PHYSICAL PROPERTIES OF LIPID MONOLAYERS ON ALKYLATED PLANAR GLASS SURFACES

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ABSTRACT A method for transferring a lipid monolayer from an air-water interface to an alkylated glass slide is described. Specific antibodies bind tightly to lipid haptens contained in these monolayers on the glass slides. We conclude that the polar head groups of the lipids face the aqueous phase. A monolayer containing a fluorescent lipid was used to show that the monolayer is homogeneous as observed with an epifluorescence microscope. A periodic pattern photobleaching technique was used to measure the lateral diffusion of this fluorescent lipid probe in monolayers composed of dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine. Different regions of the pressure-area isotherms of the monolayers at the air-water interface can be correlated with the diffusion of the fluorescent probe molecules on the monolayer-coated glass slide. Monolayers derived from the so-called "solid-condensed" state of a monolayer at the air-water interface showed a very low probe diffusion coefficient in this monolayer when placed on a glass slide, $D \leq 10^{-10}$ cm^2/s. Monolayers derived from the "liquid condensed/liquid expanded" (LC/LE) region of the monolayer isotherms at the air-water interface showed rapid diffusion ($D > 10^{-8}$ cm^2/s) when these same monolayers were observed on an alkylated glass slide. The monolayers attached to the glass slide appear to be homogeneous when derived from monolayers in the LC/LE region of monolayers at the air-water interface. There is no major variation of the diffusion coefficient of a fluorescent lipid probe when this diffusion is measured on a lipid monolayer on a glass slide, for monolayers derived from various regions of the LC/LE monolayers at the air-water interface. This is consistent with the view that the LC/LE region is most likely a single fluid phase. Monolayers supported on a planar glass substrate are of much potential interest for biophysical and biochemical studies of the interactions between model membranes and cellular membranes, and for physical chemical studies relating the properties of lipid monolayers to the properties of lipid bilayers.

INTRODUCTION

Many important cell-cell interactions involve membrane-membrane contact between two cells. In a number of studies, particularly those involving various components of the immune system, it has been proven possible to replace one cell of an interacting pair by a reconstituted membrane vesicle, or liposome. In many cases, specific functional interactions between two biological cells can be mimicked using this cell-vesicle pair. For descriptions of typical recent work, see Tom and Six (1980). Two problems have motivated us to consider the possibility of specific functional interactions between a biological cell and a lipid monolayer supported on a planar substrate. First, the question is sometimes raised as to whether in previous studies of

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specific cell-vesicle interactions the finite radius of curvature of the vesicle plays any significant role (i.e., a "focusing effect," concentrating cell receptors and specific vesicle membrane components into a limited region of contact). Second, the region of greatest interest, the region of membrane-membrane contact, is particularly difficult to observe using optical microscopy, for both cell-cell interactions, and for cell-vesicle interactions. On the other hand, a functional interaction between a cellular membrane and a planar supported lipid monolayer on a glass slide should provide optimal conditions for studying the region of membrane-membrane contact. Biological applications of these coated glass slides are described elsewhere (Hafeman et al., 1981).

The method described in the present paper for the coating of glass slides with lipid monolayers of known composition and physical properties has the great advantage that these physical properties appear to be closely related to the physical properties of the same monolayers at the air-water interface. Furthermore, our measurements of the lateral diffusion of fluorescent lipid probes on the glass-supported monolayers are quite similar to those observed in lipid bilayers. The present study may therefore help further the understanding of the relationship between bilayer and monolayer membrane physical properties. Techniques for coating different materials with various amphiphilic molecules have been described (Kuhn et al., 1972); some of these coating techniques do not appear to be directly applicable to phospholipids.

MATERIALS AND METHODS

Glass slides of the desired shape (e.g., 1.8 × 1.8 cm) were washed in hot Linbro 7X detergent (Flow Labs. Inc., Inglewood, Calif.) for 0.5 h and sonicated in a bath sonicator (80 W, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). After washing the glass slides in distilled water we soaked them for 15 min in chromic acid. The glass slides were then rinsed thoroughly before being sonicated in 3 mM sodium hydroxide for 15 min. The final rinse with distilled water took 20 min, and the glass slides were then dried for at least 0.5 h in an oven at 110°–150°C.

