How To Determine Diffusion Coefficients in Planar Phospholipid Systems by Confocal Fluorescence Correlation Spectroscopy

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Confocal fluorescence correlation spectroscopy (FCS) allows for the determination of lateral diffusion coefficients and surface densities in planar phospholipid systems. The determination of the vertical (*z*-) position of the laser focus relative to the phospholipid surface plane is of crucial importance for the accuracy of the confocal FCS experiment. In this work we determine for the first time this vertical (*z*-) position of the laser focus by a so-called "*Z*-scan", which is based on the determination of diffusion times and particle numbers in 0.2 μm steps along the vertical (*z*-) axis. Experiments on supported phospholipid bilayers composed of dioleoylphosphatidylcholine (DOPC) and small amounts of Rhodamine Red-X 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rhodamine Red-X DHPE) adsorbed onto atomically flat mica and borosilicate glass demonstrate that results obtained by the *Z*-scan approach are significantly more precise than those results obtained when the fluorescence intensity maximum is used as an indicator in the determination of the vertical (*z*-) position of the sample. In addition to this basic contribution for the investigation of planar bilayer systems by confocal FCS, the lateral diffusion coefficients of Rhodamine Red-X DHPE in supported phospholipid bilayers composed of DOPC and cholesterol as well as in DOPC or dipalmitoylphosphatidylcholine (DPPC) monolayers adsorbed at a liquid−liquid interface were determined.

Introduction

Cellular membranes are protein-loaded phospholipid bilayers, the characterization of which is still a fundamental question. Phospholipid molecules contain two highly hydrophobic acyl chains and a relatively hydrophilic headgroup. This specific feature causes self-assembly of these molecules into so-called bilayers, consisting of two adjacent sheets of molecules with the headgroups exposed to water, and the chain regions buried into the hydrophobic membrane interior. Many events occur on animal plasma membranes, and the importance of understanding them is increasing. Several model systems, mimicking cellular membranes, have been produced, and several techniques have been used for characterization of these artificial membranes. An important step forward was the production of so-called supported phospholipid bilayers (SPBs) by exposure of a solid support to phospholipid vesicle suspensions.[1] Vesicles adsorbing onto such supports will often fuse spontaneously into bilayers, and this procedure is much easier than the laborious stacking technique used earlier. SPBs have now been applied in biosensors, micro- and nanostructures, blood-compatible surfaces, medical implant devices, and production of catalytic interfaces.[2]

The development of sophisticated techniques, capable of measuring physicochemical surface parameters of membranes, increased in the last two decades. These techniques include ellipsometry,[3] total internal reflection fluorescence,[4] quartz crystal microbalance,[5] impedance spectroscopy,[6] atomic force microscopy (AFM),[7] and surface plasmon resonance (SPR).[8] Systematic studies of the mechanisms of SPBs formation, such as the conditions for fusion of adsorbed vesicles, have only been performed in the past few years.[3,5−7,9−11] Fluorescence correlation spectroscopy[12] (FCS), which was invented in the early 1970s,[13,14] was used in a series of experiments on black lipid membranes.[15−17] Fluorescence photobleaching re-

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covery (FRAP), however, was used preferentially to determine the diffusion of various membrane components both in artificial and in biological membranes.[18] Recently, confocal FCS characterizations of lipid bilayers were performed on giant unilamellar vesicles,[19] on cell and model membranes,[20] and on SPBs.[11] In the first two publications[19,20] the authors state that "the relative error in the determination of the lateral diffusion coefficient was ~20%" in the liquid-crystalline phase of giant unilamellar vesicles. Those given values in the standard deviations are in line with the values reported for lateral diffusion coefficients determined for mica supported SPBs by our group.[11] In all three contributions the vertical (*z*-) position of the phospholipid membrane was localized by focusing to the point of maximum fluorescence intensity when scanning the *z*-axis in 1 μm steps and the illuminated surface area was determined by an external measurement of a standard system with a known diffusion coefficient.

In this work we present a modified method for the determination of diffusion coefficients and phospholipid surface densities of planar phospholipid systems. The so-called "*Z*-scan" involves the determination of diffusion times and particle numbers in 0.2-μm steps along the *z*-axis. From the dependence of those parameters on the position of the focus,[21,22] diffusion coefficient and surface density are directly determined without the need of an external calibration measurement. The comparison of results on mica- and borosilicate glass-supported SPBs obtained by this approach with those obtained by the traditional method applied in ref 11 not only demonstrates the higher precision of the "*Z*-scan" but also explains the origin of the apparent slow diffusion reported for borosilicate glass-supported SPBs.[11] We apply this approach for characterizing the effect of cholesterol content on the diffusion coefficients in SPBs composed of dioleoylphosphatidylcholine (DOPC). Moreover, we present the so far first determinations of diffusion coefficients in phospholipid monolayers at the oil−water interface.

