molecule (e.g., quantum yield, fluorescence anisotropy or fluorescence lifetime) when in aqueous solution and after incorporation in the membrane; or (ii) the incorporation of the molecule in the membrane leads to a change on a fluorescence property of a membrane probe.

Both fluorescence emission intensity, I, and steady-state anisotropy (r; a parameter easily calculated from polarized emission, which contains information on fluorophores' rotation while in the excited state [49]) can be used to calculate the partition coefficient of a fluorescent molecule between lipid and aqueous phases (Eqs. (12) and (13), and Table 1).

$$I = \frac{I_W + K_p\gamma_L[L]I_L}{1 + K_p\gamma_L[L]} \tag{12}$$

$$r = \frac{r_W((\gamma_L[L])^{-1} - 1) + r_L K_p\varepsilon_L\phi_L/(\varepsilon_W\phi_W)}{(\gamma_L[L])^{-1} - 1 + K_p\varepsilon_L\phi_L/(\varepsilon_W\phi_W)} \tag{13}$$

(ϕ is the fluorescence quantum yield; ideally, I should be the integrated fluorescence emission intensity but if no significant spectral shifts occur upon increasing the lipidic concentration, [L], I may be measured at a chosen wavelength). These methods were developed for non-ionizable fluorescent partitioning molecules but can be further extended to ionizable molecules. Lopes et al. [50] adapted Eq. (13) to the study of the interaction of the protonated and ionized forms of a weak acid with a membrane model system.

In addition to the steady-state fluorescence spectroscopy methodologies, partition coefficients can also be obtained by time-resolved fluorescence spectroscopy. When carrying out a time-resolved fluorescence spectroscopic study of the interaction of a fluorescent partitioning molecule with a membrane system, ideally two exponentials would describe the experimental fluorescence intensity decay ($I(t)=a_W \exp(-t/\tau_W)+a_L \exp(-t/\tau_L)$), one corresponding to the molecules in aqueous media and the other to the molecules in lipidic environment. In this case, the relative concentration of each species could be calculated from the pre-exponential factors ratio (a_L/a_W), if the radiative rate constants and absorption coefficients ratios in both phases are known (see e.g., Ref. [51]). However, in most cases, the decays both in aqueous phase and lipidic environment are complex and the total decay is described by a sum of exponentials mixing up all the contributions. Thus, this approach is certainly critical unless global analysis is carried out [52]. In practice, it is better to study the variation of the fluorescence lifetime averaged by the pre-exponentials (i.e. integrated intensity or, equally, lifetime-weighted quantum yield), $\bar{\tau}$ (Eq. (14)), upon increasing the lipidic concentration. This is an additive parameter and, therefore, leads to a straightforward formalism for the determination of K_p (Eq. (15) and Table 1; [53]).

$$\bar{\tau} = \sum a_i\tau_i \tag{14}$$

$$\bar{\tau} = \frac{\bar{\tau}_W + K_p\gamma_L[L]\bar{\tau}_L}{1 + K_p\gamma_L[L]} \tag{15}$$

It should be stressed that the average fluorescence lifetime of a fluorophore, $\langle\tau\rangle$, is given by (e.g., Ref. [49]),

$$\langle\tau\rangle = \sum a_i\tau_i^2 \Big/ \sum a_i\tau_i \tag{16}$$

However, if $\langle\tau\rangle$ was used for K_p determination, a more complex equation would be attained, where steady-state and transient-state data must be combined, as described in detail in Ref. [53].

Eqs. (6), (10), (12) and (15) can be simplified to the general equation:

$$p = \frac{p_W + K_p\gamma_L[L]p_L}{1 + K_p\gamma_L[L]} \tag{17}$$

where p stands for A, I or $\bar{\tau}$. If $\varepsilon_W\phi_W \approx \varepsilon_L\phi_L$ and $\gamma_L[L] \ll 1$ (a condition present in most experimental conditions) are assumed in Eq. (13), then p may also stand for r. A schematic plot of Eq. (17) is presented in Fig. 1.

