Spontaneous and Reversible Switch from Amphiphilic to Oil-Like Structures

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Current theories assume that the amphiphilicity of biological membranes is always preserved. We observed that two hydrogen-bonding lipid layers in contact can spontaneously and reversibly lose their amphiphilic structure and turn into an assembly of oily complexes. This result opens a new angle for understanding the reorganization of lipids during membrane fusion, since similar complexes could fill the troubling hydrophobic voids displayed in the current models. The unique tribological properties described here may also find application in the development of novel nanolubricants.

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Biological membranes are highly stable bilayer structures, whose integrity is provided by the amphiphilic character of their constituting molecules. The way by which the interaction between the hydrophobic parts combined with the water solubility of the hydrophilic groups produces these structures is now well understood [1]. Such membranes have a good mechanical cohesion that enables them to resist various stresses, and which is physiologically extremely important since membranes are used to wall off cells and to separate them into several intracellular compartments. The disruption of a bilayer structure and its transition to a nonbilayer structure does not spontaneously occur, although it is known to be an intermediate in the mechanism of membrane fusion, which takes place during many cellular processes: membrane trafficking, neurotransmitter release, infection by enveloped viruses [2,3]. To initiate membrane fusion, an energetic barrier has to be overcome in order to remove water molecules from the space between the two membranes, bend the membranes, and lead to some intermediate lipid structures. Within the cells, this energy is provided by a set of special proteins called fusion proteins: viral proteins [4] and SNARE proteins [5]. Bilayer cohesion can be lost, and membrane fusion can also occur in reconstituted systems by the addition of perturbing agents such as polymers [6] or calcium ions [7], which induce phase separation within the bilayers and locally enhance hydrophobic interactions, or by using lipids that attract each other strongly through, for example, electrostatic forces [8,9] or hydrogen bonds [10]. The structures adopted by the lipid assembly during bilayer disruption and fusion have not been fully characterized yet, although an intermediate called the fusion stalk is now considered as the most likely intermediate of membrane fusion [11–14]. This model assumes that the amphiphilic character of the lipid layers is conserved during the whole process even though the strong curvatures and voids that it implies are not always very realistic. We propose an alternate explanation: the existence of a transition state in which the lipid assembly could switch from its intrinsic amphiphilic character to a purely hydrophobic behavior. We show that such a transition can occur between several types of hydrogen-bonding lipid layers (nucleoside and nitrilotriacetate lipid layers). We analyze the phenomenon with the surface force apparatus (SFA) by studying the interaction between these lipid layers in contact, and observe that the pairing of hydrogen-bonding lipids that reside in opposite bilayers leads to the disruption of the classical monolayer structures and to the formation of an oil-like structure between the two surfaces. Interestingly, this phenomenon is reversible and the integrity of the monolayers can be recovered upon separation of the two surfaces. The oily structure of the matter between the two surfaces in contact is confirmed by applying Darcy's law, which relates a liquid viscosity to the velocity and the pressure. From force *versus* distance curves, we deduce the viscosity η of this fluid, and the result is in good agreement with expected viscosity values of similar oils.

We have performed force measurements in pure water between hydrogen-bonding lipid bilayers prepared by the Langmuir-Blodgett deposition technique on mica surfaces [15]. The depositions were done at a surface area slightly larger than the one at collapse to ensure maximum density of the layers. The first layer, made of dimyristoyl-phosphatidyl-ethanolamine lipids (DMPE, from Avanti Polar Lipids), was used to render the mica hydrophobic. The second layer, made of lipids containing hydrogen-bonding groups in their polar head (synthesized as described elsewhere [16,17]) was deposited on the DMPE hydrophobic chains so that its headgroups were facing the aqueous solution. Three types of hydrogen-bonding lipids were used whose functional group was either thymidine (T), adenosine (A), or nitrilotriacetate (NTA). Nucleosides (A and T) are able to form two hydrogen bonds, whereas NTA groups can form up to six hydrogen bonds. In order to probe the effective contribution of hydrogen-bonding on the force curves, two sets of control lipids were also synthesized. In the first control lipid, the polar head resembles the T group except for a key H atom which is replaced by a methyl group, thus suppressing its hydrogen bond donor ability (MeT lipid). In the second control lipid,

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the NTA group is complexed with a nickel ion, *via* a chelation bond, thus blocking all the hydrogen bond donor OH groups (Ni-NTA lipid). Experiments were conducted with the surface pairs which bind strongly through hydrogen bonds: A/A, T/T, A/T, and NTA/NTA, and with the control surface pairs: T/MeT, A/MeT, MeT/MeT, and Ni-NTA/Ni-NTA. The force *versus* distance profiles of all these configurations have already been described elsewhere [18,19]. Here, the interest is mainly focused on the behavior of the layers in contact.

