Spontaneous Formation of Giant Phospholipid Vesicles

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The spontaneous formation of giant (diameter > 10 μm) vesicles from a number of phospholipids in excess aqueous solution has been studied by light-microscopy. Electrically neutral as well as charged phospholipids swell to form giant vesicles only if the lipids are in the fluid phase. This shows that electrostatic repulsion alone cannot explain the spontaneous formation of giant vesicles. The results confirm the suggestion that steric forces between extended membranes play a significant part in this process.

Introduction

In the last years there have been a number of papers describing swelling properties of several phospholipids in excess (⩾ 99%) water. Some of the authors observed the spontaneous formation of free membranes from a lump or crystal of the material with electrically neutral phospholipids, especially natural or synthetic phosphatidyl-cholines (lecithins) and phosphatidylethanolamines (cephalines) (see, *e.g.* [1–6]). In an early investigation Papahadjopoulos observed spontaneous vesicle formation also of other compounds [7]. He found that the acidic phospholipids only formed vesicles at a pH larger that the pK for the first ionization of the head group of the substance. All these experiments usually yielded a number of giant unilamellar vesicles (GUV) with dimensions from a few μm up to several hundred μm which can be readily examined by light microscopy. On the other hand results of X-ray experiments on dispersions of phospholipids in water suggested a limited swelling of neutral phospholipids [8] and an indefinite uptake of water only by electrically charged compounds [9].

The results have been discussed with respect to the different interactions between membranes, *i.e.* the competition of attractive van der Waals forces *vs.* repulsive hydration, electrostatic, and undulation forces (see *e.g.* [10–14]). Although no generally accepted theoretical framework exists today it has been stressed that undulation forces play at least a significant role for the interactions between fluid membranes.

In this report I will describe a number of simple experiments on the spontaneous vesicle formation of a number of less frequently studied phospholipids in excess water. Among the electrically neutral (at neutral pH) compounds I have studied sphingomyelin. The other phospholipids like phosphatidylserine, phosphatidic acid, phosphatidylglycerol, phosphatidylinositol, and cardiolipin have a negatively charged head group and thus experience electrostatic repulsion.

The results will be discussed with respect to the role of the thermal main transition temperature and of undulation forces. Chemically pure phospholipids have a well defined transition temperature at which the fatty acid chains melt and the substance enters a fluid crystalline phase. At this transition T_m undulation forces between extended membranes are "switched on" because the bending stiffness of the membranes is drastically reduced. (For discussion of the change of bending stiffness at T_M see ref. [15].)

Materials and Methods

All phospholipids used in this study were from Sigma (Munich, F.R.G.). As mixtures with natural composition of fatty acids I used sphingomyelin (SM) from egg-yolk (EYSM) or bovine brain (bbSM), bovine brain phosphatidyl-serine (bbPS), egg-yolk phosphatidic acid (EYPA), bovine heart cardiolipin (bhCL), bovine brain cardiolipin (bbCL), and phosphatidyl-inositol from soy beans (sbPI). Synthetic phospholipids were di-palmitoyl-phosphatidylserine (DPPS), di-myristoyl-phosphatidylglycerol (DMPG), and di-stearoyl-phosphatidylglycerol (DSPG). Most of the charged compounds were obtained as their sodium salt. Thin layer chromatograms of the sub-

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stances showed, respectively, a single spot in iodine vapor.

A small quantity of the material (some μg) was spread on an object slide. After adding a drop of about 50 μl aqueous solution the cover glass was applied and the small microscopic chamber was sealed with a glue against evaporation. As a solution I used twice distilled water containing 50 μM NaN_3 against bacterial contamination. The pH measured in the bulk solution was about 5,5 which means that in all experiments the phospholipid molecules, except SM, bear a negative charge, CL being doubly charged. I also performed experiments by buffering with 50 mM TRIS-HCl or sometimes 100 mM TRIS-HCl at pH 7. In some experiments 100 mM NaCl was used to shield electrostatic interaction by Debye screening. To test the possible influence of bivalent metal ions (especially Ca^{2+}) I have added the chelator EDTA (ethylene-diamintetraacetic-acid) as sodium salt in some experiments.

Electrophoretic experiments could be performed in a special microscopic chamber containing two parallel platinum electrodes.