To a solution of 80% hexadecane (99% pure), 12% carbon tetrachloride and 8% chloroform we added 0.1% octadecyltrichlorosilane (Petrarch Systems, Levittown, Pa.). A similar technique is described by Sagiv (1980). Fromherz (1973) also used an alkylsilylcholoride to alkylate glass surfaces. The slides were continually agitated for 10–15 min. The slides were slowly removed to the air phase (with draining) and then washed three times in chloroform for at least 5 min for each wash.

The quality of the hydrophobic coating was checked by placing a 20-μl droplet of water on the slide. The coating was considered satisfactory if the contact angle was close to 90° and if the droplet started moving freely and continuously on the slide when the tilt angle of the glass exceeded ~40°.

To prepare and study a lipid monolayer at an air-water interface we used a film balance and the procedure described in the previous paper (von Tscharner and McConnell, 1981). the surface pressure was measured with a Wilhelmy plate (0.22 μm Millipore filter) attached to a Roller Smith torsion balance (Biolar Corp., N. Grafton, Mass.). The area containing the lipids was adjusted by changing the height of a rhombus-shaped moveable frame. The water level in the temperature-controlled trough that contained the frame was 4 cm. This was deep enough to slide a petri dish or other support under the monolayer. We used a suction cup and tweezers to handle the support. A type of support frequently used is shown in Fig. 1. the cover slip was attached to the bottom of the dish with strips of one-quarter-inch wide double-coated tape (3M Co., St. Paul, Minn.).

The monolayer of the desired composition was first applied to the air-water interface from a hexane-ethanol (9:1) solution. To obtain an optically homogeneous monolayer it is important to leave the monolayer on the surface for 0.5 h at low pressure in the liquid-expanded state. The film pressure is then measured as the monolayer area is reduced. The monolayer was then left at a pressure ~40 dyn/cm for

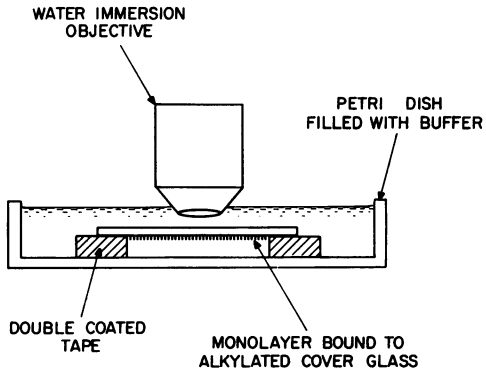

FIGURE 1 Monolayer membrane as mounted to facilitate fluorescence photobleaching and microscopic observation.

2 h before adjusting the pressure to the value to be used for coating the alkylated glass. The alkylated glass was washed with distilled water immediately before coating with lipids. Only glass slides that appeared perfectly dry after washing with water were used. We held the alkylated glass with tweezers and brought it horizontally onto the monolayer with a short, quick thrust. (This "horizontal approach" was possibly first used by Langmuir and Schaefer (1938) to coat hydrophobic surfaces with protein monolayers at an air-water interface.) It is important to avoid trapped air bubbles.

When the contact between the glass and the monolayer is made, very little change in surface pressure is observed, indicating that the monolayer is not squeezed out from under the glass. After ~10–15 s the glass was pushed through the monolayer and placed on the desired support resting at the bottom of the trough. The support together with the monolayer-coated glass was then removed from the trough with enough water to keep the monolayer-coated glass under water. The water removed from the trough was then replaced with an equal volume of water. To return to the original pressure, the monolayer has to be recompressed to compensate for the monolayer removed from the surface. Typically the monolayer area removed by coating the glass slide and the tweezers is 1.5–2 times the area of the coated slide. (This factor is not exactly 1.0 since some lipid coats the backside of the slide, and the tweezers.)