Materials and Methods

Materials. The lipids dipalmitoylphosphatidylcholine (DPPC) and DOPC were purchased from Avanti Polar Lipids (Alabaster, AL). Rhodamine Red-X 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rhodamine Red-X DHPE) and *N*-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (TRITC DHPE) were purchased from Molecular Probes (Leiden, The Netherlands). Water used in our study was purified with a MilliQ purification system. Microscope borosilicate glass slides (Paul Marienfeld GmbH & Co. KG, Germany) were cleaned with detergent (Sparkleeen, Fisher Scientific Co., Pittsburgh, Canada), boiled in chromic acid for 20 min, and extensively rinsed with water. Mica (muscovite) is a potassium aluminum silicate hydroxide fluoride and is easily cleaved into thin sheets, providing a clean, plane, molecularly smooth, hydrophilic surface with only a few imperfections.[23] It is very frequently used in AFM,[7] and it is also suitable for the determination of adsorbed phospholipid mass by ellipsometry.[11] One side of mica plates (5 mm in diameter, Methafix, Montdidier, France) was scratched, and the plates were fixed with a silicon glue onto the microscope borosilicate glass slide. Before use a few mica layers were cleaved using scotch tape. Buffers contained (A) 2 mM EDTA, 10 mM Hepes, 150 mM NaCl, pH 7.4 or (B) 2 mM $CaCl_2$, 10 mM Hepes, 150 mM NaCl, pH 7.4. All buffers were filtered through a 0.2 μm syringe filter (Schleicher & Schuell, Germany) prior to use.

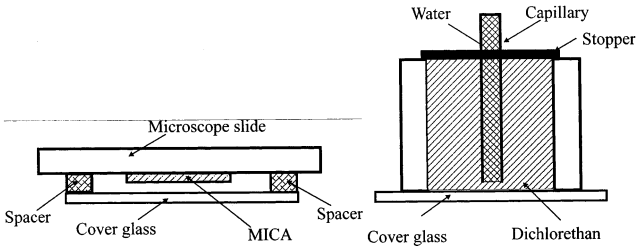

Figure 1. Schematic representation of the cuvettes used in FCS measurement for the determination of lateral lipid diffusion coefficients in supported phospholipid bilayers deposited on mica or borosilicate glass (left part of the figure) and in phospholipid monolayers formed on liquid−liquid interfaces (right part of the figure).

Preparation of Liposomes and SPBs. First a mixture of labeled and unlabeled lipids was prepared by mixing appropriate amounts of these lipids dissolved in chloroform. The solvent was evaporated with a stream of dry nitrogen, and the lipid mixture was resuspended by vortexing in an appropriate volume of buffer A, creating a turbid suspension. This suspension was sonicated for 10 min, yielding a solution of small unilamellar vesicles (SUVs). The ratios of labeled to unlabeled lipid ranged from $1:10^4$ to $1:10^6$. The size of the SUVs was estimated using FCS,[11] and the resulting values of hydrodynamic radius varied around 20 nm with about 15% of deviation in size. The cuvette (Figure 1) was filled with the solution of SUVs (diluted with buffer B to the final lipid concentration 100 μM) and incubated for 180 min. The redundant vesicles were flushed with buffer B, and the cuvette was then placed on the FCS instrument. The rough position of the support's surface was determined by moving the focus in the vertical (*z*-) direction and visualizing the reflected laser light with a CCD camera. When the approximate position of the surface was found, we performed a fast intensity scan in the vertical (*z*-) axis to determine the *z*-position corresponding to the maximum of the detected fluorescence intensity. Then the position of focus was set 1 μm below the maximum of the fluorescence intensity and a more precise "*Z*-scan" measurement was performed. During the preparation of vesicles and SPBs from DPPC, the solution and the solid support were kept heated at 60 °C. After the incubation the sample was placed on the microscope.

Preparation of Monolayers at the Liquid−Liquid Interface. The cuvette was constructed from a glass cylinder glued onto a thin microscope cover glass, and the capillary was fixed with a Teflon stopper (Figure 1). The appropriate amount of phospholipids was dissolved in 1,2-dichlorethane, which was equilibrated with water prior to use. The final phospholipid concentration used in our experiment was 1 μM, and the ratios of labeled to unlabeled lipids were in the range from $1:10^5$ to $1:10^6$. The phospholipid concentration of 1 μM is sufficient to cover the interface with the area of 0.8 mm^2 (i.e. the internal surface of the capillary with the diameter 1 mm) by a phospholipid monolayer with the density of 1.5×10^{-6} mol/m^2.[24] The lower part of the cuvette was filled with the solution of the phospholipids in 1,2-dichlorethane. The capillary, sealed on one side and filled with water which was equilibrated with 1,2-dichlorethane prior to use, was inserted into the cuvette. The Teflon stopper fixed the position of the capillary so that the position of the open end of the capillary was about 150 μm above the microscope cover glass (Figure 1).

