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Formation of supported planar bilayers by fusion of vesicles to supported phospholipid monolayers

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A technique for the production of supported phospholipid bilayers by adsorption and fusion of small unilamellar vesicles to supported phospholipid monolayers on quartz is described. The physical properties of these supported bilayers are compared with those of supported bilayers which are prepared by Langmuir-Blodgett deposition or by direct vesicle fusion to plain quartz slides. The time courses of vesicle adsorption, fusion and desorption are followed by total internal reflection fluorescence microscopy and the lateral diffusion of the lipids in the adsorbed layers by fluorescence recovery after photobleaching. Complete supported bilayers can be formed with phosphatidylcholine vesicles at concentrations as low as 35 μM. However, the adsorption, fusion and desorption kinetics strongly depend on the used lipid, NaCl and Ca^{2+} concentrations. Asymmetric negatively charged supported bilayers can be produced by incubating a phosphatidylcholine monolayer with vesicles composed of 80% phosphatidylcholine and 20% phosphatidylglycerol. Adsorbed vesicles can be removed by washing with buffer. The measured fluorescence intensities after washing are consistent with single supported bilayers. The lateral diffusion experiments confirm that continuous extended bilayers are formed by the monolayer-fusion technique. The measured lateral diffusion coefficient of NBD-labeled phosphatidylethanolamine is $(3.6 \pm 0.5) \cdot 10^{-8}$ cm^2/s in supported phosphatidylcholine bilayers, independent of the method by which the bilayers were prepared.

Introduction

Various model membrane systems have been used in the past to study the physical properties and the physiology of biological membranes. Amongst them, single phospholipid bilayers which are supported on flat solid substrates have become increasingly popular in recent years [1]. The advantage of these supported planar bilayers (SPBs) over many other model membrane systems is that they are unilamellar and geometrically well defined. Originally, SPBs have been developed for modeling cell–cell and cell–membrane interactions in the immune system (for reviews, see Refs. 2 and 3). In more recent years, they have also proven to be very useful in studies of the lateral diffusion of adsorbed macromolecules by fluorescence recovery after photobleaching [4,5], the specific binding of proteins to well-defined membrane receptors by total internal reflection fluorescence microscopy [6–9], the structure of bound molecules by atomic force microscopy [10,11], and the orientation of α-helices of membrane-bound polypeptides by attenuated total reflection infrared spectroscopy [12].

Since in general many membrane proteins and receptors are laterally mobile in biological membranes (and often change their lateral mobilities upon ligand binding and signal transduction (see e.g. Refs. 13 and 14), it would be desirable to be able to reconstitute integral membrane proteins into SPBs in a functional, laterally mobile form. Unfortunately, this has proved to

Abbreviations: FRAP, fluorescence recovery after photobleaching; LB, Langmuir-Blodgett; NBD-eggPE, egg *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; SPB, supported planar bilayer; TIR, total internal reflection; TIRFM, total internal reflection fluorescence microscopy.

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be difficult with two common techniques that have been used to prepare SPBs. The problems with the Langmuir-Blodgett technique [1] are that many proteins do not tolerate to be transfered into monolayers where some of their domains may become directly exposed to air, and that relatively large quantities of protein are required on the Langmuir trough. The direct fusion technique [15] which utilizes the fusion of small unilamellar vesicles on hydrophilic surfaces can be performed with small quantities of reconstituted protein-lipid vesicles and produces SPBs in which at least a fraction of the protein retains its physiological binding specificity. However, the proteins reconstituted by this method are generally not laterally mobile. The reasons for their immobilization could be that significant portions of their structures may protrude from the outer surface of the vesicles and may interact irreversibly with the hydrophilic surface of the solid substrate in the course of vesicle fusion.

These difficulties motivated us to investigate an alternative technique to prepare supported planar bilayers and to develop methods which will permit us to follow the reconstitution process in real time. The technique which we call the monolayer-fusion technique, uses a Langmuir-Blodgett phospholipid monolayer on a hydrophilic support as the substrate for vesicle fusion. Therefore, the vesicles to be fused will encounter the hydrophobic fatty acyl chains of the supported amphiphilic monolayer rather than the hydrophilic quartz support, i.e. a situation which may better preserve some protein structures. The adsorption, fusion and desorption kinetics of fluorescent-labeled vesicles of different compositions and concentrations are followed by total internal reflection fluorescence microscopy (TIRFM), and the lateral lipid diffusion coefficients in the resulting planar bilayers are determined by total internal reflection interference fringe pattern photobleaching (TIR/FRAP) at various

