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Bilayers of neutral lipids bear a small but significant charge

F. Pincet[1,a], S. Cribier[2], and E. Perez[1]

[1] Laboratoire de Physique Statistique de l'École Normale Supérieure[b], 24 rue Lhomond, 75231 Paris Cedex 05, France
[2] Laboratoire de Physico-Chimie Moléculaire des Membranes Biologiques, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

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Abstract. Many experiments done on neutral lipid bilayers in pure water show weak repulsions. These weak forces prevent vesicles from adhering and are generally overcome by adding some salt in the aqueous medium. They also appear as stray repulsions in surface forces measurements made on lipid bilayers. Using a surface forces apparatus in pure water and in salt solution, we have measured the forces between two stearoyl-oleoyl-phosphatidyl-choline (SOPC) bilayers and between two dimiristoyl-phosphatidyl-ethanolamine (DMPE) bilayers. The results show that the repulsions are due to a small amount of negative charges coming from impurities in SOPC. This was quantitatively confirmed by electrophoretic measurements. There are 3 times less charges in the case of DMPE layers. The effect of these charges which is negligible at high salt concentration may significantly affect the adhesion energy and behaviour of neutral lipid bilayers between 0 and ≈ 40 mM salt.

PACS. 87.15.Kg Molecular interactions; membrane-protein interactions – 87.15.By Structure and bonding

1 Introduction

The properties of lipid bilayers have been the focus of much research for many decades, as self-assembled molecular systems and as model membranes. Most of the various components of the bilayer interactions and adhesion are now fairly well understood [1,2], as are the bilayer elastic [3] and phase [4] properties, owing to the increasing number of techniques which enable to perform measurements at the molecular scale. More recently, there has been a renewed interest in vesicles, to which biologically active molecules can be attached, and whose adhesion can be obtained from contact angle measurements on micromanipulated vesicles [5]. However, in spite of the numerous studies on vesicle adhesion [5,6], there are still unresolved issues. Even though phosphatidylcholine (PC) is neutral, some salt (≈ 25 mM) is always necessary to make PC giant unilamellar vesicles (GUV) adhere, while large unilamellar vesicles (LUV) adhere on GUV without salt [7]. Surface forces apparatus (SFA) measurements between PC bilayers in the presence of monovalent salt often display double-layer forces [8] although, to our knowledge, no monovalent ion adsorption has been reported on PC bilayers. During SFA measurements with neutral lipid bilayers in pure water, the calibrations of the translations of the apparatus vary as a function of the lipid, as if occasionally some small repulsion were perturbing the measurements. For instance, with dioleoyl-phosphatidylcholine (DOPC) bilayers [9], the calibration related to the force measurement of the SFA (see experimental section) is always a few percents below the calibration obtained during experiments involving nucleoside functionalised lipids [10] while both types of lipids are uncharged. The purpose of this paper is to shed light on these questions because small double-layer forces may significantly affect the adhesion between vesicles inasmuch as the interbilayer distance during adhesion is of the nanometer scale. Given the methods of production of lipids, a very weak percentage of charged lipid impurities may be present in any lipid sample which is in general chromatographically tested. We have investigated this point by measuring interbilayer forces *versus* distance with an SFA [11] in pure water and in salt solution. As this technique in its usual setup is chiefly sensitive to forces that vary rapidly with distance while double-layer forces in pure water vary very slowly with distance, we had to carefully design the experiments to overcome this difficulty. To measure these forces, SOPC bilayers were deposited on mica. However, to be sure that the mica itself does not provide any electrostatic charge when coated by a lipid layer, a control experiment was necessary. DMPE was tentatively used for this control experiment. If the forces between mica surfaces coated by a DMPE bilayer reveal an uncharged surface, then the outer monolayer of DMPE can be removed and replaced by an SOPC monolayer (Fig. 1) in order to perform the

[a] e-mail: pincet@physique.ens.fr
[b] Associé au CNRS et aux Universités Paris VI et Paris VII