Instruments. FCS measurements were performed using a Confocor 1 (Carl Zeiss GmbH, Jena; Evotec Biosystems GmbH, Hamburg) consisting of a confocal microscope Axiovert 100 containing a 40× water immersion objective×, a 1.2 numerical

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aperture, and an He−Ne laser as the excitation source (used excitation wavelength: 543 nm). This experimental setup allows us to locate the laser focus relative to the sample plain in 0.1 μm steps in the vertical (z-) direction. The emission was detected via a band-pass filter transmitting light between 550 and 600 nm. The fluorescence signal was detected by an avalanche photodiode (EG-SPCM 200) and correlated online with an ALV-500E digital correlator board (ALV, Langen, Germany). FCS measurements were performed at room temperature (23 °C). Data fitting was performed with Microcal Origin 7.0 Professional.

FCS: Correlation Function and Models for Translational and Lateral Diffusion. FCS monitors the fluctuation of the fluorescence intensity $I(t)$ emitted during a certain period of time. The normalized autocorrelation function $G(\tau)$ is calculated from $I(t)$, representing the fluctuations of the fluorescence intensity at a given (delay) time τ (eq 1).[13]

$$G(\tau) = 1 + \frac{\langle \delta I(t)\, \delta I(t+\tau)\rangle}{\langle I(t)\rangle^2} \tag{1}$$

where the brackets describe the time average and δ is the fluctuation symbol. The actual volume element is defined by the laser beam profile, which is Gaussian in the x- and y-directions and Lorenzian in the z-direction, and by a pinhole that spatially filters the light emitted out of the focus. The intensity autocorrelation function $G(\tau)$ for Brownian three-dimensional motion in a Gaussian volume element is described by eq 2,[12]

$$G(\tau) = 1 + \frac{\gamma}{PN}\frac{1}{1+(\tau/\tau_D)}\left(\frac{1}{1+(\tau/\tau_D)(\omega_0/\omega_z)^2}\right)^{1/2} \tag{2}$$

where ω_0 is defined as the distance from the optical axis and ω_z as the distance along the optical axis, both at which the intensity has dropped by $1/e^2$, PN and τ_D represent the particle number and the diffusion time, respectively, and γ is a correction factor considering the intensity profile in the focus.[25] The dimension of the volume element can be determined by measuring a 5 nM rhodamine 6G aqueous solution with known diffusion coefficient $D = 2.8 \times 10^{-8}$ m^2 s^{-1}, fitting the data to eq 2, and using eq 3.[12]

$$\omega_0 = \sqrt{4D\tau_D} \tag{3}$$

If the diffusion is restricted in a plane (two-dimensional diffusion), then the autocorrelation function is reduced to[12]

$$G(\tau)_{2D} = 1 + \frac{\gamma}{PN}\frac{1}{1+(\tau/\tau_D)} \tag{4}$$

Since for the used dyes intersystem crossing means creating molecules in the triplet state that can be considered as nonfluorescent, an average fraction of dye molecules in the triplet state (T) and an intersystem crossing relaxation time (τ_{tr}) have to be included in the theoretical fitting functions.[27]

$$G(\tau)_{2DT} = 1 + (1 - T + Te^{-\tau/\tau_{tr}})\left(\frac{\gamma}{PN[1-T]}\right)\left(\frac{1}{1+\tau/\tau_D}\right) \tag{5}$$

If two molecules with different diffusion times τ_i, but with the same spectral characteristics (quantum yield, emission spectra, etc.), are present, eq 5 has to be changed to[12,27]

$$G(\tau)_{2DT} = 1 + (1 - T + Te^{-\tau/\tau_{tr}})\left(\frac{\gamma}{PN[1-T]}\right)\left(\left(\frac{Y}{1+\tau/\tau_A}\right) + \left(\frac{1-Y}{1+\tau/\tau_B}\right)\right) \tag{6}$$

where τ_A and τ_B are the diffusion times of components A and B, respectively, and Y is the fraction of component A.

Data Acquisition and Analysis. The desired physically relevant parameters PN and τ_D were obtained via fitting the measured correlation curve by one of the above-described theoretical models (eqs 2 and 4−6). The fitting was done by minimizing the weighted reduced χ^2 value using the Levenberg−Marquardt nonlinear least-squares routine. This routine requires the knowledge of a standard deviation for each point of the fitted autocorrelation curve. These values of standard deviations cannot be calculated analytically, so we used a method described by Wohland[28] for their experimental determination.

The Wohland method is based on splitting up one long measurement into a series of short measurements. Such a splitting provides us with a series of experimentally independent values of the autocorrelation function for every delay time τ_l. By averaging them, it is possible to reconstruct an autocorrelation curve with measurement time equal to the total measurement time of the series of the short measurements. Furthermore, their standard error is a valid estimation of the real standard deviation of the related averaged experimental data point in the autocorrelation curve. The mathematical expression used for the calculation represents an application of Wohland's more general formula:[28]

$$\sigma(\tau_i) = \sqrt{\frac{1}{L(L-1)}\sum_{l=1}^{L}\left(\frac{G_l(\tau_i)-1}{G_l(0)-1} - \frac{1}{L}\sum_{l=1}^{L}\frac{G_l(\tau_i)-1}{G_l(0)-1}\right)^2} \tag{7}$$

where $\sigma(\tau_i)$ is the standard deviation of the averaged autocorrelation function at the delay time τ_i, $G_l(\tau_l)$ is the value of the autocorrelation function of the lth short measurement at delay time τ_i, and L corresponds to the number of the short measurements. The value for $G_l(0)$ was estimated by averaging over the first data points at short delay times of each $G(\tau_i)$.