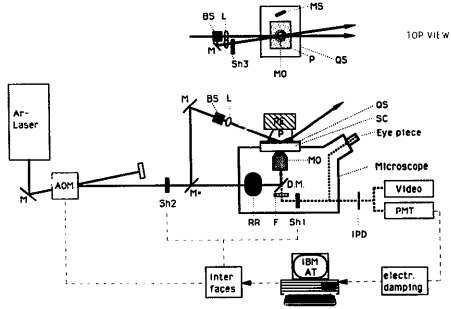

Fig. 1. Schematic diagram of the laser fluorescence microscope for total internal reflection fluorescence microscopy (TIRFM) and fluorescence recovery after photobleaching (FRAP). An argon ion laser (Innova 70-4, Coherent, Palo Alto, CA, USA) is used as the light source. The intensity of the laser beam is regulated with a computer-controlled acousto-optic modulator (AOM, 40, Intraaction Corp., Bellwood, IL, USA). A removable mirror (M*) is used to switch the instrument between TIR- and epi-illumination. For TIR-illumination the beam is split with a non-polarizing beam splitter cube (BS, Newport Corp., Fountain Valley, CA, USA) and the two beams are focused with two lenses (L, $f = 150$ mm) and intersected at the quartz/buffer interface in the image plane of an inverted microscope (IM35, Carl Zeiss, Oberkochen, Germany). A trapezoidal quartz prism (P) with one side normal to the incoming beam is optically coupled with immersion oil to the quartz microscope slide (QS). The illuminating laser beam is split in a plane (see top view) which is inclined at an angle of 72° from the vertical microscope axis. The quartz slide (40×25×1 mm) fits tightly into the sample cell (SC) with a glass coverslip bottom which is approximately 1.5 mm below the lower surface of the quartz slide. Two access holes (not shown) in the frame of the cell allow the perfusion of buffer and other materials through the cell. The contents of the cell are stirred with a little magnetic stirrer (MS) and the temperature can be controlled with a Peltier element (PE). The interference pattern is only used for measurements of lateral diffusion by TIR/FRAP and as a focussing aid. Fluorescence intensities in the TIR mode are measured with only one beam and the other beam is blocked with shutter 3 (Sh3). In epi-illumination FRAP, a Ronchi ruling (RR) is imaged at the quartz/buffer interface through the dichroic mirror (DM) and the microscope objective (MO; 40× water immersion, NA = 0.75, Carl Zeiss). An image plane diaphragm (IPM) in the optical path of the microscope is used to select for areas of interest and the fluorescence intensities in these areas are measured by a photomultiplier tube (PMT, Thorn EMI, Type 9658A, Ruislip, Great Britain) with an analog noise reduction system (designed and built by Dr. G. Haenisch). This signal is digitized and fed into an IBM-AT computer. In FRAP experiments, shutter 1 (Sh1) is closed during the bleach pulses to avoid over-exposure of the PMT. Shutter 2 (Sh2) is closed between individual fluorescence intensity measurements in kinetic TIRFM experiments to avoid excess photobleaching.

stages of bilayer completion, i.e. in the presence of large amounts of vesicles in solution. First results on the reconstitution of a membrane protein, cytochrome b_5, with the monolayer-fusion technique are reported elsewhere [16].

Materials and Methods

Materials

POPC, POPG, NBD-eggPE were from Avanti Polar Lipids (Alabaster, AL, USA) and all other chemicals were from Merck (Darmstadt, Germany) and of the highest available purity grade. Ion-exchanged ('nanopure') water was used for the preparation of all buffers.

Total internal reflection fluorescence microscopy

Fluorescence intensities of membranes which contained NBD-eggPE and which were located at the quartz/buffer interface were measured by TIRFM as described previously [6]. By this method, only the fluorescent molecules which were very close (within approx. 100 nm) of the quartz/buffer interface were excited by the evanescent wave of a total internally reflected laser beam on the stage of an inverted fluorescence microscope. Fluorescence emission was measured with a photomultiplier whose output current was electronically enhanced for maximum sensitivity and maximum signal/noise.

A number of significant modifications were made to the previously described instrument and a schematic diagram of the improved apparatus is presented in Fig. 1.