In one exception to the general rule of the increase of ε, A, ϕ, I, r and τ of a fluorophore upon incorporation on a membrane system, Vermeir et al. [45] reported a decrease on the fluorescence intensity of the partitioning molecule when in the membrane. This quenching process was used as a

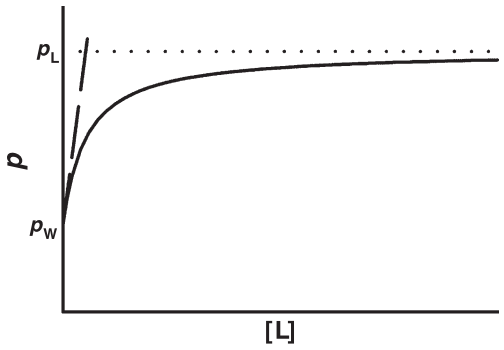

Fig. 1. Schematic representation of Eq. (17) (partition parameter, p, vs. lipid concentration, [L]). The function is hyperbolic-like if $p_L > p_W$ (solid line). The initial slope (dashed line) is $(dp/d[L])_{[L]=0} = K_p\gamma_L(p_L - p_W)$; therefore, K_p cannot be calculated from the initial regime only, unless p_L is known (p_W can be measured).

simple methodology to obtain the value of the partition coefficient, by a linear fit:

$$\frac{I_W}{I} = 1 + K_p\alpha \tag{18}$$

(α is the relative lipidic volume). This equation, apparently at odds with Eq. (12), becomes equivalent to it if one assumes that $I_L = 0$.

As stated before, the partition coefficient of a fluorescent molecule can be obtained by monitoring its fluorescence parameters in aqueous solution and after incorporation in the membrane. Using a different approach, the partition coefficient of a nonfluorescent molecule can be obtained by fluorescence spectroscopy, as long as its incorporation in the membrane leads to a change on a fluorescence property of a membrane probe. These measurements are usually based on the fluorescence quenching of the membrane probe by the partitioning molecule, as the decrease on the fluorescence intensity of the probe depends on the average number of quencher molecules (the partitioning molecule) in its vicinity. Lakowicz and Hogen [54] developed a method that was later applied to several molecular systems (e.g., [55,56]). Eq. (19) shows that a plot of $1/k_{app}$ (i.e., the reciprocal of the apparent bimolecular quenching constant; Eq. (20)) as a function of α yields a straight line with intercept $1/(k_qK_p)$ and slope $(1/k_q - 1/(k_qK_p))$, where k_q is the physically meaningful bimolecular quenching rate constant in the membrane.

$$\frac{1}{k_{app}} = \alpha\left(\frac{1}{k_q} - \frac{1}{k_qK_p}\right) + \frac{1}{k_qK_p} \tag{19}$$

$$\frac{\langle\tau\rangle_0}{\langle\tau\rangle} = 1 + k_{app}\langle\tau\rangle_0[Q]_t \tag{20}$$

(the subscript 0 indicates the average fluorescence lifetime in the absence of quencher, Q, and t refers to the average concentration over the total sample volume).

Eq. (19) can be rewritten in a way similar to the familiar Stern–Volmer equation [12,57]:

$$\frac{I_0}{I} = 1 + \frac{k_q\langle\tau\rangle_0K_p[Q]_t}{1 + K_p\gamma_L[L]} \tag{21}$$

This methodology was also adapted to the determination of the partition coefficient of an ionizable quencher [58,59] and to the calculation of binding constants [60].

The partitioning of nonfluorescent molecules was also studied by Lissi et al. [11], following the change induced on the excimer/monomer ratio of a membrane probe. This value changes due to the alteration on membrane viscosity caused by the insertion of the partitioning molecule. Despite its overall simplicity, this method has not gained a wide acceptance due to the high total concentrations of partitioning molecule needed for the effect to be noticed. Similar approaches were used to obtain partition coefficient values by following spectral changes on the fluorescence of a membrane probe [61], including change on the skewness of the emission peak (named center of spectral mass) [62], upon alteration of the polarity or lipid organization around the fluorophore caused by the insertion of the partitioning molecule.