Description of the "Z-Scan". In our previous work on SPBs adsorbed onto mica or borosilicate glass,[11] the right vertical (z-) position of the focus was localized by searching for the point of the maximum fluorescence intensity along the z-axis in 1 μm steps. In the experiments described herein, $G(\tau)$ values were determined at different positions along the z-axis in 0.2 μm steps ("Z-scans"). It was shown that in planar systems the diffusion time τ_D and particle number PN, respectively, depend on the position of the focus. In the case of a planar system parallel with the focal plane of the microscope, the dependence can be mathematically described[21,22] by equations 8 and 9:

$$\tau_D = \frac{{w_0}^2}{4D}\left(1 + \frac{{\lambda_0}^2\Delta z^2}{\pi^2 n^2 {w_0}^4}\right) \tag{8}$$

$$\mathrm{PN} = \pi c {w_0}^2\left(1 + \frac{{\lambda_0}^2\Delta z^2}{\pi^2 n^2 {w_0}^4}\right) \tag{9}$$

where w_0 is the radius of the beam in the focal plane, D is the lateral diffusion coefficient, c is the average 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 beam diameter minimum z. Thus, we measured autocorrelation functions at different values of focus position z and fitted those functions by eq 5. The obtained parameters PN and τ_D were plotted against the focus position z. The fitting of these plotted dependencies using eqs 8 and 9 directly yields[29] three parameters: (1) the lateral diffusion coefficient D; (2) the two-dimensional particle number PN at the beam radius minimum w_0; (3) and the minimum radius of the laser beam w_0.

(25) The correction factor γ is defined by Thompson,[12] and its value depends on the experimental geometry setup. In the case of the three-dimensional Gaussian volume element profile, the value of the correction factor γ is equal to $2^{-3/2}$; in the case of the two dimension Gaussian profile, its value is 0.5.

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(29) In this fitting procedure we again minimize the reduced χ^2. As for the correct results of the fitting the standard deviation for each data point is required, we used the estimations of standard errors in the diffusion time and particle number, which were obtained by fitting the corresponding $G(\tau)$'s, as the weighting factors.

When compared to the "original" method applied,[11,19,20] this approach has the following intrinsic advantages: (a) precise (in 0.2 μm steps) determination of the relative position between the sample and the focus plane along the *z*-axis instead of relying on the assumption that the maximum of the detected fluorescence intensity indicates this position (with a precision of 1 μm); and (b) direct determination of the lateral diffusion coefficient *D* instead of performance of calibration measurements using an external standard.

Results and Discussion

Choice of the Labeled Lipid System. There are several requirements for an ideal fluorescence dye, which can be successfully used in FCS. First, the molar absorbance should be quite high and the absorption maximum of the dye should be close to the laser excitation wavelength. Second, the label should exhibit a high emission quantum yield. For the determination of the relatively slow lateral diffusion in membranes, in particular, its photostability and its tendency not to form aggregates are important. We have tested the suitability of several lipids labeled by various types of fluorophores.[30] We found that Bodipy-labeled lipids show the highest suitability for SPBs for the excitation at 488 and 514 nm, and Rhodamine Red-X DHPE- and TRITC DHPE-labeled lipids do so when using 543 nm excitation. In the case of measurements on a phospholipid monolayer formed on a liquid−liquid interface, only Rhodamine Red-X DHPE appeared to be suitable when using the latter excitation wavelength.

Determination of Lateral Diffusion Coefficients in SPBs on Mica. Studies using AFM[7] have demonstrated that, when exposing freshly cleaved mica surfaces to small unilamellar vesicles composed of neutral phospholipids in the presence of calcium, phospholipid bilayers (SPBs) are formed. Thus, we are sure that when performing *Z*-scans on DOPC adsorbed on mica in buffer B (10 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4),[7] we are in fact characterizing confluent SPBs without defects (see Table 2 in ref 7). The *Z*-scans were performed with 0.2 μm steps. Figure 2 shows the obtained dependencies of PN and τ_D on Δz and the corresponding fits using eqs 8 and 9, respectively. Apparently, the described "parabolic" model fits the data very well. The obtained parameters are (1) $D = (4.2 \pm 0.4) \times 10^{-12}$ m^2 s^{-1}; (2) two-dimensional particle number PN = 0.80 ± 0.05 at the beam radius minimum w_0; (3) and the radius $w_0 = (0.25 \pm 0.03) \times 10^{-6}$ m. The relative error in *D*[31] was found to be lower than 10% for DOPC-supported phospholipid bilayers deposited on mica.