Data were acquired through an A/D converter (Metrabyte, Taunton, MA, USA) into an IBM-AT computer and calculations were performed with a program written in ASYST (Asyst Technologies, Rochester, NY, USA). Typically, fluorescence intensities were determined by averaging the measured values of 12 different spots. The standard deviation of these averaged intensities was used as a measure for the quality (homogeneity) of the prepared membrane. All experiments (except those which measured the influence of salt concentrations on the formation of SPBs) were performed in 10 mM Tris-HCl, 150 mM NaCl (pH 7.4). The sample cell was kept at 22°C, and the cell contents were stirred continuously.

Fluorescence recovery after photobleaching

The lateral diffusion of the lipids in the bilayer was determined by pattern photobleaching, either with epi- or TIR-illumination. In epi-illumination, the periodic stripe pattern was produced by focusing a Ronchi ruling in the back-focal image plane according to Smith and McConnell [17]. Usually, a Ronchi ruling of 50 lines per inch was used which resulted in a repeat distance of 12.7 μm on the sample. The periodic pattern in TIR-illumination was produced by the interference of two laser beams at the quartz/buffer interface [18]. The TIR/FRAP data were analysed following Davoust et al. [19] and the mobile fractions are expressed as the percentage of the theoretical fluorescence recovery at each given depth of photobleaching. It should be noted that the determination of the mobile fraction by the TIR/FRAP method is sensitive to the relative intensities of the two beams which produce the interference pattern. Even though a good precision cube beam splitter (Newport, model 05BC16NP.2, Fountain Valley, CA, USA) was used in our experiments, the relative intensities of the two intersecting beams were 54:46 on the sample. As a consequence, the mobile fractions determined by TIR/FRAP were only about 80% of those which were determined by conventional epi-FRAP on the same test samples. In order to account for this effect, all TIR/FRAP mobile fractions were multiplied by a correction factor of 1.2. The lateral diffusion coefficients determined by TIR/FRAP and epi-FRAP were the same within an error limit of 20% and were not corrected. Although technically more difficult, the advantage of TIR/FRAP over epi-FRAP is that surface diffusion can be measured even in the presence of relatively high fluorophore concentrations in solution. Control experiments without the periodic pattern were routinely performed in the TIR- and epi-modes in order to assure that neither membrane-solution exchange reactions nor bulk diffusion contributed to the measured fluorescence recoveries.

Lipid vesicles

Unilamellar vesicles were prepared by the extrusion method according to Hope et al [20]. Briefly, a lipid solution in $CHCl_3$ was dried in a glass tube by a stream of nitrogen, followed by a high vacuum for at least 1 h. The lipids were resuspended by vigorous vortexing in the desired buffer solution. After five freeze/thaw cycles, the lipid dispersion was extruded eight times through two 100 nm pore size polycarbonate filters (Nucleopore, Pleasanton, CA, USA). The mean diameter of these vesicles was determined by negative stain electron microscopy to be 80 to 90 nm. This is in good agreement with previously published values [21].

Preparation of planar bilayers

Quartz slides were cleaned with a 10% solution of Contrad 90 (Technosa S.A., Lausanne, Switzerland) in deionized water by boiling for 20 min, followed by hot sonication in a bath sonicator for 30 min. Subsequently, they were rinsed with deionized water, washed with methanol and dried for 1 h at 150°C. Immediately before use, the quartz slides were further cleaned in an argon plasma cleaner (Harrick Corp. Ossining, NY, USA) for 10 min. Each slide was re-used about five

times. To test for the influence of the detergent on the properties of bilayers formed by the fusion method, some of the slides were cleaned with Linbro 7 × (Flow Laboratories AG, Allschwil, Switzerland) or Contrad 90 with or without methanol treatment as indicated.

(a) Langmuir-Blodgett technique. SPBs were prepared as previously described [1]. The first monolayer was transferred vertically from an air/buffer (10 mM Tris-acetic acid (pH 5.0)) interface onto a clean quartz slide at a surface pressure of 32 mN/m. The second monolayer was transferred horizontally in a 10 mM Tris-HCl, 150 mM NaCl, pH 7.5 buffer at 32 mN/m. The use of the pH 5.0 buffer for the first coating provided for a better coupling of this monolayer to the solid substrate and consistently resulted in high quality SPBs. When ion-exchanged or glass destilled water was used in the first trough, SPBs with defects were produced quite frequently, whereas a pH 7.5 buffer in the first trough resulted most of the times in a loss of the lipid during the deposition of the coated slide onto the second monolayer.

