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ORIGINAL PAPER

Fluorescence Lifetime Tuning—A Novel Approach to Study Flip-Flop Kinetics in Supported Phospholipid Bilayers

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Abstract In the present work we introduce a straightforward fluorescent assay that can be applied in studies of the transbilayer movement (flip-flop) of fluorescent lipid analogues across supported phospholipid bilayers (SPBs). The assay is based on the distance dependent fluorescence quenching by light absorbing surfaces. Applied to SPBs this effect leads to strong differences in fluorescence lifetimes when the dye moves from the outer lipid leaflet to the leaflet in contact with the support. Herein, we present the basic principles of this novel approach, and comment on its advantages over the commonly used methods for investigating flip-flop dynamics across lipid bilayers. We test the assay on the fluorescent lipid analog Atto633-DOPE and the 3-hydroxyflavone F2N12S probe in SPBs composed of DOPC/ DOPS lipids. Moreover, we compare and discuss the flip-flop rates of the probes with respect to their lateral diffusion coefficients.

Keywords Flip-flop dynamics · Lateral diffusion · Fluorescence lifetime tuning · Fluorescent lipid analogues · F2N12S 3-hydrohyflavone

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Introduction

The plasma membrane, a complex matrix of a broad range of lipids and proteins, plays a crucial role in cell functions. The cell plasma membranes consist of two distinct leaflets that differ in lipid composition. Lipid translocation between the two leaflets of the lipid bilayer is called flip-flop. In this process, the polar lipid headgroup moves through the hydrophobic region and must thus overcome an unfavorable energy barrier. Consequently, the rate of lipid translocation across the lipid bilayer is expected to be relatively slow. The flip-flop process is facilitated in cells by a number of mechanisms that act to maintain and regulate the lipid distribution across membranes [4, 22, 23, 27, 28]. For instance, enhanced flip-flop, that leads to disruption of the lipid asymmetry across the membranes, may be induced by local defects of the bilayer structure that occur upon membrane insertion of platelets antagonists [5], proteins [29], as well as pore-forming peptides [9]. The possible mechanism of this process was proposed to be through transient defects in the lateral packing of lipids [14].

A number of studies have already aimed to access the flip-flop dynamics of lipid analogues across phospholipid bilayers in vesicles. The first trial by Kornberg and McConnell used sodium ascorbate reduction of a paramagnetic analog of phosphatidylcholine (PC) in sonicated vesicles. The flip-flop half time was 6.5 h at 30 °C [19]. Another study monitored the transbilayer movement of lipid analogs labeled with the fluorescent fatty acid, 5-(5,7-dimethyl BODIPY)-1-pentanoic acid (C5-DMB-) in 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) large unilamellar vesicles (LUVs) at 22 °C. The results showed that C_5-DMB-PC moved across the bilayer with a half time $t_{1/2}$=7.5 h, while the half times for C_5-DMB-sphingomyelin and C_5-DMB-ceramide were 3.3 h and 22 min, respectively [1].

There are only few studies of transbilayer movement of lipids or lipid analogues across planar phospholipid membranes. In case of supported phospholipid bilayers (SPBs), the asymmetry may be obtained by either Langmuir-Blodget/Langmuir-Schaefer (LB/LS) or Langmuir-Blodget/vesicle fusion (LB/VF) method. However, lipid mixing is extensive during LB/LS preparation [11] and remarkably fast flip-flop ($t_{1/2}$=1.3 min, for DMPC at 20.4 °C) was observed in case of LB/VF near or above the main phase transition temperature [20], impeding the flip-flop studies of confluent planar phospholipid bilayers. Consequently, data on transbilayer movement in SPBs is restricted to studies of liquid ordered L_o domains or gel phases. A study that employed fluorescence interference contrast microscopy on polymer tethered planar bilayers composed of POPC, asymmetrically labeled with 0.5% Rh-DPPE showed flip-flop half time $t_{1/2}$=15 h across SPBs at 22 °C [16]. However, lateral diffusion within polymer-tethered membranes was significantly slower than in confluent SPBs studied by FRAP [26] or Z-scan FCS [21, 24].