The samples were studied with a phase-contrast microscope (Leitz) sometimes equipped with a video system (Grundig) and camera (Olympus). If necessary the sample was heated on the object stage of the microscope with the help of an externally circulating water bath (Haake) or an electrical heating stage (Sensortek). The temperature was calibrated with a small thermocouple in a special chamber [3]. Unilamellar vesicles could be distinguished from multilamellar ones easily with some experience by looking for those with lowest optical contrast. The results of the visual judgement agreed well with those of photometric measurements [16].

Results

Sphingomyelin

Both sphingomyelins behaved very similar to egg lecithin although the gel-fluid transition temperature was somewhat higher. For the studied natural mixtures room temperature is just at the beginning of the broad transition between 28 °C and 40 °C. If the sample was kept at room temperature (25 °C) only a few small vesicles detached from the crystal of SM within the first few hours. Sometimes indications of a phase separation were noticed. While at 25 °C most of the material in the same lump appeared to remain crystalline for several days, single half-spherical membranes with diameters up to 50 μm detached at the border of the crystal (Fig. 1).

However, if the phospholipid was incubated at 35 °C, *i.e.* in the middle of the broad transition range, a large number of uni- and paucilamellar vesicles of various forms appeared quickly (Fig. 2). Also prominent were (often spherical) dark bodies as they have been observed before with egg lecithin [17]. The contour of these structures sometimes exhibited undulations with amplitudes of some μm. In a few samples also sponges of passages, *i.e.* tunnel-like structures connecting neighbouring bilayers, were seen irrespectively of the addition of EDTA.

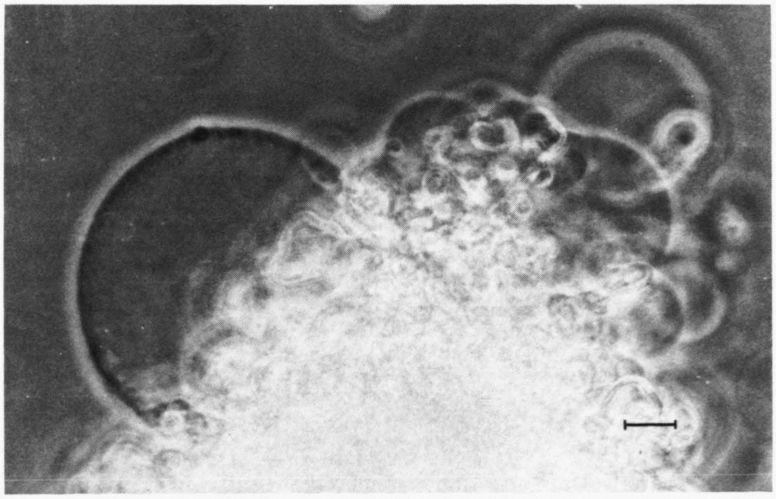

Fig. 1. Half-spherical membranes detaching from a SM crystal in water at about 25 °C. Bar indicates 10 μm.

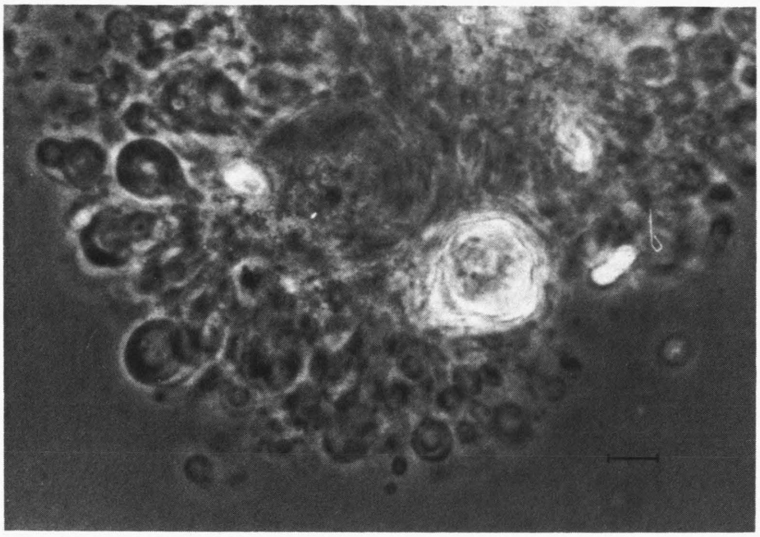

Fig. 2. At 35 °C a large number of vesicles swell from the SM crystals. Bar indicates 10 μm.