(b) Direct fusion of lipid vesicles on quartz. This procedure follows the one previously descibed by Brian and McConnell [15]. A clean quartz slide was assembled in the TIRFM cell which was then filled with buffer and mounted on the stage of the laser fluorescence microscope. A suspension of vesicles was injected and the changes in fluorescence intensity at the quartz/buffer interface were followed with TIRFM. After a constant value had been reached (after about 1–2 h), the sample cell was washed with buffer at a flow rate 20 ml/h or more. Lateral diffusion coefficients were measured before and after the wash procedure.

(c) Fusion of vesicles to supported phospholipid monolayers. A method to produce a single planar bilayer by fusion of vesicles to a supported monolayer has been previously described [22]. A monolayer of dipalmitoylphosphatidic acid was deposited on a germanium plate and the second monolayer was completed by adsorption (and fusion) of sonicated vesicles of POPC. This results in a completely immobilized bilayer. A refined protocol is described in the following. A single monolayer of POPC was transferred vertically from the surface of a 10 mM Tris-acetic acid, pH 5.0 buffer in a Langmuir-trough onto a clean quartz slide at 32 mN/m. This monolayer coated slide was assembled in the TIRFM cell which had been filled with the fusion buffer (10 mM Tris-HCl, 150 mM NaCl (pH 7.4)), or with another buffer as indicated. The cell was mounted on the stage of the laser fluorescence microscope and the fusion was started with the injection of the vesicles. The increase of the fluorescence intensity at the quartz-buffer interface was followed by TIRFM for 1 to 3 h. Subsequently, the fluorescence intensity was measured at different spots and the lateral diffusion of the lipids was measured by FRAP. The resulting membranes were then washed with at least 20 volumes of buffer. The fluorescence intensities and the lateral diffusion coefficients were again measured at the final plateau levels.

Results

Formation of supported phospholipid bilayers

A new method to prepare SPBs consists of (a) the deposition of a single phospholipid monolayer on the hydrophilic surface of a quartz slide and (b) the fusion

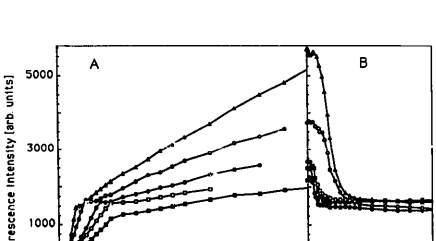

Fig. 2. Formation of supported planar bilayers by fusion of POPC vesicles to supported phospholipid monolayers. (A) Fluorescence intensity increases, measured by TIRFM, at the interface of a quartz supported planar monolayer and a 10 mM Tris-HCl, 150 mM NaCl, pH 7.5 buffer after the addition of different concentrations of small unimellar vesicles composed of POPC and 0.2 mol% NBD-eggPE. (B) Time courses of the decreases in fluorescence intensity during removal of excess adsorbed vesicles by washing of the SPBs with buffer at a continuous flow of 20 ml/h. The lipid concentrations of the vesicle suspensions are 35 μM (■), 50 μM (□), 75 μM (●), 100 μM (○), 200 μM (△).

of small unilamellar vesicles to the hydrophobic face of the supported monolayer which is exposed to a buffer solution. The adsorption and the fusion of the vesicles which contain a small percentage of the fluorescent lipid NBD-eggPE can be followed by measuring the increase of the fluorescence intensity with TIRFM. Several time courses of the adsorption of vesicles at different concentrations and composed of POPC and 0.2 mol% NBD-eggPE are shown in Fig. 2A.

The substrate was always a single monolayer of POPC on quartz. At all concentrations, two phases of adsorption were observed; i.e., an initial fast linear phase followed by a sudden transition to a slower phase. Both phases were dependent on the vesicle concentration. The initial rate of adsorption increased by roughly a factor of four when the vesicle concentration was increased from 35 to 200 μM. However, the bend occurred rather independent of the vesicle concentration always at about 1300–1500 of the arbitrary fluorescence units defined by our experimental setup. The adsorption process was probably diffusion-limited, because a change in the stirring speed had a dramatic influence on the adsorption kinetics. Without stirring, fusion was very slow. Fig. 2B shows the desorption kinetics of excess vesicles during washing the cell with a continuous flow of 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 buffer. The fluorescence intensities of all curves returned to about 1400–1600 fluorescence units within the first 15 min.

Homogeneously fluorescent surfaces were observed on the quartz slides at this stage. The fluorescence intensities were about the same as those observed when the SPBs were prepared by the LB technique in which only the second monolayer (distal to the substrate) was labeled with 0.2 mol% NBD-eggPE (Table I).