The fluorescent assay presented herein is based on fluorescence lifetime tuning by light absorbing layer of indium-tin oxide (ITO). This approach is based on energy transfer from emitting molecules in the close proximity of a partially reflecting surface [7, 8]. These phenomena result in shortening of the fluorescence lifetime of emitting molecules [6].There are two main mechanisms underlying a change in the fluorescence lifetime. The first is the rather moderate effect of optical interference leading to an oscillatory behavior at long distances (on the order of the emission wavelength). The second is a strong electromagnetic coupling between the fluorescing molecule and the absorbing layer (a metal or a semiconductor with an imaginary part of the refractive index for given emission wavelengths) leading to a strong energy transfer from the molecule to the absorbing film and resulting in a short range (tens of nanometers) quenching and lifetime decrease.

We recently applied the fluorescence lifetime tuning approach to SPBs to differentiate between 2D and 3D diffusion of identical fluorophores [3] and to demonstrate the positioning of the fluorescent dyes within two bilayer leaflets [21].

In order to study the flip-flop kinetics, SPBs are created on an oxidized surface of silica modified ITO-covered glass supports. Since the optically active layer of ITO acts as a quencher for fluorescent probes, the lifetime of emitting molecules in close proximity to the ITO surface is considerably reduced. This effect exhibits strong distance dependence. As a result, fluorescence lifetimes of head-labeled probes incorporated into the inner and outer leaflet of SPBs differ significantly [21]. In the developed assay, fluorescent probes are added to the outer leaflet of SPBs. As the fluorescent molecules undergo motion to the inner leaflet of the bilayer, their lifetime is shortened in close proximity of the quenching ITO layer. The monitored amplitude averaged fluorescence lifetime decreases with time and reaches a final equilibrium value that corresponds to the fluorescence lifetime of symmetrically labeled SPBs incubated on the same surface.

Materials and methods

Chemicals and supports Lipids 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC), 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine (DOPE) and 1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-L-Serine] (DOPS) were supplied by Avanti Polar Lipids (Alabaster, AL, USA). The flavone probe, *N*-[[4′-*N*,*N*-diethylamino-3-hydroxy-6-flavonyl] methyl]-*N*-methyl-*N*-(3-sulfopropyl)-1-dodecanaminium (F2N12S), was synthesized as described [25]. Atto633-NHS and Atto488-NHS esters were obtained from ATTO-TEC GmbH (Siegen, Germany), and were used in a standard lipid labeling procedure to obtain Atto633-DOPE and Atto488-DOPE, respectively. Solvents of spectroscopic grade were supplied by Merck (Darmstadt, Germany).

The glass supports were covered with a 173-nm layer of indium-tin oxide (ITO), 90% indium oxide In_2O_3 and 10% tin oxide SnO_2 and were supplied from GeSiM mbH, Grosserkmannsdorf, Germany. The ITO supports were covered with 6 nm thick SiO_2 layer by vacuum sputtering. The supports were characterized with a J.A. Woollam variable angle spectral ellipsometer as described elsewhere [3]. Supports were cleaned before use with 1% detergent, rinsed with miliQ water and oxidized by 10 min UV/ozone treatment. Mica stacks of 5 mm diameter (Metafix, Montdidier, France) were freshly cleaved prior to usage.

Preparation of SPBs The bilayers were prepared by SUVs fusion on silica modified ITO-covered glass supports (flip-flop measurements) and mica sheets (reference measurements). SUVs were obtained by mixing DOPC and DOPS lipids in chloroform at a 4:1 molar ratio. Labeled lipid analogue of spectrally distinct characteristic with respect to the studied flip-flop probe was added at dye to lipid ratio 1:200 000. After solvent evaporation under a stream of dry nitrogen, lipids were re-hydrated in buffer A (10 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH=7.5) to obtain a lipid concentration of 10 mM. The suspension was vortexed for 5 min and sonicated for 20 min using a tip sonicator (Sonopuls HD 2070, Bandelin electronic GmbH & Co. KG, Berlin, Germany) to produce a suspension of SUVs. SUV suspension was then incubated with buffer B (10 mM Hepes, 150 mM

NaCl, 1 mM $CaCl_2$, pH=7.5) on the hydrophilic surface (oxidized silica modified ITO-covered glass, or freshly cleaved mica) in order to obtain homogeneous Supported Phospholipid Bilayers (SPBs). The remaining vesicles were flushed away with buffer B. Before flip-flop experiments, the membranes were tested for fluidity and homogeneity by both Z-Scan FCS and confocal imaging. The measurements were performed at 24 °C.