The values of the lateral diffusion coefficient *D* obtained for DOPC adsorbed on mica may be compared with the diffusion coefficients obtained for other bilayer systems.[19,20,32] Recent confocal FCS studies on giant unilamellar vesicles composed of 100% dilauroylphosphatidylcholine at room temperature yielded a diffusion coefficient[19] of 3×10^{-12} m^2 s^{-1}. Conventional photobleaching studies using total internal reflection excitation on a 10% PS, 88% POPC, and 2% NBD-PC planar bilayer on a silicon surface at room temperature resulted in a diffusion coefficient[33] of 1.2×10^{-12} m^2 s^{-1}. Employing the same technique and using a similar lipid composition, the same authors obtained a diffusion coefficient of 2.5×10^{-12} m^2 s^{-1} for Langmuir−Blodgett monolayers.[32]

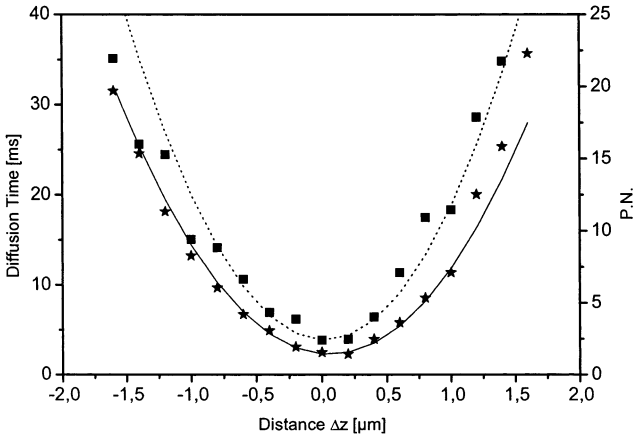

Figure 2. Dependence of the lateral diffusion time (τ_D, ■ points) and the particle number (PN, ★ points) on the *z*-position of the focus. This *Z*-scan was performed on SPBs formed from DOPC containing Rhodamine Red-X DHPE-labeled lipids in a ratio $1{:}5 \times 10^5$ (labeled to unlabeled) deposited on mica in buffer B (10 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4). The experimental data points were fitted according to eqs 8 (- - - line) and 9 (— line), respectively.

The influence of the ratio between labeled and not labeled lipids was studied in the range from $1{:}10^4$ to $1{:}10^6$ in order to find the ratio under which the dye concentration is about one particle per sample volume, if the sample and the focus plane coincide. Under this condition, the signal-to-noise ratio reaches its highest value.[34] The most suitable ratio appeared to be $1{:}5 \times 10^5$, corresponding to an experimentally determined averaged particle number PN equal to 0.8. This value is somewhat smaller than the estimated particle number 1.3 for a volume element with a radius of 0.25×10^{-6} m, using the value of 0.415 μg/cm^2 for the phospholipid mass density of SPBs.[35] This discrepancy could be interpreted as an indication for a fluorescence quenching of rhodamine chromophores facing the hydrophilic solid support. Since it is difficult to exactly prepare lipid mixtures with the above listed ratios, we believe that such a conclusion should not be made.

Moreover, measurements on DPPC at room temperature were performed. However, under these conditions photobleaching leads to a decrease in the fluorescence intensity during the time period of the experiment (6 s). Thus, the photobleaching prevents the determination of $G(\tau)$ within the gel state of the bilayers.

Determination of Lateral Diffusion Coefficient in SPBs on Borosilicate Glass. In our previous work we reported that addition of small unilamellar vesicles containing 20% dioleoylphosphatidylserine (DOPS) and 80% dioleoylphosphatidylcholine to an oxidized borosili-

(30) The tested labeled lipids were: 2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β-BODIPY 500/510 C_{12}-HPC), 2-(5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene-3-nonanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β-C_4-BODIPY 500/510 C_9-HPC), 2-(4,4-difluoro-5-octyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β-C_8-BODIPY 500/510 C_5-HPC), *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), Oregon Green 488 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Oregon Green 488 DHPE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE), and 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD C_6-HPC) (purchased from Molecular Probes).

(31) The relative error was determined from 15 independent measurements performed on 15 different sample preparations.

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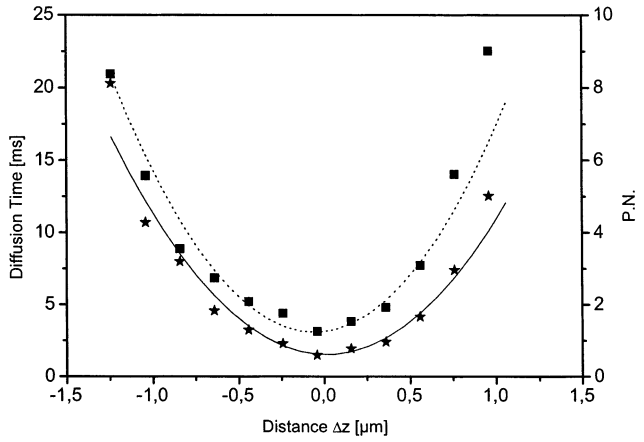

Figure 3. Dependence of lateral diffusion time (τ_D, ■ points) and particle number (PN, ★ points) on the *z*-position of the focus. The method of *Z*-scan was performed on SPBs formed from DOPC containing Rhodamine Red-X DHPE labeled in a ratio $1{:}5 \times 10^5$ (labeled to unlabeled) adsorbed onto borosilicate glass. The experimental data points were fitted according to eqs 8 (- - - line) and 9 (— line), respectively.

