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Adhesion of lipid membranes induced by $CrCl_3$

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Abstract. – We describe the apparently first reversible unbinding transition of fluid phosphatidylcholine bilayers. It is induced by minute concentrations of $CrCl_3$ in aqueous 0.1 M NaCl solution. Mutual adhesion sets in at a threshold of 1.1 μM. The adhesion energy increases steeply with $CrCl_3$ concentration up to 20 $\mu J/m^2$. It continues to rise with a much smaller slope until the transition becomes irreversible at ca. 40 μM.

Introduction. – Fluid lipid bilayer membranes often serve as simple model systems of biological membranes. Nevertheless, they already possess interesting and complex physical properties. One of them is the reversible transition of membranes from a bound to an unbound state, well known as the unbinding transition. Two identical lipid bilayers in water interact by the attractive van der Waals force and the repulsive electrostatic, hydration and undulation forces. The control parameter of an unbinding transition could be, *e.g.*, temperature or the Hamaker constant. A second-order unbinding transition was predicted several years ago [1]. However, experimental observation is quite rare and the order of the transition is difficult to determine. An unbinding transition was found for the first time in a phase contrast light microscopy study on digalactosyldiacylglycerol (DGDG) membranes [2]. Visibly separate bilayers went into spontaneous adhesion when the temperature was lowered and separated without apparent hysteresis when it was raised again.

Irreversible unbinding of fluid membranes has been found in the case of phosphatidylcholine (PC) bilayers, which in general are regarded as electrically neutral. They peel off, single or perhaps in bunches, from the multilayer system in excess water when upon water uptake its repeat distance has reached a characteristic value in the range of 65 Å. The first experiments demonstrating that the so-called equilibrium period is not a stable state, in contrast to former ideas, were reported by Hartung *et al.* [3]. Studying prehydrated disordered multilayer systems of palmitoyloleoyl PC (POPC), they found the X-ray diffraction signal of the fixed repeat distance in most samples to decrease and vanish within hours or days. In parallel optical studies various water-rich structures, including single bilayers, were seen to form along the borders of the decaying multilayer systems. Employing dioleoyl PC (DOPC), Thimmel *et al.* [4] did similar experiments in a more controlled way ensuring disintegration of the multilayer system

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and allowing X-ray and optical studies on the same sample. They distinguished two stages of swelling, increase of the period to a final value and peeling off, which sometimes overlap. Also, they measured an increase of the speed of disintegration with temperature. Vogel *et al.* [5] in initially independent work examined very highly ordered POPC and dimyristoyl PC (DMPC) multilayer systems by X-ray diffraction. When heated, the systems disintegrated rather abruptly at 80 °C and 95 °C, respectively. However, later on POPC multilayer systems were found to disintegrate at any chosen temperature (down to 14.8 °C), though very slowly at the lower values [6]. Pabst *et al.* [7] studied both planar and vesicular POPC multilayer systems in D_2O by means of neutron scattering. Their experiments confirm that unbinding of these multilayer systems occurs via irreversible disintegration processes whose speed depends on temperature and experimental circumstances. Obviously, in all those studies the multilayer systems represented a metastable state of the fluid bilayers. The reversible unbinding transition found by Pozo-Navas *et al.* [8] in multilayer systems of a mixed lipid coincides with the melting ($L_\beta \rightarrow L_\alpha$) transition of the two-dimensional lattice. In the following we describe the apparently first reversible unbinding transition of fluid PC bilayers. It is induced by the triply charged chromium ions of $CrCl_3$ dissolved in aqueous 0.1 M NaCl solution.

Experiments. – POPC was purchased from Avanti Polar Lipids (Alabaster, Alabama) and used without further purification. Vesicle preparation [9], micromanipulation and pressure adjustment [10–13] were done as described in the literature. Electroformation began with depositing ca. 1 μg of lipid in chloroform onto an ITO-coated glass slide serving as an electrode. Subsequently, the sample was dried under vacuum overnight to remove traces of the organic solvent. An identical slide was mounted on top to build a swelling chamber resembling a plate capacitor. The chamber was filled with ca. 300 μl of aqueous 180 mOsm sucrose solution and a voltage of 1 V/mm and 10 Hz was applied between the 2 mm distant slides. After about 5 h of incubation giant vesicles were formed. For changing into the experimental chamber, selected vesicles were aspirated by a micropipette and shielded by a transfer pipette. Both chambers were mounted on the stage of a light microscope with phase and differential interference contrast (DIC). The sucrose solution in the swelling chamber was slightly hypotonic, its osmolarity being approximately 5 mOsm less than that of the 0.1 M NaCl solution in the experimental chamber, so that the vesicle membranes relax after transfer.