For each flip-flop measurement, a small aliquot containing approximately 1 nmole of fluorescent analogue in methanol solution was added to the preformed SPBs. The final methanol concentration did not exceed 0.5%. The solution above the SPB was continuously stirred in order to facilitate the dye incorporation into the membrane. Just before the addition of the fluorescent dye, we focused at the membrane and started a set of point measurements in order to capture the flip-flop dynamics.

Instrumentation The measurements were performed using the time-resolved fluorescence confocal microscope MicroTime 200 (PicoQuant GmbH, Berlin, Germany). The system consists of an inverted confocal microscope (Olympus IX81) with a water immersion objective (1.2 NA, 60x). The PDL-828 "Sepia II" driver unit with pulsed diode lasers (LDH-D-C-405B, 405 nm, LDH-P-C-470, 470 nm, and LDH-DC-635B, 635 nm, PicoQuant) having a pulse FWHM of 80 ps and a repetition rate up to 40 MHz, SPAD detectors (Perking-Elmer, Fremont, USA and MPD Micro Photon Devices, Bolzano, Italy) and PicoHarp 300 TCSPC board were used. For each dye, adequate laser lines, dichroic mirrors (405 RDC, Z473/635 RPC), emission filters (500LP, HQ685/50), and emission dichroic (Z635) (Omega Optical, Brattleboro, VT, USA) were employed.

Data treatment Experiments were carried out in the TTTR mode. TCSPC fluorescence decays were tail-fitted using the SymPhoTime software. At any point of the time course of the flip-flop, the decays were fitted by a two-exponential function as two distinct lifetimes were present corresponding to inner and outer leaflet of the bilayer (e.g. for the case of Atto633 in SPBs on silica modified ITO-covered glass support the two lifetimes were $\tau1$=2.5 ns and $\tau2$=1.4 ns). As the fit variables amplitudes and lifetimes are correlated, the flip-flop kinetics analysis based only on the time evolution of relative amplitudes is susceptible to fitting artifacts. For that reason, we used the more robust parameter, amplitude weighted average lifetime.

The spontaneous partitioning of lipid analogues into the lipid bilayers was observed as an initial increase of fluorescence intensity after dye addition. Due to continuous stirring of the solution, the incorporation of the dyes into the membrane was much faster than their flip-flop kinetics, and it appeared that probe incorporation was almost complete within approximately 60 s. After probe incorporation, the time evolution of the amplitude weighted average fluorescent lifetime $\tau_{av}(t)$ was fitted with a mono-exponential function in the form $\tau_{av}(t)=A^* \exp(-\lambda t)+\tau_{av0,}$ where A corresponds to the difference in the amplitude weighted average fluorescent lifetime between asymmetrically (at t=0) and symmetrically labeled SPBs (at $t\rightarrow\infty$), λ is the sum of transbilayer motion rate constants (from outer to inner leaflet and from inner to outer leaflet) and τ_{av0} is the amplitude weighted average fluorescent lifetime of the probe in symmetrically labeled SPBs. The flip-flop half time, $t_{1/2}$ corresponds to half of the signal change, caused by the transbilayer motion of fluorescent molecules from one bilayer leaflet to another, hence $t_{1/2}$ can be written as $t_{1/2}=\ln 2/\lambda$.

A Z-scan approach [2] was applied for lateral diffusion measurements within symmetrically labeled SPBs. A set of point measurements was performed at various z-positions of the focal plane and the bilayer spaced by 0.15 μm (Z-scan approach). Particular measurements were treated as described in [2] and the resulting FCS curves were fitted using one component 2D diffusion model accounting for triplet state transition. The obtained particle number (*PN*), which is the average number of particles in the illuminated membrane surface, and the diffusion time (τ_D) showed parabolic dependences on the z-position of the objective as can be expected from the Gaussian-Lorentzian excitation intensity profile. The parabolic dependences were fitted with the following equations:

$$PN = \pi c \omega_0^2 \left(1 + \frac{\lambda_0^2 \Delta z^2}{\pi^2 n^2 \omega_0^4}\right) \quad (1)$$

$$\tau_D = \frac{\omega_0^2}{4D} \left(1 + \frac{\lambda_0^2 \Delta z^2}{\pi^2 n^2 \omega_0^4}\right) \quad (2)$$

where D is the lateral diffusion coefficient, ω_0 is the radius of the beam in the focal plane, c is the average surface concentration of diffusing fluorescence molecules in the illuminated area, n is the refractive index of the medium, λ is the wavelength of the excitation light, and Δz is the distance between the sample position z_0 and the position of the focal plane. The curve fitting was performed using the Levenberg-Marquardt nonlinear least-squares routine implemented in OriginPro 7.0 software.

Theoretical calculations of quenching efficiency Theoretical calculations based on classical electrodynamics used a model of an oscillating dipole located in stratified media [3, 12]. The input parameters were position and orientation of the dipole (fluorescing molecule) within the layered

system, thicknesses (d_{ITO}=173 nm, d_{SiO2}=6 nm, d_{SLB}= 5 nm), refractive indices of the layers (n_{SLB}=1.45, n_{water}= 1.333, n_{glass}=1.5188, n_{ITO}=1.951 + 0.041i, n_{SiO2}=1.456) and the emission wavelength (670 nm) as well as quantum yield (0.64). The relative lifetime change is derived by comparing dipole emission rates in the supported lipid bilayers with free standing membrane. The source code for the calculations was kindly provided by Joerg Enderlein.

Results

Our aim was to determine the flip-flop kinetics of the standard fluorescent analogue, Atto633-DOPE and the environment-sensitive membrane probe F2N12S, by exploiting the lifetime quenching phenomenon. The latter probe has been demonstrated to have a great potential for ratiometric detection of apoptosis in cells [25], as well as for visualization of lipid domains in giant unilamellar vesicles [17].

An important step in applying the lifetime tuning approach was the choice of the quenching support on which the SPBs are formed. In our previous work [3, 21], we used ITO-covered glass support (glass microscopic slide covered with a 173-nm layer of indium-tin oxide). However, the fluorescence quenching of that support proved to be too strong for the experiments presented herein. To decrease the quenching efficiency we decided to modify the ITO-covered glass support by covering it with a

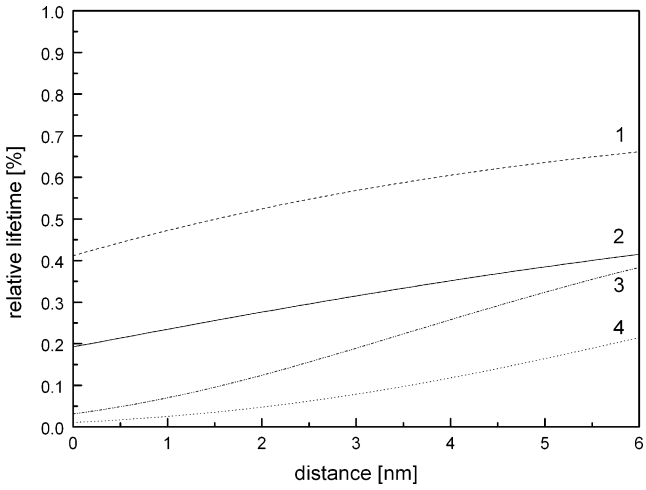

Fig. 1 Relative lifetime as a function of the position of the dye within the SPBs on the quenching support (distance 0). Calculations were performed for the wavelength of 670 nm and 0.64 quantum yield. A thin ITO film of thickness d_{ITO}=173 nm and a complex refractive index n_{ITO}=1.951 + 0.041i is deposited on a glass substrate with refractive index n_{glass}=1.519. Above the film, fluorescing molecules are placed at either distance z=0 from the metal's surface (lines 3 and 4) or at a distance z=6 nm (the 6 nm layer of SiO_2 spacer deposited on the ITO film, n_{SiO2}=1.456) (lines 1 and 2). The fluorescing molecules are incorporated into a supported lipid bilayer of thickness d_{SLB}=5 nm, and a refractive index n_{SLB}=1.45. The supported bilayer is placed in a liquid environment with refractive index n_{water}=1.333. The reference lifetime is the lifetime of the same dye in free standing membranes in solution. Results are shown for vertical (2, 4) and parallel dipole orientation (1, 3) for ITO support (3, 4) and ITO support with a 6 nm silica spacer (1, 2)