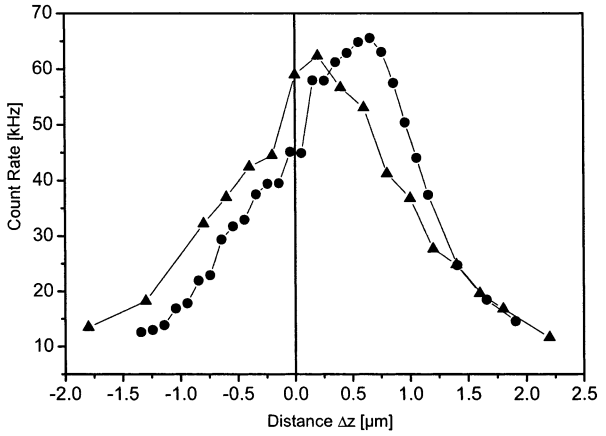

Figure 4. Dependences of the fluorescence intensities (count rate) on the *z*-position of the focus. Shown are those for DOPC-supported phospholipid bilayers containing Rhodamine Red-X DHPE-labeled lipids in the ratio $1{:}5 \times 10^5$ (labeled to unlabeled) deposited on mica (▲) and borosilicate glass (●) in buffer B (10 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4). The solid line demonstrates the z_0 position of the focus, where the parameters particle number PN and diffusion time τ_D, respectively, become minimal.

cate glass surface results in a planar lipid system characterized by lateral diffusion coefficients which are three time smaller than those obtained for SPBs formed on mica. In this work we studied SPBs formed from DOPC adsorbed on borosilicate glass by applying the method of the *Z*-scan and fitting the experimental points according to eqs 8 and 9 (Figure 3). The obtained parameters are (1) the lateral diffusion coefficient $D = (4.0 \pm 0.5) \times 10^{-12}$ m^2 s^{-1}; (2) the two-dimensional particle number PN = 0.8 ± 0.1 at the beam radius minimum w_0; and (3) the radius $w_0 = (0.26 \pm 0.03) \times 10^{-6}$ m. In contradiction to our previous work, the results obtained for borosilicate glass are in quite good agreement with the results obtained for mica. In Figure 4 we compare the dependencies of PN and τ_D on the *z*-position with the dependence of the detected fluorescence intensity on the *z*-position of the focus. It can be clearly seen that the positions of the minima in PN and τ_D ($\Delta z = 0$), respectively, do not correspond with the position of the maximum in the detected fluorescence intensity, which appears at the relative *z*-position Δz equal to 0.7 μm for SPBs on borosilicate glass. Under the given experimental conditions, the illuminated area at that position of the maximum fluorescence intensity is about 3 times larger than it would be at the position of the beam radius minimum $z = z_0$. The diffusion time obtained at this position is 11.8 ms, which is about 3 times more than the value of 4.0 ms obtained in the z_0 position. Thus, it becomes clear that the diffusion time and coefficients presented in our previous work, where we believed to observe anomalously slow diffusion for phospholipids adsorbed onto borosilicate glass surface, have to be considered as an artifact. This artifact is caused by the intrinsic uncertainty in the "original" method of the determination of the right *z*-position of the focus along the *z*-axis.[11,19,20] The dimension of the discrepancy between $z = z_0$ and the position of the fluorescence intensity maximum varies depending on the used solid support (the shift determined for SPBs on mica is about 0.2 μm; see Figure 4), laser intensity, choice of the dye, and other experimental conditions. The results show that borosilicate glass is a suitable support for forming SPBs and might suggest that reported cases of anomalously slow diffusion might have to be re-examined by using the *Z*-scan method.

The relative error in the determination of D[31] was lower than 15% for DOPC adsorbed on borosilicate glass. The higher error and the smaller diffusion coefficients D observed on the borosilicate glass surface compared to mica might be explained by imperfections present on the surface, while the mica surface shows unique flatness at the molecular level. The conclusion that regular SPBs are formed on mica as well as on borosilicate glass under the above-mentioned conditions is supported by ellipsometry measurement on the identical systems, respectively.[36]