In a typical adhesion experiment two vesicles were aspirated with micropipettes. One of them (substrate vesicle) was held under high tension (1 mN/m) and therefore maintained its spherical shape during the whole experiment. The vesicles were maneuvered into close proximity and the second vesicle (test vesicle) was allowed to adhere to the substrate vesicle. A typical experimental situation is illustrated in the DIC micrograph of fig. 1 together with parameters describing the geometry of the experiment. Note that raising the suction pressure P in the pipette of the test vesicle increases the penetration of the membrane meniscus into the pipette and decreases the area of adhesion.

Analysis of the experiment and calculation of the energy density of adhesion γ were carried out with two independent methods. The average spacing of the ions on the bilayer is different inside and outside of the contact area (see below). First, a balance of energy is considered. The work done by the micropipette is compensated by a change in adhesion energy $\gamma \Delta A_c$ so that

$$\gamma = -\pi R_p^2 \frac{\Delta L_p}{\Delta A_c} \Delta P, \tag{1}$$

where R_p denotes the inner radius of the pipette, ΔL_p and ΔA_c the shift of the meniscus in the pipette and the change of area of adhesion, respectively. ΔP is the suction pressure difference between two subsequent configurations with different L_p and A_c. Second, a balance

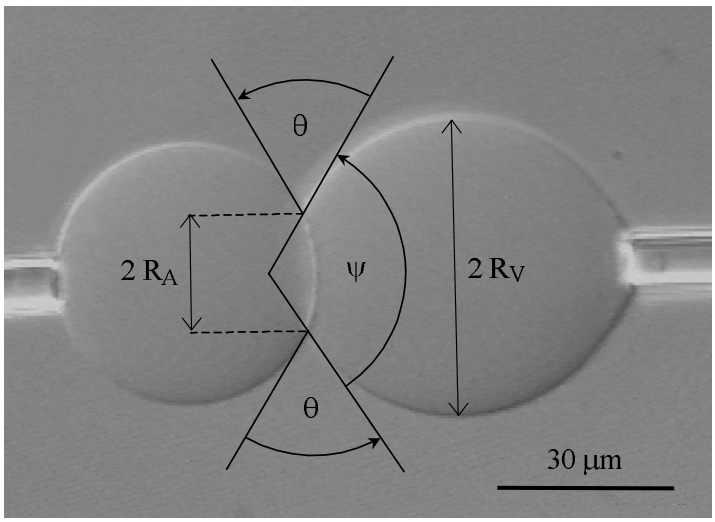

Fig. 1 – Differential interference contrast micrograph of the adhesion test. Note the membrane meniscus of the test vesicle inside the pipette on the right ($\Delta P \approx 10\,\mathrm{Pa}$).

of stresses is utilized, the well-known Young-Dupré equation containing contact angle θ and membrane tension σ,

$$\gamma = \sigma\,(1 - \cos\theta)\,. \tag{2}$$

In each experiment the $CrCl_3$ concentration was fixed. Different suction pressures were applied to the test vesicle and the respective geometric parameters were measured. In fig. 2

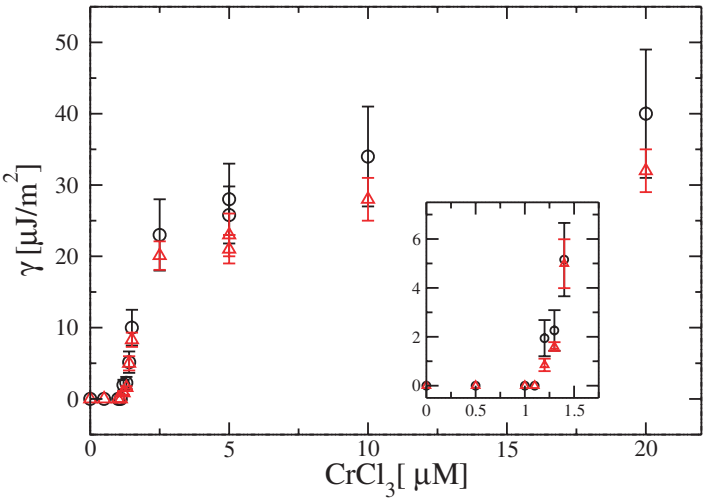

Fig. 2 – The dependence of the energy density of adhesion on the $CrCl_3$ concentration as determined by energy (○) and stress balances (△). The sodium concentration was 100 mM in all experiments. The inset is a closeup of the vicinity of the transition. Each data point represents a measurement with one pair of vesicles except for the points at 1.2 and 1.3 μM (two pairs) and 0 μM (three pairs). The γ values for the circles were calculated by averaging all values obtained from eq. (1) and are shown with the corresponding errors. In both types of measurement there was no significant dependence of γ on σ.