The average lateral diffusion coefficient of the lipids, measured on a large number of adsorbed membranes was $(4.0 \pm 0.5) \cdot 10^{-8}$ cm^2/s, i.e. very close to the diffusion coefficient which was observed in the LB bilayers $((3.4 \pm 0.3) \cdot 10^{-8}$ cm^2/s). Most significantly, high fractions of mobile lipid (80–90%) were observed with both preparation techniques (Table I). Taken together, the microscopic observations, the fluorescence intensity and the diffusion measurements support the conclusion that single bilayers were formed on the quartz slides and that the labeled vesicles had disintegrated and fused on the supported monolayer to form large continuous bilayers which exhibited long range (i.e. over tens of μm) diffusive lateral lipid transport. It can be further concluded from these data that the sharp bend in the adsorption curve always occurred when single bilayers were completed and that the second slow phase represented the reversible adsorption of further vesicles. When still higher concentrations of lipid vesicles (such as 1 mM) were used, multiple bends were observed, suggesting that multiple bilayers had been formed. However, these additional layers could be washed away with an excess of buffer.

In addition to the here described monlayer-fusion technique and the LB technique [1], the direct fusion technique on plain quartz slides, first described by

TABLE I

Final fluorescence intensities, mobile fractions, and lateral diffusion coefficients in supported planar phospholipid bilayers prepared by three different methods

Preparation technique	Vesicle composition	Fluorescence intensity (rel. units)	Mobile fraction (%)	Diffusion coefficient (10^{-8} cm^2/s)
Neutral vesicles				
Ves. fusion on SPM	POPC, 0.2% NBD-PE	1550 ± 80	83 ± 7	4.0 ± 0.5
Ves. fusion on plain quartz	POPC, 0.2% NBD-PE	2800 ± 40	–	$< 10^{-3}$
		2800 ± 20 [a]	80 ± 5 [a]	3.5 ± 0.5 [a]
Ves. fusion on labeled SPM	POPC	1350 ± 60	78 ± 4	3.6 ± 0.5
Langmuir/Blodgett		1420 ± 30	90 ± 4	3.4 ± 0.3
Negatively charged vesicles				
Ves. fusion on SPM	80% PC/20% PG, 0.2% NBD-PE	640 ± 80	89 ± 8	4.0 ± 0.5
Ves. fusion on plain quartz	80% PC/20% PG, 0.2% NBD-PE	1470 ± 40	–	$< 10^{-3}$
Langmuir/Blodgett		610 ± 70	95 ± 4	3.5 ± 0.3

[a] Quartz slides washed with Linbro 7× with or without methanol treatment, or washed with Contrad 90 without methanol treatment. All other quartz slides were washed with Contrad and then treated with methanol. SPM, supported phospholipid monolayer.

Brian and McConnell [15], is very popular to prepare SPBs. The monolayer and the direct fusion techniques are compared in Fig. 3.

At the same lipid concentration of 100 μM, supported bilayers were formed within similar time ranges with both methods. A sharp bend in the adsorption and fusion time course was also observed with the direct fusion method. However, the fluorescence intensity at the bend was about twice as high in bilayers which were formed by direct fusion, because in this case, both leaflets of the SPB contained labeled lipids.

Negatively charged lipids

Most biological membranes contain negatively charged lipids and in many applications of SPBs, the use of negatively charged lipids is desired. Therefore, we tested the effect of such lipids on the formation of SPBs by vesicle fusion to supported monolayers. Pure POPC was again used for the first monolayer coating. When the vesicles which were used for the fusion step were composed of 80% POPC and 20% POPG, the adsorption/fusion kinetics were different to those observed with pure POPC vesicles (Fig. 4). A nonlinear, monophasic increase of the fluorescence intensity was observed which saturated when one bilayer was formed. This kinetic behavior was most likely due to increasing electrostatic repulsion forces between the supported bilayer and the vesicles as the supported bilayer approached completion. Presumably, repulsive electrostatic and hydration forces also prevented the adsorption of further vesicles. The same forces are known to determine the high lamellar repeat distances in multilayered structures of negatively charged lipids [23]. When these bilayers were washed, no decrease of the

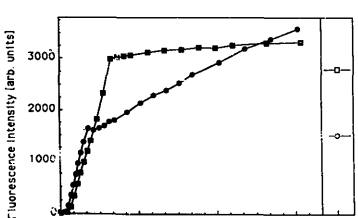

Fig. 3. Comparison of the fusion kinetics of POPC vesicles on plain and monolayer-coated substrates. Fluorescence intensity increases on plain quartz microscope slides (■) and on a quartz slide which has been coated with a single monolayer of POPC (●) are shown as a function of time after the addition of 100 μM POPC (plus 0.2 mol% NBD-eggPE) vesicles in 10 mM Tris-HCl, 150 mM NaCl (pH 7.5). The open symbols indicate the fluorescence intensities after the membranes have been washed with buffer.

