Biol. Chem., Vol. 383, pp. 337 – 341, February 2002 · Copyright © by Walter de Gruyter · Berlin · New York

Short Communication

Muscovite (Mica) Allows the Characterisation of Supported Bilayers by Ellipsometry and Confocal Fluorescence Correlation Spectroscopy

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We demonstrate for the first time that ellipsometry and confocal fluorescence correlation spectroscopy (FCS) are complementary methods for the characterisation of supported planar phospholipid bilayers (SPBs) formed on mica, a mineral used in atomic force microscopy investigations of SPBs. Addition of small unilamellar vesicles containing 20% dioleoyl-phosphatidylserine (DOPS) and 80% dioleoyl-phosphatidylcholine (DOPC) to an oxidised borosilicate surface, on the other hand, results in a planar lipid system characterised by lateral diffusion coefficients which are three time smaller than those obtained for SPBs. Moreover, seven labelled phospholipids were tested for their suitability in the FCS characterisation of vesicles as well as of SPBs.
Key words: Bodipy / Borosilicate glass / Lateral diffusion / Microscopy / Oregon green / Phospholipid vesicles.

Cellular membranes are protein-loaded phospholipid bilayers, separating two electrolyte solutions. The phospholipid molecules in these membranes contain two highly hydrophobic acyl-chains and relatively hydrophilic headgroups. These specific features cause 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. Several model systems mimicking cellular membranes have been produced, but the study and application of these systems has long been hampered by the lack of techniques for membrane research in electrolyte solutions.

During the last two decades this situation has radically changed with the development of sophisticated techniques, capable of measuring physicochemical surface parameters of membranes at molecular resolution in buffers. These techniques include ellipsometry (Cuypers *et al.*, 1980), total internal reflection fluorescence (Axelrod *et al.*, 1983), quartz crystal microbalance (Okahata and Ebato, 1989), impedance spectroscopy (Stelzle and Sackmann, 1989), atomic force microscopy (AFM; Singh and Keller, 1991) and surface plasmon resonance (Salamon *et al.*, 1994). An important step forward was the production of so-called supported phospholipid bilayers (SPBs) by exposure of a solid support to phospholipid vesicle suspensions (Brian and McConnell, 1984). Vesicles adsorbing onto such supports will often fuse spontaneously into bilayers, and this procedure is much easier than the earlier-used laborious stacking technique. SPBs have now been applied in biosensors, micro- and nano-structures, blood-compatible surfaces, medical implant devices, and production of catalytic interfaces (Sackmann, 1996). Systematic study of the mechanisms of SPB formation, such as the conditions for fusion of adsorbed vesicles, have only been performed in the last few years (Giesen *et al.*, 1995; Nollert *et al.*, 1995; Rädler *et al.*, 1995; Steinem *et al.*, 1996; Csúcs and Ramsden, 1998; Keller and Kasemo, 1998; Reviakine *et al.*, 2000; Reviakine and Brisson, 2000).

Fluorescence correlation spectroscopy (FCS) was developed in the early 1970`s (Magde *et al.*, 1972; Elson and Magde, 1974; Ehrenberg and Riegler, 1974). Immediately after the introduction of FCS a series of experiments on black lipid membranes was published (Koppel *et al.*, 1976; Webb, 1976; Fahey *et al.*, 1977). However, following these first FCS experiments a different technique, fluorescence photobleaching recovery was used preferentially to determine the diffusion of various membrane components both in artificial and in biological membranes. Recently confocal FCS characterisation of lipid bilayers was performed on giant unilamellar vesicles (Korlach *et al.*, 1999) and on living cells (Schwille *et al.*, 1999). In this work we investigate the adsorption of phospholipids onto mica and borosilicate surfaces in order to find a solid support, which guarantees the formation of SPB's and allows for their characterisation by confocal FCS and ellipsometry. Moreover, seven fluorescently marked phospholipids are tested for their suitabilities in the characterisation of unilamellar vesicles as well as supported bilayers by FCS.

