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# Transformation of phosphatidylcholine multilayer systems in a large excess of water

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**Abstract**

The swelling of similar samples of prehydrated phosphatidylcholine in excess water (> 99 wt/wt%) was studied microscopically and by X-ray diffraction. The Bragg peaks of the lamellar repeat distance were monitored for up to ten days. After a short period of rapid water uptake, the peaks remained stable in width and position, thus indicating that the so-called equilibrium distance was established in the fluid membrane stack. Within the next days we usually found the peaks to decrease continuously before they vanished in the background. The results suggest that the multilayer system desintegrates because the membranes peel off from it. Their separation may be driven by a tendency of the unstressed fluid membranes to develop a superstructure.

*Keywords:* Swelling process of phosphatidylcholine; X-ray scattering; Membrane adhesion; Structural instability

## 1. Introduction

Dry lipids such as the electrically neutral phosphatidylcholines (PC) spontaneously take up water. Some studies suggest that the swelling of PC multilayer systems in excess water stops when the so-called equilibrium spacing of $\approx 3$ nm is reached, which corresponds to a repeat distance of $\approx 7$ nm [1,2]. On the other hand, swelling has been used repeatedly and in many laboratories to obtain large single membranes and giant vesicles of the same lipids, the recipe dating back to Reeves and Dowben [3].

In the following we describe an attempt to resolve this contradiction. To this end, we studied the swelling of similar samples of prehydrated PC in excess water by either light microscopy or X-ray diffraction. All the optical samples displayed the usual generation of extended single membranes and giant vesicles. Most of the multilayer systems in the X-ray samples were also found to disintegrate. We infer from the X-ray data that the membranes peel off from the stacks of membranes. Attributing the separation to the simultaneous formation of a superstructure, we discuss possible direct evidence for the super-

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structure and an associated membrane roughness. An X-ray micrograph showing an unusually rough PC membrane is shown for the first time.

## 2. Material and methods

Egg-phosphatidylcholine (EPC) and palmitoyl-oleyl-phosphatidylcholine (POPC) were deposited in a chloroform solution (20 mg/ml) onto a clean glass slide for light microscopy or into a glass capillary (diameter: ≈ 2 mm, thickness: 0.01 mm) for X-ray diffraction. The chloroform was completely removed by slow evaporation and subsequent exposure of the specimen in an oven to reduced pressure (≈ $10^2$ Pa) at 40°C for about 12 h. At the end, the pure lipid formed a central spot on the glass slide or a ring in the capillary. The samples were then incubated for prehydration in water vapor (40°C) for about 1 h and weighed. Finally, bulk water was added with a syringe, so that the lipid content of the sample cells was about 1%. Subsequently, the samples were sealed (with glue or paraffin for optical or X-ray studies, respectively) and mounted on the respective instruments as quickly as possible.

Light microscopy was performed with a Leitz Ortholux microscope equipped with phase contrast, in polarized light and with an achromatic lens (40 × 0.75).

For X-ray diffraction a Marconi rotating anode generator was used (Cu-Kα line; λ = 1.54 Å; 35 kV, 55 mA) in combination with an electronic linear counter and a multichannel analyzer. The samples were thermostated at a constant temperature of about 25°C, far from the main phase transitions at −20°C or less.

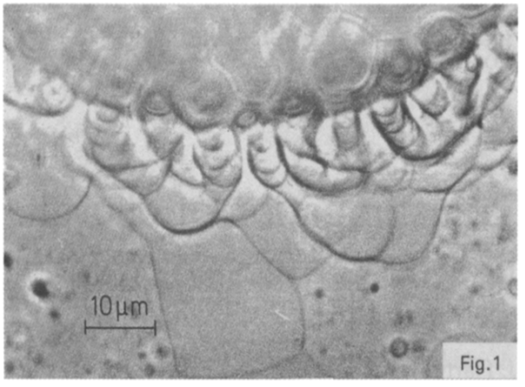


Fig. 1. Swelling from the multilamellar phase of prehydrated phosphatidylcholine in excess water at ≈ 25°C. Single vesicles begin to form and separate from the lipid.

## 3. Results

### 3.1. Light microscopy

As soon as the prehydrated PC was exposed to water in a sample cell, the first single membranes could be recognized at the border of the lipid

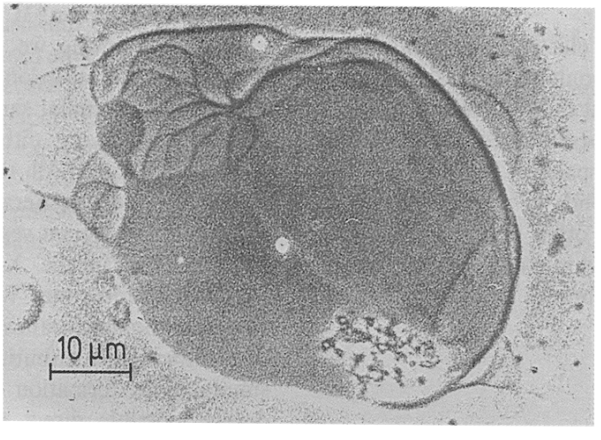


Fig. 2. A grey region next to the swelling multilamellar phase.