No movement of most vesicles could be seen in DC-fields of up to 7 V/cm which means that they were not charged.

Experiments with SM at elevated temperatures in solutions of larger ionic strength, 100 mM NaCl or 100 mM TRIS at pH 7 yielded very few vesicles. Mainly compact structures like thick walls containing many lamellas and myelin-like cylinders grew out of the crystal (Fig. 3).

Phosphatidylserine

If synthetic DPPS is incubated in water or excess buffer at room temperature the crystals might disperse but remain rigid without any sign of detachment of membranes at their rim (Fig. 4). The crystals obviously have imbibed water to a certain extent in the first few hours since they appeared slightly enlarged and the contour of them became smoother.

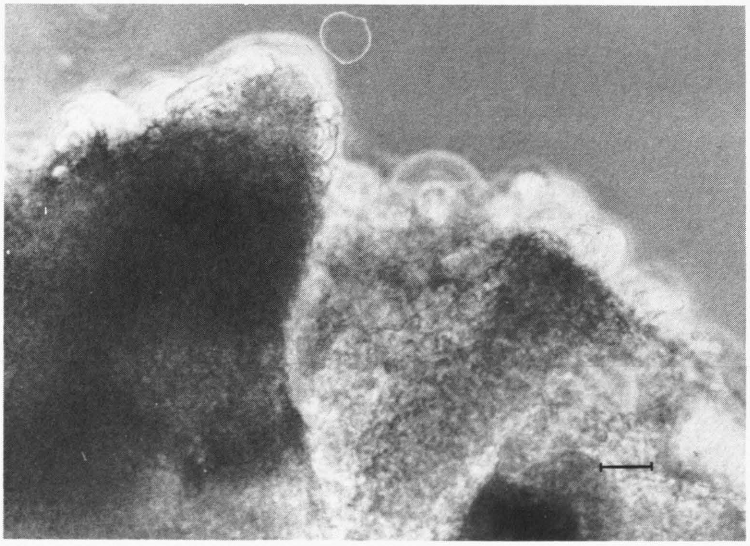

Fig. 3. In 100 mM NaCl the structures growing out of the crystal of SM are more compact. Bar indicates 10 μm.

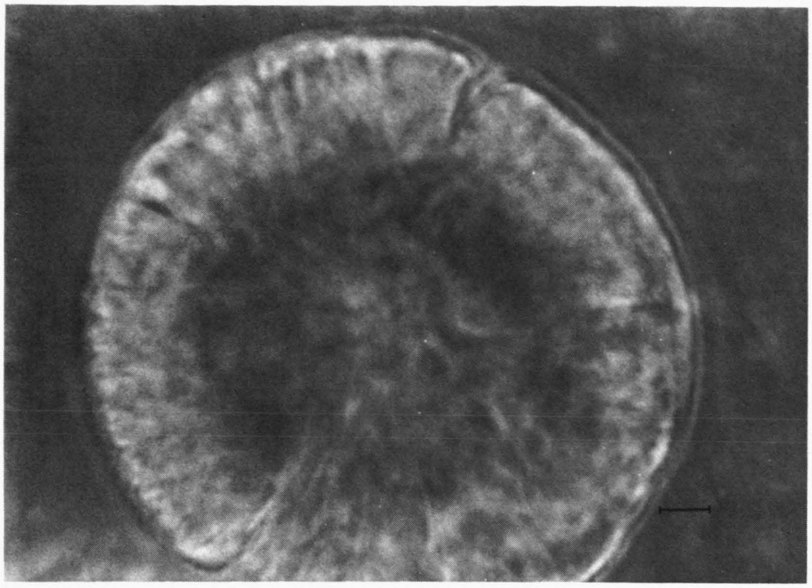

Fig. 4. Crystal of DPPS in TRIS-HCl (pH 7) at $T < T_m$. Bar indicates 10 μm.