FCS measurements were performed using a Confocor 1 (Carl Zeiss GmbH, Jena, Germany; Evotec Biosystems GmbH, Hamburg, Germany) containing an Ar ion laser as the excitation source (used excitation wavelength: 488 nm). The volume of the detection volume element, created by the laser beam, was determined by calibration measurements of rhodamine 6G to be in average about 0.5×10^{-15} l. FCS monitors the fluctuation of the fluorescence intensity I(t) emitted over a certain period of time. The normalised autocorrelation function G(t) is calculated from I(t), representing the fluctuations intensity at a given (delay) time τ [equation (1)].

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

The brackets denote temporal averages, and δ is the fluctuation symbol. The autocorrelation function G(t) is fitted to equation (2), where N and τ_D represent the particle number and the diffusion time, respectively. The parameter S is the three-dimensional structural parameter defined as a ratio between the length of vertical ω_z and horizontal ω_{xy} axes.

$$G_{3D}(\tau) = 1 + \frac{1}{N} \cdot \frac{1}{1 + (\tau/\tau_D)} \cdot \frac{1}{\sqrt{1 + \frac{\tau}{S^2 \tau_D}}} \quad (2)$$

The volume of the volume element was determined by measuring a 5 nM rhodamine 6G aqueous solution with known diffusion coefficient $D=2.8 \times 10^{-8}$ $m^2 s^{-1}$, by fitting the data to equation (2) and using equation (3).

$$\varpi_{xy} = \sqrt{4D\tau_D} \quad (3)$$

If the diffusion is restricted in a plane (two-dimensional diffusion) then the autocorrelation function is reduced to (Elson and Magde, 1974):

$$G(\tau)_{2D} = 1 + \frac{1}{N} \cdot \frac{1}{1 + (\tau/\tau_D)} \quad (4)$$

Since for the dyes used intersystem crossing means creating molecules in the triplet state that can be considered as non-fluorescent, an average fraction of dye molecules in the triplet state (T) and an intersystem crossing relaxation time (τ_{tr}) has to be included in the theoretical fitting functions (Schwille *et al.*, 1996).

$$G(\tau)_T = (1 - T + Te^{-\tau/\tau tr}) \cdot G(\tau) \quad (5)$$

Combination of equation (4) and (5) leads to equation (6), which has been used when fitting G(τ) obtained on vesicles adsorbed onto solid support.

$$G(\tau)_{2DT} = (1 - T + Te^{-\tau/\tau tr}) \cdot \left(1 + \frac{1}{N} \cdot \frac{1}{1 + \tau/\tau_D}\right) \quad (6)$$

For the investigation of lipid adsorption onto borosilicate surface we used microscope cover glass (thickness: ~100 μm; Menzel-Gläser, Braunschweig, Germany). Microscope cover glass was boiled in a solution of potassium dichromate (80 g) in 1 l (4.6 M) sulphuric acid (20 min) and then rinsed with water. Mica (muscovite, a potassium aluminum silicate hydroxide fluoride; gift of A. Brisson) is easily cleaved into thinnest sheets providing a clean, plane, molecularly smooth, hydrophilic surface with only a few imperfections (Kawaniski *et al.*, 1990). It is very frequently used in AFM (Reviakine and Brisson, 2000; Reviakine *et al.*, 2000). The 5 mm diameter mica discs were attached by silicon glue onto conventional microscope slides (thickness: approx. 1.1 mm; Knittel-Gläser, Mannheim, Germany). The microscope slides were mounted to cleaned microscope cover glass by ~100 μm spacers (see Figure 1).

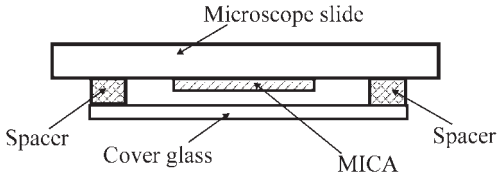

Fig. 1 Schematic View of the Sample Cell Used in Mica Experiments.
The spacers consists of 3 to 4 layers of double sticking tape (one layer approximately 30 μm thick).

