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Interpretation of passive permeability measurements on lipid–bilayer vesicles. Effect of fluctuations

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Abstract

A stochastic model for migration dynamics of solute molecules from the bulk aqueous phase to the intravesicular water pool is developed with a goal to interpret recent passive permeability measurements of bilayer membranes. Previously neglected fluctuations of the number of solubilized species in the inner water pool of a vesicle are naturally incorporated into the model. For a homogeneous one-phase bilayer, the model predicts exponential long-time asymptotics of the migration dynamics with a rate constant given by a sum of the frequencies of exit and entry of a solute molecule from/into the intravesicular water pool into/from the bulk aqueous phase. The long-time constant is directly related to the membrane permeability. © 1997 Elsevier Science B.V.

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1. Introduction

Although ion transport in biological membranes is primarily mediated by specific protein channels or pumps, passive diffusion through the underlying lipid matrix is also viable and can be of notable importance [1]. Most of the studies of the ion permeability have been performed on lipid bilayer vesicles which are regarded as models for biological membranes. The permeability of the membrane is related to the molecular packing in the bilayer and is, therefore, very sensitive to the density fluctuations [1–3], whatever is the microscopic mechanism for passive transport along the lipid hydrocarbon chains (involving quantum-mechanical tunneling or kink-defect carriers).

One of the most powerful techniques to measure the transport and partitioning of ions within bilayer membranes is based on fluorescence quenching [4–8]. The method is not only simple in practical realization but is effective and sensitive to very small chromophore concentrations. In a typical experiment [7,8], one uses unilamellar vesicles labeled with the fluorescent probe (a head group labeled lipid analog). Addition of a quencher (a metal ion, for instance) to the bulk aqueous phase leads to almost immediate reduction of fluorescence from the outer leaflets of the vesicles. Further decrease of the fluorescence intensity is due to the ability of the quencher to penetrate the bilayer and quench the fluorophores located on the inner leaflets. The rate of this process is a measure of the passive permeability.

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In analyzing the fluorescence decay kinetics, most authors assume the intravesicular water pool to be macroscopic, i.e., they introduce an average concentration of quencher molecules in the intravesicular water pool and apply the laws of the conventional chemical kinetics. However, it is well established that the number of solubilized species fluctuates from vesicle to vesicle, and when the solubilizate concentration is low such fluctuations are comparable to the average [9]. Adequate description of the kinetics of solibilization requires invoking a stochastic approach which properly accounts for the bilayer-crossing events. Detailed theoretical analysis of migration dynamics of solutes between lipid vesicles was performed some time ago with a focus on the fluorescence stopped-flow observables [10]. The purpose of this paper is to extend the stochastic approach to describe the passive permeability measurements of the bilayers. We also compare the predictions of the stochastic model versus the results of the conventional approach and illustrate an important role of fluctuations at low solute concentrations.

Related and recently very active area of research concerns chain exchange dynamics in block copolymer micelles [11–13]. In experiment the dynamics are monitored, e.g., by nonradiative energy transfer between donor and acceptor chromophore groups attached to different polymers. The results derived in this paper are also applicable to such systems.

2. Stochastic model

A conceivable mechanism for ion transport across the bilayer membrane involves three stages. The first is ion binding to the outer surface of the vesicle from the bulk aqueous solution. Then ions have to penetrate through the bilayer. And finally, the ions are released into the water pool inside the vesicle. All processes are, of course, reversible. In accordance with experimental observations, the limiting stage in the overall transport process is essentially ion penetration through the bilayer. Therefore, in our consideration we distinguish only between 'solubilized' (i.e., located in the inner water pool of the vesicle) and 'free' (i.e., located in the bulk aqueous phase) ions without explicitly specifying whether they are bound to the corresponding leaflet of the vesicle or not. Without plunging into molecular details, ion penetration through the bilayer can be associated with a diffusive barrier crossing and also with a passage through a fluctuating bottleneck. Except at very short times, both processes are well approximated by the exponential kinetics [14,15]. Let us consider the simplest possible situation where transport is characterized by a single rate constant thus neglecting any nonhomogeneities of the bilayer structure. This is a good approximation for biological membranes in the fluid state. When the system is close to the gel-to-fluid phase transition and domains of the two phases are formed within the bilayer, one has to ascribe different permeability to each phase and also to the associated interfacial regions between gel and fluid domains [3].