Determination of Lateral Diffusion Coefficients on Phospholipid Monolayers at the Liquid−Liquid Interface. The experimental setup for the determination of lateral diffusion coefficients of phospholipids located at a 1,2-dichlorethane−water interface is shown in Figure 1. Though the thin capillary is stabilizing the position of the interface, we observed that the dichlorethane−water interface was still moving along the *z*-axis, with a speed of about 6 μm/h. Though we have made several attempts to minimize this effect, we could not find experimental conditions guaranteeing a perfectly stable localization of the interface. Thus, it was not possible to perform a classical *Z*-scan as used above. Nevertheless, this experimental alignment allowed the performance of a "time dependent *Z*-scan". Since the interface appeared to move constantly in the *z*-direction, the focus was set about 1 μm close to the position of the interface and a series of measurement was performed. As the interface was moving in time toward the position of beam radius minimum, a "time dependent *Z*-scan" was obtained (Figure 5). Since it is difficult to determine the exact speed of the *z*-move of the interface and thus the absolute magnitude of the *z*-position, we omitted a fitting of the experimental data points by eqs 8 and 9, respectively. However, this "time dependent *Z*-scan" yields information on the two-dimensional diffusion time at the position of the beam radius minimum. The radius of the laser beam in the minimum was calculated from the diffusion time obtained from a calibration measurement of Rhodamine Red-X DHPE in 1,2-dichloroethane and the diffusion coefficient ($D = 1.2 \times 10^{-10}$ m^2 s^{-1}) of this dye−solvent system[37] according to eq 3. Using the same equation, the lateral diffusion coefficients of Rhodamine RedTM-X DHPE in monolayers consisting either of DPPC or DOPC were calculated (Table

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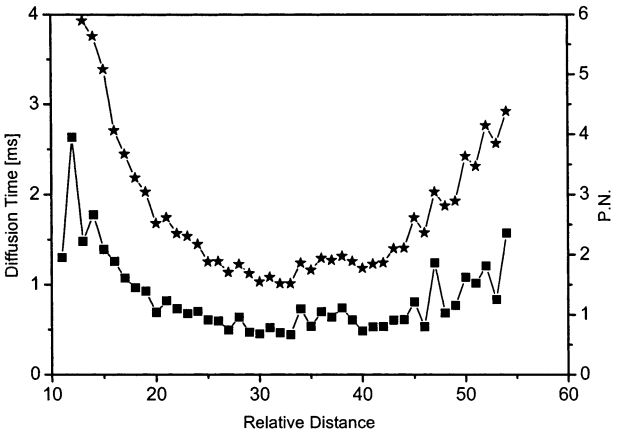

Figure 5. Dependences of the lateral diffusion time (t_D, ■ points) and particle number (PN, ★ points) on the relative *z*-position of the focus. The parameters were obtained by the method of "time dependent *Z*-scan" performed on a DOPC monolayer containing Rhodamine Red-X DHPE in a ratio $1:10^5$ (labeled to unlabeled) adsorbed onto the 1,2-dichlorethane−water interface.

Table 1. Summary of Parameters Obtained from FCS Measurements on SPBs Adsorbed onto Mica and Borosilicate Glass as Well as on Phospholipid Monolayers at the 1,2-Dichlorethane−Water Interface using Rhodamine Red-X DHPE as the Labeled Lipid[a]

mica	D^b ($\times 10^{-12}$ m^2 s^{-1})
DOPC	4.2 ± 0.4^c
DOPC + 10% cholesterol	2.3 ± 0.2^c
DOPC + 30% cholesterol	1.1 ± 0.2^d
DOPC + 60% cholesterol	0.5 ± 0.1^d
DPPC[e]	
borosilicate glass	**D^b ($\times 10^{-12}$ m^2 s^{-1})**
DOPC	4.0 ± 0.5^c
DPPC[e]	
liquid−liquid interface	**D[f] [$\times 10^{-12}$ m^{2}s^{-1}]**
DOPC	36 ± 8^c
DPPC	18 ± 7^c

[a] The ratio between labeled and unlabeled lipids was equal to $1:5 \times 10^5$ and $1:10^5$ in the case of SPBs and monolayers, respectively. The three-dimensional diffusion coefficient of Rhodamine Red-X DHPE has been determined to be equal to $D = 120 \times 10^{-12}$ m^2 s^{-1} in 1,2-dichloroethane. [b] Determined from experimental data points fitted by eq 8. [c] The relative error was calculated from 15 independent measurements. [d] The $G(\tau)$'s were fitted with a model with two different diffusing species. Listed is the diffusion coefficient determined for the slowly diffusing species. The relative error was calculated from five independent measurements. [e] Not determinable due to strong photobleaching. [f] Determined by the "time dependent *Z*-scan" at 100 μm above the glass surface; the radius w_0 was determined by a calibration measurement of Rhodamine Red-X DHPE in 1,2-dichlorethane ($D = 1.2 \times 10^{-10}$ m^2 s^{-1}).

1). The determined diffusion coefficients in DOPC monolayers have been proofed to be roughly 10 times larger than those determined in SPBs consisting of DOPC but still considerably smaller than the diffusion coefficient for three-dimensional diffusion in neat solvents. The diffusion coefficient determined for DPPC monolayers is about 2-fold smaller when compared to that of the DOPC system.