The optical appearance of the interior of the crystals became more inhomogeneous. Since the crystals appeared as bright objects in phase contrast it followed that they were relatively densely packed with DPPS. Above $T_m = 51$ °C the crystals transformed into giant most often unilamellar membranes (Fig. 5). The membranes fluctuated with readily visible amplitudes. Upon lowering the temperature the membranes froze and the formerly smooth contours of vesicles became jagged. Collapsed structures returned to separated fluctuating membranes if the sample was heated again to $T > T_m$. The natural extract of phosphatidylserine from bovine brain readily formed fluctuating membranes in TRIS buffer (pH 7) at room temperature.

I noticed that in some samples where DPPS was

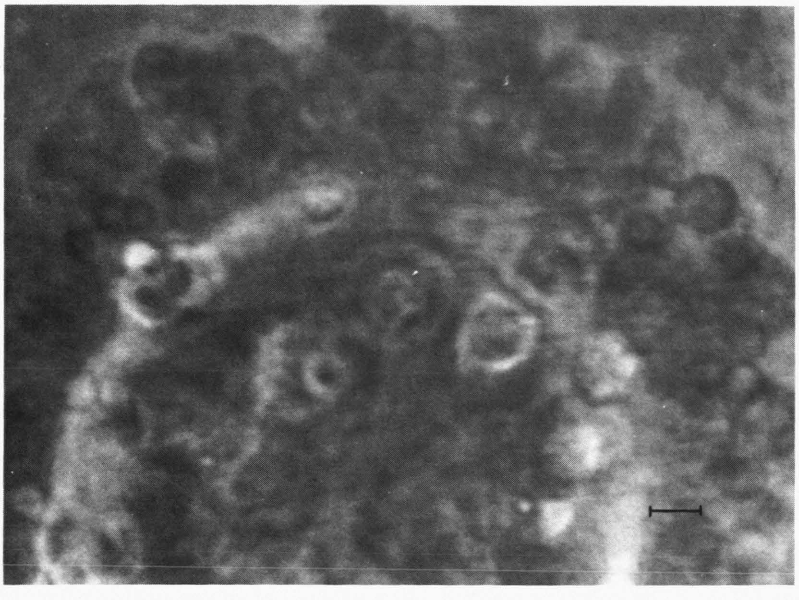

Fig. 5. Vesicles spontaneously grow out of the DPPS crystal if $T > T_m$. Bar indicates 10 μm.

immersed in water containing 0.5% EDTA after a few days vesicles with diameters up to 10 μM and smooth contours were found at temperatures a few degrees below T_m.

Phosphatidylglycerol

Phosphatidylglycerol crystals were a very hygroscopic powder which dispersed into a milky cloud very fast if water was added at room temperature. PG crystals seemed to be more hygroscopic than the lecithins or also PS crystals. As with DPPS the crystals changed their form to a smoother appearance which suggested that water was imbided below T_m.

At about 60 °C the crystals of DSPG were starting to swell into lipsomes and vesicles with unilamellar walls which exhibited strong fluctuations. This happened irrespectively of the special medium, *i.e.* no differences between water, 100 mM NaCl and TRIS at pH 7 have been detected. Upon lowering the temperature below 60 °C the membranes froze to rigid structures with an irregular appearance.

Basically the same behaviour was found with DMPG in 50 mM TRIS, only the characteristic temperature being now at about 23 °C. One striking feature was the appearance of large clouds of μm-sized vesicles after incubation above T_m for at least one day.

A somewhat strange effect with DSPG was that after one day of incubation I have observed crystals with melting temperatures above at least 65 °C.

Phosphatidylinositol

As in the case of PG at room temperature the uptake of water was very quick and the opaque crystal of phosphatidylinositol became milky. Within a few minutes small vesicles with diameters of a few μm as well as liposomal structures appeared at the border of the crystals. Myelin like fingers sometimes formed intertwined helical structures. Some of the 10 μm spheroids in front of the more compact crystal appeared grey in phase contrast and eventually an onion like structure of lamellas was resolved. A few unilamellar spherical vesicles and short tubes have also been found in the first hours. Judging by eye the fluctuations of their walls were not different from those of lecithin membranes. Again all these features were independent of the medium. However, in 100 mM NaCl all swelling structures were more compact, *i.e.* the extensions of free membrane parts were much smaller.