The following labelled lipids were used: 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) (all compounds were purchased from Molecular Probes, Leiden, The Netherlands). In all experiments the mixture of 20% DOPS and 80% DOPC (purchased from Avanti Lipids) was used. The ratios of labelled to unlabelled lipid ranged from 1:10 000 to 1:1 000 000. Small unilamelar vesicles (SUV) and large unilamelar vesicles prepared by extrusion (LUVET) were prepared by sonication for 20 minutes or extrusion through filters with pores size of 1 μm using Lipofast extruder (Avestin, Inc., Alabaster, Canada), respectively, from the turbid suspension of lipids in 50 mM Tris-100 mM NaCl buffer pH 7.4 in presence of 3 mM Ca^{2+}. The surfaces were exposed to the solution contained SUV (100 μM) and incubated for 30 minutes. The redundant vesicles were flushed by the same buffer, placed on the FCS instrument and measured.

Ellipsometry is based on the measurement of polarisation changes in light reflected by silicon (Speijer *et al.*, 1996) and mica surface (this work). These changes are sensitive to the presence of thin surface films of organic materials, like proteins or phospholipids, on the reflecting surface and allow detection of changes in membrane surface mass of 3–5 ng/cm^2, that is, about 1% of initial membrane mass. A round mica plate of 12 mm diameter was fixed with melted wax on an aluminium slide of 40 × 13 mm. The mica covered a hole of 8 mm diameter in the aluminium slide. In order to prevent light reflection from the backside of the mica plate, this side was made dull with emery paper before it was fixed on the aluminium slide. The ellipsometer cuvette, containing the reflecting mica surface, was filled with 5 ml of rapidly stirred buffer containing SUV's (composition see FCS experiments above). All procedures were carried out at room temperature unless stated otherwise.

FCS measurements on LUVETs as well as on SUVs labelled with the previously mentioned marked phospholipids in ratio of 1:100 000 were performed. Three-dimensional evaluation of autocorrelation functions (equations 2 and 5) led to diffusion coefficients of about 1.3×10^{-11} m^{2}s^{-1} in case of SUV and 5.9×10^{-12} m^{2}s^{-1} in case of LUVETs. From the calculated hydrodynamic radius we determined the number of phospholipids per vesicle. When averaging the results for the used Bodipy and Oregon green dyes we found 5500 phospholipid molecules per vesicle with 9% error deviation in case of SUVs and 30000 phospholipid molecules per vesicle with 29% error deviation in case of LUVETs. These apparent differences in the reproducibility are caused by different standard deviations in diffusion times, approximately 14% in case of LUVETs compared to 4.5% in case of SUV. Although we are aware of a recent publication discussing the resolution of FCS experiments (Meseth *et al.*, 1999) we do not have a clear explanation for such obtained differences. The comparison of the seven fluorescently marked phospholipids (see also Table 1) shows that the diffusion coefficients obtained on vesicles labelled with Oregon green 488 DHPE have the smallest standard deviations (5% for SUV) and the highest reproducibilities. NBD-marked phospholipids, on the other hand, show large discrepancy in obtained results and photobleaching is observed. When using freshly prepared vesicles containing the above listed Bodipy-labelled phospholipids, the results were, in terms of standard deviations and reproducibility, comparable to those obtained by Oregon green 488 DHPE. However, we observed that the obtained particle number is changing within a time period of 24 hours, indicating self-aggregation of Bodipy-marked phospholipids (Slavik, 1994).

Extensive studies using AFM (Reviakine *et al.*, 2000; Reviakine and Brisson, 2000) have demonstrated that, for the phospholipids and calcium concentrations (20% DOPS / 80% DOPC and 3 mM Ca^{2+}, respectively) used in the present study, freshly cleaved mica surfaces exposed to SUV's are covered with confluent phospholipid bilayers (SPBs). Thus we are sure that when performing FCS and ellipsometry experiments on mica described below (see also Figure 2A and Figure 3, respectively), we are in fact characterising SPBs.