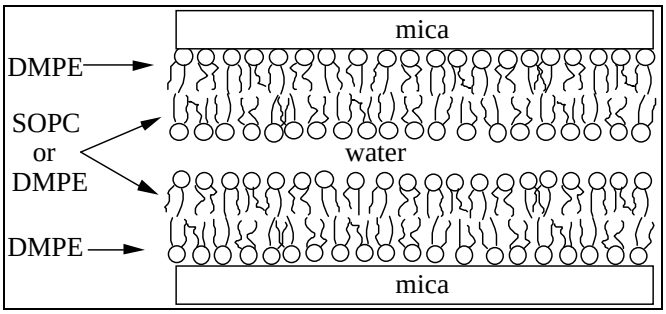

Fig. 1. Configuration of the lipid layers for forces measurements. The inner monolayers are always DMPE, and the outer layers are either SOPC or DMPE.

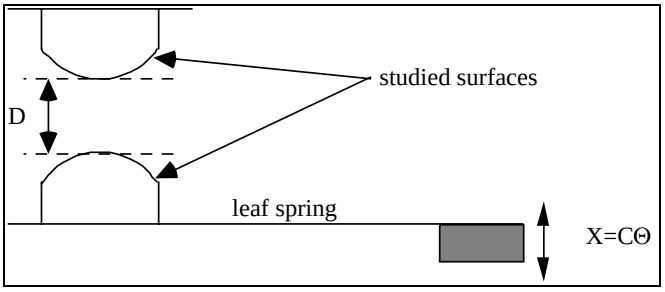

Fig. 2. Schematic view of the surface force apparatus. θ is the rotation angle of the micrometric screw that controls X.

same measurements on these new surfaces and ascribe any possible charges to the SOPC monolayer. This is what we have done in a series of independent experiments.

2 Experimental

The SFA has been described in detail in many papers [11] and exists in several versions. It will not be described here except for the part which is essential to the present measurements. The SFA enables to measure the force (± 0.1 μN) between two surfaces as a function of distance (± 10 pm) measured by multiple beam interferometry. One of the surfaces is fixed at the end of a leaf spring which is used to measure the force. The base of this leaf spring can be moved by a distance X by means of a differential spring system. This system is made up of two springs having a stiffness ratio of 1 000, so that when a micrometric screw bends the supple spring by 1 μm, the stiff spring is bent by 1 nm and therefore the base of the leaf spring is moved by the same distance [11]. The rotation angle of this micrometric screw is called θ, and X is proportional to θ (Fig. 2). In the absence of forces between the two surfaces, the leaf spring does not bend and the displacement ΔX of its basis is equal to the displacement ΔD of its end measured interferometrically: $\Delta X/\Delta\theta = \Delta D/\Delta\theta = C$ where C is the calibration of the translation. If there is a force between the surfaces, the leaf spring undergoes a flexion to balance the force and we have: $\Delta X = \Delta D +$ flexion and $C = \Delta X/\Delta\theta$ is therefore different from $\Delta D/\Delta\theta$. In the SFA, $\Delta X/\Delta\theta$ is not measured directly. ΔD is measured by the change of the intersurface distance; C is usually obtained by measuring ΔD and $\Delta\theta$ at a distance at which the surface force is zero, generally in the range 100 to 200 nm for bilayer experiments, and for which the equality $C = \Delta D/\Delta\theta$ is valid. However, the forces between two weakly charged surfaces at zero salt concentration are very long-ranged (> 100 nm), vary slowly with distance and are difficult to detect and measure. In this case, the usual way to obtain the calibration may give a wrong value of C. In order to circumvent this difficulty, we use the following experimental approach.