In most of the samples after a few days giant spheres with diameters up to 50 μm, most of them including smaller vesicles, were found. After one week the giant spheres were garnished with small μm-sized particles (Fig. 6).

In some samples I observed after three days tubular structures which were packed in close aggregates and also sponges of passages.

In TRIS buffered solutions during the first day in liposomal fingers water-rich regions segregated frome more closely packed lamellas (Fig. 7). The

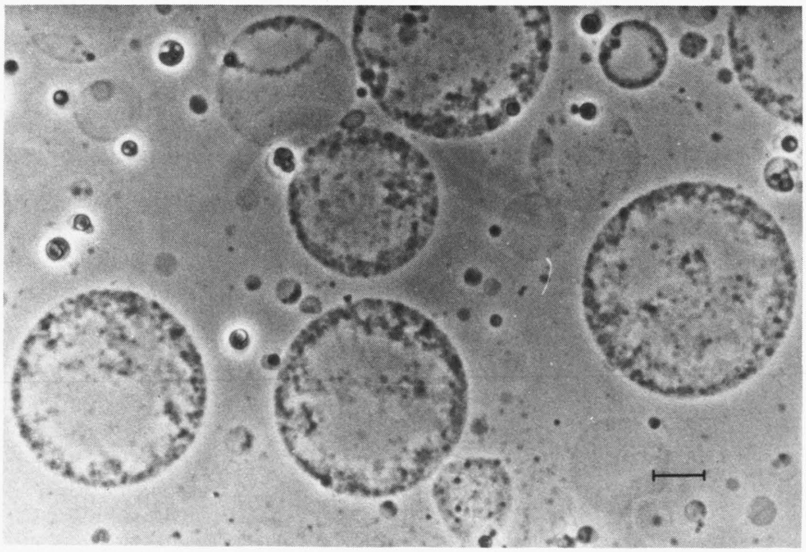
Fig. 6. PI vesicles are sometimes garnished with small vesicles. Bar indicates 10 μm.

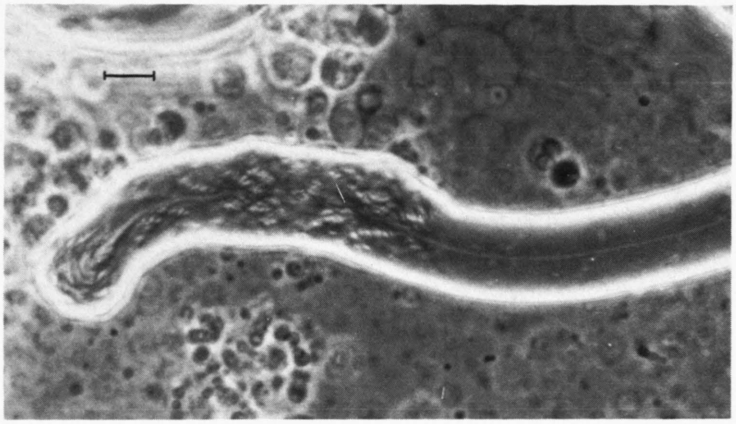

Fig. 7. Phase separation in liposomal fingers of PI. Bar indicates 10 μm.

water bags were separated by uni- or multilamellar sheets of PI. In extended regions membranes were seen adhering to each other. Raising the temperature to 60 °C was not sufficient to separate adhering membranes and the aggregates were drifting like rigid bodies through the sample.

Phosphatidic acid

Phosphatidic acid in water, TRIS buffer or NaCl at room temperature formed only μm-sized vesicles and liposomes which slowly segregated from the crystals. GUVs with diameters above 10 μm have been found very rarely. Sometimes thick liposomal fingers grew from the crystals into the bulk solution. In a few cases onion-like structures developed in their interior.

Cardiolipin

Most of the experiments were performed with cardiolipin from bovine heart (bhCL). At room temperature in unbuffered water the crystals of bhCL swelled into approximately spherical objects with smooth contours. In phase contrast the spheroids with diameters of a few dozen μm appeared homogeneously grey. Less prominent were μm-size vesicles and some myelin-like helices. After one day I observed in some samples sponges of passage with very small diameters (~ 1 μm) of the passages. After about four days a number of color scattering objects [18] were found in dark field microscopy. Besides the common blue spheres also scatterers of other colors appeared. The latter were closely packed arrays of μm-sized tubes.