The distance D between the surfaces is defined as the distance between the hydrophobic midplanes of the bilayers [Fig. 1(a)]. All the surface pairs which are able to bind strongly through hydrogen bonds display the same force *versus* distance features. When the surfaces are approached, no force is measured between 200 nm and 100 nm, and an attractive force sets in at a distance of about 100 nm. When the gradient of this attractive force is larger than the spring constant, the two surfaces jump into close contact [Fig. 1(b)]. The distance reached after this jump in of the surfaces (D_0) is about 6 nm, (e.g., 5.3 ± 1.5 nm for A/A and 5.8 ± 1.2 nm for T/T). The expected thickness of the outer monolayers can be deduced from the volume and the molecular area of the lipids. The volume of the lipids is estimated from crystallography data on nucleosides [20,21] and nitrilotriacetate [22] compounds, and from the volume of the aliphatic chains [23]. This gives approximately 1.7 nm^3 for the A lipid, 1.6 nm^3 for the T lipid, and 1.8 nm^3 for the NTA lipid. Given the molecular area of the lipids at the deposition pressure (0.63 nm^2, 0.56 nm^2, and 0.56 nm^2 for the A, T, and NTA lipids, respectively), this leads to an expected thickness of the outer leaflets of the order of 5.5 nm in the case of the nucleoside lipids and 6.4 nm in the case of the NTA lipid. This is very close to D_0, which corresponds therefore to the distance between two intact and fully hydrated monolayers. From the shape of the interference fringes (not shown), one observes that the two surfaces, which were initially two crossed cylinders, flatten immediately on a circular area having a diameter of about 10 μm over which the thickness variation is less than 1 nm. Under an extremely small pushing force (~ 50 μN), the distance continues to slowly decrease in time and becomes much smaller than D_0 (during this phase, the surfaces remain flattened in the contact area). If the small pushing force is maintained long enough, the distance will eventually reach zero (contact between the hydrophobic tails of the inner monolayers). When a pulling force is applied, the distance slowly increases back to D_0 (e.g., 4.7 ± 1.9 nm for A/A and 6.0 ± 0.5 nm for T/T) and the surfaces separate. The energy necessary to separate the surfaces (adhesion energy) is 1 to 2 orders of magnitude higher than the one observed with nonfunctionalized neutral lipids [19,23]. The minimum distance depends on how long the pushing force has been applied. However, the value of the adhesion energy is independent of this minimum distance. The behavior described above can be reproduced in the course of subsequent approaching/separation cycles. In contrast, control surface pairs also come into contact at the distance D_0 but this distance remains roughly constant in time when a small pushing force is applied [Fig. 1(b)]; the application of a pulling force leads eventually to the separation of the two surfaces at a distance of approximately D_0.