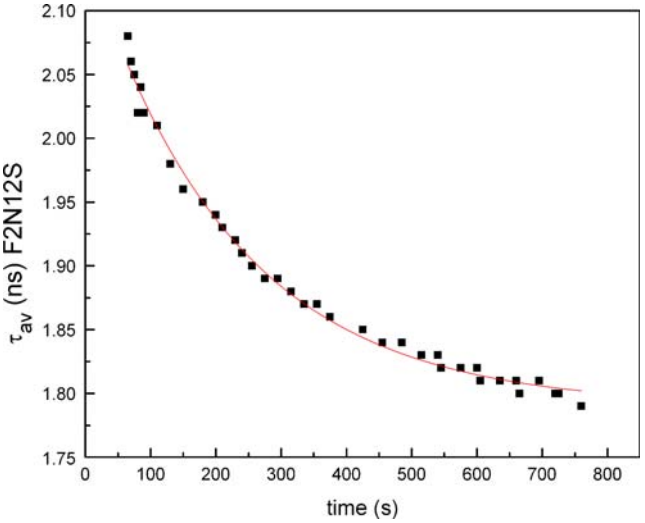

Fig. 2 Exponential decay of the amplitude weighted average fluorescence lifetime for the flavone probe F2N12S added to DOPC/DOPS 4:1 SPBs on ITO-covered glass with a 6 nm SiO_2 layer. The last point represents the average fluorescence lifetime of F2N12S in symmetrically labeled SPBs. The excitation wavelength was 405 nm, emission filter was 500LP. The data were fitted with a mono-exponential decay (*solid line*) and the half time of the transbilayer movement of F2N12S is 155 +/− 8 s

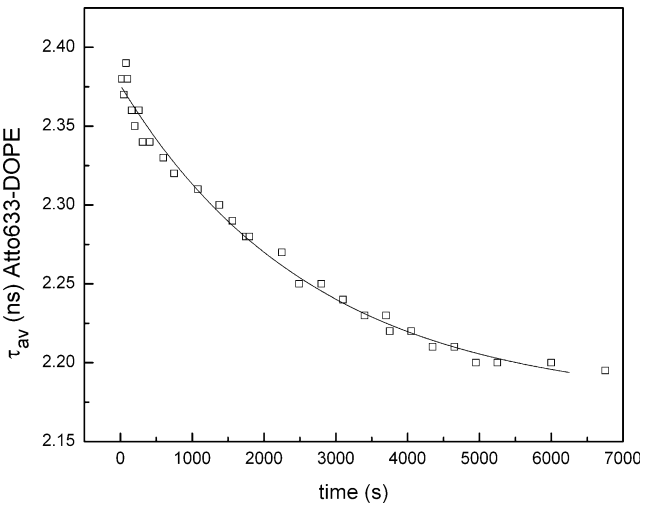

Fig. 3 Changes of the amplitude weighted average fluorescence lifetime of Atto633-DOPE after addition to DOPC/DOPS 4:1 SPBs on ITO-covered glass with a 6 nm SiO_2 layer. The last point represents the average fluorescence lifetime for the same probe in symmetrically labeled SPBs. The excitation wavelength was 635 nm, emission filter was HQ685/50. The data were fitted with a mono-exponential decay yielding the half time of the transbilayer movement of the probe $t_{1/2}$=1,900 +/− 170 s

