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A liposomal fluorescence assay to study permeation kinetics of drug-like weak bases across the lipid bilayer

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ABSTRACT

Lipid bilayer permeation is considered the major route for *in vivo* barrier passage of drugs. Despite this fact, no technique is currently available to measure the kinetics of permeation across a single lipid bilayer of structurally unrelated drug-like solutes. We developed a liposomal fluorescence assay capable to determine permeation kinetics of basic drug-like solutes across lipid bilayers. The assay is based on the hypothesis that permeation of a weak base along a concentration gradient results in net proton release at the *cis*-side and net proton capture at the *trans*-side of the bilayer. The resulting pH changes were monitored with pH-sensitive fluorophores: Test compounds were incubated with liposomes containing a pH-sensitive fluorophore at the bilayer surfaces or in the aqueous lumen and fluorescence changes were monitored with a stopped-flow apparatus in solution or by total internal reflection fluorescence microscopy with surface-captured liposomes on a microfluidic platform. Incubation with lipophilic basic drugs resulted in the expected fluorescence changes while incubation with compounds without basic functionality or high polarity did not affect fluorescence. Kinetics of fluorescence changes followed bi-exponential functions. Logarithmic permeation coefficients ($\log Perm_{app}$) determined in solution and by microfluidics technology showed a good correlation ($r^2 = 0.94$, $n = 7$) and $\log Perm_{app}$ increased with increasing lipophilicity. Neither diffusion in the aqueous phase nor partitioning into the bilayer was rate-limiting. PEGylation of 2% of the liposomal lipids reduced $Perm_{app}$ by a factor ~300. In conclusion, the presented liposomal fluorescence assay is capable to determine permeation kinetics of weak basic drug-like solutes across lipid bilayers. The method is adaptable to microfluidics technology for high-throughput measurements and can potentially be modified to work for weak acid solutes.

1. Introduction

The kinetics of lipid bilayer permeation and protein-mediated transport of amphiphilic solutes is of high interest in drug development and toxicology. Membrane permeation is a prerequisite for the solute to reach an intracellular target and determines in addition its pharmaco- or toxicokinetics, also referred to as its ADMET properties. Absorption (the A in ADMET), Distribution across tight barriers such as the blood–brain barrier and distribution into cells, Metabolism to active, inactive or reactive, toxic (Toxicity) metabolites by intracellular enzymes, re-absorption in the renal tubules reducing Excretion, all these processes depend on membrane permeation. In drug development, lipid bilayer permeation is the preferred pathway for drugs to cross *in vivo* barriers as this process is unspecific, allowing a broad structure variability, and non-saturable at typical drug concentrations, excluding transport-related saturation phenomena and drug–drug interactions [1].

Currently, lipid bilayer permeation of a solute is predicted and optimized based on various *in silico* (computational) and *in vitro* assays. *In silico*, compounds are filtered for structural properties that influence barrier permeation, the most important ones related to hydrogen bonding capabilities and molecular size [2]. *In vitro* methods include the determination of the logarithmic concentration ratio of neutral species

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(log*P*) or total compound (log*D*) in n-octanol/buffer, passage of lipid- or solvent-impregnated filter pores such as the parallel artificial membrane permeability assay (PAMPA) and permeation across cultured epithelial or endothelial cell layers [3–6]. These parameters are either surrogate measures or, in the case of cultured cells, result from a combination of several barrier mechanisms. There is currently no method available to measure permeation kinetics across a well-defined single lipid bilayer of a wide variety of structurally unrelated drug-like compounds. Such a method would allow to investigate questions related to the kinetics and mechanisms of lipid bilayer permeation of drug-like compounds in more depth.

The thermodynamically most stable form of a bilayer consisting of cell membrane lipids, is a vesicle or liposome. Fluorescence spectroscopy offers several approaches to study membrane permeation of fluorescent solutes into liposomes, out of liposomes or across barriers formed of fused liposomes. Examples are permeation studies with compounds with self-quenching fluorescence. After encapsulation at high concentration into liposomes, permeation kinetics can be determined from the increase in fluorescence upon permeation out of the vesicles. This technique was applied by Jespersen et al. to determine the influence of fatty acids on doxorubicin permeation [7] and by Shimanouchi et al., to study permeation kinetics of calcein [8]. Kolesinska et al. presented a method to follow lipid membrane permeation of fluorescent-labeled cell-penetrating peptides with a nanoFast chip with lipid-membrane sealed cavities. Florescence in the cavities resulting from peptide permeation was measured with a fluorescence microscope [9]. Confocal microscopy allows to follow permeation of fluorescent low molecular weight compounds and peptides out of giant unilamellar vesicles as shown by Li et al. and in one of our recent works [10,11]. The major drawback of such approaches is that the solute under investigation must be fluorescent or carry a fluorescent moiety.

Our groups have studied membrane permeation of peptides, aromatic carboxylic acids and tetracyclines with terbium-containing liposomes in solution and europium-containing liposomes immobilized on a microfluidic chip, respectively [12–16]. Photoluminescence occurs when energy is transferred from the aromatic system of the permeated solute to the liposome-entrapped lanthanide. These lanthanide-based systems have the advantages of low background luminescence and large stokes shifts. However, the solute requires a suitable absorption spectrum and a structural feature that chelates the lanthanide for energy transfer.

