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# CHANGES IN SIZE AND SHAPE OF LIPOSOMES UNDERGOING CHAIN MELTING TRANSITIONS AS STUDIED BY OPTICAL MICROSCOPY

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**Changes in the shape and size of dipalmitoylphosphatidylcholine liposomes at the phase transition at 41.5°C have been monitored by light microscopy. All liposomes change size or shape at the transition and those with simple topologies such as spheres and cylinders can be readily measured. The surface area of these is some 24% greater above the transition than below. This surface area change is virtually identical to that predicted by crystallographic measurements on this system. Also, the rate of transition from one state to another is seen to proceed more rapidly in the smaller liposomes. Optical microscopic observation provides a rapid simple method for monitoring the dependence of the lipid bilayer area on temperature.**

## Introduction

We have recently completed a series of studies of the statistical mechanics and kinetics of the chain melting transition of phospholipid membranes induced by pressure and temperature changes as monitored by Raman spectroscopy [1,2]. The kinetic studies led us to question what processes in the liposomes might be responsible for the slow relaxations of the liposomes. In particular, it seemed possible that mechanical processes in the liposomes might be the rate-limiting steps in the interconversion between the 'liquid' and 'solid' states of the bilayer around the chain melting transition temperature. Change in the molecular area per headgroup at this transition should induce related changes in liposome shape and size.

Light microscopy is potentially an attractive method for the study of liposome size and shape changes. The measurements can be made instantaneously on intact structures, the samples are neither wet nor stained nor frozen, and the same liposomes can be cycled through phase transitions innumerable times. Early studies of the structure of hydrated phospholipids by light microscopy [3–5], were made of extracts of naturally-occurring lipids. The sharp chain-melting phase transitions of the synthetic phosphatidylcholines were not yet known, and most of the natural products had heterogeneous mixtures of hydrocarbon chains dominated by the unsaturated fatty acids. The lipids studied had fluid hydrocarbon chains at all temperatures at which observations were made [4,5].

The chain melting transition in liposomes made of the saturated hydrocarbon chain phosphatidylcholines have been observed by phase contrast microscopy [6], a technique which appears to be capable of observing single lipid bilayers on edge [7]. Liposome dimensions have been observed by

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microscopy to change under osmotic stress [8], but no quantitative studies of area changes at the transition temperature have been made. There have been attempts to quantify the temperature-induced size changes of small sonicated unilamellar vesicles using various techniques other than microscopy [9–11], but the results for the sonicated system may not directly apply to dispersed lipids which do not have such conformationally-strained bilayers. It is obvious from previous microscopic studies that multilamellar and even large unilamellar liposomes are of sufficient size to be monitored by optical microscopy, and that substantial changes in dimensions are expected. In this work, which has been presented elsewhere in preliminary form [12] we demonstrate that observation by light microscopy can be used to ascertain changes in surface area of lipids in bilayers.

## Materials and Methods

Acetone-precipitated L-α-dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids, Inc. and used without further purification. Dispersions were prepared by drying the lipid-solvent mixture in a rotary evaporator, and subsequently removing traces of solvent in high vacuum for 12 h. Dispersions had a sharp endotherm at 41.4°C and a pretransition with enthalpies identical to literature values as determined by differential scanning calorimetry. The calorimeter used was a Perkin-Elmer DSC-2, with techniques identical to those used in other publications from this laboratory [13]. The dry DPPC was then dispersed in pure water (prepared with a Millipore Milli-R/Q system) at 60°C by gentle swirling of the water over the solid lipid. The samples were readied for microscopic examination by placing a drop of dispersion on a clean glass slide, and then covering the drop with a 100 μm polyethylene spacer and a cover slip. The spacer prevented crushing of the liposomes. Bees wax was used as a sealant to prevent evaporation, and such samples were stable indefinitely if stored at or below 4°C.

The microscope used was a Reichert Zetopan equipped with a Polaroid camera back and Nomarski interference optics. Temperature control of the sample was achieved with a thermoelectric cooling and heating stage (model TS-2ER) manufactured by Bailey Instruments, Inc. We found that the stage could accurately control the temperature of the sample to within half a degree centigrade of the meter reading. This was only true, however, as long as the objective lens was kept more than about a millimeter away from the cover slip; otherwise the thermal mass of the objective lens itself overrode the temperature control. Consequently, we found that for high power observation (15 × or greater) it was necessary to use long working distance objectives (Leitz and American Optical Corp.). Because of this use of non-standard lenses it was not always possible to use the Nomarski interference optics to their fullest advantage.