the evaluated energy densities of adhesion obtained from both methods of analysis are shown. The membrane tension σ in (2) is related to the suction pressure by the extended Laplace equation $\sigma = (2/R_\mathrm{p} - J)^{-1}\,\Delta P$, where J is the sum of the principal curvatures of the test vesicle and was determined from the geometric relation $J = (R_\mathrm{V} - R_\mathrm{A}\cos\psi/2)\left(R_\mathrm{V}^2 - R_\mathrm{A}^2\right)^{-1}$. Both ways of determination are in reasonable agreement and indicate that adhesion sets in between concentrations of $1.1\,\mu$M and $1.2\,\mu$M $CrCl_3$. At lower concentrations we could not find adhesion, not even when pressing the vesicles against each other for minutes. At $CrCl_3$ concentrations higher than $\approx 40\,\mu$M the experiment was no longer reversible, *i.e.* area of adhesion and penetration length of the meniscus in the pipette did not react reproducibly when the suction pressure was decreased or increased. Note that due to the small number of lipid molecules relative to the chromium ions in the chamber, the concentration of the latter in bulk solution and hence their chemical potential may be regarded as constant in an experiment. Even at the adhesion threshold the total number of lipid molecules in the sample cell was smaller by roughly an order of magnitude than the number of Cr ions.

In additional experiments we locally injected a small volume of a fairly concentrated $CrCl_3$ solution into a swollen vesicle suspension in the swelling chamber, thus inducing mutual adhesion of adjacent vesicles near the region of injection (experiments not shown). If the averaged $CrCl_3$ concentration in the sample was clearly below $1\,\mu$M, adhesion vanished in the course of hours. This was another sign of a reversible unbinding transition in the μM range, this time in sucrose solution.

Discussion and conclusions. – The plot of adhesion energy γ *vs.* $CrCl_3$ concentration allows no definitive decision on the order of the unbinding transition. More data points and less scatter would be required for this purpose. However, it seems that γ starts with a finite slope rather than a parabolic dependence, which speaks for a first-order transition. Another perhaps more convincing argument for a first-order transition is provided by the frequently observed metastability of PC multilayer systems in excess water [3–5, 7]. It should be noted that the lateral tensions in the experiments are powers of ten above the range of so-called weak adhesion of vesicles [14,15]. A large electrostatic energy barrier just outside the potential well responsible for binding would cause first-order membrane unbinding [16]. Such a barrier could in principle be produced by the Cr^{3+} ions bound to the membrane surfaces, but this is unlikely because of the small Debye length of 1 nm in 100 mM NaCl solution which is much less than the estimated distance between the bound Cr ions. Another relatively simple model that leads to discontinuous unbinding has been described in the context of membrane adhesion by local stickers [17]. They may aggregate if they increase locally the bending rigidity of the membrane. This can result in discontinuous unbinding as the bulk concentration of the stickers and, thus, their chemical potential is being varied. In the present context the stickers are provided by the trivalent ions which adsorb onto the membrane.

Let us propose a different model of first-order unbinding which seems to explain as well other anomalies of PC bilayers. It makes use of non-Hookean bending elasticity, *i.e.* energy terms of higher than quadratic order in the principal curvatures. The bending energy per unit area, g, is restricted in all those cases to a reduced set of terms, taking in general the form

$$g = \frac{1}{2}\kappa J^2 + \bar{\kappa}K + \bar{\kappa}_2 K^2 + \kappa'\left(\nabla J\right)^2 + \bar{\kappa}_4 K^4. \tag{3}$$

The complete set of moduli up to fourth order has been given by Mitov [18]. In (3), $J = c_1 + c_2$ is again the sum of principal curvatures c_1 and c_2, $K = c_1 c_2$ is the Gaussian curvature, while the κ's are elastic moduli. The first two terms of (3) represent Hookean elasticity, κ being the bending rigidity and $\bar{\kappa}$ the modulus of Gaussian curvature. The $\bar{\kappa}$ term drops

