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Biophysical Chemistry 129 (2007) 120–125

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

A fluorescence method for determining transport of charged compounds across lipid bilayer

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Received 3 November 2006; received in revised form 15 May 2007; accepted 15 May 2007
Available online 24 May 2007

Abstract

There is a constant need for simple, economical and time-efficient methods which allow evaluating a compound's ability to penetrate the biological membrane, one of the key parameters needed to characterize biologically active compounds. In the paper we propose a new method of permeability determination. Instead of detecting the compound's concentration directly, we employ an approach in which the membrane interface is labeled with a fluorescein lipid probe; the probe is sensitive to the presence of charged compounds. The fluorescence intensity changes of the dye permanently attached to both sides of a model lipid bilayer are measured. Specifically, the time course of the fluorescence intensity changes following a rapid induction of a non-equilibrium state of the sample allows the evaluation of the membrane permeability for the compound. The method was validated by the determination of the phenyltin compound's transport through the model phosphatidylcholine unilamellar liposome bilayer.

Keywords: Fluorescence method; Membrane permeability; Charged compounds

1. Introduction

The ability to cross biological membranes (permeability) is one of the most important functional parameters for a compound which is considered biologically active. The membrane permeability allows predicting a compound's toxicity [1] or determining its capability to become a pharmacological agent [2]. There are continuous efforts to develop an efficient, cheap and reliable method which allows estimating the ability of a compound to penetrate biological membranes. Biological membranes are complex structures and there are no simple experimental models available at present [3]. Consequently, to determine the ability of a compound to penetrate biological membranes, tests on live cells are frequently used. Different variations of this approach are available but they are relatively expensive and time-consuming [4]. These difficulties spur the search for in vitro tests applicable to a raid screening of a large number of compounds. Therefore, a well-defined and stable model membrane, mimicking relevant biological membrane parameters, needs to be employed. Since majority of pharmaceuticals are small molecular mass molecules, which cross the biological membranes via passive diffusion, a biological membrane can be safely reduced to a model lipid bilayer. The major challenge in the development of such models is the determination of a compound's flux across the membrane. To achieve that, changes of its concentrations on both sides of the lipid bilayer should be sampled. There are two in vitro methods currently

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doi:10.1016/j.bpc.2007.05.010

applied for permeability measurements, namely Parallel Artificial Membrane Permeation Assay (PAMPA) and the liposome-based, spectroscopic method which determines non-symmetrical oriented dipoles distribution using the second harmonic signal generation spectroscopy (SHG). The PAMPA is the application of the filter — supported lipid membrane system developed for industrial purposes. The assay allows for an evaluation of the compound's permeability, but, in addition to problems with the lipid bilayer stability, it requires the additional measurement for the determination of the compound's concentration in the trans-compartment with the help of the appropriate analytical technique [5,6]. The SHG method, on the other hand, is capable of measuring molecular transport across model lipid bilayers (liposomes) in real time and without the need for direct compound detection [7,8]. According to the conditions of the SHG signal detection, it allows differentiating molecules present in the bulk solution from those molecules in the region of interest, namely the interface of bilayer and water. Since the SHG signal directly responds to the water molecules polarized by the charged liposome surfaces it may also serve as a complementary technique to other methods in investigating the electrostatic properties of lipid bilayer surfaces. Although the technique provides straightforward results, its main disadvantages are: high cost and complexity of the instrumentation. In the paper we present a fluorescence method to determine the permeability of a model lipid bilayer to charged amphiphilic compounds. The method is fast, can be performed on commercially available fluorescence equipment and automated for high throughput screening (HTS) applications.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (eggPC) and cholesterol were purchased from Avanti Polar Lipids Inc. (Birmingham, AL, USA) and *N*-(5-Fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Fluorescein-PE) was obtained from Molecular Probes (Eugene, OR, USA). Organotin compounds — triphenyltin chloride ($(C_6H_5)_3SnCl$ or Ph_3SnCl) and diphenyltin chloride ($(C_6H_5)_2SnCl_2$ or Ph_2SnCl_2) were purchased from Alfa Products (Karlsruhe, Germany).