Photographs were taken with Polaroid type 667 film (3000 ASA) at exposure times of from 0.5 to 0.1 s. At such slow shutter speeds the smaller particles which could be resolved by eye were smeared out because of excessive Brownian motion. The size of the liposomes was determined by measuring their outermost dimensions directly from the Polaroid prints with a pocket comparator and reticle from Edmund Scientific Company. Three liposomes which could be described as spheres or cylinders terminated by hemispherical sections were measured, and their dimensions were converted to surface areas.

## Results

A dispersion of phospholipids in water prepared as we have done contains a very heterogeneous population of liposomes. This is because the liposomes are formed by shearing off myelin figures which spontaneously form as the lipids hydrate at the surface of dry lipid. Increased shear forces seem to reduce the size of the largest liposomes, which are initially multilamellar, and also seem to produce a greater number of unilamellar structures. In our hands the sizes of individual liposomes have ranged from tens of micrometers down to the limit of resolution of light microscopy. Liposomes in which many bilayers are stacked in close apposition are strongly birefringent by most microscopic techniques, and these are essentially the only liposomes which can be seen under direct transmitted light microscopy. The Nomarski interference technique greatly en-

hances the visualization of small changes in the index of refraction such as those seen in liposomes with large spacing between bilayers or those with only a few bilayers. Consequently, it is possible to visualize liposomes which are topologically much simpler than the complex large multilamellar structures which are the dominant product of the dispersion procedure.

We have, unfortunately, no simple means for determining if a particular liposome is truly unilamellar beyond the observation that with the Nomarski illumination there is a large range of birefringent intensities for structures ranging down to a discrete lowest level for the liposomes which we believe to be unilamellar. Such a belief is supported by previous microscopic studies [7]. It does not matter in our case whether a liposome is unilamellar or not as it is the area change of the outermost bilayer only that is measured. As the liposomes are more dense than water, they settle to the bottom of the 100 $\mu$m thick sample volume. Prolate ellipsoids and cylinders lie with their long axes in the focal plane, thus facilitating measurement. It is the rare liposome in the observable size range which has a regular shape, and, as only the regular shapes are readily amenable to accurate measurement, one must search for simple liposomes. This is not difficult, as the field of view in a microscope slide may readily contain of tens of thousands of non-overlapping liposomes.

When a dispersion sample has been equilibrated at temperatures below the pretransition temperature for a few minutes or more, raising the temperature rapidly to above the chain melting transition temperature (41.4°C) produces two distinct changes in liposome conformation, one at each transition temperature. As there is an appreciable hysteresis in the lower transition, making it difficult to observe on lowering the temperature, we have chosen to make a rigorous study of only those changes in liposome size which occur at the main transition. In principle, however, the pretransition, or even the subtransition can be monitored with this method. We have also found that more reproducible measurements can be made when the liposomes are first equilibrated at high temperatures, and are then brought to successively lower temperatures. Crumpling or folding of the oligolamellar liposomes is often seen on raising the temperature through the pretransition. The changes observed on heating the liposomes always involve an increase in the apparent surface area of the constituent bilayers. Gradual small changes occur in the surface areas within single phase regions of the bilayer phase diagram, and large changes at the pretransition and the main transitions, the main transition having by far the larger effect. Multilamellar structures are often seen to become transiently ruffled at the main transition temperature, as if the surface areas of parts of te liposomal bilayers were changing at different rates. Elongated structures can be seen to elongate and shrink in a more orderly fashion, reminiscent of the expansion of a balloon inflated for the first time. That is, the growth begins at some structural defect and then the conversion to a different shape proceeds along a growth ring which moves as the conversion is accomplished. This is exactly the model which we have used earlier to treat the statistical mechanics of the phase transition [14]. In the melting region there are two phases separated by a continuous perimeter.

The rate at which liposomes change shape depends strongly on the size and shape of the liposomes. If the temperature of the stage is rapidly changed through the main phase transition at 41.4°C a wave of motion passes through the sample as the vesicles change shape. Under these circumstances the small vesicles (those of 1 $\mu$m or less in diameter) can be seen to convert rapidly from one state to the other in less than a second, whereas larger multilamellar liposomes may continue to change shape for up to several seconds.