Once we assume that each event of solute crossing the bilayer is characterized by a single first-order rate constant, the overall solute migration dynamics in a monodisperse ensemble of vesicles can be described by the following scheme [16]

$$V_n + Q_{\mathrm{aq}} \underset{(n+1)k_-}{\overset{k_+}{\rightleftarrows}} V_{n+1} \tag{1}$$

where V_n stands for a vesicle incorporating n solute molecules in the inner water pool, Q_{aq} denotes a solute in the bulk aqueous phase, k_+ is the rate constant for entry of a solute molecule from the bulk aqueous phase into the intravesicular water pool, while k_- is the rate constant for the reverse process. In what follows, we shall be concerned with a particular fluorescence quenching experiment and thus focus on the quencher solubilization dynamics (explaining our motivation for the notation Q_{aq} introduced). In Eq. (1), we have neglected possible dependence of either k_+ or k_- on the number of quenchers already contained in the inner water pool of a given vesicle. This is a reasonable approximation at low occupancy numbers.

Let $[Q]$ be the total concentration of quenchers in solution and $[V]$ denote the concentration of vesicles. The average number of quenchers in the inner water pool of a vesicle $\bar{n}(t)$ is related to the concentration of quenchers in the bulk phase $[Q_{\mathrm{aq}}](t)$ at any time as $[Q_{\mathrm{aq}}](t) = [Q] - [V]\bar{n}(t)$. Let us further define the time-dependent probability for a vesicle to contain n quenchers in the inner water pool as $\nu_n(t) = [V_n](t)/[V]$, where $[V_n](t)$ is the concentration of

such vesicles. Eq. (1) corresponds to the following set of stochastic rate equations

$$\frac{d\nu_n}{dt} = k_+[Q_{aq}]\nu_{n-1} - \left(k_+[Q_{aq}] + nk_-\right)\nu_n + (n+1)k_-\,\nu_{n+1} \tag{2}$$

In a typical experiment, the quencher is added to the bulk aqueous phase. Therefore, the initial condition for Eq. (2) is $\nu_n(0) = \delta_{n,0}$, where δ is the Kronecker delta.

By using the generating function defined by $F(s,t) = \sum_{n=0}^{\infty} s^n \nu_n$, Eq. (2) can be reduced to the partial differential equation

$$\frac{\partial F(s,t)}{\partial t} + k_-(s-1)\frac{\partial F(s,t)}{\partial s} = k_+([Q] - [V]\bar{n}(t))(s-1)F(s,t) \tag{3}$$

Taking into account the initial condition, $F(s,0) = 1$, and the conservation constraint, $F(1,t) = 1$, we transform Eq. (3) to the following form (Appendix A)

$$\frac{\ln F(s,t)}{s-1} = \frac{k_+[Q]}{k_-}(1 - e^{-k_- t}) - k_+[V]e^{-k_- t} \otimes \bar{n}(t) \tag{4}$$

where $\otimes$ denotes the convolution product. Noting that $\bar{n}(t) = \partial F(s,t)/\partial s|_{s=1}$, we obtain from Eq. (4)

$$\bar{n}(t) = \bar{n}_\infty\{1 - e^{-kt}\} \tag{5}$$

where

$$\bar{n}_\infty = \frac{k_+[Q]}{k_- + k_+[V]} \text{ and } k = k_- + k_+[V] \tag{6}$$

The generating function is given by $\ln F(s,t) = (s-1)\bar{n}(t)$ from which we find that the probability distribution is Poissonian

$$\nu_n(t) = \frac{\{\bar{n}(t)\}^n}{n!}\exp\{-\bar{n}(t)\} \equiv P_n\{\bar{n}(t)\} \tag{7}$$

with a time-dependent average.

Poisson statistics are inherent in a random system of noninteracting particles ('ideal gas') [17]. As soon as the density of the system is increased, interactions generally manifest themselves skewing the equilibrium distribution. Attractive interactions have an effect of broadening the distribution while repulsion causes its narrowing. Similarly, interactions also influence the solute migration dynamics. For instance, repulsion favors escape of solutes from the inner water pool of a vesicle. This effect can be included by an appropriate Boltzmann factor in k_-. Long-range interactions (e.g., between ionic species) require special approach, at least on the level of the Poisson–Boltzmann approximation. Here we neglect such effects for simplicity assuming that the average occupancy number in the inner water pool of a vesicle is sufficiently low.