2.2. Labeled liposome preparation

Liposomes were prepared by the extrusion method as described elsewhere [9]. In short, appropriate amounts of phosphatidylcholine (eggPC) (and cholesterol) were mixed with Fluorescein-labeled phosphatidylethanolamine (Fluorescein-PE) in chloroform, giving probe: lipid molar ratio 1:250. The organic solvent was evaporated under the stream of argon followed by a two-hour incubation in the vacuum to remove remnants of the solvent. Finally, the dry lipid film was hydrated with PBS buffer (pH = 6.5, 147 mM NaCl) and the sample was vortexed for 4 min. The milky multilamellar vesicle suspension obtained was subsequently passed through the polycarbonate filter with the pore size of 100 nm.

2.3. Fluorescence measurements

Unless otherwise specified, 150 μl of both the labeled liposome suspension and the phenyltin solution were rapidly mixed in the stopped-flow chamber and fluorescence intensity was measured as a function of time. Changes in fluorescence intensity were detected at the right angle to the incident light beam at the temperature of 22 °C ± 1°. The excitation wavelength was 485 nm and the emission light was collected using a cut-off filter. To ensure the reproducibility of the experimental kinetic traces, each measurement was repeated at least three times. The fluorescence intensity was normalized according to the following expression:

$$F_i = \frac{I_i - I_{min}}{I_{max} - I_{min}}, \quad (1)$$

where F_i is the normalized fluorescence intensity corresponding to I_i (the measured fluorescence intensity at the specific time point), I_{min} and I_{max} are the minimal and maximal fluorescence intensities, respectively. All the measurements were performed on the SF-61 stopped-flow spectrofluorimeter from Hi-Tech Scientific (Salisbury, UK) with dead time of 1.6 ms.

2.4. Experimental design

All approaches, except SHG method, developed for the membrane permeability evaluation are based on the direct measurements of the amount of a compound passing through the membrane. Such an experimental design requires the model system to consist of two macroscopically well-defined compartments separated by the membrane and passing compound quantities at various time points need to be determined. For example, the two most frequently used permeability assays, namely PAMPA and cell monolayer systems, are constructed according to this scheme [4,10].

In this paper, we propose the method based on the assumption that the membrane compound permeability can be measured with the properly selected fluorescent probe whose location within the membrane is well-defined. The application of fluorescent probes opens the possibility to use liposome as a model membrane system, therefore, simplifying the experimental design. Consequently, the two distinct compartments can be precisely identified and monitored with an appropriately selected fluorescent probe sensitive to the presence of the compound.

To evaluate the transfer of a compound through a lipid bilayer, the fluorescence stopped-flow technique has been implemented and the membrane-associated fluorophore, sensitive to changes of its local environment, applied. The liposome suspension is driven out of equilibrium in the stopped-flow apparatus chamber while mixed with the isotonic solution of the compound. The resulting concentration difference across the lipid bilayer induces the flux of the compound which is detected with the changing fluorescence intensity of the fluorophore covalently attached to a lipid at both sides of the lipid bilayer.

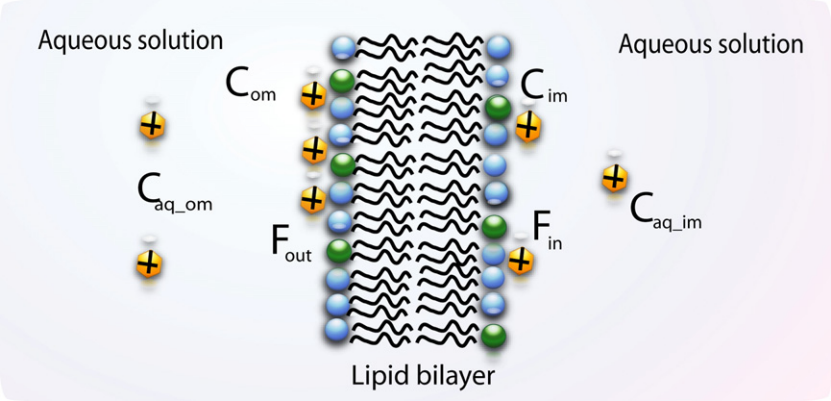

Fig. 1. A schematic diagram of a labeled lipid bilayer. Both fluorescence (F_{out}, F_{in} fluorescence intensity originating from the outer and inner lipid layer) and relevant concentrations of a charged compound (C_{om}, C_{im} concentration of the compound adsorbed at the outer and inner lipid layer, respectively, C_{aq} concentration in the aqueous compartment), are indicated.