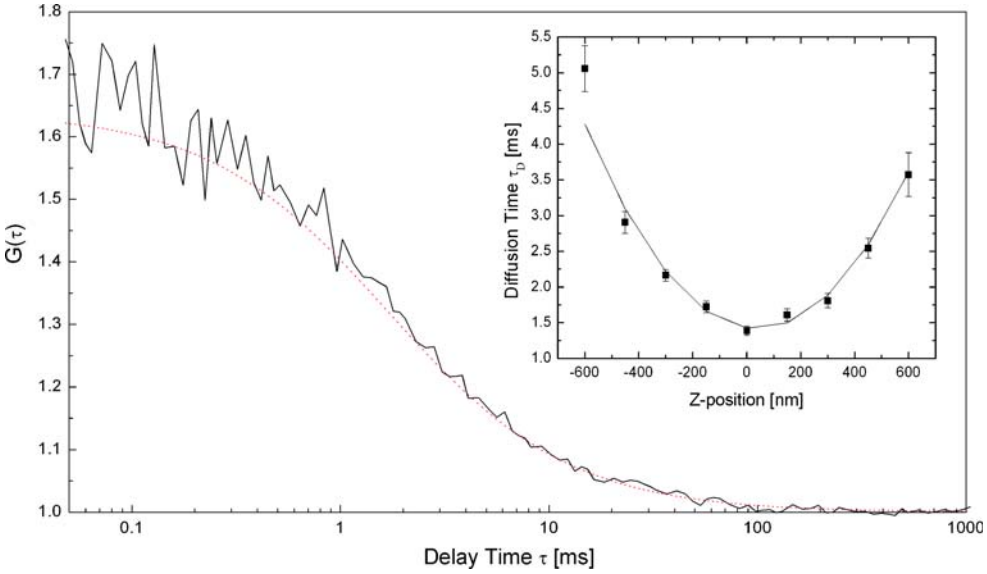

Fig. 4 The characteristic autocorrelation function G(τ), resulting from single point FCS measurement. The measurement was performed for flavone probe, F2N12S incorporated into the DOPC/DOPS 4:1 SPBs created on mica. The data was fitted with a two dimensional model that included the inter-system crossing (*dotted line*). Each measurement in the Z-scan yields the corresponding diffusion time (*inset*). Fitting of the Z-scan measurements (*line in the inlet*) according to eq. 2 allows determining the diffusion coefficient within planar systems with high precision

thin silica layer. To find an optimal thickness of the silica layer theoretical calculations were performed.

Theoretical calculations based on classical electrodynamics [3, 12, 13] enabled us to estimate the thickness of the spacer that would yield optimal fluorescence quenching for both dyes. As it can be seen from the results of calculations depicted in Fig. 1, lines 3 and 4, dyes in the inner leaflet of the SPBs on ITO-covered glass support would be almost entirely quenched and hence, we would not observe any changes in the amplitude weighted average lifetime upon transbilayer movement of the fluorescent dyes across SPBs. In order to maximize the decrease in the amplitude weighted average fluorescence lifetime, the ITO-covered glass support must be covered with a spacer layer. The results obtained for ITO-covered glass support covered with a 6 nm layer of silica suggest that this support should offer an adequate quenching range for Atto-633-DOPE fluorescence at 670 nm (lines 1 and 2, Fig. 1) as well as for the flavone dye (data not shown).

The calculations presented in Fig. 1 show that the use of 6 nm thick silica spacer decreases the overall quenching efficiency, giving a detectable signal also from the molecules located in the inner leaflet of the bilayer. Although less quenched (in comparison to ITO covered glass), the relative lifetimes for inner and outer leaflet remain sufficiently different, making the support with 6 nm silica spacer optimal for measuring flip-flop kinetics.

The fluorescence lifetime quenching on the silica modified ITO surface described above was tested for the two probes by comparison with the SPBs created on a mica support. After addition of the probes to the unlabeled SPBs on mica support, the amplitude weighted average lifetime did not show any significant changes over time (data not shown), while in case of silica modified ITO-covered glass support, two-exponential decays with a clear decrease in the amplitude weighted fluorescence lifetime were observed (Figs. 2–3). For both fluorescent probes, the amplitude weighted average fluorescence lifetimes converged to a plateau value that corresponds to the amplitude weighted average fluorescence lifetime of the probe in symmetrically labeled SPBs prepared directly by fusion of labeled vesicles.

Analysis of the time evolution of the amplitude weighted fluorescence lifetime yielded the half times of the transbilayer motion for each probe. The flip-flop half times obtained for F2N12S (Fig. 2) and Atto633-DOPE (Fig. 3) were 155 s and 1,900 s, respectively. The error of the method was calculated as the standard deviation of the fit and was not exceeding 10%.