The used ratio of labeled to nonlabeled lipids for FCS measurement on a liquid−liquid interface was $1:10^5$. The experimentally determined averaged particle number PN for this ratio at the minimum of the "time dependent *Z*-scan" was equal to 1.5 ± 0.5 (Figure 5). This value might be compared with the estimated particle number of 2.1 for the volume element with a radius of $(0.27 \pm 0.06) \times 10^{-6}$ m using the value used for the phospholipid mass density of the monolayer, 1.5×10^{-6} mol/m^2 determined by the surface tension measurement using the aqueous pendant drop electrode.[24]

Influence of Cholesterol on the Lateral Diffusion Coefficients in SPBs on Mica. Investigations into the physical chemistry of lipid−cholesterol mixtures have turned out to be quite difficult. It has been shown that large concentrations of cholesterol promote a new bilayer phase, the so-called liquid-ordered phase. At low concentrations the effects of cholesterol on the bilayer phase behavior are quite subtle. It has been accepted that, in the low cholesterol concentration range, increasing cholesterol content leads to a decrease in the lateral diffusion coefficient in the liquid-disordered phase. In this work we have investigated the influence of 10%, 30%, and 60% cholesterol on the lateral diffusion properties of Rhodamine Red-X DHPE in SPBs composed of DOPC adsorbed on mica. The $G(\tau)$ values for the 10% cholesterol system can be well fitted with eq 5, representing the diffusion of one species. The diffusion coefficient resulting from the "*Z*-scan" appears to be about half of the value obtained for the cholesterol-free system. Fitting the $G(\tau)$'s recorded for the two SPBs with higher cholesterol content by eq 5 became more difficult, and fitting with a model with two different components appeared to be more suitable (eq 6), leading to values of reduced χ^2 close to 1 for both cholesterol systems and for the examined ranges of Δz. The dependencies of the particle number and the diffusion time of the slow diffusing species on Δz can be well fitted according to eqs 8 and 9. The obtained diffusion coefficients are $D = (1.1 \pm 0.2) \times 10^{-12}$ m^2 s^{-1} and $D = (0.5 \pm 0.1) \times 10^{-12}$ m^2 s^{-1} for 30% cholesterol and 60% cholesterol in DOPC SPBs, respectively. Moreover, the ratio between slow and fast diffusing species appeared to be almost constant for all examined Δz positions (approximately 75:25 for 30% cholesterol as well as for 60% cholesterol in DOPC SPBs). Although the dependencies of the diffusion times of the fast diffusing species on Δz can be fitted according to eq 8, the resulting diffusion coefficients are unreasonably large when compared to those obtained for DOPC SPBs ($D = (8 \pm 4) \times 10^{-12}$ m^2 s^{-1} and $D = (14 \pm 7) \times 10^{-12}$ m^2 s^{-1} for 30% cholesterol and 60% cholesterol, respectively). Moreover, the given diffusion coefficients show large errors, which indicates that the model comprising two diffusing species is not suitable and possibly a modified mathematical model for an anomalous diffusion should be used.[20]

General Consideration

The precision in the determination of lateral diffusion times and surface densities, respectively, of planar phospholipid systems by fluorescence correlation spectroscopy is strongly dependent on a correct estimation of the *z*-position of the focus. Using the "original" method, the maximum in fluorescence intensity was taken as an indicator of the relative position of the focus in the

(37) The diffusion coefficient of Rhodamine Red-X DHPE in 1,2-dichlorethane was estimated by following procedure: First, the radius of the illuminated area (w_0) was determined by using a 5 nM solution of Rhodamine 6G in water (known diffusion coefficient) by assuming a cylindrical volume element. Second, the diffusion coefficient of Rhodamine Red-X DHPE (not soluble in water) in methanol was obtained by FCS measurements of a 5 nM solution of Rhodamine Red-X DHPE in methanol and by the obtained value of w_0. The hydrodynamic radius of Rhodamine Red-X DHPE was calculated according to the Einstein−Stokes equation. Using the same equation and the known viscosity coefficient for 1,2-dichlorethane, the diffusion coefficient of Rhodamine Red-X DHPE in 1,2-dichlorethane was calculated.

z-direction.[11,19,20] In those experiments the position of the sample is determined by focusing in 1 μm steps in the *z*-direction. Using this approach, the error in the determination of the maximum fluorescence intensity can be theoretically up to 0.5 μm when focusing in *z*-direction. Assuming a random *z*-position of the sample when searching for the maximum in the fluorescence intensity in 1 μm steps, the obtained displacement between the position of the focus and the sample plane varies within the interval from -0.5 to 0.5 μm. The integration of eq 8 over Δz within this interval leads to values for the diffusion time and the particle number, respectively, which are 1.36 times higher than the values obtained for the case that the displacement is equal to zero. Please note that those considerations are valid when investigating planar lipid systems, which are characterized by perfect matching locations of maximum fluorescence intensity and minimum of the waist of the laser focus, and when determining the *z*-position of the laser focus with a 1 μm resolution.

Conclusions

Reproducibility and accuracy of the results obtained from confocal FCS of planar bilayer systems is the main requirement for its broader application. We believe that the presented "*Z*-scan" approach is an indispensable tool for fulfilling this requirement. Due to its atomic flatness and its compatibility with alternative methods such as ellipsometry and AFM, mica is certainly the material of choice when working with supported bilayer systems. The focus position at the maximum fluorescence intensity is not necessarily matching the position with the minimum of the illuminated area. This fact might lead to a wrong interpretation of the apparent anomalously slow diffusion properties.

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