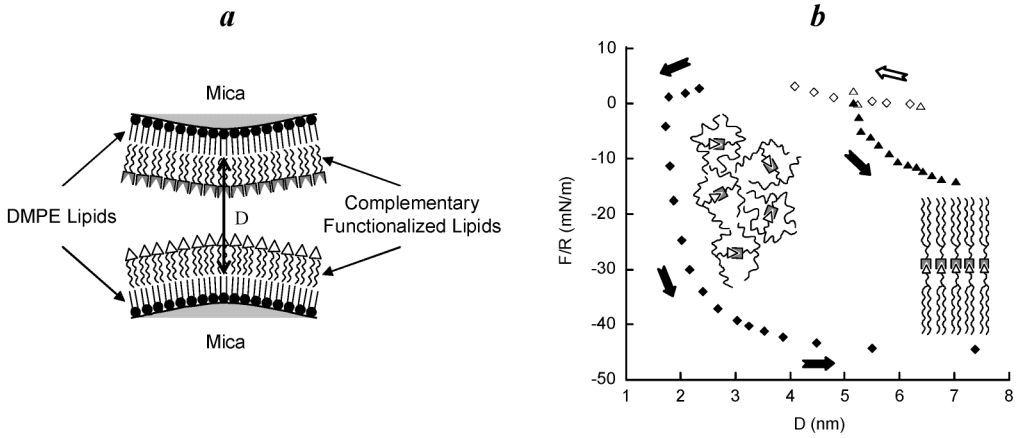

FIG. 1. (a) Geometry of the interacting bilayers during SFA experiments. (b) Typical force versus distance profiles (open symbols: approaching phases, solid symbols: separation phases) between two hydrogen-bonding surfaces in contact (example of T/T interaction, diamonds), and between two surfaces in which the hydrogen bonds have been blocked (example of T/MeT interaction, triangles). When the surfaces reach contact, the two outer monolayers are still compact and fully hydrated. Under small compression, two facing functionalized lipids bind tightly to form a complex with reduced hydrophilicity. The assembly of these complexes behaves like oil which flows away, inducing the observed decrease in distance. When the surfaces are pulled apart, the lipid complexes flow back, and the bilayer structures are recovered. The arrows indicate the directions of the approaching/separation cycles. Here, the force was not kept constant during the compression phases (upper part of the curves where F/R is positive).

In all cases, the integrity of the two outer monolayers is therefore preserved immediately after they come into contact. In the case of control surface pairs, this integrity is preserved throughout the whole experiment. For surfaces which form strong hydrogen bonds, the significant decrease of the distance shows that the bilayer structure is disrupted. If the waiting time is sufficient, this decrease continues down to the complete vanishing, in the contact area, of any matter coming from the outer monolayers: the two outer monolayers slowly fuse. This fusion process is different from what is observed when nonbonding lipid bilayers are forced to fuse by the local application of strong pressuring forces [24]. First, it occurs spontaneously, since no strong pushing force is required. Second, the phenomenon is reversible, since the integrity of the monolayers is recovered by pulling on the surfaces. Finally, the adhesion energy is independent of the minimum distance reached before the pulling force is applied. In the case when nonbonding surfaces are forced to fuse, strong pushing forces are required, and, in the course of several approaching/separation cycles, the bilayers expose more and more hydrophobic groups. Therefore, as the number of contacts increase, the pushing force required for fusion decreases and the adhesion energy increases. In that case, the integrity of the bilayers is therefore not fully restored after each approaching/separation cycle, and the fusion process is not reversible.

Hydrogen bonds thus seem to be responsible for the unusual reversible fusion phenomenon observed between two monolayers of nucleoside or NTA lipids. When these monolayers adhere, hydrogen bonds are formed between lipid headgroups at the expense of hydrogen bonds with water molecules, which are broken. Since the water molecules that were hydrating the polar heads are removed and since the hydrogen-bonding groups are involved in the pairing, the effective size of the lipids hydrophilic part is substantially reduced, and the lipid complexes formed in the contact zone display a global hydrophobic character. The matter between the two surfaces thus behaves like an ordinary viscous liquid which flows out of the contact area under pressure. Since lipid molecules are made of aliphatic chains, the subsequent liquid can be assimilated to oil. This process is similar to what was theoretically described by Safran *et al.* [6]. Because of the dehydration of the polar heads, there must be a slight depletion of functionalized lipids between the surfaces in order to equilibrate the chemical potentials inside and outside of the contact zone. Then, hydrophobic attraction between newly exposed aliphatic chains can enhance the interaction between hydrogen-bonding lipids and therefore the formation of lipid complexes.

During the separation of the surfaces, the fluid character of this oil-like structure helps the gathering of lipid complexes in the contact region and the reformation of compact lipid monolayers: the resulting monolayers are intact, and the phenomenon is reversible [Fig. 1(b)]. In contrast, nonfunctionalized lipids, whose fusion has been forced, do not form a cohesive structure in the space between the two surfaces. In that case, only a fraction of the lipid molecules flows back toward the contact area during the separation process: the resulting monolayers are depleted, and the phenomenon is irreversible.