(fig. 1). Numerous onion-like structures developed along the border. Membrane fluctuations were well visible and, occasionally, membranes were seen to adhere to each other. This adhesion is not spontaneous, but demonstrates the frequently studied adhesion induced by lateral tension [4,5].

Big membrane pockets grew gradually from the border resulting in many different unilamellar structures which were still connected. In the course of time, giant unilamellar vesicles were released into the aqueous phase. Vesicle production could be promoted by alternatingly raising and lowering the temperature (e.g. between 10 and 40°C). Constant elevation of the incubation temperature favoured the formation of onions with well distinguishable, strongly fluctuating membranes.

In the swollen samples there were usually a few rather uniformly looking grey regions, similar to the dark bodies of earlier studies [6,7], with a typical extension of 50–100 μm (fig. 2). Rapid, fine-grained fluctuations of the light intensity indicated the presence of membranes, but there were no signs of a parallel ordering in these regions. As in the dark bodies, the membranes probably form a sponge, i.e. a disordered highly self-connected structure, at a scale not much below the limit of optical resolution. The grey regions were rather stable; single membranes could swell from the border but usually did not, and normally no vesicles were released. However, in the course of time holes could develop in the grey regions (fig. 3). Usually, the holes were not circular and they were traversed by single membranes.

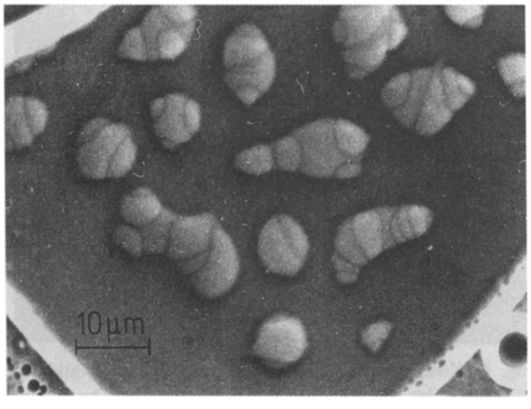


Fig. 3. A grey region displaying several holes. Single fluctuating membranes are spanning the holes.

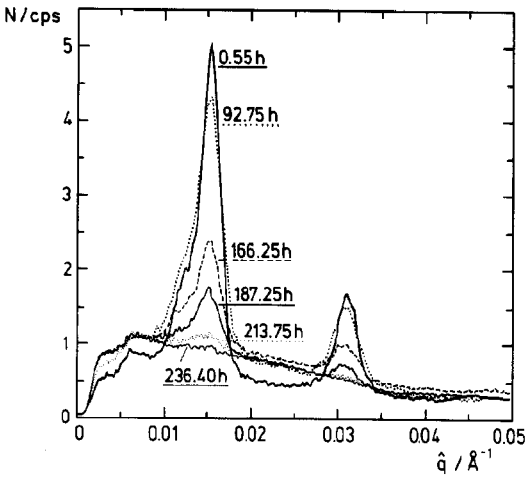


Fig. 4. Debye–Scherrer diagrams ($\hat{q} = q/2\pi$) taken at different times from a swelling system of POPC in pure excess water ( ≈ 1% lipid by weight; at 25°C).

### 3.2. X-ray diffraction

From the very beginning of the experiment, the Bragg peaks characteristic of a lamellar order could be observed (fig. 4). During the first minutes the peak maxima moved towards lower diffraction angles, thus indicating that the multilayer systems were still swelling. The repeat dis-

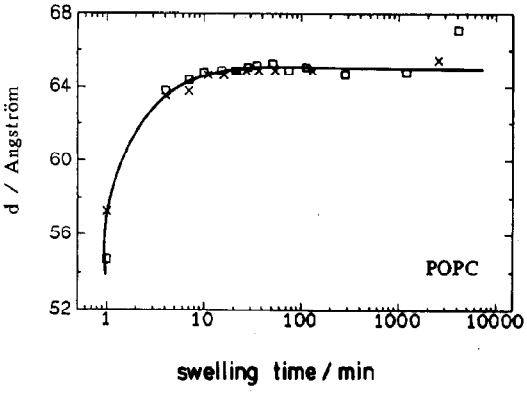


Fig. 5. Time dependence of the lamellar repeat distance obtained from X-ray diffraction of swelling POPC/water systems.

tance as a function of time is plotted for two examples in fig. 5. These results were the same in all samples investigated and in good agreement with other experiments [1,2]. In the case of POPC the final "equilibrium repeat distance" for the lamellar phase was about 6.5 nm.

