Lipid Vesicle Adsorption versus Formation of Planar Bilayers on Solid Surfaces

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ABSTRACT The adsorption and spreading behavior of lipid vesicles composed of either palmitoyloleoylphosphatidylcholine (POPC) or *Escherichia coli* lipid upon contact with a glass surface was examined by fluorescence measurements. Fluorescently labeled lipids were used to determine 1) the amount of lipid adsorbed at the surface, 2) the extent of fusion of the vesicles upon contact with the surface, 3) the ability of the adsorbed lipids to undergo lateral diffusion, and 4) the accessibility of the adsorbed lipids by external water soluble molecules. The results of these measurements indicate that POPC vesicles spread on the surface and form a supported planar bilayer, whereas *E. coli* lipid vesicles adsorb to the surface and form a supported vesicle layer. Supported planar bilayers were found to be permeable for small molecules, whereas supported vesicles were impermeable and thus represented immobilized, topologically separate compartments.

INTRODUCTION

Supported planar bilayers (SPBs) are phospholipid membranes adsorbed to a planar hydrophilic solid support. Apart from lipid vesicles and black lipid membranes (BLMs), SPBs have been used as model membranes, e.g., to study cell-cell recognition in the immune system (McConnell et al., 1986), adhesion of cells (Stelzle and Sackmann, 1989; Poglitsch et al., 1991; Tendian et al., 1991), adhesion of membrane patches (Seifert et al., 1993), protein binding to lipid ligands (Kalb et al., 1990; Schmidt et al., 1992), and membrane insertion of proteins (Ramsden and Schneider, 1993).

SPBs are prepared by essentially two different methods: 1) consecutive transfer of two lipid monolayers onto a surface via the Langmuir-Blodgett technique and 2) spreading of lipid vesicles on a surface (McConnell et al., 1986; Kalb et al., 1992). For the preparation of SPBs, lipids with different head groups have been utilized, such as neutral phosphatidylcholine and phosphatidylethanolamine (Zasadzinski et al., 1991), negatively charged phosphatidylglycerol (Kalb et al., 1992; Pearce et al., 1992), or positively charged dioctadecyldimethylammonium bromide (DODAB) (Stelzle et al., 1993). Various hydrophilic substrates have been used as support, e.g., silica (Naumann et al., 1992), quartz (Kalb et al., 1990; Esumi and Yamada, 1993), oxidized silicon (Tamm and McConnell, 1985), and mica (Zasadzinski et al., 1991; Mou et al., 1994).

The common picture of an SPB is that of an ordinary bilayer that covers the surface of the support homogeneously and is separated from it by a water layer of about 2 nm thickness. This picture derives from a number of experimental data. Brian and McConnell (1984) reported that an SPB is homogeneous over distances of at least microns and that the lipid molecules in an SPB exhibit the same lateral mobility as in conventional fluid bilayers. The homogeneity of SPBs was confirmed recently by atomic force microscopy measurements (Mou et al., 1994). Electric measurements indicate that SBPs represent a permeation barrier, although their electrical resistance does not reach the value of BLMs (Stelzle et al., 1993). The thickness of the water layer of about 2 nm was determined by neutron reflection experiments (Johnson et al., 1991). The same conclusion was derived from ^{1}H-NMR experiments with SPBs spread on small glass beads (Bayerl and Bloom, 1990). In this case, the data provided evidence for the existence of an aqueous compartment inaccessible to ions. This compartment was assigned to the water layer between the SPB and the glass surface permitting an estimate of the thickness of the water layer.

In our own studies aimed at developing a biosensor based on a transport protein, we were also able to demonstrate the existence of a compartment shielded from the exterior by a permeation barrier (Kiefer et al., 1991; Klee et al., 1992). However, when we determined the volume of this compartment, it turned out to be too large to be identified with the space between the SPB and the glass surface. The idea arose that the inaccessible compartment might be provided by vesicles adsorbed on the glass surface. Adsorbed lipid ves-

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Abbreviations used: POPC, palmitoyloleoylphosphatidylcholine; POPE, palmitoyloleoylphosphatidylethanolamine; POPG, palmitoyloleoylphosphatidylglycerol; N-SRh-PE, *N*-(Texas Red sulfonyl)-1,2-dihexadecanoylglycero-3-phospho-ethanolamine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-1,2-dihexadecanoylglycero-3-phosphoethanolamine; F-PE, *N*-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; TMA-TEMPO, 4-trimethylammonium-2,2,6,6-tetramethylpiperidin-1-oxyl; FRAP, fluorescence recovery after photobleaching; BLM, black lipid membrane; SPB, supported planar bilayer; SVL, supported vesicle layer.

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icles have been suggested as intermediates in the process of forming SPBs (Jackson et al., 1987; Xia and van de Ven, 1992) but to the best of our knowledge have not been considered as stable structures on the surface of solid supports.

This stimulated us to study the interaction of lipid vesicles with glass surfaces, paying special attention to the possibility of forming a supported vesicle layer (SVL). We asked three questions: 1) Do vesicles upon contact with a glass surface always spread and form SPBs, or do they sometimes adsorb and form SVLs? 2) What are the parameters that govern the formation of SPBs or SVLs? 3) What are the characteristic properties of SPBs and SVLs? To answer these questions, we performed fluorescence measurements with labeled lipids to determine 1) the amount of adsorbed lipid, 2) the extent of vesicle fusion, 3) the ability of the adsorbed lipids to diffuse laterally, and 4) the accessibility of the adsorbed lipids to external probe molecules. Essentially two types of lipid were used, palmitoyloleoylphosphatidylcholine (POPC) and *Escherichia coli* lipid, because they were found to represent the extreme cases in the formation of either SPBs or SVLs.