In order to validate this description, we have conducted experiments in which quasiconstant small pushing forces were applied to hydrogen-bonding monolayers. The decrease in distance as a function of time can then be predicted by Darcy's law, which describes the flow of viscous liquids under stresses. The oil does not oppose any resistance to the decrease in distance. Therefore, in the present configuration, the experiment can be schematized by two disks (representing the flattened part of each monolayer) separated by oil, on which a constant normal pushing force F is applied. The subsequent decrease in separation distance follows the relation

$$Ft = \frac{3\pi}{8}\eta\left(\frac{D_{\max}^4}{D^4} - 1\right)\frac{R^4}{D_{\max}^2}, \tag{1}$$

where η is the viscosity of the oil, $D_{\max}$ is the separation distance at $t = 0$, and R is the radius of the disks. This model can only be applied to the approaching parts of the force *versus* distance profiles since the oil has to return to the contact area when a pulling force is applied.

Our system accurately follows Darcy's law (Fig. 2), which proves that the two bound monolayers behave like an ordinary liquid. The apparent viscosity as deduced from Eq. (1) is not perfectly reproducible from one experiment to the other. We attribute this variation to few water molecules that can be trapped in the contact area. Nevertheless, we did not observe any correlation between the viscosity

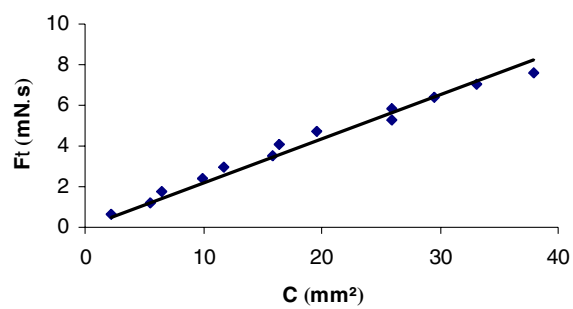

FIG. 2 (color online). SFA experiment performed at quasiconstant force F (22 ± 7 μN in this case). Ft is plotted as a function of the parameter $C = \frac{3\pi}{8}(\frac{D_{\max}^4}{D^4} - 1)\frac{R^4}{D_{\max}^2}$, which represents the decrease in distance. R was taken equal to 3 μm. The data points correspond to the compression phase of a T/T experiment. The linear dependence indicates that the system follows accurately Darcy's law. The slope equals the viscosity η of the fluid between the two surfaces (of the order of 1000 Pa s), which is in good agreement with viscosity values of similar oils.

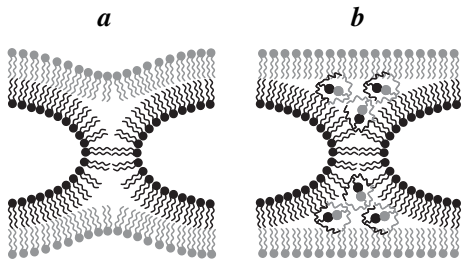

FIG. 3. (a) The current stalk model of membrane fusion intermediate assumes that the amphiphilicity of the interacting bilayers is conserved, which leads to the existence of hydrophobic voids. (b) Oily complexes such as the ones observed here between hydrogen-bonding lipids could locally fill these voids.

and the applied force F. The viscosity always remained between 200 and 2000 Pa s, which is similar to viscosities of common oils. This result, along with the behavior following closely the prediction of Darcy's law, therefore validates our model.

The unusual transition from amphiphilic to oil-like structures observed here between hydrogen-bonding lipid layers opens a new angle for understanding membrane fusion. In the current models, membrane hemifusion and fusion proceed through the formation of intermediate nonbilayer structures called stalks. Such structures, which assume that the amphiphilic character of the fusing bilayers is always preserved, lead to some discrepancies in the geometry of the lipid assembly (strong local curvatures and presence of voids), and therefore to some unrealistically large energies of the stalk intermediates. Recent theoretical works tried to solve this problem by calculating a new shape of the stalk, involving compression of monolayers thickness and tilting of lipids [11–13]. However, the local disruption of bilayer structures, such as the one observed here between two hydrogen-bonding layers, has never been considered. We anticipate that such nonbilayer structures could also be obtained from other types of molecular interactions, and could locally fill the troubling hydrophobic voids (Fig. 3). The formation of oily domains within lipid membranes has also been observed after the conversion of phospholipids into diacylglycerols (DAG) through phospholipase C activity [25]. DAG molecules, which can reach significant local concentrations in biological membranes, could therefore induce similar nonbilayer hydrophobic structures.

Hydrogen-bonding lipid layers, which turn into oil when they come into contact, could also be of great interest for the development of nanolubricants presenting unique tribological properties. Direct applications will be found, for instance, in the lubrication of microelectromechanical systems using mixtures of bound and mobile phases [26].

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