In the steady-state fluorescence experiment, the contribution to the total signal due to fluorophores residing on the inner leaflets of the vesicles is given by [9,10]

$$I_{in}(t) = I_{in}(0)\sum_{n=0}^{\infty}\frac{\nu_n(t)}{1+\alpha n} \tag{8}$$

where $\alpha = k_q\tau$ with k_q being the rate constant for quenching in a vesicle incorporating one quencher in the inner water pool and τ the excitation self-decay lifetime. In deriving Eq. (8), we have multiplied the probability for the excitation to be deactivated in a vesicle with n quenchers, $[1+\alpha n]^{-1}$, by the probability $\nu_n(t)$ that the vesicle in fact contains n quenchers, and then summed the result over n. Here we have assumed, on the basis of previous experimental and theoretical findings, that quenching in the inner water pool of a vesicle obeys pseudo-first-order kinetics [9]. This implies taking only the lowest eigenvalue from the spectrum of the underlying restricted diffusion problem. In other words, this is a long-time approximation which proves to work very well for vesicles of relatively small size (almost perfectly well for micelles). As the vesicle size is increased, more eigenvalues have to be taken into account, and eventually one will use a free diffusion model to describe the quenching kinetics.

It is important to realize the difference between the stochastic approach and the conventional formalism of chemical kinetics as applied to solubilization dynamics. Stochastic approach considers the full time-dependent distribution of quenchers among vesicles, and the steady-state fluorescence intensity is calculated 'microscopically' for each occupancy number

and then averaged over the quencher distribution. In contrast, conventional theory considers only the average and thus leads to the following expression for $I_{in}(t)$

$$I_{in}(t) = \frac{I_{in}(0)}{1 + \alpha\bar{n}(t)} \tag{9}$$

Time dependence of the average $\bar{n}(t)$ can be derived without any information about the form of the quencher distribution. One just writes the corresponding rate equation, $d\bar{n}/dt = k_+[Q_{aq}] - k_-\bar{n}$, and solves it subject to the conservation constraint, $[Q] = [Q_{aq}] + [V]\bar{n}(t)$. The result is Eq. (5), as expected.

Apparently, Eq. (9) should work well for small α, where $\sum_n \nu_n(t)[1 + \alpha n]^{-1} \approx \sum_n \nu_n(t)(1 - \alpha n) = 1 - \alpha\bar{n} \approx [1 + \alpha\bar{n}]^{-1}$. Small α virtually means small k_q, and since k_q is inversely proportional to the vesicle radius squared [9], this limit implies large vesicle size. Eq. (9) must also be a good approximation for large values of $\bar{n}$, since then one can write $\sum_n \nu_n(t)[1 + \alpha n]^{-1} \approx \sum_n \nu_n(t)/\alpha(n + 1) = \sum_n \nu_{n+1}(t)/\alpha\bar{n} \approx [1 + \alpha\bar{n}]^{-1}$. The second equality follows from Eq. (7). The assumption of $\bar{n} \gg 1$ is implicit in the conventional approach where vesicular interior is treated as a macroscopic phase.

In Fig. 1, we compare Eq. (8) versus Eq. (9) for

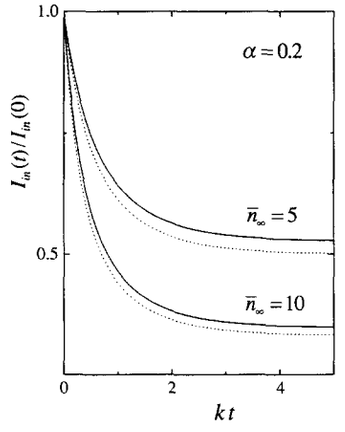

Fig. 1. Time-dependent normalized fluorescence intensity of excited probes inside vesicles after quenchers are added to the bulk phase, calculated within the framework of the stochastic model (Eq. (8), full curves) and the conventional model (Eq. (9), dotted curves) for $\alpha = 0.2$ and $\bar{n}_\infty = 5,10$.