MATERIALS AND METHODS

Materials

E. coli lipid was extracted according to the method of Radin (1981) from wild-type strain B grown to the stationary phase. It was purified by silica gel column chromatography as described previously (Wright and Overath, 1983).

POPC, palmitoyloleoylphosphatidylethanolamine (POPE), and palmitoyloleoylphosphatidylglycerol (POPG) were purchased from Avanti (Alabaster, AL). *N*-(Texas Red sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanol-amine, triethylammonium salt (N-SRh-PE), *N*(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylamonnium salt (NBD-PE), *N*-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, trimethylammonium salt (F-PE), and 4-trimethylammonium-2,2,6,6-tetramethylpiperidin-1-oxyl iodide (TMA-TEMPO) were obtained from Molecular Probes (Eugene, OR). Phosphatidylethanolamine-1-palmitoyl-2-[1-^{14}C]linoleoyl (C^{14}-DPPE) was from Amersham (Braunschweig, Germany). All other chemicals were of analytical grade as provided by Merck (Darmstadt, Germany).

Suprasil quartz glass plates (45 × 12.5 × 1.25 mm) and a triangular-shaped cuvette of the same material were provided by Hellma (Müllheim, Germany). The surface quality of various commercial quartz glasses varied with respect to smoothness. Whenever possible we used flame-polished Suprasil quartz glass to reduce effects from surface defects.

Silicon wafers were a gift of Wacker Chemie (Burghausen, Germany) and of H. Gaub (Technical University, München, Germany). Glass beads of diameter 0.64 μm were a gift of T. Bayerl (Technical University, München, Germany).

Lipid vesicle preparation

Unilamellar vesicles were prepared by extrusion. Required amounts of lipid stock solutions in chloroform were put into a glass tube, and solvent was removed by evaporation at room temperature. The resulting lipid film was further dried at reduced pressure for more than 30 min and agitated in buffer, yielding a 1 to 10 mg/ml solution. The large multilamellar vesicles obtained were extruded 31 times through 50-nm pore size polycarbonate filters (Nuclepore, Pleasanton, CA) with a LiposoFast extruder from Avestin (Ottawa, ON). The size of the resulting lipid vesicles was measured by photon correlation spectroscopy with a Coulter N4/SD submicron particle analyzer (Hialeah, FL). The mean diameter obtained was 75 to 90 nm.

Multilamellar vesicles were prepared by sonication of a hydrated lipid film of 1 mg/ml final lipid concentration. This procedure yielded vesicles with a mean diameter of 70 to 90 nm.

Lipid concentrations of stock lipid vesicle dispersions were 0.1 to 1 mg/ml. The NBD-PE, F-PE, or N-SRh-PE added was 0.5 to 2 mol% of total lipid content. For measurements at standard conditions, the buffer used was prepared always of 10 mM sodium phosphate titrated to pH 7.4 and of 100 mM Na_2SO_4. Only in the case where *E. coli* lipid vesicles were prepared for subsequent Ca^{2+}-induced fusion experiments was 10 mM HEPES buffer used instead.

Surface treatment

For cleaning purposes, coverslips, Suprasil quartz plates, and silicon wafers were boiled in butanol or methanol or treated with 2% Hellmanex II (Hellma, Müllheim, Germany) and sonicated. The cleaning procedures did not influence the results. Before use, surfaces were rinsed extensively with deionized water with electrical resistivity of more than 15 M[Ωohm]cm.

Application of lipid vesicles to surfaces followed the procedure described by Brian and McConnell (1984). Lipid vesicle dispersions (lipid content, 0.1–1 mg/ml) were incubated on different substrates for more than 4 h at room temperature and finally rinsed extensively with buffer solution. Ca^{2+} (20 mM) was used to fuse *E. coli* lipid vesicles on the surface. The lipid-coated surface never came in contact with air.

Lipid adsorption assay

Suprasil quartz plates were incubated with vesicle dispersions prepared by sonication and washed thoroughly. The procedures on quartz plates were carried out with forceps in clean, homemade teflon containers. As observed by fluorescence microscopy, the damage caused by this handling was scratches in the lipid layer not exceeding 10 mm^2 in area. Membranes were labeled with 0.9% F-PE or C^{14}-DPPE. Adsorbed membrane was solubilized with 0.1% sodium dodecylsulfate, and the amount of solubilized labeled lipid was determined. F-PE fluorescence was measured with a Perkin-Elmer MPF-3 fluorimeter at an excitation wavelength of 495 nm and an emission wavelength of 520 nm, both at 5 nm bandwidth. The radioactivity of C^{14}-DPPE was measured with a 1600 CA Packard liquid scintillation analyzer (Downers Grove, IL), yielding about 200 cpm in the case of adsorbed POPC. The lipid adsorption procedure was controlled without a quartz plate. Rinsing the teflon container with sodium dodecylsulfate solution yielded less than 1% of the signal obtained with quartz plates. When quartz plates had been covered with nonlabeled lipid first, less than 5% of the signal with clean quartz plates was obtained.

