Lipid Diffusion in Giant Unilamellar Vesicles Is More than 2 Times Faster than in Supported Phospholipid Bilayers under Identical Conditions

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The lateral diffusion coefficients of a BODIPY tail-labeled lipid in two model systems, namely, free-standing giant unilamellar vesicles (GUVs) and supported phospholipid bilayers (SPBs), were determined by fluorescence correlation spectroscopy (FCS) using the Z-scan approach. For the first time, the performed measurements on 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) bilayers maintain exactly the same experimental conditions for both systems, which allows for a quantitative comparison of lipid diffusion in these two commonly used model membranes. The results obtained revealed that the lipid mobility in free-standing bilayers ($D = 7.8 \pm 0.8\ \mu m^2\ s^{-1}$) is significantly higher than in the bilayer created on the solid support (mica) ($D = 3.1 \pm 0.3\ \mu m^2\ s^{-1}$).

Introduction

Lateral diffusion of biological membrane components is one of the key parameters responsible for cell processes such as peptide aggregation or cell adhesion. Native cell membranes present a complex and heterogeneous system, which makes the description of their biophysical properties quite difficult. Thus, a number of model systems with simplified lipid compositions and structures are used. In particular, giant unilamellar vesicles (GUVs)[1] and supported phospholipid bilayers (SPBs)[2] belong among the most popular model systems for mimicking the cell membrane. The main advantage of GUVs is that their bilayer is free-standing and they are about the size of a cell. Nevertheless, their limitations lie in quite demanding methods of preparation, which excludes their formation at higher ionic strengths.[3] Moreover, GUVs are usually stabilized by sugars such as sucrose and glucose, the presence of which may decrease diffusion coefficients up to a factor of 3.[4] The second model system (SPBs) is formed with the help of hydrophilic surfaces (e.g., mica, oxidized silicon, silica, and glass[5]). The mica represents a preferable surface that is completely flat even on the atomic scale, minimizing the presence of bilayer defects.[2] Nevertheless, objections are often raised that the interaction of the lipid molecules with the surface affects the behavior of the membrane significantly.[6] Therefore, in this letter we address the issue of whether the diffusion of the fluorescent tail-labeled lipid is altered by the presence of the hydrophilic support and to what extent the effect occurs. For this purpose, we decided to compare the GUVs and SPBs by means of fluorescence correlation spectroscopy (FCS).[7] We used the so-called Z-scan approach, originally developed for SPBs[8] and recently applied for cells,[9] which enables us to determine the diffusion coefficients in 2D systems with high accuracy. In comparison to the conventional FCS approach, the Z-scan approach has following advantages: (1) no need for external calibration;[8] (2) precise positioning of the bilayer within the volume element;[8] and (3) spot-size-dependent determination of diffusion coefficients that may identify hindered diffusion phenomena.[9] On the whole, we are convinced that the diffusion coefficient (D) in SPBs and GUVs can be determined at the best with a precision between 7 and 10% by means of the Z-scan approach.[8] In the study[10] using identical instrumentation as in ref 8, we employed conventional FCS for the determination of diffusion coefficients in SPBs and found errors of more than 20%. This point of view might be confirmed by an average error of about 20% obtained by averaging over the errors in the D values determined by standard FCS for single-component phospholipid bilayers given in the herein cited publications.[12,16−18]

To maintain exactly the same conditions for both the systems, SPBs as well as GUVs were prepared from the same lipid mixture; moreover, the measurements were carried out in one cuvette. Thus, the ionic strength and osmolality of the medium were identical, and the lipid diffusion in both systems can be compared quantitatively.

Previous publications[8,11−18] do report diffusion coefficients (Supporting Information) of labeled lipids or lipid analogues in GUVs as well as in SPBs formed by single lipids determined by various fluorescence methods such as FCS or fluorescence recovery after photobleaching (FRAP). However, none of these studies was performed on both systems simultaneously (i.e., under

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identical conditions) and thus addressed the issue of this work. Moreover, in our opinion therein reported diffusion coefficients[8,11−18] do not allow consistent conclusions to be drawn on the differences in diffusion between GUVs and SPBs.