To compare the flip-flop rates of the probes with respect to their lateral diffusion coefficients, Z-scan Fluorescence Correlation Spectroscopy approach was applied [2]. The lateral diffusion coefficients of the probes within symmetrically labeled SPBs on mica were measured. A typical autocorrelation curve resulting from an FCS measurement is presented in Fig. 4. The inset (Fig. 4) represents the principle of the Z-scan approach. In brief, consecutive FCS measurements spaced by Δz=150 nm were performed.

Table 1 Comparison of the lateral diffusion coefficients and flip-flop half times of the fluorescent probes F2N12S and Atto633-DOPE

Fluorescent probe	Lateral diffusion coefficient [a] [$\mu m^2/s$]	Flip-flop half times [b] [s]
F2N12S	6.8 +/− 0.5	155 +/− 8
Atto633-DOPE	5.3 +/− 0.4	1,900 +/− 170

Experiments were performed on DOPC/DOPS 4:1 SPBs created on mica [a] or on ITO-covered glass with a 6 nm SiO_2 layer [b] at 24 °C. The flavone derivative, F2N12S, exhibits faster lateral diffusion and flip-flop in comparison to the fluorescent lipid analogue Atto633-DOPE

Each autocorrelation curve was analyzed and yielded the corresponding diffusion time, τ_D [2]. The diffusion times resulting from consecutive measurements of each Z-scan were fitted to parabola, according to eq. 2. The resulting values of diffusion coefficients are resistant to artifacts due to accurate positioning and beam waist determination and hence allows for precise determination of lateral diffusion in planar systems.

The lateral diffusion and flip-flop data are summarized in Table 1. It is visible that the F2N12S probe within SPB is significantly faster than fluorescent lipid analogue, Atto633-DOPE in terms of both the lateral and transbilayer mobility.

Discussion

The observed flip-flop dynamics of the fluorescent lipid analogue Atto633-DOPE is more than one order of magnitude faster than those reported for PC-analogues in either sonicated egg PC vesicles [19] or POPC LUVs [1]. There are numerous factors that facilitate lipid flip-flop [10, 15, 18, 29] and may thus, explain the observed differences. For instance, transient defects in lipid membranes have been shown to be involved in transbilayer movement [14]. Hence, surface induced imperfections within SPBs may well explain the increased transbilayer movement within SPBs as compared to vesicles. These imperfections may also account for the much faster flip-flop dynamics observed for F2N12S in SPBs as compared to cell membranes, since preferential location of the probe in the external leaflet of plasma membranes was observed for at least one hour (Shynkar et al, 07).

Interestingly, the flavone F2N12S probe exhibits approximately an order of magnitude faster flip-flop in comparison to the Atto633-DOPE lipid analog in SPBs. Moreover, the lateral diffusion of the flavone probe is also significantly faster than that of the fluorescent lipid analog measured in this study or published elsewhere [2, 21, 26]. We may speculate that interactions of the F2N12S probe with surrounding lipids are weaker and hence the flip-flop motion energy barrier is comparatively lower than in the case of the fluorescent phospholipid analogs. It seems feasible, that the weaker dye-lipid interactions are also responsible for the faster lateral diffusion of the F2N12S dye within the SPBs. The weaker interactions and faster diffusion of F2N12S are probably because it bears only one hydrophobic (dodecyl) chain in contrast to the lipid derivative Atto633-DOPE that bears two longer chains (dioleoyl).

To summarize, we have devised and applied an easy to use method that can be applied to investigate the transbilayer movement of head-labeled fluorescent lipid analogs across confluent supported phospholipid bilayers. Up to our knowledge, it is the first suitable method for flip-flop studies of SPBs prepared by SUVs fusion.

The application of this method to natural cell membranes should, in principle, be possible and would certainly be of great interest. However, some complications may be expected that stem from the complexity of natural membranes. First of all, the distance of the cell membrane from the support should not exceed several nanometers in order to have sufficient quenching characteristics. This may be hampered by large membrane proteins which disable direct contact of the membrane with the support. Moreover, as stirring could not be exploited, the incorporation of the dye should be included in the flip-flop fitting.

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