To determine the number of adsorbed lipid molecules and the extent of coverage of the surface, the following simplifying assumptions were made. A POPC or *E. coli* lipid molecule has a molecular weight of 760 Da and occupies an area of 60 Å^2. The surface area of a Suprasil quartz plate was 1270 ± 10 mm^2.

To determine the quantity of adsorbed lipid on glass beads, 1 mg of glass beads was incubated in 2 ml of a 0.02 mg/ml POPC, 1% NBD-PE-labeled vesicle dispersion. The glass/vesicle dispersion was centrifuged and the fluorescence intensity of the supernatant was recorded.

Vesicle fusion assay

Vesicle fusion was investigated by monitoring fluorescence energy transfer between NBD-PE and N-SRh-PE directly on the surface (Struck et al., 1981). Two extruded vesicle populations, labeled either with 2% NBD-PE or with 2% N-SRh-PE, were mixed and incubated in a triangular cuvette. The cuvette content was exchanged by buffer until no further decrease of

fluorescence intensity was detectable. Hence, the detected fluorescence originated exclusively from surface-adsorbed lipid.

Fluorescence measurements applying the surface-front technique were carried out using a triangular-shaped quartz cuvette held with a homemade cuvette holder. The geometry used is shown in Fig. 1. The fluorescence light is detected under 90° to the incident light and opposite the direction of geometric reflection. For these measurements, a Perkin-Elmer fluorimeter LS50B (Beaconsfield, UK) was used. Fluorescence spectra were recorded upon excitation at 460 nm. The excitation and emission slits were 5 nm. The background signal due to scattered light was about 30% of the signal and was subtracted.

For calibration, fluorescence was also recorded of adsorbed lipid containing 2% NBD-PE or 2% N-SRh-PE, or a mixture of 1% NBD-PE and 1% N-SRh-PE.

The fraction of lipid fused was determined in the following way. When $I_{\text{NBD-PE}}$ and $I_{\text{N-SRh-PE}}$ denote, respectively, the fluorescence intensities at 520 nm of vesicles labeled separately with 2% NBD-PE or N-SRh-PE, the fluorescence intensity of mixed but not fused vesicles labeled separately with 1% NBD-PE or 1% N-SRh-PE follows as $I_o = (I_{\text{NBD-PE}} + I_{\text{N-SRh-PE}})/2$. The fraction f of fused vesicles is obtained from the relation

$$f = 1 - \frac{I - I_f}{I_o - I_f}. \quad (1)$$

where I_f denotes the fluorescence intensity of the mixed vesicles after complete fusion and I the observed fluorescence intensity. When vesicles labeled separately with NBD-PE and N-SRh-PE were mixed in dispersion, they did not undergo fusion for the concentration and time period chosen.

Lipid lateral diffusion assay

Fluorescence microscopy and fluorescence recovery after photobleaching (FRAP) were used to study the ability of adsorbed lipids to undergo lateral diffusion.

Sonicated vesicles labeled with 0.9% NBD-PE were incubated on cleaned coverslips and rinsed with buffer. Lipid-coated coverslips were observed with the fluorescence microscope Axioplan (Zeiss, Oberkochen, Germany) using an oil immersion 63× Neofluar objective and a 10× ocular (filter set II, ex. 450–490, em. FT 510, LP 520). A bleached 160 μm octagonal-shaped spot was produced by continuous illumination for 2 min. The bleached spot was briefly observed immediately, after 2 and 10 min. If the bleached spot disappeared, the lipids could diffuse freely; if it remained, their mobility was restricted. This rather coarse way of monitoring FRAP allowed the detection of lateral motion of fluorescently labeled lipid molecules over distances on the order of hundreds of microns.

Lipid accessibility assay

Accessibility of membrane leaflets was determined by a method adopted from McIntyre and Sleight (1991). The fluorescence of NBD-PE can be destroyed chemically by treatment with dithionite. Both reacting species, dithionite ion $S_2O_4^{2-}$ and SO_2^- radical, are rather membrane-impermeable on a time scale of minutes, so that the reaction allows a quantitative distinction between accessible and nonaccessible NBD-PE incorporated in membranes.

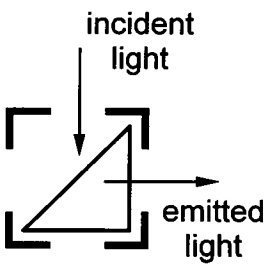

FIGURE 1 Geometry of the fluorescence measurements on vesicle fusion and lipid accessibility.

Fluorescence of surface-adsorbed membrane was detected by applying the surface-front technique described above. Extruded vesicles containing 1% NBD-PE were incubated in the 1.5-ml triangular cuvette. Again, the cuvette bulk volume was replaced by buffer until there was no further change of fluorescence intensity and all fluorescence detected originated from adsorbed lipid. Twenty microliters of 1 M sodium dithionite in 1 M Tris (pH 10) was applied, and fluorescence intensities were measured before and after reaction. The fluorescence of NBD-PE was excited at 460 nm and detected at 520 nm; the excitation and emission slits were 5 nm wide. Accessibility was defined as the relative decrease of fluorescence intensity after the dithionite reaction had come to completion. The fraction of fluorescence remaining after reaction with dithionite reflects the fraction of nonaccessible membrane leaflet.