The influence of the ratio between labelled and unlabelled lipids has been studied in the range from 1:10 000 to 1:100 000. The most suitable ratio for FCS measurement appeared to be 1:100 000 corresponding to an experimentally determined averaged particle number N of 3.7. This value is well corresponding to the estimated particle number of 4.8 for a cylindrical volume element with a radius of 0.22×10^{-6} m using the value of 0.415 μg/cm^2 for the phospholipid mass density of DOPS/DOPC SPBs (Speijer *et al.*, 1996). Using the 1:100 000 ratio we compared Oregon green 488 DHPE with the Bodipy-labelled phospholipids for their suitability for FCS measurements of SPBs (see also Table 1). Oregon green 488 DHPE showed considerably photobleaching and thus is less suitable for SPB's studies. The diffusion coefficients obtained for all four Bodipy-labelled lipids are reproducible. The error in diffusion time was about 20%. The determined results were consistent, however, within this error. The observed lateral diffusion time was about 5 ms (Table 2), which results in a corresponding diffusion

Table 1 Suitability of Labelled Lipids for Studying Small and Large Unilamellar Vesicles (SUV and LUVET, Respectively) as Well as Supported Planar Bilayers (SPB's) on Mica by Fluorescence Correlation Spectroscopy Using Ar Ion Laser Excitation.

	(β-BODIPY 500/510 C_{12}-HPC	(β-C_4-BODIPY 500/510 C_9-HPC	(β-C_8-BODIPY 500/510 C_5-HPC	BODIPY FL DHPE	Oregon green 488 DHPE	NBD-PE	NBD C_6-HPC
SUV and LUVET	good	good	good	good	very good	not suitable	not suitable
SPB's	very good	very good	very good	good	fair	not suitable	not suitable

Suitability was evaluated according to reproducibility of the results, quantum yields, photostability, and self-aggregation tendencies of the compounds.

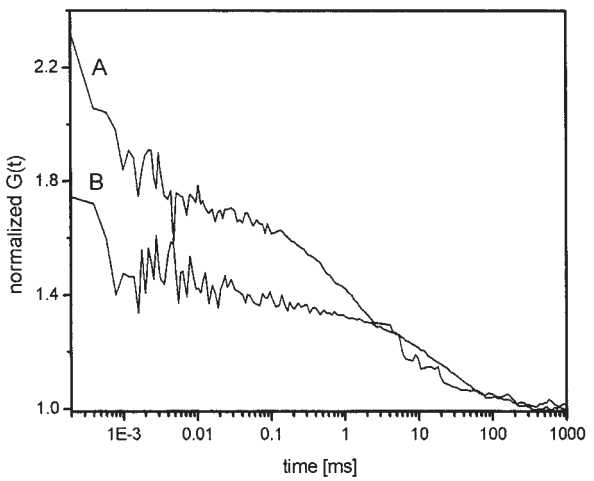

Fig. 2 Autocorrelation Curves G(τ) Obtained for Bodipy (β-C_4-BODIPY 500/510 C_9-HPC) in Adsorbed Phospholipids (20% DOPS and 80% DOPC; Ratio Labelled to Unlabelled Lipids 1:100 000) on Mica Surface (A) and on Microscope Cover Borosilicate Glass (B).
As excitation source the Ar ion laser (488 nm) with a power of 1 mW was used. The corresponding lateral diffusion times were in the case of mica 4.5 ms and in the case of borosilicate glass 14.8 ms.

Table 2 Comparison of Lateral Diffusion Coefficients of Four Differently Labelled Lipids in Adsorbed Phospholipids on Mica Surface at Room Temperature.

Sample	Diffusion time [μs]	D [$\times 10^{-12}$ m^2 s^{-1}]
β-BODIPY® 500/510 C_{12}-HPC	5100±1000	2.5
β-C_4-BODIPY® 500/510 C_9-HPC	4900±1000	2.6
β-C_8-BODIPY® 500/510 C_5-HPC	4500±1100	2.8
BODIPY® FL DHPE	5500± 400	2.0

The ratio 1:100 000 of labelled lipids to unlabelled in mixture of 20% DOPS and 80% DOPC was used.

coefficient of 2.5×10^{-12} m^{2}s^{-1}. Recent confocal FCS studies on giant unilamellar vesicles composed of 100% dilauroyl phosphatidylcholine at room termperature yielded diffusion coefficient of 3×10^{-12} m^{2}s^{-1} (Korlach *et al.*, 1999). Conventional photobleaching studies using total internal reflection excitation on 10% PS, 88% POPC and 2% NBD-PC planar bilayer on silicon surface at room temperature resulted in a diffusion coefficient of 1.15×10^{-12} m^{2}s^{-1} (Huang *et al.*, 1994). Employing the same technique and using a similar lipid composition, the same authors obtained diffusion coefficient of 2.54×10^{-12} m^{2}s^{-1} for Langmuir-Blodgett monolayers (Huang *et al.*, 1992). Thus, we are quite confident that the diffusion coefficient for planar bilayers on mica determined in this study reflects regular lateral phospholipid diffusion within the liquid crystalline state.