The generalized polarization method (GP) [63] has also been used to obtain partition coefficient values [64], by extending the GP concept to a three-wavelength excitation generalized polarization (3wGP). However, the formalisms used in this method are quite peculiar and left out of the scope of the present review. Equally peculiar and not dealt within detail is the method by Polozov et al. [65], which is based in previous knowledge of the spectrum in water and in the membrane (see below).

3.3. Artifacts

As described, when deviation from a hyperbolic-like rational function is experimentally observed, this can be due to either experimental artifacts or otherwise to the restricted assumptions of a simple model. Most important artifacts are:

(1) Light scattering is a severe restriction in absorption methodologies. Fluorescence data may also be affected. Large unilamellar vesicles (LUV) should be preferred to SUV to avoid membrane curvature effects; however, the scattered intensity is proportional to the squared volume of the scattering particle and the scattering effect can be critical. In addition, gel phase data is more distorted, due to the higher refractive index of these bilayers as compared to the fluid phase. Subtracting a blank is only an approximate correction [66–68]. Absolute fluorescence intensities may be affected, but fluorescence lifetimes are much less sensitive. Therefore, we suggest that if possible, instead of I, the lifetime-weighted quantum yield, $\bar{\tau}$ (Eq. (14)), should be used. Anisotropy measurements are strongly affected by light scattering and this induces a deviation of the hyperbolic-like fitting at the highest lipidic concentrations such as shown in, e.g., Fig. 8 of Ref. [2]. This eventually is the greatest restriction to the otherwise very sensitive fluorescence technique, once that for a correct recovery of meaningful p_L parameter (and also K_p, due the strong correlation of the two parameters), a quasi-plateau region on the plots should be obtained.

(2) Bimolecular photophysical interactions are an eventual complicating factor, essentially in situation of overcharged membranes. Although membranes are viscous media even in the fluid phase, diffusion controlled processes cannot be ignored, and static mechanisms can also be operative. Self-quenching would affect all the measurements related to fluorescence intensity, lifetimes (downward deviations in the initial regime of the hyperbolic-like plots), as well as anisotropy (upward deviation via the decrease in fluorescence lifetime). A likely process to affect anisotropy

data (downward deviation) is nonradiative energy homotransfer (energy migration) or donor–donor energy transfer [69]. Förster radius up to 50 Å, for instance, are not uncommon due to the strong spectral overlap of absorption and emission. Dyes absorbing in the visible spectral range are typical examples. It should be stressed that fluorescence intensity is not affected by this process, at variance with a common belief (e.g., [26]).

4. Additional remarks

At this point, a question should be raised: What is(are) the best optical spectroscopy method(s) to quantify the partition coefficient of a fluorescent molecule? Table 1 presents general rules for the selection of the method depending on the spectroscopic properties of the probe, e.g., an anisotropy-based methodology cannot be used for a molecule that does not fluoresce in aqueous phase. Moreover, a maximized difference between p_L and p_W should be sought.

Several other aspects regarding the abovementioned spectroscopic methods should be stressed:

(i) Molecular partition is a dynamic event, where equilibrium may take a long time to be achieved. Obviously, K_p should be calculated after equilibrium is reached, unless it is to be calculated from kinetic data [70]. A time scan of the chosen spectroscopic parameter (ε, ϕ, r, etc.) can be used to reveal the time it takes to reach equilibrium.

(ii) The spectroscopic determinations are usually carried out by titration, i.e., addition of successive amounts of lipid to the solution keeping the solute concentration constant (except for the dilution effect). In the case of photophysical methodologies some fluorophores can bleach easily (e.g., tryptophan in protein and peptides and some linear polyenes). In this case, separate samples with constant solute concentrations and different lipid amounts should be used.