Accessibility to TMA-TEMPO was probed by applying 2.5 mM TMA-TEMPO instead of dithionite. Accessibility measurements with TMA-TEMPO were performed with the emitted light detected in the direction of geometric reflection (Fig. 1), to avoid any inner filter effects of the bulk volume, and the triangular cuvette was then rotated slightly to circumvent geometric reflection.

As a control, the accessibility of extruded 1% NBD-PE-labeled vesicles in dispersion was determined. Fluorescence intensities of solutions containing stirred fluorescent samples were measured in a 1 × 1 cm quartz cuvette using a thermojacketed sample holder.

The accessibility experiment was also performed with supported spherical bilayers (SSBs) on glass beads. Various amounts of glass beads (diameter, approximately 0.64 μm) were given to a 1% NBD-PE-labeled extruded POPC vesicle dispersion (0.02 mg/ml). The fluorescence was recorded in the same way as with vesicles in dispersion.

Fluorescence lifetime measurements

Fluorescence lifetimes of NBD-PE in POPC membranes were determined to study the possibility of fluorescence quenching at the surface. Collisional quenching and energy transfer by the Förster mechanism would decrease the mean fluorescence lifetime. Extruded POPC vesicles containing 1% NBD-PE and a mixture of these vesicles with glass beads (surface ratio membrane/glass, 1:0.8) were used as samples. Time-resolved fluorescence measurements were performed as described (Döring et al., 1995). NBD-PE fluorescence was excited at 305 nm and detected at 520 nm.

RESULTS

Amount of lipid adsorbed

Our first aim was to determine the amount of lipid adsorbed on a planar surface under different experimental conditions. For this purpose, fluorescently or radioactively labeled lipid was mixed to the lipid under study, the resulting vesicles were incubated on a glass plate for several hours, the glass plate was washed several times, and the fluorescence or radioactivity of the adsorbed lipids was detected. The result for the amount of adsorbed POPC or *E. coli* lipid as a function of salt concentration is shown in Fig. 2. For POPC, the amount of adsorbed lipid is independent of salt concentration; for *E. coli* lipid it increases.

The amount of 5.3 μg adsorbed POPC corresponds, with an area of the plate of 1270 mm^2, to a surface density of 4.2 ng/mm^2 and, with a molecular weight of 760 Da, to a surface particle density of 5.5 pmol/mm^2 or an area per particle of 30 Å^2. Because the area occupied by a lipid

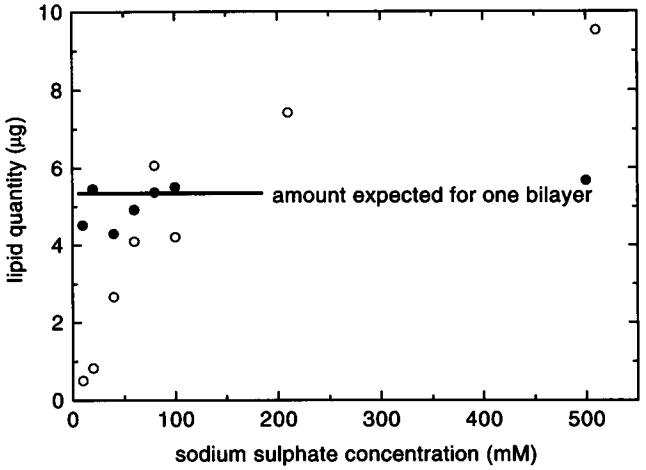

FIGURE 2 Amount of lipid adsorbed on quartz plates as a function of sodium sulfate concentration determined by fluorescence measurements. Lipid vesicle dispersions of 0.1 mg/ml POPC (●) or *E. coli* lipid (○), both labeled with 0.9% F-PE, were incubated on quartz plates, the plates were washed thoroughly, and the remaining fluorescence was detected.

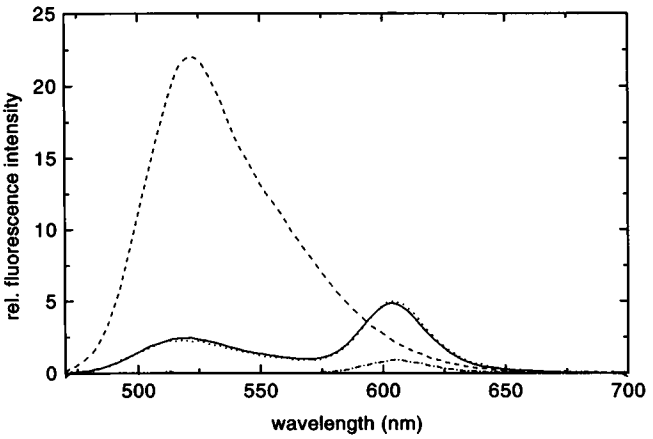

FIGURE 3 Fluorescence emission spectra of labeled POPC adsorbed on quartz plates. POPC was labeled with 2% NBD-PE (- - -) or 2% N-SRh-PE (-·-·-), or with 1% NBD-PE and 1% N-SRh-PE (····). To study vesicle fusion, the vesicles labeled separately with 2% NBD-PE and 2% N-SRh-PE were incubated together on a quartz plate, the plate was washed, and the spectrum was recorded (——). The excitation wavelength was 460 nm.

molecule is about 60 Å^2, this amount of lipid corresponds to a single bilayer.