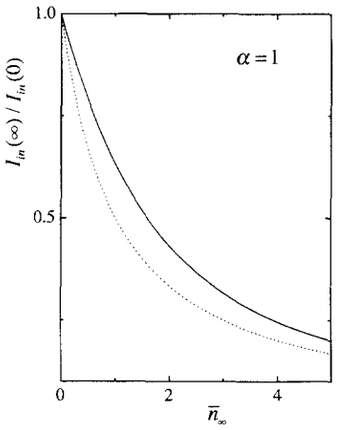

Fig. 2. Normalized steady-state fluorescence intensity of excited probes inside vesicles as a function of quencher concentration at equilibrium for $\alpha = 1$: stochastic model (full curves), conventional model (dotted curves).

$\alpha = 0.2$ and $\bar{n}_\infty = 5,10$. The role of fluctuations proves to be significant for these parameter values. In Fig. 2, we plot the long-time limit of the fluorescence intensity (after complete equilibration) as a function of $\bar{n}_\infty$. The difference between the stochastic model and the conventional kinetics is quite pronounced for low quencher occupancy numbers, though it disappears when $\bar{n}_\infty$ becomes very large.

At long times, Eq. (8) can be approximated by the following simple expression (it can be derived using $P_n\{\bar{n}(t)\} \approx P_n(\bar{n}_\infty)[1 + (\bar{n}_\infty - n)e^{-kt}]$ for small e^{-kt})

$$I_{in}(t)/I_{in}(\infty) \cong 1 + (\bar{n}_\infty - \langle n\rangle_\infty)e^{-kt} \tag{10}$$

where we have introduced a new definition

$$\langle n\rangle_\infty = \frac{\sum_{n=0}^{\infty} nP_n(\bar{n}_\infty)/(1 + \alpha n)}{\sum_{n=0}^{\infty} P_n(\bar{n}_\infty)/(1 + \alpha n)} \tag{11}$$

Fig. 3 illustrates how the final exponential stage is reached.

Apparently, fluorescence intensity of the fluorophores on the outer leaflets grows with time, as $[Q_{aq}](t)$ is decreased. Assuming Stern–Volmer law to hold, i.e.,

$$I_{out}(t)/I_{out}(0) = 1/\left(1 + k_q^a\tau\left[Q_{aq}\right]\right) \tag{12}$$

which is quite reasonable for sufficiently low

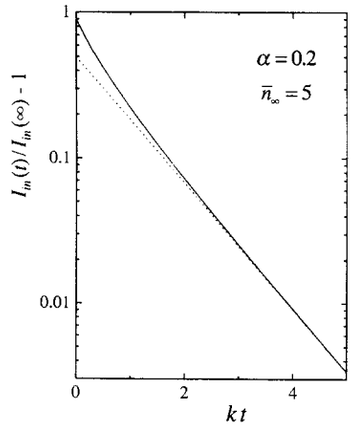

Fig. 3. Plot of $I_{in}(t)/I_{in}(\infty)-1$ versus kt calculated by using the stochastic approach for $\alpha = 0.2$ and $\bar{n}_\infty = 5$. Dotted line shows the asymptotics (Eq. (10)).

quencher concentrations, we can obtain in the long-time limit

$$I_{out}(t)/I_{out}(\infty) \cong 1 - \frac{K_{eq}[V]k_q^a\tau[Q]}{1 + K_{eq}[V] + k_q^a\tau[Q]}e^{-kt} \tag{13}$$

where $K_{eq} = k_+/k_-$ is the equilibrium partitioning constant of quenchers and k_q^a is the second-order rate constant for diffusion-controlled quenching of the fluorophores on the outer leaflets of the vesicles by quenchers dissolved in the bulk aqueous phase. Total fluorescence intensity is proportional to the sum of $I_{in}(t)$ and $I_{out}(t)$ each weighted with the corresponding partitioning coefficient of the fluorophores between vesicle leaflets.

3. Concluding remarks

We have shown on the basis of a simple stochastic model that fluctuation of the number of solute molecules among vesicles can be of considerable importance in describing the solubilization dynamics and lead to significant deviations from conventional kinetics when the average number of solutes per vesicle is sufficiently low. Therefore, particular care should be taken of the fluctuations when dealing with small unilamellar vesicles.