Experimental Section

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti) was mixed with tail-labeled fluorescent lipid 2-(4,4-difluoro-5-octyl-4 bora 3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3 phosphocholine (C8-BODIPY 500/510 C5-HPC, Invitrogen) in a ratio of 200 000:1 in chloroform. For SPBs samples, the headgroup-labeled fluorescent lipid *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (BODIPY FL DHPE, Invitrogen) and the lipid analogue 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD, Invitrogen) were also used.

GUVs were prepared by a modified electroformation method originally developed by Angelova.[1] The solution of lipid and dye was gently applied to the platinum electrodes and kept for at least 3 h under vacuum. The obtained dry lipid film was further hydrated with a 150 mOsm sucrose solution, and an AC electrical field was applied to the electrodes. The medium was then exchanged for a 148 mOsm glucose solution. The presence of glucose in the final solution allowed the sedimentation of liposomes and decreased the movement of vesicles.

The SPBs were prepared by spreading small unilamellar vesicles (SUVs) on the freshly cleaved mica surface[5] or cleaned glass covered with indium tin oxide (ITO). Ellipsometry measurements gave clear evidence that under the used conditions bilayers were formed and no adherent vesicles were present. SUVs were prepared from the above-mentioned chloroform solution of dye and lipid identically to the method reported by Beneš et al.[5] with either Hepes buffer (10 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$) or glucose solution (150 mOsm) as a medium. The mica or ITO-covered glass was attached to a holder 200 μm above the bottom of the cuvette as described in Benda et al.[8] The formed GUVs were transferred to the cuvette containing the glucose solution and SPBs formed on mica attached to the holder. In this case, the GUVs are lying on the bottom of the cuvette, and the SPBs are kept in solution by the dipped holder.

FCS measurements were carried out on a MicroTime 200 inverted epifluorescence confocal microscope (Picoquant, Germany). We used a configuration containing a pulsed diode laser (LDH-P-C-470, 470 nm, Picoquant, and LDH-DC-635, 635 nm, Picoquant) providing 80 ps pulses at a 40 MHz repetition rate, a proper filter set (clean up filter 470/20, dichroic mirror 505DRLP and band-pass filter 525/50) (Omega Optical), and a water immersion objective (1.2 NA, 60x) (Olympus). Low power of 5 μW at the back aperture of the objective was chosen to minimize the effects of photobleaching and saturation.[19] The setup was adjusted to avoid all potential artifacts.[20] A set of the FCS curves was measured at various z positions of the focal plane and the bilayer spaced by 0.2 μm (Z-scan approach). Particular FCS curves were treated as described in Benda et al.[8] The standard deviation was estimated for each lag time and consequently used for weighted fitting.[21] The obtained particle number (*PN*), the average number of particles in the illuminated membrane surface, and the diffusion time (τ_D) showed a parabolic dependence 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 fluorescent 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.

We also plotted τ_D versus PN/PN_0.[9] Such a dependence follows the equation

$$\tau_D = \tau_0 = \frac{\omega_0^2}{4D_{eff}} \frac{PN}{PN_0} \quad (3)$$

where PN_0 corresponds to the number of molecules observed when the sample is in the focal plane (i.e., in the beam waist). The linear regression of this dependence yields the effective diffusion coefficient D_{eff} and the intercept t_0, which is 0 for free diffusion.[9] This analysis is based on the work of Wawrezinieck et al.[22] It allows us to distinguish between free and hindered diffusion and can confirm the validity of the Z-scan approach. The MicroTime 200 additionally provides time-correlated single-photon-counting histograms representing the fluorescence decay of the probe. In the additional experiments on SPBs adsorbed onto ITO glass, these histograms were tail fitted to multiexponential decay laws. The experimental temperature (289 K) was chosen to render the undulation motions of the free-standing bilayer.