We have picked out three liposomes (see Figs. 1, 2 and 3) as exemplary of these which can be used for measurement of surface area. For those liposomes shown in Figs. 2 and 3 only those measurements shown in the figure captions were made, whereas for the liposome in Fig. 1 six measurements were made, three above and three below the chain melting temperature ($T_m$). The mean area for the Fig. 1 liposome just above $T_m$ was 23% greater than the mean area just below $T_m$, whereas for the liposomes in Figs. 2 and 3 there was an apparent 25% increase in both cases. The measurements from these liposomes are, then, in close agreement, despite the possible measuring errors. At the main phase transition in these liposomes

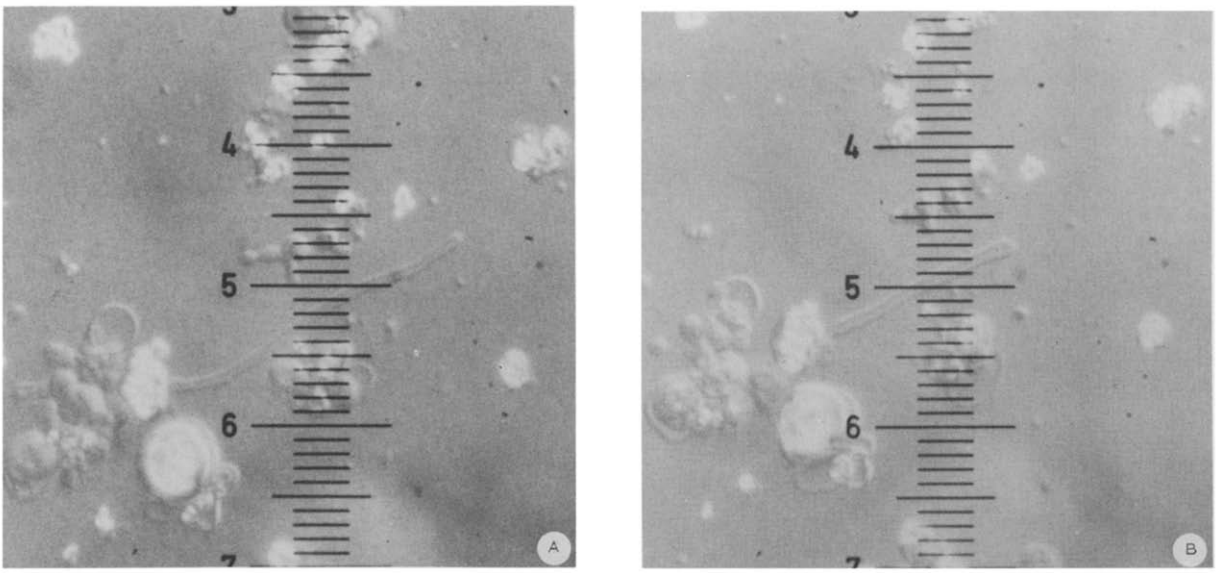


Fig. 1. The first two in a series of photographs monitoring the size changes of the long tube-shaped liposome crossing the frames. In part A at 43°C the liposome has a surface area of approx. 406 $\mu m^2$, while in part B at 40°C the surface area is approx. 323 $\mu m^2$. The spacing between the graticle marks is 3.1 $\mu m$.

there is an increase of 24% in the surface area of each bilayer with a standard deviation of 1.2%. This value corresponds quite well with those determined by X-ray crystallographic and monolayer studies of about 48 $Å^2$ below the transition, and 58 $Å^2$ above, for a change of 21% [15].

It is difficult to tell by eye whether a liposome is, for example, a sphere or an oblate ellipsoid with