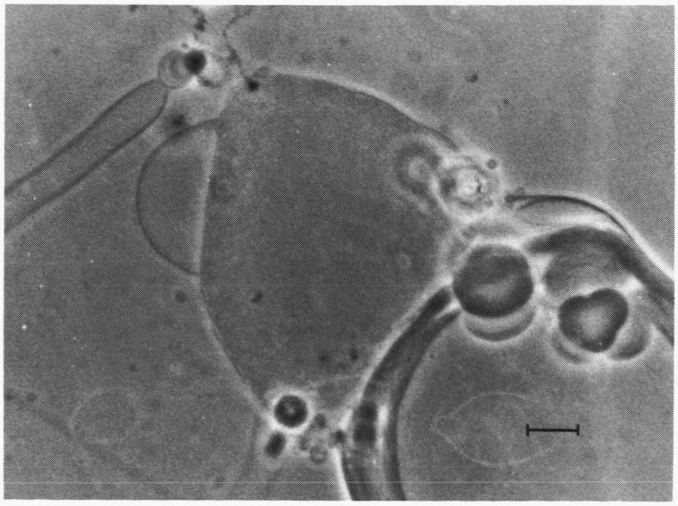

Fig. 8. Passage sponge in CL-water systems. Bar indicates 10 μm.

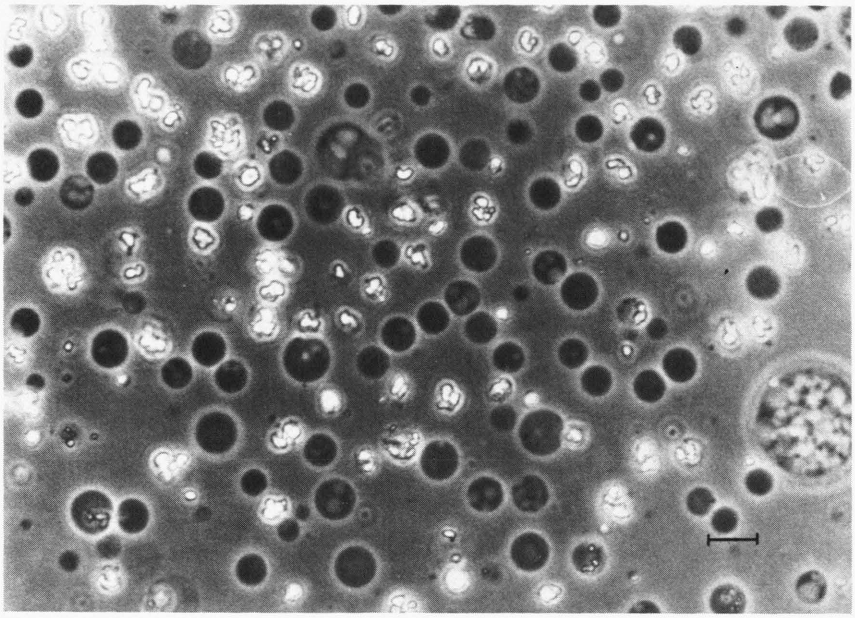

Fig. 9. Dark bodies occurring during swelling of Cl. Bar indicates 10 μm.

bhCL also mixed very well with lecithins. Experiments with palmitoyloleoyl-phosphatidylcholine (POPC), the most abundant fraction of natural egg lecithin, suggested that the larger the fraction of bhCL the more abundant were the dark bodies, passage sponges and color scatterers.

In a test series of experiments with CL from bovine brain no differences with respect to bhCL were noticed.

Discussion

The results of the simple swelling experiments showed that, with the possible exception of PA, all of the substances in contact with much excess water spontaneously formed giant vesicles above a certain temperature which equals the main transition (chain-melting) temperature T_m (see [19]) of the compounds. In some samples all of the material was converted into vesicles if it was incubated above T_m for one week. At $T < T_m$ light microscopy revealed that the crystals obviously imbibed water and grew slightly larger but never dispersed indefinitely. All of the main characteristics were qualitatively the same for electrical neutral SM, charged and doubly charged (CL) phospholipids. Only minor differences were noticed from the behaviour of lecithin for which a wealth of experimental studies exists and has been published elsewhere (see, *e.g.* [6, 20]). Superstructures of membranes, *i.e.* sponges of passages or 'dark bodies', appeared quite often with SM and CL and were much less frequently observed with PI. It is possible that in the early stages of swelling these lipids may favour non-lamellar fusion sites between adjacent lamellas, and accept a lamellar bicontinous structure at larger water content.