Table 1. Diffusion Coefficient Obtained for GUVs in the Glucose Solution and for the SPBs in the Glucose Solution and in the Buffer for C8-BODIPY 500/510 C5-HPC[a]

system (T = 289 K)	D(10^{-12} m^2 s^{-1})	D_{eff}(10^{-12} m^2 s^{-1})
GUVs[b] (glucose solution)	7.8 ± 0.8	7.6 ± 1.1
SPBs[c] (glucose solution)	3.1 ± 0.3	3.0 ± 0.7
SPBs[c] (buffer)	2.7 ± 0.3	2.7 ± 0.9

[a] The lipid used was DOPC. Values of D and D_{eff} were obtained from a classical Z scan and by plotting PN/PN_0 versus τ_D, respectively [b] The calculated diffusion coefficient for GUVs was taken as the average of 24 measurements (among them, 18 on different GUVs). [c] The calculated diffusion coefficient for SPBs was taken as the average of 18 measurements (SPBs were prepared 6 times, and the Z scans were performed at 3 different positions for each sample).

Results and Discussion

Measurements on BODIPY tail-labeled lipid in GUVs and SPBs were performed in the above-mentioned one-cuvette setup as well as in the isolated systems. In all cases, the obtained FCS curves could be satisfactorily fitted with a model derived for one particle freely diffusing in the planar system.[8] The measurements performed on the isolated SPBs and GUVs gave the same results as the measurements carried out in the one-cuvette setup, which confirms that both investigated systems were independent and did not influence each other in the one-cuvette setup. Furthermore, in the case of isolated GUVs, the experiments were done on both the bottom and the top membrane, and no significant difference was observed. This shows that the bottom membrane of GUVs is not affected by the presence of the support, in contrast to the situation for SPBs. Consequently, the top and bottom membranes of GUVs can be considered to be free-standing.

The determined values of the diffusion coefficient are summarized in Table 1. Obviously, the value of the diffusion coefficient obtained for GUVs is approximately 2 times higher compared to that for the SPBs. A similar trend and a significantly more pronounced effect were observed when comparing SPBs formed on the glass and the free-standing bilayer of the black

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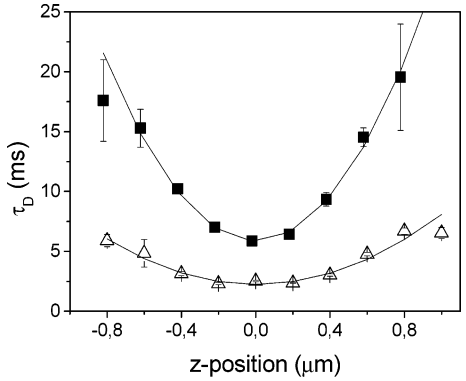

Figure 1. Typical dependence of the diffusion time τ_D on the z position of the laser beam for GUVs (△) and SPBs (■).

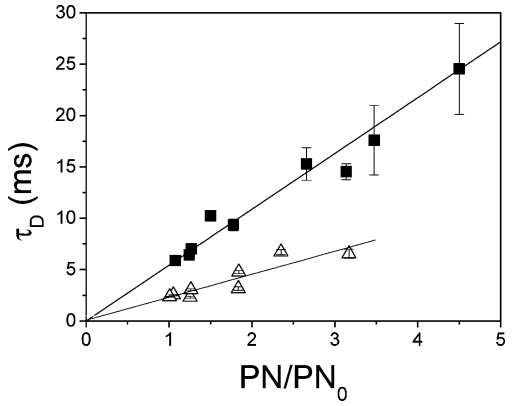

Figure 2. Dependence of PN/PN_0 on τ_D obtained for GUVs (△) and SPBs (■).

lipid membrane.[23] The effect of the presence of ions (10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$) or glucose solution (148 mOsm) does not seem to alter the diffusion in the supported bilayer within the experimental error (Table 1). The typical dependence of diffusion time on the z position of the objective is illustrated in Figure 1. The dependence of particle number PN is almost identical for GUVs and SPBs (not shown). In addition, an alternative evaluation method of plotting τ_D versus PN/PN_0 (Figure 2) was used. This method monitors the dependence of the diffusion time on the size of the illuminated area. A linear dependence with zero intercept together with the fact that the effective diffusion coefficient D_{eff} is equal to the diffusion coefficient obtained from the Z scan confirms the fact that we investigate free 2D diffusion for both GUVs and SPBs because the intercept t_0 for all of the measured samples lies in the interval 0 ± 0.5 ms.[22]