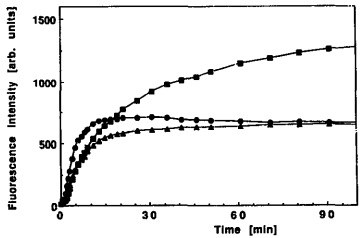

Fig. 4. Fusion kinetics of negatively charged vesicles on plain and monolayer-coated substrates. The kinetics of fluorescence intensity changes on quartz slides which have been coated with a POPC monolayer are shown after the addition of 200 μM (▲) and 900 μM (●) lipid vesicles composed of 80% POPC, 20% POPG and 0.2% NBD-eggPE in 10 mM Tris-HCl, 150 mM NaCl (pH 7.5). The squares (■) show the fusion kinetics of 200 mM vesicles of the same composition on plain quartz slides.

fluorescence intensity could be detected which provided further proof that only the lipids which were needed for the completion of the second leaflet of the bilayer had adsorbed to the surface.

The lateral diffusion coefficients and the mobile fractions of 0.2 mol% NBD-eggPE in the negatively charged bilayers were as high as those observed in SPBs which were composed of POPC only (Table I). These observations further confirmed the formation of complete supported bilayers also for the case of negatively charged phospholipids. However, the fluorescence intensities of the negatively charged bilayers were about half of the intensities that were observed when no negatively charged lipids were present. We attribute this to a change in the quantum yield of the fluorophore in the electrostatically different environments on the surfaces of POPC and POPC/POPG (4:1) bilayers, respectively. The same effect was observed when negatively charged SPBs were prepared by the direct fusion or the LB techniques (Table I).

Fig. 4 also shows a comparison of the monolayer-fusion kinetics with the direct fusion kinetics. As it was the case with pure POPC, the final fluorescence intensity after direct fusion was about twice the intensity after monolayer fusion or when the LB technique with only the second leaflet labeled was used to prepare SPBs.

Influence of Na^+ and Ca^{2+} on the fusion of vesicles to supported monolayers

The fusion of POPC vesicles to supported monolayers depends on the ionic strength of the buffer. Fig. 5 shows the fusion and wash kinetics at various concentrations of NaCl. No fusion occurred in 10 mM Tris-HCl

and in the absence of NaCl. Very slow adsorption and fusion was observed with 20 mM added NaCl. In the presence of 50 mM and 150 mM NaCl the adsorption/fusion was fast and the curves exhibited the sharp bend after completion of the SPB. The kinetic behavior in the presence of 1 M NaCl was different. A bend and saturation occurred only when one monolayer plus the fluorescence equivalent of one additional bilayer were adsorbed to the supported monolayer. Also, the wash kinetics extended over a much longer time than at the lower salt concentrations and about 3 h elapsed before the fluorescence intensity returned to the level of a single bilayer (Fig. 5B). In the presence of 150 mM NaCl and 2 mM $CaCl_2$, a mixed behavior was observed. The adsorption/fusion kinetics followed those of 50 and 150 mM NaCl (without added Ca^{2+}), whereas the desorption process paralleled the one in the presence of 1 M NaCl. Also, after washing, the SPBs which were formed in the presence of 1 M NaCl or 150 mM NaCl and 2 mM $CaCl_2$ showed some brighter areas by fluorescence microscopy which probably represented patches of adsorbed lipid vesicles which were difficult to remove by the 3-h wash procedure. When the lateral diffusion coefficients were measured in these areas, they were unchanged compared to those measured in the areas with a single bilayer. However, the mobile fraction was reduced roughly by a factor which was inversely proportional to the fluorescence intensity. This observation suggests that the membranes in these adsorbed patches did not fuse to form extended sheets of bilayers and did not permit long range lateral lipid diffusion.