There are a few reports on spontaneous formation of GUV from acidic phospholipids which agree with my observations. Mueller and coworkers found GUVs if a film of bbPS was brought into contact with water of low ionic strength [5]. Micrometer sized complex vesicles of bbPS were also obtained by Rand and coworkers during simple swelling in buffer [21].

The results of the swelling experiments seem to contradict experiments where intermembrane forces have been measured directly. With the osmotic stress technique [22] or with a surface force apparatus [23] the properties were studied of either a bulk dispersion of the lipid or a stiff lipid film supported by mica. The distances were measured up to at most a few dozen nm. The systems which were studied in this report were somewhat different. I have looked mainly at the behaviour of the "boundary" lipid where part of the substance is in contact with bulk excess water. With light microscopy the change of intermembrane distances below 1 μm could only be inferred quite indirectly and the separation of membranes could be detected unambiguously only above this limit.

I will discuss the results with respect to changes of the intermembrane interactions between membranes which might occur at T_m. f and g will denote the pressures and area densities of the free energy of interaction, respectively, and d will be the distance between the surfaces of the membranes. For simplicity the discussion is restricted to planar geometries.

The hydration force f_h is very short ranged and will only be relevant at distances d of less than about 3 nm [22]. It could not be studied by light microscopy and will thus further be neglected.

For not too large distances attractive van der Waals forces f_v between phospholipid membranes in water vary with d^{-3}

$$f_v = -\frac{H}{6\pi} d^{-3}. \tag{1}$$

The same power law holds for repulsive undulation forces f_u [10]. If the lipids have a hydration shell the distances in both power laws might slightly differ because the undulation forces "see" the hydration shell as a more or less steric hindrance. In non-ionic solutions the main contributions to van der Waals interaction comes from the zero-frequency term which drops off slowly with d [24]. The higher frequency terms lead to a steeper decline of f_v than d^{-3} at distances of some dozen nm. In ionic solutions the leading zero frequency term is screened which leads to a reduction of f_v to less than 50% even at small separations [25]. From force measurements at large distances (a few nm) the head-group dipoles were suspected to give rise to a similar screening [26].

The prefactor in the van der Waals power law depends on the value of the Hamaker coefficient H. Taking the theoretical estimate for close distances of $H = 6 \times 10^{-14}$ erg [24, 25] we obtain (in cgs units) $f_v = 3 \times 10^{-15} \cdot d^{-3}$. Measurements in various lipid water systems give, however, a range of H from extremely large values of about 3×10^{-13} erg in PE [27] and SM [28], $0.28 - 0.7 \times 10^{-14}$ erg for PS in 400 mM NaCl [29], $0.13 - 3.8 \times 10^{-14}$ erg of PG in 100 mM NaCl [29] (inferred from osmotic stress experiments), or $8 - 10 \times 10^{-14}$ at smaller separations [30] (inferred from surface force apparatus experiments), down to 1.3×10^{-14} erg in PC and PE at larger separations (of a few nm) [26]. With these values the substances studied would have covered a range of f_v over more than two orders of magnitude. This is unlikely and it seems better to assume the short-range Hamaker coefficient to be about 6×10^{-14} erg or 50% less if the zero-frequency term in it is screened by ionic solutions [25]. At the transition between the gel and the fluid phase no effect on the Hamaker coefficient has been found experimentally, although in studies on synthetic PCs below and above T_m Marra has reported changes in the geometric parameters of the molecules [23].