The limiting value at high salt concentration for the amount of adsorbed *E. coli* lipid is about 10 μg, which would correspond to roughly two bilayers on the plate. As an alternative, one may assume that vesicles of *E. coli* lipid form an SVL on the glass surface. The amount of 10 μg adsorbed lipid would then suffice to cover 47% of the surface of the plate by spherical vesicles (viewed from above). Such an incomplete coverage seems unrealistic (because of the negative charge of the lipids the vesicles will repel each other, but not over distances of their size). Thus, keeping the assumption that vesicles are adsorbed, the vesicles would be predicted to become flattened upon adsorption, approaching the case of coverage by two bilayers.

The amount of lipid adsorbed on glass beads was also determined, and 1 mg of glass beads was found to adsorb 23 μg of POPC. Using 0.64 μm for the diameter of the beads and 2.5 g/cm^3 for their density, this amount of lipid corresponds to about 1.5 bilayers covering the beads.

Fusion of lipid vesicles

The degree of lipid mixing, equivalent to vesicle fusion, was determined by fluorescence energy transfer assay (Struck et al., 1981). Two kinds of lipid vesicles were prepared, which differed only in the labeling with either NBD-PE, serving as fluorescence donor, or N-SRh-PE, serving as acceptor. The two kinds of vesicles were mixed and incubated on a glass surface; then the glass surface was washed several times, and a fluorescence emission spectrum was recorded. The result for POPC is shown in Fig. 3. Upon excitation of the donor at 460 nm, donor fluorescence was detected at 520 nm and acceptor fluorescence at 605 nm. This indicated fluorescence energy transfer and hence mixing of the labeled lipids due to fusion of the vesicles. As a control, the labeled lipids were mixed before preparation of the vesicles, and the double-labeled vesicles were treated as before (Fig. 3). The two spectra essentially coincide, indicating complete fusion of the adsorbed vesicles. From Eq. (1), the fraction of fused vesicles is 98% (Table 1).

The same measurements were performed with *E. coli* lipid (data not shown). At 40 mM salt, no fluorescence energy transfer between donor and acceptor was observed, indicating that the labeled lipids do not mix and the vesicles do not fuse (Table 1). Presumably, the negatively charged lipids contained in *E. coli* lipids prevent the vesicles from coming into close contact. With increasing salt concentration, a weak fluorescence energy transfer appears. This effect may be explained by energy transfer between labels on different vesicles, which increases when the vesicles come in closer contact, due to increased screening of the charges at higher salt concentration. It is known that by adding Ca^{2+} ions the electrostatic repulsion can be overcome completely and turned into attraction, stimulating the vesicles to fuse (Cohen et al., 1984; Marcelja, 1992). In-

TABLE 1 Properties of lipid adsorbed on surfaces: fraction of vesicle fusion, ability of lateral diffusion, accessibility to dithionite

Lipid type	Condition	Fraction of fusion*	lateral diffusion‡	accessibility to dithionite*
POPC	100 mM salt§	0.98	Yes	0.99
E. coli lipid	40 mM salt	0	No	0.53
E. coli lipid	100 mM salt	0.29	No	0.62
E. coli lipid	Ca^{2+} added	1.01	Yes	0.99

*Surface: flame-polished glass.
‡Surface: glass coverslips.
§Salt was Na_2SO_4.

deed, when vesicles of *E. coli* lipid were incubated on the glass surface and washed, and Ca^{2+} ions were added, fluorescence energy transfer was detected, as in the case of POPC vesicles (i.e., the vesicles fused) (Table 1).

Lateral diffusion of adsorbed lipid

The ability of adsorbed lipids to diffuse laterally in the plane of the surface was investigated by detecting fluorescence recovery after photobleaching. When vesicles of POPC labeled with NBD-PE were incubated on a planar support and the plate was washed several times and then viewed under the fluorescence microscope before bleaching, a uniform fluorescence was observed, giving the impression of a continuous planar bilayer. This observation was made for a number of different supports such as quartz slides of different quality, glass coverslips, or Si wafers covered with SiO_2, Ta_2O_5, Si_3N_4, or Al_2O_3. When the fluorescence was bleached to some extent, it recovered as demonstrated in Fig. 4, *A–C*. Only in the case of Al_2O_3-covered wafers was no recovery observed. This implies that in all other cases the POPC lipids form a topologically continuous structure such as an SPB.

Usually, a few bright spots were visible on the plates that might arise from adsorbed vesicles. When glass of inferior quality was used, the number of such spots increased. This phenomenon has been described already by Brian and McConnell (1984). Adsorbed vesicles may also be responsible for the immobile fraction of lipid observed in quantitative FRAP experiments (Brian and McConnell, 1984; Tamm and McConnell, 1985). This view is supported by the result of an experiment (data not shown), in which an SPB of POPC labeled with NBD-PE was exposed to dithionite and the residual dispersed fluorescence was subjected to photobleaching. As will be shown below, dithionite permeates through an SPB so that the residual fluorescence arises only from adsorbed vesicles and should not recover after photobleaching. This was indeed observed.

When vesicles of *E. coli* lipid were incubated on a planar support, the visual impression was that of a more grainy layer. After photobleaching, the fluorescence did not recover, as shown in Fig. 4, *D–F*. This implies that the *E. coli* lipids are not organized in a topologically continuous structure such as an SPB; the result would, however, be compatible with the formation of an SVL. When Ca^{2+} ions were added, the fluorescence again recovered completely, indicating that fusion of the vesicles led to a continuous structure formed by the *E. coli* lipids.