When liposome suspension is mixed with the isotonic solution of the compound, first, the molecules adsorb onto the liposome outer surface. This process is diffusion-controlled and should be completed within a few milliseconds (Fig. 2a). Next, the compound crosses the lipid bilayer and appears at the inner liposome surface. It will last as long as the electrochemical potentials on both membrane surfaces equilibrate. Each of the above mentioned processes will result in corresponding changes in the fluorescence intensity. Therefore, its continuous recording should contain information on both adsorption and permeation processes.

Initially, the overall signal acquired from the sample is a sum of the fluorescence intensities originating from two distinct populations of fluorescent probe; one located on the inner and the other on the outer side of the liposome membrane (Fig. 1).

$$F^0_{tot} = F^0_{in} + F^0_{out}, \tag{2}$$

where $F^0{}_{tot}$ indicates the overall fluorescence intensity, and $F^0{}_{in}$ and $F^0{}_{out}$ the fluorescence intensities of probes located on the inner and the outer leaflets of the untreated liposomal lipid bilayer, respectively.

In the subsequent analysis, we assume that any changes of the fluorescence intensity are the sum of the independent changes taking place at the inner and outer membrane surfaces, therefore:

$$F^0_{out} + \Delta F_{tot}(t) = F^0_{in} + \Delta F_{in}(t) + F^0_{out} + \Delta F_{out}(t). \tag{3}$$

This equation is valid only if F^0_{in} and F^0_{out} are independent of time, meaning that the photobleaching effect can be ignored. Independent experiments show that there is no significant decrease of the total fluorescence intensity when fluorescently labeled vesicles are present in the measurement chamber for relevant extent of time.

The process of compound permeation through the lipid bilayer can be divided into stages which occur on different time scales. The compound diffusion to and adsorption onto the membrane surface requires a few milliseconds (Fig. 2a). This process is reflected by the change of the fluorescence intensity of fluorophores located at the outer vesicle surface, whereas the membrane crossing by the compound occurs within tens of seconds followed by its rapid dissociation from the other membrane surface. Therefore, when the membrane permeability is analyzed, the adsorption onto and desorption from the membrane can be considered as instantaneous. The initial

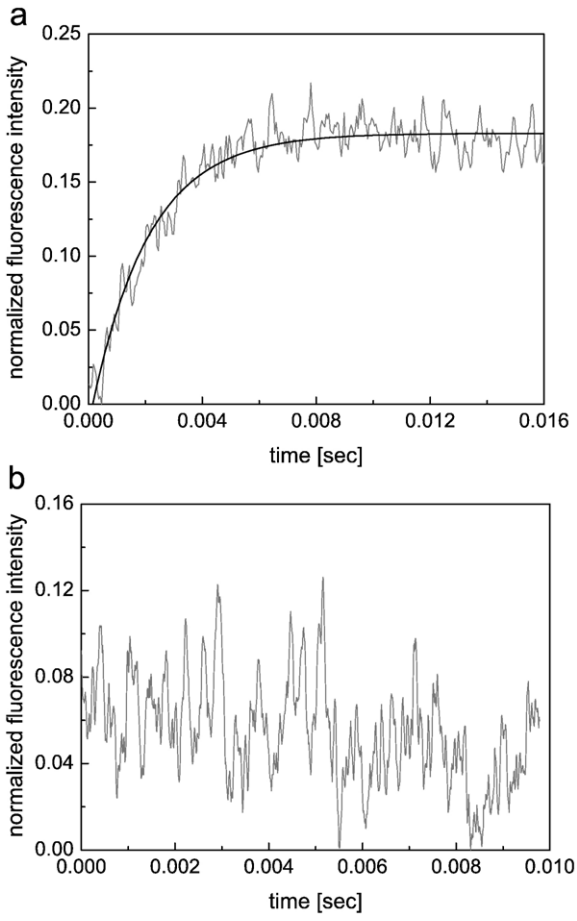

Fig. 2. (a) Fluorescence kinetic trace acquired in the millisecond range after the Fluorescein labeled liposome solution was mixed with the 20 μM isotonic solution of triphenyltin. (b) Fluorescence signal obtained after mixing lidocaine hydrochloride with Fluorescein labeled eggPC liposomes of the same concentration as for the example (a).

fluorescence of the sample, shortly after compound adsorption, can be described by the equation:

$$F(t)|_{t\approx 0} = F^0_{\text{in}} + F^0_{\text{out}} + \Delta F_{\text{out}}(t). \quad (4)$$