The undulation forces vary as

$$f_u = A_u \frac{(kT)^2}{k_c} d^{-3} \tag{2}$$

where kT is the thermal energy and A_u a dimensionless coefficient. k_c is the curvature elastic modulus (bending stiffness) of the membranes. The range of f_u may be reduced by finite-size effects [15, 31] and by mechanical lateral tensions in the membranes [12, 14]. In the original derivation of undulation forces the prefactor of d^{-3} was 4×10^{-16} erg [10] which was calculated with a bending stiffness of 2×10^{-12} measured in the fluid phase of lecithin [32]. Below T_m, k_c has not been measured yet but can be expected to be at least one order of magnitude larger (see also ref. [15]). k_c in the fluid phase is not known for the substances used in this study. However, since the main contribution to the bending stiffness comes from the hydrocarbon core of the membranes [33], one would expect variations of k_c less than one order of magnitude [33]. Because of the lack of data for the lipids used in this study I will use the value of the lecithins in this discussion. The strength of f_u has not been measured directly in phospholipid systems and has been debated theoretically (see [10, 12, 34]). Measurements on related lamellar phases [35], however, seem to confirm the original theoretical prediction of $A_u = 0.46$ [10]. It is tempting to explain the experimental results by the competition of f_u and f_v alone because since $f_u \sim 1/k_c$ this force is very sensitive to T_m. A straightforward comparison of f_v and f_u shows that even in the fluid phase van der Waals attraction will be stronger than undulation repulsion and noncharged membranes are not expected to separate. A more rigorous treatment, however, must allow for hydration forces (or unequal distances in the power laws (1) and (2)). The faster decay of f_v at large distances and possibly smaller rigidities k_c [36] will also favour separation. Furthermore, it cannot be assumed that the forces are additive. An approach to handle this problem was reported by Evans and Parsegian [13]. Their result predicts stable cohesion between lammellas if $H = 1.1 \times 10^{-13}$ erg and $k_c = 10^{-12}$ erg are used.

One may suspect then that electrostatic repulsion is the driving force in vesicle generation. If charged planar membranes interact through pure water electrostatic forces f_e fall off with d^{-1} at small spacings and small surface charges. At larger spacings and surface charges a cross-over to a d^{-2} dependence will take place [34]. These forces are screened in ionic solutions and drop off steeply at d larger than the Debye length of the solution.

Even if only a small fraction (*e.g.* 10^{-4} of the membrane molecules is charged) f_e will dominate over f_v and f_u at $d > 10$ nm [42]. Since all of the acidic phospholipids studied in this report carry a surface charge which is much larger f_e is sufficiently large to drive membranes indefinitely apart in water. The present experiments, however, showed that spontaneous vesicle formation is not prevented by 100 mM NaCl with the Debye length being as small as 1 nm. Further, electrically neutral phospholipids can be charged by specific adsorption of cations if they are present even in very dilute concentrations. Surface force apparatus studies with synthetic PCs showed that the association constant of bivalent cations is larger in the gel phase than in the fluid phase. For Ca^{2+} this difference is almost one order of magnitude [23]. If electrostatic forces are the relevant factor for membrane separation to distances larger than 1 μm one would expect the effect to be stronger at $T < T_m$, which disagrees with the own experimental results. It should be stressed also, that chelation of Me^{2+} with EDTA did not prevent vesicle formation in the experiments reported in this paper.

For completeness I should mention a report where spontaneous formation of vesicles depended critically on temperature. Gershfeld *et al.* [37] reported that this happens with DMPG at a temperature T^* about 8 °C higher than T_m. T^* equals the critical temperature of surface bilayer formation from surface monolayers at the air-water interface. This process, however, is most likely not swelling of vesicles from a crystal but an assembly of molecules from solution. In the present experiments I obtained vesicles from DMPG at T just above T_m.

In summary, it cannot be easily understood why spontaneous formation of vesicles from the compounds takes place at $T > T_m$. However, shielding by ionic solutions did not prevent vesicle formation. f_u is the only force which is sensitive to T_m, but the theoretical estimate of its strength gives a value much too small if compared with the simple van der Waals law (Eqn. (1)). It is possible, however, that other steric forces operate between the membranes. (This is also proposed for the lecithins on the basis of a study of adhesion induced by mechanical tension [38].)

Conclusion

It has been shown by light microscopic experiments that neutral as well as charged phospholipids in excess aqueous solutions spontaneously can swell into giant unilamellar vesicles. For synthetic compounds this only happened if the sample was incubated above the chain melting transition temperature T_m of the lipid. A discussion of the possible intermembrane forces showed that neither electrostatic repulsion nor undulation forces with a strength predicted by theory can account for this process. It is suggested that further steric forces play a significant part in this process.

Acknowledgements

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