Lateral diffusion of lipids during the fusion process

TIR/FRAP was used to measure the lateral diffusion of NBD-eggPE in the vicinity of the quartz-buffer interface. POPC vesicles were allowed to adsorb and fuse with the supported monolayer as described above, but instead of measuring the increase of the fluorescence intensity as a function of time, the fusion process was followed by measuring lateral diffusion in 1-min time intervals. Although the fluorescence intensities which were measured prior to each bleach pulse increased during the first 15 min as expected from the TIRFM experiments, no measurable lateral lipid diffusion could be detected ($D < 10^{-10}$ cm^2/s and/or mobile fractions < 5%) during this period. At about 15 min after the addition of the vesicles, the lipids suddenly started to diffuse with a diffusion coefficient of about $4 \cdot 10^{-8}$ cm^2/s which was identical to the average diffusion coefficient which was measured after washing (Table I). The initiation of long-range lateral diffusion occurred at the same time point as the sharp bend in the fluorescence intensity kinetics. Also beginning at this time, the amount of mobile lipid always corresponded to the equivalent of 1 lipid monolayer. These data indicate that during the first phase of lipid binding, the vesicles either adsorbed without fusion or fused with the monolayer as distinct individual entities. Only when a critical surface concentration was achieved (at about 15 min), these individual units or patches became connected very suddenly and perhaps, cooperatively to form the extended supported phospholipid bilayer.

Pretreatment of the quartz slides

When the lateral diffusion of lipids was measured in SPBs which were prepared by direct fusion to the quartz slides, the values obtained for the mobile fractions depended strongly on how the quartz slides were

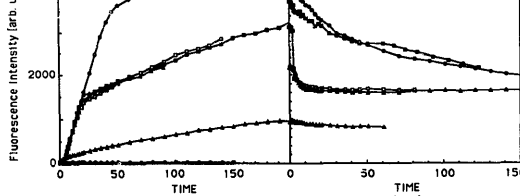

Fig. 5. Influence of salt concentrations on the fusion of POPC vesicles to supported monolayers. (A) Kinetics of vesicle fusion and adsorption to POPC-coated quartz slides at different concentrations of NaCl and $CaCl_2$. (B) Kinetics of the desorption of excess lipid during continuous washing with buffer at 20 ml/h. The buffer compositions are: 10 mM Tris-HCl (pH 7.5) and no salt (●), 20 mM NaCl (▲), 50 mM NaCl (□), 150 mM NaCl (△), 150 mM NaCl and 2 mM $CaCl_2$ (■), and 1 M NaCl (○) added. The vesicle concentration in all experiments is 50 μM.

cleaned. If the original protocol [15] was used and the slides were washed with Linbro 7 × and destilled water prior to drying and argon plasma cleaning, the adsorbed vesicles fused and high mobile fractions were measured. The same results were obtained when Contrad 90 was used as the detergent. However, when the Contrad 90 treated slides were washed with methanol and then dried, the mobile fraction dropped to zero. This did not happen when Linbro 7 × was used as a detergent and subsequently treated with methanol. A possible explanation for these observations is that some detergent molecules which remained on the surface were required for the fusion of vesicles on 'clean' quartz slides and that methanol was able to completely remove residual Contrad 90, but not Linbro 7 × from the surface.

Properties of the first leaflet

The properties of the first (quartz-exposed) leaflet of the supported bilayer were examined by labeling the monolayer which was transferred from the Langmuir trough with NBD-eggPE and by a subsequent fusion of unlabeled POPC vesicles. The LB deposition of the POPC monolayer was routinely carried out under mildly acidic buffer conditions (pH 5.0), which assured good coupling of this monolayer to the substrate. Once deposited, this monolayer was very stable, even when transferred to the usual fusion buffer of pH 7.5. The fluorescence intensity distribution in this monolayer was very homogeneous as judged from fluorescence microscopy. After completion of the bilayer, the lateral diffusion coefficient of the lipids in the quartz-exposed leaflet of the bilayer was $(3.6 \pm 0.5) \cdot 10^{-8}$ cm^2/s and the mobile fraction was $78 \pm 4\%$. Both of these values were close to the corresponding values when the leaflet distal to the quartz surface was labeled (Table 1). Before the addition of unlabeled POPC vesicles, the fluorescence intensity of the supported monolayer was about 1800 of our relative fluorescence units. As a result of the fusion with plain POPC vesicles, the fluorescence intensity decreased by about 10%, and a further 10% decrease of the fluorescence intensity was observed after washing the completed SPB with buffer. The final fluorescence intensity was roughly the same as when only the second leaflet of the bilayer was labeled (Table 1). The origin of the observed fluorescence intensity decreases could be a small reordering of the lipids in the first leaflet upon bilayer completion, and/or the expulsion of some excess lipid from this leaflet. A few bright spots which probably represented adsorbed fluorescent vesicles remained after washing the SPBs which had been labeled in the first leaflet. This suggests that a limited (roughly 10%) lipid exchange could occur between the first monolayer and the adsorbed vesicles.