(iii) In most cases (fluorescence intensity, anisotropy, etc.), the formalisms lead to a hyperbolic-like three-parameter dependence: parameter value in water (p_W), in the membrane (p_L), and K_p (Eq. (17)).[2] As p_W can be directly measured, the problem is simply to a two-parameter fitting.[3] Moreover, an additional advantage of spectroscopic methodologies for K_p determination is to attain information on the parameter value in the membrane, p_L, which contains structural and/or dynamic information about the incorporated solute. The more relevant cases are those of the fluorescence anisotropy, r_L (dynamic information; usually lower rotational diffusion coefficients), and fluorescence lifetime, τ_L (usually increases upon incorporation in a more hydrophobic medium).

(iv) Obviously, knowledge on the partition coefficient, K_p, is essential to obtain correct spectral information about the solute in interaction with the membrane and in this way derive structural information, e.g., about the solute microenvironment. Two examples are the following:

(a) *Fluorescence spectrum of the solute in the membrane*. In case that K_p is not too high, the molar fraction of solute in water, x_W ($x_W = 1 - x_L$; Eq. (3)), can be significant. In case that the solute fluoresces in water (e.g., tryptophan), the experimental spectrum, $I(\lambda)_{L+W}$, is the sum of the fractions both in water, $I(\lambda)_W$, and in the membrane, $I(\lambda)_L$. This last one can be obtained from Eq. (22) [71], e.g., for discussing spectral shifts upon membrane incorporation,

$$I(\lambda)_L = C\left(I(\lambda)_{L+W} - x_W \frac{1}{1+\bar{\tau}_L/\bar{\tau}_W} I(\lambda)_W\right) \quad (22)$$

The spectra to be used in the above equation are the normalized ones (unit area). $\bar{\tau}_L$ is obtained from the fitting procedure described above (Section 3.2; Eq. (15)) and $\bar{\tau}_W$ is directly experimentally accessible. C is a normalization constant. Polozov et al. [65], instead of determining K_p from fluorescence intensity at a single wavelength, favor the utilization of multiple information from spectral composition, which consists basically in using Eq. (22) for determining K_p when the spectra in water and in the membrane are known.

(b) *Time-resolved fluorescence anisotropy*. Under the conditions previously described for the correction of fluorescence spectra (simultaneous emission of the two species), the total anisotropy decay is eventually difficult to analyze. In the most common case of complex decay (two or more components), of both free and bound species, the number of needed fitting parameters would be too big. However, even in the case that the dynamic information contained in the initial part of the decay cannot be recovered, the limiting anisotropy of the bound species, $r_{\infty,L}$, is readily obtained from Eq. (23) [71], which allows an easy determination of the order parameter of the system [49],

$$r_{\infty,L} = \left(1 + \frac{x_W \bar{\tau}_W}{x_L \bar{\tau}_L}\right) r_\infty \quad (23)$$

where r_∞ is the experimentally determined value in the presence of lipid at time = ∞.

4.1. Most important model limitations

(1) Although most cases can be rationalized according to the described two-state model (free and bound monomers), the situation can be more complex when there is aggregation of the solute in water or in the membrane. Examples of this situation are compounds that self-assemble in the membrane (cooperativity mechanisms), such as those involved in the

[2] Alternative formalisms, which do not require p_L to be known, are presented in Ref. [11].

[3] In order to have a correct error distribution on the data points, and using today's computational tools, all the nonlinear equations presented should be directly used in a nonlinear fit to the experimental p vs. [L] data points (Eq. (17)). Linearization may bias the results.

formation of membrane channels. This is the case of the polyene antibiotic nystatin [72] and of the peptide melittin [73]. Moreover, monomer-aggregate equilibrium can exist in the aqueous phase; melittin is again an example [74]. Direct incorporation of oligomers in the membrane, in addition to monomers, may also happen. In these cases, specific models should be developed taking into account the described multi-equilibria. Sometimes there is no analytical solution and numerical methods should be used.