Similar experiments were performed with mixtures of synthetic lipids, which to some extent mimic *E. coli* lipid, especially the negative charges. These were POPC/POPG and POPE/POPG, both at a molar ratio of 80:20. In both cases, a partial but nonuniform recovery of the fluorescence after photobleaching was observed (data not shown). Hence, these mixtures behave differently from *E. coli* lipid, which did not show any recovery, as well as POPC, which showed a uniform recovery.

In all cases in which the fluorescence recovered, this ability was lost after air was allowed to contact the lipid-covered surface. (Therefore, electron microscopic or atomic force microscopic pictures taken of desiccated samples of a membrane-covered surface do not reflect the original situation.)

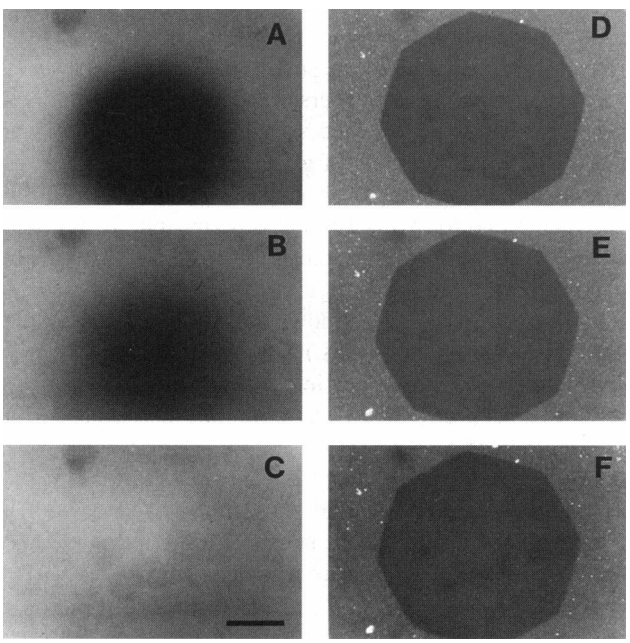

FIGURE 4 Microscopic pictures of POPC (*A–C*) and *E. coli* lipid (*D–F*) adsorbed on a coverslip and labeled with 0.9% NBD-PE, taken immediately (*A, D*) and at 2 min (*B, E*) and 10 min (*C, F*) after photobleaching of a spot. Bar = 50 μm.

Accessibility of adsorbed lipids

The accessibility of adsorbed lipids was investigated by using fluorescently labeled lipids and adding a fluorescence quencher. If the quencher has access to the labeled lipids, a decrease in the fluorescence is detected. As a fluorescence label we used NBD-PE and as a quencher dithionite, which chemically destroys the NBD fluorescence. When POPC vesicles labeled with NBD-PE were incubated on a glass plate, the plate was washed, and dithionite was added, the fluorescence vanished completely, as shown in Fig. 5. This implies that all lipid molecules are accessible from the external medium. This finding was astonishing, because one would have expected POPC to form a perfect planar bilayer, with only half of the lipid molecules being accessible from the external medium. When the same experiment was performed with *E. coli* lipid, the relative fluorescence decreased from 1 to 0.38 (Fig. 5). A value of 0.5 would be expected for large vesicles, because half of the lipids are inaccessible from the external medium. For highly curved vesicles, the number of lipid molecules in the outer leaflet is slightly larger than in the inner leaflet, so that the fluores-

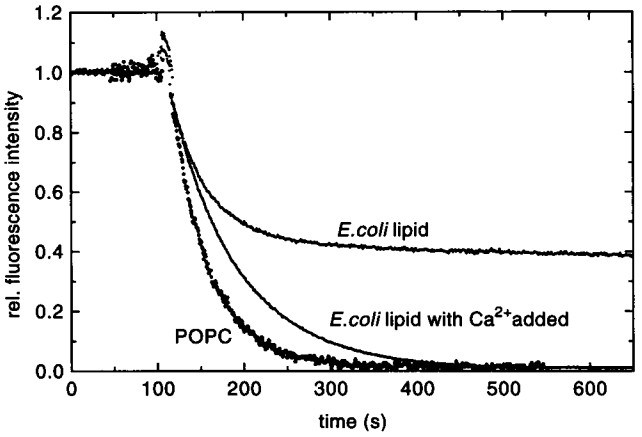

FIGURE 5 Time course of the fluorescence of POPC and *E. coli* lipid adsorbed on glass surfaces after the addition of dithionite (at 110 s). Adsorbed *E. coli* lipid was investigated without and with 20 mM $CaCl_2$. Accessibility was determined from the intensity at 500 s after the addition of dithionite. The excitation wavelength was 460 nm, the emission wavelength 520 nm.

cence may decrease to a value below 0.5. The above result, therefore, is again consistent with the formation of an SVL. Sixty-two percent of the lipid molecules were accessible to dithionite (Table 1). In the presence of Ca^{2+} ions, again all lipids were accessible to dithionite, indicating Ca^{2+}-induced fusion of the *E. coli* lipid vesicles and formation of an SPB (with almost 100% accessibility as observed for POPC). As a control, the experiment was repeated with vesicles in solution. Upon addition of dithionite, the relative fluorescence decreased to 0.44, i.e., 56% of the lipids were accessible.