The goal of the work presented here was to develop a liposomal permeation assay that can be used for a wide variety of structures, in particular for drug- and toxin-like compounds. Most pharmacologically or toxicologically active compounds contain a weak base or acid function. As the protonation/deprotonation equilibrium is dependent on the environment, these solutes change their degree of protonation when passing across the lipid bilayer [17]. We aimed at monitoring these changes with pH-sensitive fluorophores. Fluorescent pH indicators were used before to study transfer of protons across lipid bilayers by single-molecule total internal reflection fluorescence (TIRF) microscopy [18] and permeation of carboxylic acids across black lipid membranes or into giant unilamellar vesicles by confocal laser scanning microscopy as recently published by Grime et al. and Li et al. [19,20]. However, no method so far was suitable to determine the permeation kinetics of drug-like compounds across lipid bilayers. Here we present a new approach for permeation studies with drug-like weak bases, expandable to weak acids. We evaluated the methodology with liposomes in solution and immobilized on a microfluidic chip, amenable to high-throughput measurement and investigated the influence of lipid polyethyleneglycol modification (PEGylation) on the permeation kinetics with the goal to increase permeation half-lives from the millisecond to the second range.

Fig. 1 describes the principle of our liposomal permeation assay. At an outside-to-inside concentration gradient of a weak base across the lipid bilayer of a membrane vesicle, partitioning from the outside aqueous phase into the hydrophobic core of the bilayer results in a net proton release at the outer side of the bilayer while partitioning out of the hydrophobic core into the vesicle lumen results in a net proton capture by the basic solute at the inner bilayer/water interface (Fig. 1a, b). We show here, that these changes in pH can be monitored in real time with an adequate pH-sensitive fluorophore. As fluorophores we used either 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) in the liposomal lumen (Fig. 1a) or *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (F-DHPE) with the fluorophore located at the membrane surfaces (Fig. 1b). The protons released at the outer membrane surface were buffered by 2-(*N*-morpholino)ethanesulfonic acid (MES), a zwitterionic buffer with low membrane permeability [21], at pH 5.9, that is slightly acidic mimicking the pH in the lumen of the duodenum. The liposome lumen contained no buffer but NaCl at isoosmolarity, equilibrated to the extraliposomal pH. Within this unbuffered environment, proton capture by permeating solutes caused an increase in fluorescence. These changes in fluorescence were measured in a stopped-flow apparatus (Fig. 1a and b) and with microfluidics technology (Fig. 1c), respectively.

2. Methods

2.1. Liposomes

Large unilamellar liposomes (LUV) were prepared by extrusion [22]. In brief, a thin film of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was prepared from a methanol/chloroform stock solution by solvent evaporation at 37 °C in vacuum. For F-DHPE liposomes, 0.05% of total lipids (w/w) F-DHPE was added before evaporation. For liposomes used in the microfluidic device, 2% (w/w) 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG-biotin) and 0.025% (w/w) 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were added to the lipid solution. The lipid film was flushed with nitrogen and 1 mL per 10 mg lipids of the respective inner aqueous phase (200 mM NaCl, pH 5.9; MES 10 mM, NaCl 190 mM (MES/NaCl), pH 5.9 or 100 μM HPTS in 200 mM NaCl, pH 5.9) was added. Lipid swelling and formation of multilamellar vesicles (MLV) were induced for 20 min at 37 °C. MLVs were freeze-thawed ten times in liquid nitrogen and in a water bath at 37 °C respectively, and LUVs were prepared by twenty extrusion passages through a 200 nm membrane (Whatman Nuclepore Track-Etch Membrane, 25 mm, 0.2 μm). The average hydrodynamic diameter and size distribution were measured by dynamic light scattering (Zetasizer 3000 HAS, Malvern Instruments). Liposomes were stored at 4 °C for maximal one week.

2.2. Exchange of outer aqueous phase

For stopped-flow experiments, the outer aqueous phase was exchanged by size exclusion chromatography (Sephadex G-25 PD-10, GE Healthcare). The column was equilibrated with the desired outer phase (MES/NaCl, pH 5.9 or 100 μM HPTS in 200 mM NaCl, pH 5.9) and 1 mL liposome solution was placed on the column, washed with outer phase and collected. The column was washed for reuse and stored at 4 °C. For microfluidic experiments, the outer aqueous phase was exchanged on-chip after vesicle immobilization.

2.3. Stopped flow fluorescence measurements

An SX17MV stopped-flow apparatus (Applied Photophysics) was used. The fluorophores were excited at 450 nm and fluorescence emission was monitored with a long pass filter 475 nm. All measurements were performed at 25 °C. Drug solutions, 20 μM, in the respective outer aqueous phase (MES/NaCl, pH 5.9 or 100 μM HPTS in 200 mM NaCl, pH 5.9) were mixed in a 1:1 ratio with the liposomes in the cuvette, resulting in a final lipid concentration of about 2.5 mg/mL.