(2) Even in the situation of a two-state model, the thermodynamic framework implies that the partition coefficient is used in situations of dilute solution. Deviations can happen when the membrane is overcharged with solute, i.e., the partition coefficient is no longer a constant and depends on the number of solute molecules per lipid. One common situations is the interaction of charged species with charged membranes, where anti-cooperative effects are due to the decrease of electrostatic interactions. The case of neutralization of negatively charged lipids by cationic peptides results in a decrease of the Gouy–Chapman potential and formalisms that allow a correct data analysis are available, as previously described.

4.2. Membrane inter-domain partition

The concept of membrane/water partition coefficient can be extended to the partition of a molecule between two different lipid phases. This area has gained an increased importance during the last decade due to the rising awareness for the biological relevance of the existence of membrane domains and lipid rafts (for reviews see, e.g., Refs. [75–77]) and is now a very active field of research [22].

The precise knowledge about phase-coexistence (e.g., gel/fluid or different types of fluid such as liquid-ordered/liquid disordered) is essential for characterizing lipidic systems. The partition coefficient of a molecule between two coexisting membrane phases can be defined similarly to Eqs. (1) and (4). If $K_p \neq 1$ the molecule is preferentially incorporated in one of the phases; if $K_p \approx 1$ the molecule is distributed randomly between the two phases. All the previously discussed methods can be applied in this context. With nowadays fluorescence microscopy techniques, it is possible to directly observe the appearance and extinction of membrane domains and fluorophore partition between them [78].

Similarly to water/lipid distribution, the relative partitioning of a fluorescent molecule between two membrane phases can also be estimated from fluorescence quenching by a membrane quencher known to be incorporated preferentially in one of the domains, in a binary [79,80], ternary or higher order mixture [81,82]. Fluorescence Resonance Energy Transfer (FRET) experiments can also be used for determining partition coefficients. However, the recovered values may be biased due to the relative topology of donors and acceptors because FRET efficiency depends on donor–acceptor distance. Nevertheless, this methodology allows detailed structural information about membrane domains to be obtained [83].

Finally, it should be stressed that (i) in most biophysical studies of phase coexistence in membranes, the goal is not the determination of K_p, but instead to use this information to, e.g., obtain phase diagrams [84,85]—a recent work describing partition constants and its molecular rationalization is available [51]; (ii) in most cases, the solute incorporation in gel phase is on structural membrane defects [86]; (iii) when the partition coefficients of a solute between gel phase/water and fluid phase/water are known, the gel/fluid partition coefficient can be calculated from their ratio.

5. A brief survey of nonoptical techniques

As previously described, most partition coefficients determinations using non-spectroscopic techniques are based on studies that involve physical separation of aqueous and lipidic phases. Filtration [87–92] and centrifugation [1,8,12,61,89,93–98] are the most commonly used techniques for such purpose. Solute quantification is carried out in one of the phases: either the lipid retained by the filter or the supernatant (aqueous phase). Radiometry and UV–Vis absorption are generally used for concentration determination but other techniques are occasionally used (e.g., electron paramagnetic resonance, EPR; [95]). However, filtration methodologies were criticized [88] and centrifugation techniques are only accurate if the trapped buffer in the pellet is accounted for [89].

Dialysis techniques are also commonly used [10,12,93,99–102]; they do not have the drawbacks of complete phase separation and the aqueous phase is easily accessible for solute titration. Dialysis cells made of two chambers separated by a dialysis membrane are used. One half-cell is filled with the solution and the other half-cell with buffer (reference or blank cell) or vesicles. A time is given for equilibration, after which solute is quantified in the first half-cell. Dialysis membranes have cut-off sizes that enable free diffusion of solutes but prevent vesicles from passing to the other chamber. Solute quantification has been done by UV–Vis absorption (e.g., [12]), sometimes combined with HPLC [10,98,100,102]. It is worth mentioning that dialysis is not the only technique that allows selective sampling from the aqueous phase. Solid phase microextraction was also applied to partition coefficient determinations [99].