Because the accessibility of all NBD-PE molecules in POPC membranes was unexpected, we tested several modifications of the standard procedure, such as variation of the incubation temperature, sonication in the presence of lipid vesicles, addition of different amounts of negatively or positively charged lipids or detergents, and variation of surface treatments (e.g., argon plasma cleaning). None of them led to a relative accessibility of 0.5 as expected for a perfect SPB. So we conclude that an SPB of POPC is permeable to small molecules such as dithionite.

To put this conclusion on a firm basis, other possible interpretations of the data must be excluded. We considered the following four possibilities, all of which would permit an explanation under the assumption of a perfect SPB: 1) the fluorescence labels facing the glass surface may be quenched by components of the surface; 2) the labels may be distributed asymmetrically in the SPB with the majority being in the outer layer; 3) the labels may undergo fast flip-flop between the inner and outer layer, so that the irreversible quencher dithionite can destroy all labels when they are on the outside; and 4) dithionite may diffuse into the space between the surface and the SPB at the boundary of the SPB.

The following control experiments were performed: 1) The fluorescence lifetimes of NBD-PE in an SPB and in vesicles of POPC were measured and found to be 5.27 ns and 5.28 ns, respectively. This confirms that the fluorescence is not quenched by components of the supporting surface. Furthermore, the fluorescence did not reappear after solubilization of the dithionite-reacted membrane with detergent. 2) A dispersion of POPC vesicles with the two labels NBD-PE and N-SRh-PE distributed symmetrically was prepared and spread on a glass surface to form an SPB. The fluorescence emission spectra of the vesicle dispersion and the SPB were measured and found to be identical (data not shown). A higher concentration of the labels in the outer leaflet would have led to a higher efficiency of fluorescence energy transfer. This implies that in the SPB the labels are distributed in the same way as in the vesicles, i.e., symmetrically with respect to both leaflets. 3) Dithionite acting as an irreversible quencher of fluorescence was replaced by the reversible collisional quencher TMA-TEMPO. If the labeled lipids in an SPB would undergo fast flip-flop, the fluorescence would be destroyed completely by dithionite, but not by TMA-TEMPO (even at high concentrations). In the experiment, the fluorescence was quenched by TMA-TEMPO to the maximum extent, which is about twice as much as for adsorbed vesicles of *E. coli* lipid. Hence, flip-flop cannot explain the data; the result instead demonstrates that TMA-TEMPO also permeates the SPB. Nakanishi et al. (1985) have shown that phospholipid flip-flop in an SPB occurs with a half-life of 10 h to 1 day. This is much longer than the time span of our measurements. 4) Vesicles of POPC were labeled with NBD-PE and incubated with small glass beads, the beads were washed, and dithionite was added. (The leakiness of SPBs on glass beads might be a consequence of mechanical damage during the washing procedure, which in contrast to the washing of a plate, includes a centrifugation step. This possibility was excluded by an experiment in which small portions of glass beads were added to a stirred dispersion of NBD-PE-labeled vesicles of POPC and the accessibilities to dithionite were measured. Small amounts of glass beads led to an increase of membrane accessibility, because vesicles adsorb on the glass beads and form an SPB that is permeable to dithionite. When the surface area of the glass beads added became equal to that of the vesicles in the dispersion, the accessibility was close to 100% and remained at that level upon addition of more glass beads.) The fluorescence again vanished, indicating that even in the case of SPBs on glass beads that lack any boundary, dithionite has access to all lipid molecules.

Hence, the proposed alternative interpretations of the data on the accessibility of lipid molecules in SPBs can be excluded; small molecules such as dithionite or TMA-TEMPO are able to permeate through SPBs.

DISCUSSION

Our results clearly demonstrate that phospholipid membranes are able to adopt different structures upon contact

with a hydrophilic surface. We confirmed the well-established feature of POPC vesicles to spread and form SPBs on glass surfaces (Brian and McConnell, 1984; Tamm and McConnell, 1985; Kalb et al., 1992). Another behavior, however, was found when *E. coli* lipids were used. They organize in a structure that deviates from an SPB in the amount of lipid molecules adsorbed, the extent of lipid mixing or vesicle fusion, the lateral mobility of lipid molecules, and the accessibility of lipid molecules. We tried to replace *E. coli* lipid with mixtures of lipids such as POPC/POPG or POPE/POPG, expecting to find the same behavior, but did not succeed. The mixtures exhibited a partial recovery in photobleaching experiments, in contrast to *E. coli* lipid.

The quantity of POPC lipid adsorbed on a glass plate is in good agreement with the amount expected for a single bilayer. In the case of glass beads, the amount of adsorbed lipid was slightly larger and corresponded to 1.5 bilayers. The quantity of lipid adsorbed on glass beads has been determined by various groups, but the results scatter considerably and range in value from those corresponding to one monolayer to those corresponding to two bilayers (Jackson et al., 1986; 1987; Tenchov et al., 1989; Esumi and Yamada, 1993). This variation, including our own result, may be explained by different experimental conditions and the difficulties in determining the size of the glass beads. Planar glass plates are advantageous in this respect, because their surface area can easily be determined.