Discussion

The present work demonstrates the feasibility and defines the experimental conditions for the formation of planar supported phospholipid bilayers by vesicle fusion to supported monolayers. This technique offers an alternative to the existing techniques for future attempts to reconstitute integral membrane proteins into SPBs. Specifically, it combines the following two advantages: (i) relatively small amounts of lipid vesicles can be used for bilayer formation, and (ii) the vesicles face a lipid monolayer during fusion rather than a plain quartz surface, i.e. an interface which may help to preserve the structure of membrane proteins during fusion. Also, the sensitivity and electronic control of our TIRFM/FRAP apparatus has been considerably improoved and a TIR interference fringe pattern photobleaching option [18,24,25] has been installed. This latter method permits us to measure the lateral diffusion coefficients and mobile fractions in the adsorbed layers, even in the presence of relatively large amounts of fluorescent dye in solution. We believe that with these improovements, we have all the necessary tools at hand to rapidly screen and find the optimal conditions for the reconstitution of proteins into planar membranes in a functional and laterally mobile form. In fact, we have already been able to reconstitute cytochrome b_5 in a partially mobile form into SPBs by the monolayer-fusion and the direct fusion techniques [16].

The present work also introduces TIRFM as a method to monitor the kinetics of monolayer self-assembly. Self-assembled monolayers on hydrophilic and hydrophobic supports have been produced with several different amphiphilic molecules [26–28]. Escpecially when used in conjunction with covalent polymerization on electrodes and when doped with ion-selective amphiphilic chelators, such monolayers show great promise for the fabrication of biosensors and other devices. It has been pointed out [27] that under certain conditions self-assembled monolayers are more stable than built-up LB films. The supported monolayer provides a reactive surface for hydrophobic or amphiphilic molecules in solution. Most likely, the fusion of the vesicles to the supported monolayers is driven by hydrophobic interactions between the lipid fatty acyl chains. A plausible, but certainly not unique pathway for vesicle fusion to a supported monolayer is suggested in Fig. 6. When, in this model, the vesicles approach the supported monolayer, a defect occurs in the vesicle outer monolayer to permit hydrophobic contact between the vesicle inner monolayer and the supported monolayer. Monolayer spreading then causes the rupture and disintegration of the vesicle on the supported monolayer. The hydrophobic coupling between the two leaflets of the bilayer is strong and not

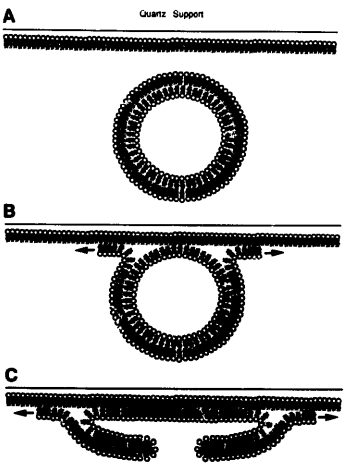

Fig. 6. Possible pathway for vesicle disintegration and fusion with a supported phospholipid monolayer. A vesicle approaches the supported monolayer (A) and a defect in the vesicle outer monolayer leads to close contact between the vesicle inner monolayer and the supported monolayer (B). The second monolayer spreads out on the hydrophobic surface of the first (supported) monolayer and causes vesicle rupture at some point in solution (C). Further spreading leads to the completed supported planar bilayer (D). In this model, strong hydrophobic interactions are believed to provide the major driving force for vesicle disintegration and fusion.

reversible by extended wash procedures. A prediction of this model is that proteins which are oriented 'right-side out' *or* 'inside out' in the vesicle would end up 'right-side out' in the planar bilayer.

Our results show that during or after bilayer formation, vesicles of the zwitterionic lipid phosphatidylcholine become adsorbed in a reversible fashion to the supported bilayer. No vesicles adsorb when negatively charged vesicles are used, probably because of electrostatic repulsion between the negatively charged surfaces of the planar bilayer and the vesicles. The adsorbed vesicles can be washed off easily from the planar bilayer at low to intermediate salt concentrations and in the absence of calcium. It is well known that electrostatic, hydration, van der Waals, steric and entropic fluctuation forces govern the equilibrium distance between closely apposed lipid bilayers [23,29–31]. Our adsorption and desorption kinetic results are in qualitative agreement with these concepts.

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