Moreover, the formation of phospholipid bilayers on mica surfaces using nearly identical conditions as in the

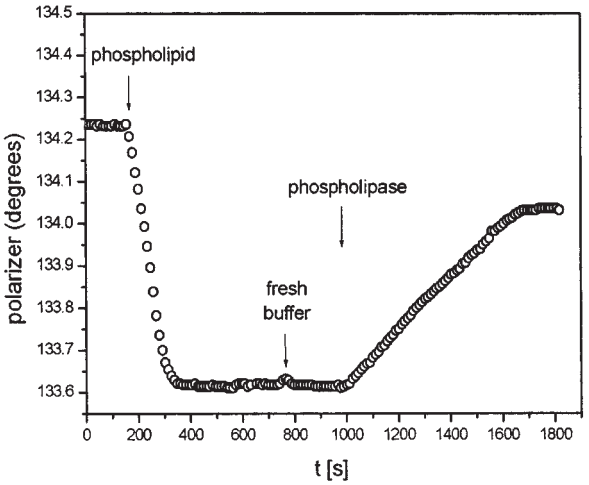

Fig. 3 Formation of a Phospholipid Membrane on Freshly Cleaved Mica, as Measured by Ellipsometry at Room Temperature.
Transport-limited formation of the membrane is apparent from the linear adsorption phase after addition of 20 μM of sonicated vesicles of 20% DOPS and 80% DOPC. After washing away excess vesicles, the membrane remains stable in fresh buffer, but is degraded after addition of 100 ng/ml of phospholipase from snake (Naja-naja) venom. The phospholipid degradation products (fatty acids and lyso-phospholipids) are much more soluble than intact phospholipids and therefore rapidly desorp from the mica surface.

FCS experiments and subsequent degradation of these bilayers by phospholipase A2 (from Naja-naja; 100 ng/ml) were measured by ellipsometry. In Figure 3 we demonstrate for the first time that formation of SPBs and their degradation by phospholipase A2 can be followed in real time on mica. We conclude that the mica surface appears to be well suitable surface for the characterisation of SPBs by FCS, AFM and ellipsometry. Since to our knowledge mica is the only solid support that allows the characterisation of SPBs by three techniques, we suppose that this finding will initiate further studies on mica combining those complementary techniques. We believe that the mica, because of its unique flatness at the molecular level, will solve reproducibility problems occurring with SPBs on silica and glass surfaces, which have considerable surface roughness.

For the characterisation of the lipid system adsorbed to oxidised borosilicate glass again the 1:100 000 labelled (Oregon green 488 DHPE as well as Bodipy compounds) to unlabelled lipid ratio was used. The obtained diffusion times were three times higher than on mica surface, in average 15.1 ms, which corresponds to diffusion coefficient of 8.5×10^{-13} m^{2}s^{-1}. This observation may argue against the formation of regular SPBs on oxidised borosilicate glass. For a comparison the autocorrelation functions, measured on (A) mica surface and (B) on borosilicate microscope cover glass, are presented in Figure 2. Moreover, it should be mentioned that we also performed analogous FCS measurements on phospholipid vesicles adsorbed onto detergent-cleaned borosili-

cate glass as well as oxidised silicon wafers. While in the first case we have indication for the formation of adherent vesicles (Reviakine and Brisson, 2000) by FCS experiments, ellipsometry clearly demonstrates the formation of SPBs on silicon wafers (Speijer *et al.*, 1996). FCS measurements on the latter system, however, did not gave reproducible results, possibly due to the presence of highly intensive reflecting light from the surface.

Acknowledgements

We thank A. Brisson for discussion and providing the mica slides as well as the Ministry of Education, Youth and Sports of the Czech Republic (*via* LN 00A032) and the Dutch Heart Foundation for financial support.

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Received June 22, 2001; accepted December 6, 2001