For POPC, the amount of lipid adsorbed is independent of salt concentration. This is different for *E. coli* lipid. At low salt concentration, the amount of *E. coli* lipid adsorbed is too small for coverage of the surface by an SPB, and at high salt concentration it is too large. This indicates formation of a structure different from an SPB. The salt dependence in the case of *E. coli* lipid suggests that electric charges play a role in adsorption. Indeed, *E. coli* lipid contains about 25% negatively charged lipids, mainly phophatidylglycerol, and a glass surface is supposed to have a negative surface potential (H. Gaub, personal communication). One therefore expects electrostatic repulsion to play a role in the interaction between the lipids and the glass surface. At high salt concentration, the repulsion would be reduced because of shielding of the charges, and more lipids would be permitted to adsorb to the surface.

The saturation value of adsorbed *E. coli* lipid at high salt concentration may give a hint on the shape of the vesicles. The amount of lipid was interpreted as corresponding to coverage of the surface by flattened vesicles. Such flattening of the vesicles seems conceivable in the sense that the attractive interaction between the vesicles and the surface, which originates in the van der Waals interaction and gives rise to adsorption, tries to maximize the contact area between vesicle and surface and thus renders the vesicles flat on one side. Their shape would then resemble a half sphere, as proposed by Seifert and Lipowsky (1990). Ellipsometric studies of *E. coli* lipid vesicles incubated on a glass surface may also be interpreted as reflecting a layer of more or less deformed vesicles (Striebel et al., 1994).

To provide further evidence for the existence of an SVL in the case of adsorbed *E. coli* lipid, the extent of vesicle fusion, lateral diffusion of the lipid molecules, and accessibility of the lipid molecules was investigated. POPC vesicles fused and spread upon contact with a glass surface (or spread and fused), whereas vesicles of *E. coli* lipid did not fuse unless a fusogen such as Ca^{2+} ion was added. Hence, *E. coli* lipid vesicles adsorb on the surface without undergoing fusion; they maintain their integrity, which is consistent with the model of an SVL. From these findings, one may get the impression that the negative charges of *E. coli* lipid prevent them from forming SPBs. However, mixtures of synthetic lipids with negative charges were found by us and others to spread at least partially on planar surfaces (Tendian et al., 1991; Pearce et al., 1992). Hence, *E. coli* lipids must contain components that have a stabilizing effect on vesicles. Vesicles of *E. coli* lipid thus have the strongest tendency to form SVLs, vesicles of POPC/POPG are intermediate, and vesicles of POPC have the weakest tendency and prefer to form SPBs.

When the lateral diffusion of adsorbed lipids was investigated, POPC lipids were found to diffuse over large distances, but *E. coli* lipids were not. This again is consistent with the model of POPC forming a homogeneous SPB and *E. coli* lipids forming an SVL in which diffusion of the lipid molecules is restricted to distances of the size of the vesicles. In the case of POPC, fluorescence recovery was not complete, indicating an immobile fraction of lipids. This fraction may arise from a small number of adsorbed but nonfused vesicles, even in the case of POPC.

When the adsorbed lipids were investigated for their accessibility from the external medium, all POPC lipids were found to be accessible to dithionite, whereas in the case of *E. coli* lipids only about half of them were accessible. The latter result is again compatible with the model of adsorbed vesicles, the lipid molecules in the inner leaflet of the vesicles being inaccessible from the medium. In the case of POPC, one might have expected that the lipid molecules in the surface-exposed leaflet of the SPB are also inaccessible from the medium. This was not observed, leading to the conclusion that an SPB of POPC is permeable to small molecules such as dithionite. Several other interpretations of this experimental observation could be ruled out by control experiments.

Our conclusion is at variance with the finding of Bayerl and Bloom (1990). These authors performed ^{1}H-NMR experiments on SPBs on glass beads and used manganese ions as a probe for accessibility to water molecules. Their ^{1}H-NMR experiments yielded a nonaccessible volume, which was attributed to the water layer between membrane and surface and led to a thickness of this layer of 1.7 nm. However, this nonaccessible volume might also be attributed to the interior of a small number of adsorbed vesicles, which were always seen in our FRAP experiments (see Fig. 4 *A*). It is worth mentioning in this context that a number of

studies have demonstrated that SPBs have a relatively high electrical conductivity, about 100-fold higher than for BLMs (Stelzle et al., 1993). This would be compatible with our finding of a considerable permeability of SPBs for small molecules. The reason for this high conductivity and permeability of SPBs compared to vesicle membranes and BLMs may lie in the roughness of the supporting surface, but further studies are required to put this suggestion on a firm basis.

CONCLUSION

Taken together, we propose two different topological structures that form upon contact of lipid vesicles with a surface: 1) Supported vesicle layers result from vesicles that adsorb to a surface and do not fuse. 2) Supported planar bilayers originate from the adsorption of vesicles followed by fusion and spreading, or spreading and fusion. Obviously, these structures are two extremes, each being present in different amounts, depending on lipid composition, ionic milieu, and surface quality.

SPBs are permeable to small molecules and, therefore, seem to be useful for applications in which membrane components are to be exposed to a medium without any requirement on permeability. Whenever a separate compartment is required, however, SPBs are not suitable and should be replaced by SVLs. The adsorbed vesicles are impermeable to small molecules and permit, for example, the study of transport across membranes (Kiefer et al., 1991; Klee et al., 1992). Their advantage compared to vesicles in solution is the immobilization of the vesicles on the support, which permits measurements in flow-through cells.

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