out in many calculations because its integral over surface area depends only on membrane topology. The next two terms are of fourth order. Model calculations for PC bilayers yielded $\bar{\kappa}_2 = -1 \cdot 10^{-36}\,\mathrm{Jm}^2$ [19,20] and $\kappa' = 2 \cdot 10^{-38}\,\mathrm{Jm}^2$ [21]. The last term is of eighth order. Its size can be guessed from dimensional analysis to be about $1 \cdot 10^{-71}\,\mathrm{Jm}^6$ [20], while its sign is taken to be positive to restrain the curvatures. The third term of (3) is crucial; it destabilizes the flat membrane when $\bar{\kappa}_2$ is sufficiently negative. The only modulus measured to date is the bending rigidity. Since it varies strongly from method to method, it was measured again for POPC by vesicle aspiration as part of the present work; the result is $(5.2 \pm 1.6) \cdot 10^{-20}\,\mathrm{J}$ [22]. The two quartic moduli occurring in (3) are easier to derive from simple bilayer models than those omitted. Fortunately, the $\bar{\kappa}_2$ term may be expected to dominate because the main effect of saddle curvature is a shrinkage of the lipid/water interfaces of high interfacial tension, combined with a rather mild and uniform deformation of the hydrocarbon chain regions. The reduced bending energy (3) has been used to qualitatively explain an optically unresolvable roughness [23], a graininess [24] and a fragmented state [25], all found with PC membranes. Also, bilayer peeling was attributed to non-Hookean bending, either to a superstructure that exists only in the free state [3] or to a decrease of the effective bending rigidity when the membrane unbinds [4]. Monte Carlo simulations starting from the flat bilayer produced a tetragonal structure resembling an egg carton [20,26] and, as its "melt", a graininess with some orientational and positional short-range order [26]. In the most thoroughly studied example of graininess the moduli were $\kappa = 5 \cdot 10^{-20}\,\mathrm{J}$, $\bar{\kappa}_2 = -0.9 \cdot 10^{-36}\,\mathrm{Jm}^2$, $\kappa' = 1 \cdot 10^{-38}\,\mathrm{Jm}^2$ and $\bar{\kappa}_4 = 0.9 \cdot 10^{-72}\,\mathrm{Jm}^6$, while the temperature was $T = 470\,\mathrm{K}$. The simulated pattern closely resembles the occasional graininess found in electron microscopy of DOPC vesicles frozen from room temperature [24]. The theoretical result can be adjusted from 470 K to ca. 300 K by multiplying all the moduli by 2/3.

Encouraged by the results of simulation, we now apply (3) to the problem of reversible first-order unbinding. We expect the single bilayer not to take on a superstructure but to undergo enhanced, non-Hookean bending fluctuations. The mean-curvature fluctuations are restrained all over the membrane in the bound states where the bilayer is linked to the substrate by Cr^{3+} ions. Similar restraints exist in any state in which the fluctuating membrane feels the substrate. Discontinuous (un)binding is possible because non-Hookean elasticity can produce a barrier in the free energy of the fluctuating bilayer when it is made to approach the substrate at a given c. This is related to the increase of the effective bending rigidity with decreasing fluctuation amplitude, to be considered next.

For convenience, the bending fluctuations are described in terms of the hat model [19,26]. Each hat consists of a spherical cap of molecular area $A_0 \approx 1\,\mathrm{nm}^2$ and a brim of zero J. The hats fluctuate independently of each other in the absence of non-Hookean terms. With a purely harmonic bending potential the equipartition theorem leads to

$$\langle J^2 \rangle = \frac{k_\mathrm{B} T}{\kappa A_0} \tag{4}$$

for each spherical cap and thus the membrane as a whole. In the nonharmonic case, eq. (4) gives the effective bending rigidity κ_{eff} instead of the "bare" value κ as a function of $\langle J^2 \rangle$. However, the hat model is now only an approximation because the hats are (weakly) coupled by the non-Hookean terms. Neglecting this and dropping the (relatively small) gradient term in (3), we may write for a single cap

$$g = \frac{1}{2}\kappa \left(\frac{2}{R}\right)^2 + \bar{\kappa}_2 \left(\frac{1}{R}\right)^4 + \bar{\kappa}_4 \left(\frac{1}{R}\right)^8, \tag{5}$$