The lateral diffusion of the lipid on the lipid-coated alkylated glass was measured using pattern photobleaching (Smith et al., 1979) with a Zeiss photomicroscope III (magnification 800x).

L-α-Diplamitoyl phosphatidylcholine (DPPC)[1] and L-α-dimyristoyl phosphatidylcholine (DMPC) were purchased from Sigma Chemical Co. (St. Louis, Mo.; 98% pure) and were used without further purification. *N*-4-Nitrobenzo-2-oxa-1,3-diazole-L-α-dimyristoyl phosphatidylethanolamine (NBD-PE) was purchased from Avanti Biochemicals Inc. (Birmingham, Ala.) and was used without further purification. The lipid hapten was synthesized in this laboratory by D. Carter according to a modification of Brûlet's original synthesis (1977). The chemical formula of the lipid hapten is given in Hafeman et al. (1981). The fluorescent IgG antibodies against the hapten were a kind gift from D. Hafeman. They were prepared in our laboratory according to a procedure similar to the one described by Humphries and McConnell (1976).

Organic solvents have been checked for amphiphilic impurities by applying an excess amount on the film balance. A reduction of the area to 15% of the starting one resulted in no increase in surface pressure.

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[1] *Abbreviations used in this paper:* π, surface pressure in dyn/cm; A, average surface area per lipid molecule; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; NBD-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazole L-α-dimyristoylphosphatidylethanolamine; SC, solid condensed; LC, liquid condensed; LE, liquid expanded; LC/LE, liquid condensed/liquid expanded.

RESULTS

A monolayer at the air-water interface can be transferred at all pressures onto an alkylated glass slide. In the following discussion, we will always refer to the monolayer on the glass slide simply as "the monolayer" in contrast to the monolayer at the air-water interface. Air exposure of the monolayer for a fraction of a second is long enough to disrupt it; the slide again becomes hydrophobic. The slide will preferentially stay at the air-water interface. If a monolayer-coated slide is kept on an ordinary microscope slide it will remain firmly attached because of the hydrophilic properties of the lipid headgroups. This indicates that the headgroups face the water as they do at the air-water interface. The handling of the monolayer under water is not critical. The lipids were never washed away.

The difference in the π-A diagram between a pure DPPC monolayer and a DPPC monolayer containing 1% hapten is small (Fig. 3). We coated an alkylated glass slide with a 1% hapten-containing monolayer at a pressure of 35–40 dyn/cm and a temperature of 26 ± 0.2°C. We added an excess of fluorescent antibodies to the solution and incubated the monolayer for 10 min. The fluorescence as observed under the microscope was intense. If the solution around the monolayer was changed at least 10 times within 0.5 h, the fluorescence was still as intense as at the beginning. The antibodies did not wash off. The same experiment but on a monolayer without haptens showed no fluorescence after it was washed. This specific antibody binding to the haptens indicates that the headgroups of the monolayer are exposed to