If the water/membrane partition coefficient of the compound is large and its flux through the membrane is slow, it can be safely assumed that the amount of adsorbed compound at the outer surface is constant in the course of the measurement. This will result in the initial fluorescence increase on the outer membrane surface to the maximum value F_{max} and ensure that it will not change as long as the amount of the compound in the outer aqueous phase is large compared to that in the liposome interior. The initial amount of the compound on the outer membrane surface can be evaluated if the partition coefficient is predetermined. Consequently, cross-membrane flux of the compound will result in increase of its concentration on the inner side of the vesicle, whereas its quantity on the outer surface will not change. This will induce corresponding fluorescence intensity changes:

$$F(t)_{t>0} = F^0_{\text{in}} + \Delta F_{\text{in}}(t) + F_{\text{max}}, \quad (5)$$

where ΔF_{in} and F_{max} represent fluorescence changes at the inner liposome surface and fluorescence intensity originating from the outer surface, respectively. When the amount of the adsorbed compound on the outer surface is constant the fluorescence intensity does not change. Therefore, its flux across the membrane will result in the change of the total fluorescence originating exclusively from the inner liposome surface. When the quantity of the passing compound is small the change of the fluorescence intensity will be proportional to the concentration of the compound appearing on the inner liposome surface. Consequently, the change of the total fluorescence intensity will reflect the flux of the compound through the membrane:

$$F(t)_{\text{tot}} \approx \text{const.} + \Delta F_{\text{in}}(t). \quad (6)$$

Assuming that the time needed to cross the membrane is much longer than the diffusion time to the liposome surface, the increase of the fluorescence intensity can be directly correlated with the compound concentration change:

$$\Delta F(t)_{\text{tot}} \approx \Delta F_{\text{in}}(t) = a\Delta C^{\text{in}}_m(t), \quad (7)$$

where a is a constant which can be independently determined. Consequently, the fluorescence change can be approximated with a single exponential function:

$$\frac{\Delta F(t)_{\text{tot}}}{F^0_{\text{tot}}} \approx \frac{\Delta F_{\text{in}}(t)}{F^0_{\text{tot}}} = A\ \exp(-\tau t), \quad (8)$$

where τ is a measure of the membrane permeability for the compound.

3. Results

To test the proposed method both the fluorescence probe and an example of a compound, whose permeability will be determined, have been chosen. Phenyltins are chemicals with well-documented biological activity and with the capability to cross the lipid bilayer by passive diffusion [1,11,12]. It has been shown previously that adsorption of such compounds results in the electrostatic surface potential generation which can be detected with the fluorescent probe located at both membrane surfaces. Fluorescein-PE is a probe which satisfies the two conditions. Firstly, the fluorophore is hydrophilic and its covalent attachment to the lipid molecule forces it to stay close to the membrane surface, and secondly, its sensitivity to local proton concentration suits it for the detection of an amount of the surface charges [13–15]. The model lipid bilayer in the form of liposomes has a number of advantages; they are relatively easy to form and when formed from eggPC there is no phase transition in the relevant temperature range. This guarantees that the fluorescence intensity changes are due exclusively to the phenyltin presence on the membrane surface [13]. In addition, liposomal system has two well-defined compartments, i.e. the bulk aqueous phase and the liposomes interior separated by the tight lipid bilayer [13].

The cationic compound added to the liposome suspension initially will adsorb onto the outer lipid layer generating the surface potential and subsequent increase of the fluorescence intensity of Fluorescein-PE, which will respond to increasing local pH induced by the surface potential [16,17]. The process of adsorption will be completed within a few milliseconds as shown in Fig. 2a. At the same time the fluorescence of probes located at the inner lipid layer will not be altered. Subsequent changes of the fluorescence intensity will result exclusively from the phenyltin flux. Experiments performed using lidocaine hydrochloride, where no signal change was observed (Fig. 2b), justified the above interpretation of fluorescence changes. An example of the fluorescence intensity dependence on time and the lipid composition is presented in Fig. 3. The analysis of these plots shows that the curves can be fitted sufficiently with an exponential function with the time constant being proportional to the phenyltin permeability through the membrane. Since the addition of cholesterol stiffens the lipid bilayer