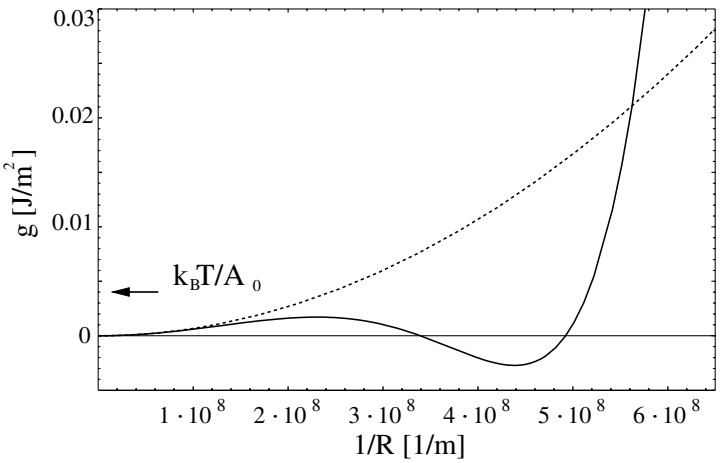

Fig. 3 – Plot of g for $\kappa = (2/3) \cdot 5 \cdot 10^{-20}\,\mathrm{J}$, $\bar{\kappa}_2 = (2/3) \cdot 10^{-36}\,\mathrm{Jm}^2$, $\bar{\kappa}_4 = (2/3) \cdot 10^{-71}\,\mathrm{Jm}^6$ and $\kappa' = 0$. Hookean elasticity alone is represented by the dashed curve. See main text for $k_\mathrm{B}T/A_0$ and the factor 2/3.

where R is the radius of cap curvature. To deal with the present situation we substitute for the moduli the values listed above, but multiplied by 2/3. The resulting $g(1/R)$ is plotted in fig. 3 together with the harmonic part of the bending energy and the divisor $k_\mathrm{B}T/A_0$ of g in the exponent of the Boltzmann factors of the partition function. Inspection of the figure indicates that both the internal energy and the entropic part of the free energy of bending should diminish in changing from the harmonic to the nonharmonic case. We estimate the total decrease of the free energy of bending caused by the non-Hookean terms to be on the order of $-k_\mathrm{B}T/A_0 \approx -4 \cdot 10^{-3}\,\mathrm{Jm}^{-2}$. Figure 3 contains the ingredients, except for a compressive force, to derive in complicated calculations a plot of the free energy of the membrane as a function of its mean distance from the substrate. In such a plot the free energy increases as the distance decreases, traverses an unstable interval of opposite slope and then rises again.

There need be no contradiction in taking the same values of the elastic moduli to simulate graininess and to treat strongly enhanced bending fluctuations. An energy barrier delaying the transition to a superstructure may be inferred from the coexistence of vesicles with grainy and smooth membranes in the same sample [24] and was also noticed in Monte Carlo simulation [26].

Finally, we try to estimate the fraction of phospholipid molecules covered by Cr ions, distinguishing between ions adsorbed to a single, ideally separate bilayer and ions linking two bilayers. These coverages are denoted by X and X_link, respectively, and not directly related when both are $\ll 1$. To obtain an approximate value for X, the binding constant $K_\mathrm{eq} \approx 10^3\,\mathrm{M}^{-1}$, as measured for Gd^{3+} ions facing a PC bilayer [27], and the threshold value $c_\mathrm{th} \approx 1\,\mu\mathrm{M}$ of the Cr concentration, are inserted into the relationship $X = cK_\mathrm{eq}$ which is a good approximation for $X \ll 1$. The resulting value of $X = 10^{-3}$ corresponds to a lateral mean distance of bound Cr ions on the membrane of ca. 30 nm. The steep rise of γ in fig. 2 may be used to roughly estimate X_link at the transition. Putting $\gamma(c) = (X_\mathrm{link}/A_0)\, k_\mathrm{B}T \ln(c/c_\mathrm{th})$ and expanding ln linearly at $c_\mathrm{th} = 1.1\,\mu\mathrm{M}$, one finds $X_\mathrm{link} \approx 10^{-2}$. Focusing on the dependence of the chemical potential of Cr^{3+} on c, expressed by $k_\mathrm{B}T \ln(c/c_\mathrm{th})$, this estimate disregards among other things the increase with c of X_link and, concomitantly, of steric repulsion. Peeling off from PC multilayer systems in pure water apparently starts from a more tightly bound membrane state.

Divalent ions such as Ca^{2+} are well known to give rise to mutual adhesion of bilayers containing negatively charged lipid. Akashi *et al.* found slightly contaminated PC bilayers to adhere to each other when the surface charge was neutralized by adsorbed Ca^{2+} [28]. They assume that this is an adhesion of pure PC bilayers. In view of the absence of adhesion in 0.1 M NaCl solution, the binding might instead be caused by Ca^{2+} linkages between negatively charged lipid molecules. In any event, divalent ions appear incapable of binding pure PC membranes, while trivalent Cr^{3+} ions were found to be highly effective.

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