There might be a debate as to whether the choice of the probe influences the obtained diffusion coefficients. For example, it is known that some labeled lipid preferentially locates in one of the leaflets of the SPB[24] or the location of the chromophore might be different than anticipated from the chemical structure of the labeled lipid.[25] In this context, a reviewer pointed out that a part of the BODIPY chromophores might loop back and thus be located in the headgroup region. We have addressed this issue by fluorescence lifetime measurements of BODIPY tail- and headgroup-labeled lipids on SPBs adsorbed onto glass covered with indium tin oxide (ITO). The diffusion coefficients determined by FCS were identical to those measured for SPBs on mica, and the formation of SPBs was further confirmed by ellipsometry.[4] ITO quenches fluorescence, and the steep distance dependence of the quenching rate (and consequently, fluorescence lifetimes) gives information on dye-support distances.[26] The fluorescence lifetimes of the BODIPY headgroup- and tail-labeled lipid in SUVs were determined as the (unquenched) reference ($\tau_F = 5.6$ ns for both dyes). The fluorescence decay of headgroup-labeled lipid in the SPBs could not be fitted to a monoexponential decay. The biexponential fit was satisfactory and yielded two lifetimes ($\tau_{F1} = 1.3$ ns and $\tau_{F2} = 2.4$ ns), which demonstrated that two dye populations with different distances to the support were present. However, the decay recorded for the tail-labeled lipid in the SPBs was satisfactorily fitted by a monoexponential model ($\tau_F = 1.8$ ns).[26] These results clearly indicate that only a minor fraction of the BODIPY fluorophore in the tail-labeled lipid is located within the headgroup region of the SPB. Moreover, in Table 2 the diffusion coefficients in SPBs for the BODIPY tail-labeled lipid are compared with those for the BODIPY headgroup-labeled lipid and the lipid analogue DiD. Because the determined differences are within experimental error, the choice of the dye appears to have a minor impact on the diffusion coefficients determined in DOPC bilayers.

Table 2. Diffusion Coefficients Obtained for Various Dyes in the SPBs Formed on Mica[a]

dye	$D(10^{-12}$ m^2 s$^{-1})$	$D_{eff}(10^{-12}$ m^2 s$^{-1})$
C8-BODIPY 500/510 C5-HPC	3.1 ± 0.3	3.0 ± 0.7
BODIPY FL DHPE	3.5 ± 0.3	3.4 ± 0.6
DiD	3.8 ± 0.4	3.9 ± 0.9

[a] Measurements were carried out in the glucose solution at 289 K.

A large discrepancy exists in the literature regarding the frictional coupling of the inner and outer leaflets of the phospholipid bilayer. If the two monolayers were completely independent as suggested by Hetzer et al.,[27] then the diffusion of a labeled lipid in the outer leaflet would show the same diffusion characteristics as in GUVs. Because the apparent diffusion in SPBs is more than 2 times slower than in GUVs, the diffusion within the inner leaflet would have to be slowed more than 4 times because of the interaction with the support. However, such a huge effect does not seem feasible because the autocorrelation curves were fitted satisfactorily with the one-particle model. Thus, strong coupling between the bilayer leaflets appears to be more plausible, as suggested in other work.[6,28] Nevertheless, in contrast to the latter publication,[28] we observe a significant influence of the hydrophilic support on the lipid diffusion. Therefore, in the case of SPBs, we assume coupling between the support and the inner leaflet as well as coupling between the inner and outer leaflets of the bilayer.

In conclusion, in this letter we address the issue of whether and to what extent lipid mobility in the bilayer is altered when the bilayer is being deposited on the support. We compare the diffusion coefficient of a labeled lipid in the free-standing lipid bilayer of GUVs with that of the bilayer interacting with the mica surface (SPBs) in the glucose solution. The results clearly show that the diffusion is slowed by more than 2 times for the interaction with the support. Moreover, we believe that the quantitative comparison of lipid diffusion in two frequently used

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model membranes can be helpful when comparing data reported in the literature.

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Supporting Information Available: Values of diffusion coefficients (D) in GUVs and SPBs composed either of DOPC or DLPC determined by conventional FCS and FRAP. The listed values are based on the literature search. This material is available free of charge via the Internet at http://pubs.acs.org.

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