We may conclude by inspecting Eq. (10) and Eq. (13) that total fluorescence intensity of the labeled vesicle solution, while having complex kinetics at earlier stages, must have an exponential tail at long enough times after a quencher is added. This was indeed observed experimentally [5–7]. We have derived from our stochastic model that the rate constant of this final exponential stage is given simply by a *sum* of the frequencies of exit, k_-, and entry, $k_+[V]$, of a quencher molecule from/into the intravesicular water pool into/from the bulk aqueous phase. The rate constant for exit is directly related to the membrane permeability P with a coefficient of proportionality simply being the surface-to-volume ratio [18]. For spherical vesicles of radius R, $k_- = 3P/R$. The measurable long-time rate constant also includes $k_+[V]$, and thus depends on the vesicle concentration as well as on the equilibrium quencher partitioning between the outer aqueous phase and the inner vesicular microphase. If one assumes, for example, that the quenchers are equipartitioned between the two phases, namely, their number density is the same inside and outside the vesicles, $\bar{n}/V_0 = [Q_{aq}]$, one obtains $K_{eq} = V_0$, where V_0 is the volume of the vesicle. As a consequence, $k = k_-(1 + \phi)$, with ϕ denoting the volume fraction of the vesicles in solution. It is important to realize the physical meaning of what is actually obtained from experiment. The long-time rate constant k may certainly serve as a measure of membrane permeability, as it has been assumed previously by several authors [5–8], but one has to clearly distinguish its two components originating from the inward and outward fluxes of the solute species.

Complex nonexponential quenching kinetics have been observed in a stopped-flow experiment at high ionic quencher concentrations [5]. This is because ion binding to the vesicle outer leaflet creates a concentration-dependent gradient in surface potential in addition to the concentration gradient. Ion binding may also induce defects on the packing of the lipids. Nonexponential kinetics with two well-separated stages have also been observed in the vicinity of the main phase transition due to the formation within the bilayer of domains of two different phases (fluid and gel) having different permeabilities [8]. These effects can be readily incorporated into the framework of the stochastic model using the recipe presented in this paper. Another source for nonexponential kinetics is vesicle polydispersity. Recent stopped-flow fluores-

cence quenching experiments with polydisperse phospholipid vesicles have revealed stretched exponential rather than simple first-order kinetics [18]. Using stretched exponentials normally implies dispersive transport and/or fractal structure of the underlying diffusion space. Neither seems to be relevant to the problem at hand, and the physical meaning of the stretching factor obtained remains obscure. A detailed stochastic model with size-dependent rate constants for migration and quenching would apparently be more realistic, yet hardly tractable analytically.

Stochastic model discussed in this paper can also be used to describe chain exchange dynamics in block copolymer micelles [11–13]. Since micelles are normally rather small as compared with vesicles, fluctuations prove to be of particular importance in such systems [9]. Only a simple kinetic model has been devised so far to interpret stopped-flow experiments [12]. It considers only the average in a standard fashion with Eq. (5) type of kinetics. We have discussed above the applicability of the conventional kinetic approach to membrane permeability studies. All arguments remain absolutely the same here. The existence of two well separated relaxation stages in the measured kinetic curves has been reported for certain block copolymer micellar systems [11,12] that cannot be explained in terms of a single mechanism of free chain transfer between independent micelles. Another mechanism of exchange may presumably take place, such as via collisions between micelles [12]. Collisional exchange can readily be incorporated into the framework of the stochastic model [17].

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Appendix A

A common way to solve a first-order partial differential equation is by employing the method of characteristic curves, along which an unknown function satisfies an ordinary differential equation. The characteristic curves for Eq. (3) are given by

$$\frac{ds}{dt} = k_-(s-1)$$
$$s = s(c,t) = 1 + ce^{k_- t} \tag{A1}$$

where c is the integration constant. The corresponding differential equation for $F(s,t)$ is

$$\frac{dF}{dt} = k_+ c([Q] - [V]\bar{n}(t))e^{k_- t}F \tag{A2}$$

This equation is readily solvable giving

$$\ln F = k_+ c\int_0^t ([Q] - [V]\bar{n}(\theta))e^{k_-\theta}d\theta \tag{A3}$$

Now we only have to back substitute $c = (s-1)e^{-k_- t}$ from Eq. (A1) and finally transform Eq. (A3) to Eq. (4).

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