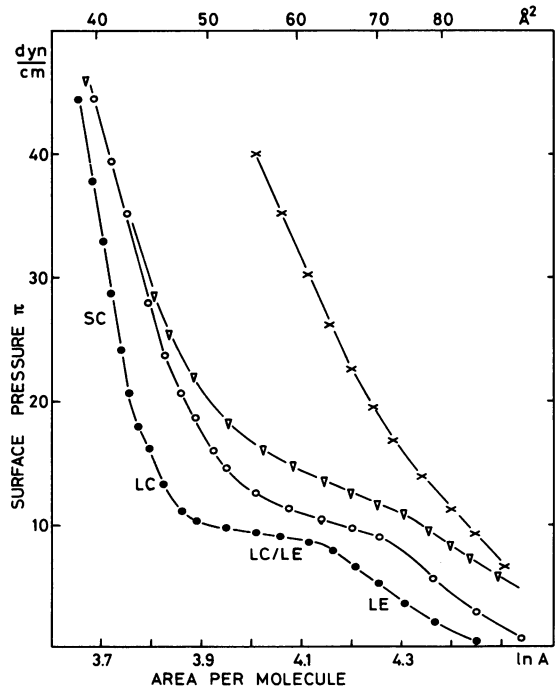

FIGURE 2. Pressure-area isotherms for phospholipid monolayers. ●, ○, DPPC monolayers at increasing and decreasing areas, respectively. ▽, DPPC and DMPC monolayers containing 1 mol/100 mol (mol %) of the fluorescent lipid probe NBD-PE. Equivalent results are obtained when 1 mol % of lipid hapten (see text) is included in the monolayer.

the aqueous phase. The haptenated monolayer with the antibodies bound to it showed a homogeneous fluorescence. <5% difference in intensity over the whole coated glass slide was measured with the photomultiplier fixed onto the microscope.

The homogeneity of the lipid monolayer was further tested by introducing the fluorescent lipid probe NBD-PE into the monolayer. The coating was performed as with the hapten-containing monolayers. Again the intensity of the fluorescence showed no variation over the whole coated area and was optically perfectly homogeneous. Note that the fluorescence intensity of the lipid probe NBD-PE is strongly dependent on the relative orientation of the monolayer (or bilayer) plane relative to the direction of propagation of polarized exciting radiation. See, for example, Smith et al. (1981). The orientation of the relevant transition dipole matrix element is such that, when a monolayer membrane is viewed from above as with an epifluorescence microscope, imperfections in monolayer (or bilayer) structure are strongly fluorescent, and easily detected. For an example, see Fig. 2 in Smith et al. (1981).

The monolayers were taken from the trough at different pressures and at 26°C (Fig. 2). The diffusion coefficients for NBD-PE molecules (1%) in a DPPC monolayer were measured at 23°C (Fig. 3). Coating from the SC region of the π vs. A diagram resulted in a diffusion constant $D < 10^{-10}$ cm^2/s. This corresponds to diffusion constants known for DPPC in bilayers. The diffusion constant increases rapidly as the area per molecule is increased to a value at which the monolayer at the air-water interface is in the liquid-condensed (LC) state. It is in this LC region and especially at the smaller area per molecule that the diffusion constant increases rapidly. Within the LC/liquid-expanded (LE) region and in the LE region the diffusion constant is of the order of 10^{-8} cm^2/s. There is a small decrease of the diffusion constant in the LC/LE region as we increase the area. At the beginning of the LE region the diffusion becomes slightly larger again. The main difference of the monolayer in the LE

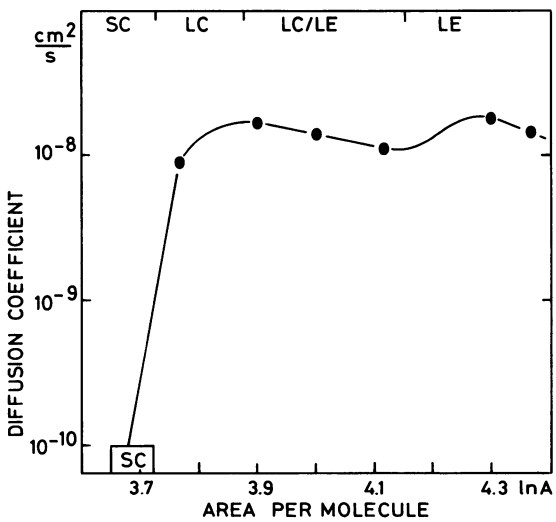

FIGURE 3 Lateral diffusion coefficients of a fluorescent lipid probe (1 mole % NBD-PE) in DPPC lipid monolayers bound to an alkylated glass slide at 23°C. Each monolayer on the glass slide was derived from a monolayer at the air-water interface at the stated area per molecule. The diffusion coefficient derived from the SC phase was 10^{-10} cm^2/s.

region is that it bleaches much faster than in the other regions. Diffusion could be measured only at the lowest possible level of the excitation (observation) beam. The reason for the faster bleach is probably a change in the structure of the monolayer which makes the NBD-PE more accessible to oxygen or which tilts the transition dipole moment of the probe in such a way that it absorbs the light more efficiently.