Table 1. These values are averages over a large number of runs and experiments. C is the calibration of the translation.

	outer layer: DMPE inner layer: DMPE	outer layer: SOPC inner layer: DMPE
$(\Delta D/\Delta\theta)/C$ without salt	0.992	0.940
$(\Delta D/\Delta\theta)/C$ with 10^{-3} M KNO_3	1	1

With 10^{-3} M salt concentration, the double-layer vanishes beyond 50 nm [11,12], and therefore above this distance, the equality $C = \Delta D/\Delta\theta$ is valid. The same measurements are performed in pure water. If $\Delta D/\Delta\theta < C$, there is a weak double-layer repulsion indicative of surface charges. If $\Delta D/\Delta\theta = C$ there is no charge present on the surfaces. It is technically simpler to add salt after the experiments in pure water and to compare the results subsequently, which is how we proceed. The lipids are obtained from Avanti Polar Lipids, the salt is KNO_3, and the pH is ~ 5.5 (pure water). The lipid bilayers are deposited at a constant pressure of 38 mN/m.

3 Results

With a DMPE bilayer on the surfaces, the change in $\Delta D/\Delta\theta$ upon the addition of salt is very small (0.8%). With salt, the measured force remains within the experimental error. Consequently, no decay-length may be deduced from the data. Without salt, a very small force can be seen with a decay-length of 162 nm. This shows the quasi-absence, but not the total absence, of charges on the DMPE bilayer coated mica surfaces. When the outer monolayers are made of SOPC, the change is much larger (6%), indicating a significant double-layer repulsion (see Tab. 1). The decay-lengths of the exponential repulsions are equal to the Debye-length corresponding to the salt concentration (see Fig. 3). This is characteristic of a double-layer repulsion. After rinsing with pure water, the decay-length increases significantly. A reliable value of C can only be obtained with added salt which guarantees the absence of long-distance repulsion.

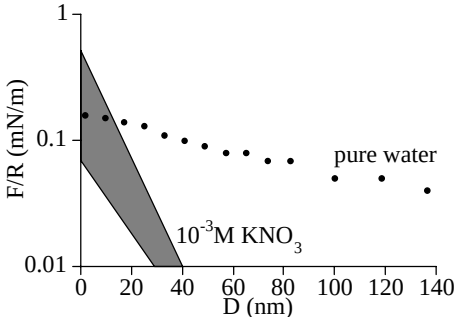

Fig. 3. Force measurements for SOPC (with DMPE as the inner monolayer). As F_0 cannot be experimentally determined in pure water, the value 0.03 mN/m has been chosen here for F_0/R. The force in 10^{-3} M KNO_3 is represented by the shaded area because it is very sensitive to the charge (the force is proportional to σ^2, it varies from one experiment to another).

4 Discussion

The control experiment establishes the absence of charges due to the mica. Those observed with SOPC can only come from the SOPC monolayer. The surface charge is obtained by using the linearised relationship between the double-layer interaction energy W of two surfaces at distance D, their charge σ and the Debye length κ^{-1} [13]:

$$W(D) = 2\sigma^2 \exp(-\kappa D)/\kappa\varepsilon. \quad (1)$$

W is related to the force F measured between curved surfaces by the Derjaguin approximation. In pure water, there is already an existing repulsion F_0 at the starting point of the force measurement (200 nm). F_0 is not measured. Consequently, the actual force is $F(D)+F_0$ where $F(D)$ is the experimental value of the force obtained by assuming that the force is zero at the onset of the measurements (200 nm).

Therefore:

$$W(D) = [F(D) + F_0]/2\pi R \quad (2)$$

where R is the radius of curvature of the surfaces. Three unknowns have to be determined: F_0, κ, σ.

In pure water, the SOPC force data is obtained by using the calibration determined in salt solution. It is fitted to an exponential for different values of F_0. Each fit provides a value for κ and σ. For a pH of 5.5, κ^{-1} is at most 170 nm and the actual F_0 value lies between 0 and the value at which $\kappa^{-1} = 170$ nm. As shown in Table 2, the value of σ (one charge per 850 SOPC molecules, accuracy: $\pm 15\%$) is not sensitive to this range of F_0 values which justifies the used procedure. The same procedure is followed in the case of DMPE to obtain the force in pure water in the range 0–200 nm. F_0 can be neglected because κ^{-1} is found equal to 162 nm when the raw data is directly fitted to an exponential (*cf.* Tab. 2). The charge of DMPE is three times smaller than that of SOPC.

