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

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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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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.