However, in most of our samples this was not the end of the swelling process. The measurements were repeated after long intervals to control the stability of the lamellar phase. In the course of time, the heights of the Bragg peaks decreased but their positions and widths did not change and no additional peaks developed. Instead, the underground was slightly increased and at the end the decreasing peaks vanished completely (fig. 4). The time for the Bragg peaks to disappear varied from sample to sample (from about one hour to days). In a few samples, the Bragg diffraction pattern remained stable for up to two weeks, the total time of observation.

## 4. Discussion and conclusion

Both the pictures obtained from light microscopy and the small-angle Bragg diffraction patterns indicate that the stack of membranes is not a stable state in dilute mixtures of phosphatidylcholine and water. Also, the peaks characteristic for the lamellar crystalline order are not replaced by others that would suggest the buildup of a cubic or any other periodic phase. This agrees with the fact that we never saw cubic phases in cryo-transmission electron microscopy of similar systems. If grey regions were formed, i.e. sponges with a relatively large mesh size, we would not detect them by X-ray diffraction. However, the optical observations indicate that sponges are rather an exception.

Instead of forming another ordered phase, the outer membranes of the multilamellar system appear to separate from the stack by peeling off. This can be concluded from the fact that the Bragg peaks change neither position nor width after the first period of swelling. Hence there is a well defined and constant repeat distance in the remaining stack. A gradual penetration of water would result in a very different behaviour of the Bragg peaks: they would broaden because of a nonuniform membrane spacing, smallest in the center of the stack, and they would move towards lower scattering angles. Since this was never observed in any of our experiments, we infer that the membranes peel off one by one or at least in small numbers.

The time required for the complete desintegration of the lamellar order was found to differ very much from sample to sample. We suspect that the speed of disintegration depends on the concentration of defects, possibly dislocations, in the multilayer systems. A disintegration of stacks into single membranes has also been detected for PCs in narrow temperature intervals a few K above their main transition temperatures [8,9]. It may have been promoted by other defects such as local phase boundaries which are known to exist near the main transition [10]. However, our experiments were carried out in the fluid phase at temperatures far above the main transition.

The discovery that in most of our X-ray experiments the multilayer system disintegrates agrees with the light-microscopic observation of single membranes and vesicles in similar samples. The separation of the membranes from a multilayer system with an apparent equilibrium spacing is surprising. It may be related to the buildup of a membrane superstructure as will be discussed

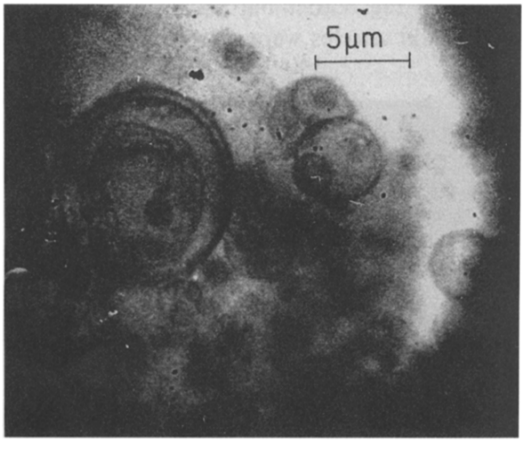


Fig. 6. X-ray micrograph showing part of a population of POPC vesicles in pure water (1% lipid content). The striking surface roughness of one of the vesicles was stable during several exposures (about 10–12 min).

immediately. Probably, the superstructure develops only in the outermost membranes, thus enabling them to separate from the stack when there is no lateral tension.

A superstructure of the bilayers of PC and other common electrically neutral lipids has been postulated originally to account for a substantial roughness of these membranes on a scale below optical resolution. The roughness, i.e. a substantial storage of membrane area far exceeding the storage by the usual thermal undulations, was inferred from the analysis of observations of mutual adhesion induced by lateral tension [4,5]. A few years ago, we have started a search for direct evidence of a superstructure and the roughness supported by it. Cryo-transmission electron microscopy revealed some grainy membranes [11,12] and angular vesicles [12,13] of egg yolk PC which may represent superstructure and roughness, respectively. Also, the deformation of PC vesicles by electric fields indicated, in some cases, reservoirs of area too large to originate from undulations [14].

Recently, the idea of a new surface roughness was substantiated when we investigated populations of gently swollen palmitoyloleoyl PC vesicles in water by X-ray microscopy [15]. With this method, the samples can be observed at room temperature and directly in their aqueous surrounding, at a resolution of about 30 nm. An example is shown in fig. 6. On a vesicle enclosed by two others, an unusual roughness is seen which remained unchanged for at least several minutes (during several exposures) and, therefore, cannot be attributed to the well-known thermal undulations.

Our central result is the proof that the multilayer system existing at the beginning of PC swelling in excess water is not a stable state. It can be continuously disassembled by a process peeling off the membranes. We suppose that this transformation is driven by the formation of a superstructure of the membranes which breaks up their quasi-spontaneous adhesion.

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