In 10^{-3} M KNO_3, $F_0 = 0$. For the SOPC data, the decay-length of the force is 10 nm, as expected, considered the value of the Debye length, and σ is 5 times larger than without salt (one charge per 150 SOPC molecules). The origin of this increase remains unknown. In the case of DMPE the force is too small to be distinguished from the experimental error. Therefore any charge present in the bilayer is too weak to be observed. A rough estimate shows that it cannot be detected with this technique at this salt concentration if less than one charge per 500 DMPE molecules.

Table 2. Estimate of the surface charge σ from the exponential fit of the repulsion for different F_0 values. However, by varying F_0/R, for SOPC in pure water, σ varies by 15% which is the accuracy of this estimate.

	F_0/R (mN/m)	κ^{-1} (nm)	σ (charge per lipid)
SOPC pure water	0	54	1/700
	0.03	95	1/900
	0.1	177	1/1 000
SOPC in 10^{-3} M KNO_3	0	10	1/145
DMPE pure water	0	162	1/2 900

These results do not give any information on the sign of the charges, but a simple electrophoresis experiment can easily give the answer. A capillary tube (section 0.3×3 mm^2) is filled with a suspension of SOPC GUV. A tension is applied between both ends. The movement of the GUV observed through a microscope shows that the SOPC GUV bear a negative charge both in pure water and in 10^{-3} M KNO_3 in agreement with negative zeta potentials obtained on PC LUV [14,15]. From our measurements, we can estimate the charges of the vesicles. In electrolyte free solution, the mobility was $4 \times 10^{-8} \pm 1.5 \times 10^{-8}$ m^{2}s^{-1}V^{-1} giving c.a. one charge per 1 100 lipids. In 10^{-3} M KNO_3 solution, the mobility was $1.9 \times 10^{-8} \pm 10^{-8}$ m^{2}s^{-1}V^{-1} giving c.a. one charge per 125 lipids. It is interesting to note that Mac Laughlin *et al.* [16] obtained a small negative charge with egg PC vesicles in 0.1 M NaCl (giving c.a. one charge per 600 lipids) that they include in their error bar. It therefore seems that this small charge phenomenon is general to phosphatidylcholine lipids. The zwitterionic headgroup of PC comprises a phosphate group (ionisation pK = 2) and a choline group which is always positively charged. At the experimental pH, pure SOPC naturally bears a very small positive charge. As in our experiments, the overall charge of SOPC bilayers is negative, it must come from impurities.

How can one reconciliate the non-adhesion of GUV with a LUV-GUV adhesion [7] at zero salt concentration? One possible explanation is that LUV membrane is under tension while GUV membrane is tensionless. This implies a reduction in the repulsive undulation forces [17] that may generate adhesion [18,19]. Simple diffusion considerations can also explain the GUV-GUV and LUV-GUV discrepancy. The typical Brownian motion speed of LUV scales to 1 μm/s and the diffusion coefficient of an

amphiphilic impurity in an SOPC bilayer is 1 $\mu m^2/s$. As the size of LUV is typically 0.1 μm, it is easy to show that the charges in the GUV membrane have the time to diffuse away from the coming LUV, decreasing therefore substantially the double-layer repulsion.

5 Conclusion

There are always residual negative charges in SOPC coming from impurities ($\approx 0.1\%$) in contrast to DMPE which hardly bears any charge. These charges can be neglected in experiments involving purposely charged vesicles. It is worthwhile noting that 0.1% impurities is undetectable by thin layer chromatography which is the usual purity test for lipids. The adhesion energy of these weakly charged vesicles results mainly from van der Waals and double-layer forces. It increases with salt concentration. These charges explain why salt is necessary to make GUVs adhere. They can also explain the stray repulsion observed in SFA experiments with neutral lipids in pure water.

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