Ion-selective electrodes were developed and applied to partition coefficients determinations [103,104]. This technique does not require the physical separation of aqueous and lipidic phases and is particularly useful when both charged and uncharged species of the solute are involved in partition [101,104–106]. The pK_a value shifts in response to the partitioning of some of the solutes into a lipidic phase [107], the partition coefficient being calculated there from. However, electrostatic saturation phenomena cannot be fully

ruled out due to the relatively high concentrations of compounds that have to be used in this kind of techniques [101]. Equilibration kinetics and lipid-to-water volume ratio limitations are additional problems [101].

Spectroscopic techniques, mainly EPR, were used in similar systems (spin labeled fatty acids; [108]). The spectra consist of two components: a sharp three-line component from the spin labels tumbling rapidly in water and a broad anisotropic component from the spin labels intercalated in the membrane. The ratios of the fractions of the total spectral intensity in the lipid-bound and free components are related to the partition coefficient [108]. The dependence of this ratio on pH enables the study of interfacial ionization of membrane-bound fatty acid. EPR has proven a powerful technique in partition studies [109–113], even when complex equilibria involving ionic species are present. Despite some differences between them, EPR spectra deconvolution analysis in partitioning studies aims at the measurement of free and membrane-associated spectral components. Lissi et al. [11] and de Paula and Schreier [61] developed data analysis methodologies that can be used with a wide range of techniques and illustrated their application with EPR data. NMR has been scarcely used (e.g., Refs. [114,115]).

Other techniques, based on the perturbation of the membrane properties upon the presence of the solutes, although not very sensitive, are not severely limited by the need for molecules having specific spectroscopic characteristics. The refractive index [116] and the gel to liquid crystalline phase transition temperature [13,117] are membrane properties that can be used for partition coefficient determination. Bánó [13] reduced the problem of the measurement of the partition coefficient to the measurement of the beginning and end of the phase transition in the lipid + solute system. Moreover, no assumption is made on the independence of the partition coefficient on the lipid concentration, allowing the direct study of saturation effects. Only one assumption is necessary: the pseudo-binary phase diagram used is independent of lipid concentration. Isothermal titration calorimetry (ITC) [118] can also be used to calculate partition parameters [29,118,119]. As the lipid concentration increases (constant solute concentration), the reaction enthalpies decrease in magnitude with the decrease of the solute available for partition. The partition isotherm is derived from the heats of reaction and can be analyzed in terms of partition models. Volume changes associated with the partitioning of foreign molecules into lipidic bilayers are also related to partition coefficients [120].

A quite different approach is used in chromatographic techniques. Monolayers of phospholipids or phospholipid analogues are covalently bonded to the hydrophobic end to the surface of silica particles and used as stationary phase in liquid chromatography [7,101,121,122]. This process is named immobilized artificial membrane (IAM) chromatography. However, the electrostatic interactions between residual charged groups in silica and charged solutes make the technique adequate to study only the partition of neutral solutes [101]; if neutral solutes are used, partition coefficients are identical to those obtained with free vesicles [121]. There are commercial alternatives to overcome this limitation in applicability [101]: TRANSIL consists of large porous silica particles noncovalently coated with single lipid bilayers. Miyake et al. [123–125] have recently developed a similar approach: immobilized liposome chromatography (ILC). Unilamellar phospholipidic vesicles are stably but noncovalently immobilized in hydrophilic gel beads (stationary phase) through avidin–biotin binding. Partition coefficients can be calculated from retention volumes of the solutes measured in both zonal and frontal modes.

Other, more specific, techniques appeared in the literature regarding partition studies, for instance electrokinetic chromatography [126], but are not commonly used and are, therefore, left out of the scope of this review.

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