For a DMPC monolayer coated at 35 dyn/cm and a temperature of 25.5°C (Fig. 2), we measured a diffusion coefficient of 3.0×10^{-8} cm^2/s at 24°C. This is of the same order of magnitude as the diffusion coefficient in a DPPC monolayer when it is not in the SC region.

DISCUSSION

The alkylated glass surface forms an ideal counterpart for the acyl-chains of the lipids. A homogeneous lipid monolayer forms a structure similar to a bilayer with the alkylated glass. Because the supporting layer is covalently bound to the glass there is only lateral diffusion in the lipid monolayer.

The solid and fluid properties of lipid monolayer are similar to their properties at the air-water interface. The diffusion coefficient, for example, for NBD-PE in DMPC is very close to the one observed in liposomes (Smith et al., 1979). The friction between the monolayer and the alkyl-chain is therefore very limited. The fluid monolayer can diffuse laterally on its nondiffusing counterpart. This suggests that the lipids diffuse quite independently on both sides of a natural bilayer. From this result we can conclude that the phase transitions of the individual layers of a lipid bilayer are not necessarily strongly coupled and might occur separately, at least in extended planar bilayers where "end effects" may be neglected.

The diffusion properties of the DPPC monolayer at the air-water interface remain as the monolayer is transferred onto the glass. Monolayers coated from the solid-condensed (SC) region show the structure similar to gel known for bilayers.

The largest change in diffusion (two orders of magnitude) is observed between monolayers coated from the SC region and the LC region. The LC/LE region shows only very little change in diffusion.

From the diffusion constant we can find the jump frequencies ν. For $D = 1.6 \times 10^{-8}$ cm^2/s and an area of 49 Å^2/molecule we calculate the jump frequency ν to be 1.3×10^7 s^{-1}. This corresponds rather well with the 1.9×10^7 s^{-1} measured for a lipid probe in DPPC bilayers at 45°C (Galla et al., 1979). The decrease in diffusion with increasing area must result from a decrease in jump frequency. This suggests that the "activation energy" for the jumps is increased as the packing density is decreased. Because there is no sign of a gradual increase in diffusion in the LC/LE region we doubt very much that this region represents the solid-fluid transition of the lipids.

If large areas of solid monolayer and fluids would coexist we would see the local disappearance of the bleach pattern in the fluid areas. If coexisting areas much smaller than the width of our bleach pattern were solid or fluid we would observe their mutual diffusion (Owicki and McConnell, 1980). As more and more of these areas become fluid we would expect an increasing diffusion which we did not observe. This supports the view that the LC/LE region is probably not a transition region between solid and fluid, and that the solid-fluid transition starts at the end of the SC region and occurs within the LC region of the

pressure-area diagram (von Tscharner and McConnell, 1981). It is of interest to note that Teissie et al. (1978) have attempted to make quantitative measurements of lipid lateral diffusion of fluorescent lipid probes in DPPC monolayers at the air-water interface. Although there are admittedly some uncertainties in the accuracy of their diffusion coefficient, it is quite interesting that they do report an abrupt change in lateral diffusion in just the same region of the π-A curve where we infer a large change in the diffusion coefficient.

For biological applications one should use monolayers that behave most similarly to natural bilayers. Therefore it is advisable to perform the coating at a pressure >35 dyn/cm where DPPC and DMPC are solid and fluid, respectively. With too high a pressure one risks having ruptures in the monolayer.

The facts that lipid monolayers can be transferred homogeneously onto an akylated glass slide and that the molecules diffuse as on a natural bilayer provide a very powerful tool for future biological investigations of membrane-membrane interactions. This is especially true because the optical observation of such interactions with a microscope is now possible. Biophysical and biochemical applications of these monolayer-coated glass slides are described elsewhere (Hafeman et al., 1981).

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