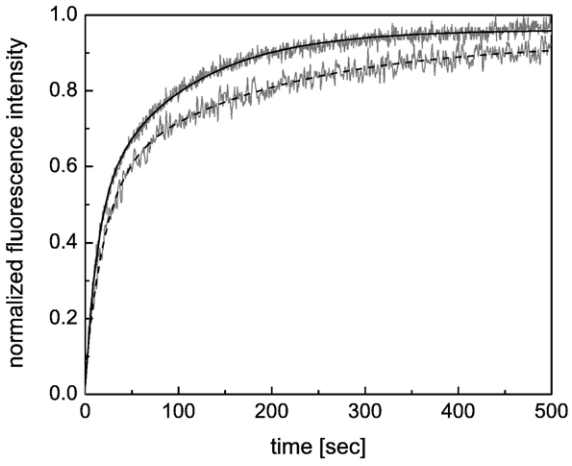

Fig. 3. The kinetic traces of fluorescence changes in the time range of hundreds of seconds approximated with an exponential function in the absence (straight line) and presence (dashed line) of cholesterol (30 mol%) in the eggPC lipid membrane after addition of 80 μM Ph_2SnCl_2 are shown.

the kinetics for Ph_2SnCl_2 observed are slower with respect to eggPC vesicles, as expected. The prediction of the presented theory was also confirmed by similar results obtained for another compound, namely triphenyltin chloride (data not shown).

4. Discussion

Estimation of a compound's capability to penetrate the biological membrane is a major parameter used by the pharmacological industry to select drug candidates from a pool of chemicals. Due to the large number of compounds tested, the financial and time efficiency are important requirements. Therefore, simple and fast tests in vitro are preferred for that purpose.

A method for the permeability determination of biological membranes is based on Caco-2 cell monolayer which requires a time-consuming cells growth stage and relatively complex and labor intensive measurements [18,19]. Due to those problems, the permeability tests on model membrane systems are preferred. Such an experimental setup has been constructed and is based on a lipid bilayer formed on porous material (PAMPA) [18]. All available permeability tests relay on determination of the tested compound concentration. This imposes certain limitation on the experimental systems — namely probing of solutions from well-defined compartments should be possible. The application of fluorescence spectroscopy is well-established for the measurements of the membrane permeability and/or integrity. The most frequent methods include the leakage of the fluorescent tracers [20–23], the permeability of the fluorescent probe quencher [24,25], and the utilization of the energy transfer between entrapped probes [26]. However, each of these methods has weak points. The major problem is that each of them requires the permeating molecule to be fluorescent. As a result these approaches are used to study the membrane itself rather than the permeability to compounds of interest. Therefore, they were not employed as a method of choice in the pharmaceutical industry for permeability determination.

The method presented in the paper proposes a different approach, namely using fluorescently labeled lipids having fluorophores located on both surfaces of the model membrane. Therefore, there are two populations of probes and each of them is sensing the compound concentration in different water compartment. Such an experimental design allows for the application of economically attractive and convenient to use liposomal membrane models. The method should be applicable to a wide range of compounds since most biologically active and pharmacologically relevant molecules are weak acids or bases. In addition, different fluorescent probes can be used, which allows detecting changes in different membrane parameters [27–30]. Such an approach is exemplified by a similar detection system designed by Melchior, where hydrophobic fluorescent probe was used to monitor insertion of various molecules into a model lipid membrane [31]. In contrast to our method, the fluorescent assay proposed by Melchior permits measuring the membrane entry of compounds only. Since DPH (1,6 diphenyl-1,3,5-haxatrien), probe used in this design [31] carries a very hydrophobic group and exhibits a rigid rod-like shape, which allows to pack well in the fatty acyl chains, it is located deeply within membranes [32]. Therefore, DPH is able to probe the interior of the lipid bilayer and to monitor the entrance of a molecule. Nevertheless, it provides no direct evidence of the molecular transport across the lipid bilayer. Summarizing, the charge-sensitive fluorescent probe, fluorescein-PE, positioned within the lipid bilayer interface and distributed uniformly on its both sides, serves as an excellent tool for the real time monitoring of an amphiphilic compound flux across model membranes. Such an experimental setup allows for rapid evaluation of the ability of a compound to cross the lipid bilayer barrier, an important parameter used to evaluate the candidates for pharmacological application. The presented method allows for a frequent probing of a large number of samples and its simplicity makes the method a good candidate for automated screening systems.

Acknowledgments

This work was possible thanks to the financial support from the Centre of Biomedical Engineering at Wroclaw University of Technology, Institute of Physics, and the Grant Agency of the Czech Republic (A.O. via 203/05/H001).

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