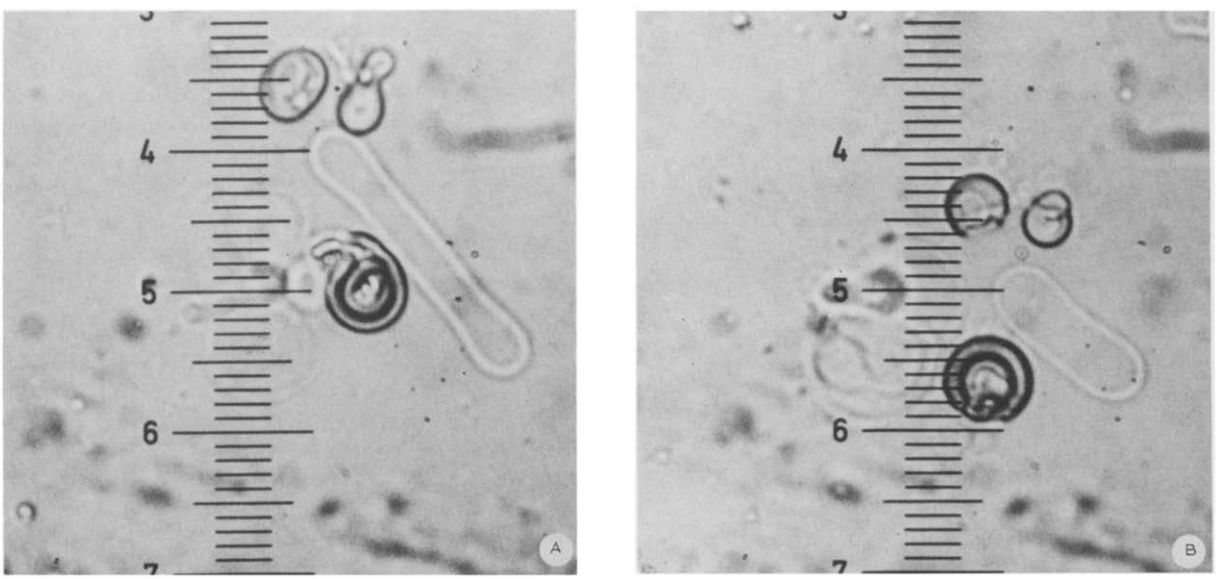


Fig. 2. The measured liposome is the open loop-shaped one at the right of center. In part A at 44°C the liposome has an area of 1272 $\mu m^2$, and in part B at 39.5°C the area is 1019 $\mu m^2$. The graticle marks are spaced 2.8 $\mu m$ apart.

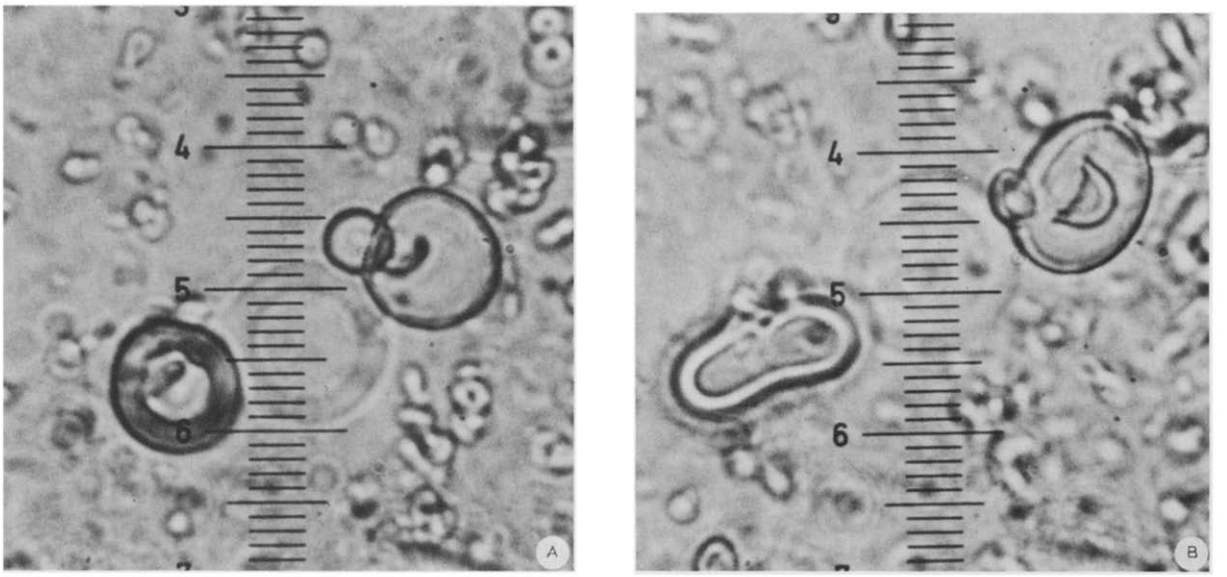


Fig. 3. The measured liposome is at the center of the frame along the graticle marks (which are spaced 1.7 $\mu$m apart). In part A at 39°C, the liposome area is 1307 $\mu m^2$, and in part B at 44°C the area is 1019 $\mu m^2$.

its minor axis normal to the plane of the slide. It might be expected that this uncertainty would adversely affect one's ability to determine changes in surface area from the dimensions of the object projected on the image plane. This is not the case, as for even the extreme cases of a perfect sphere and a coin-shaped object lying flat on the slide, the surface areas have the same dependence on the square of the radius, and hence the change in surface area for a given change in radius is the same. But partly because of this uncertainty of shape along the optical axis, we have not found a regular dependence of the volume of the liposomes with temperature. Some liposomes have a strong dependence of the volume on temperature, while we have measured others which, within the time of the measurements, have shown none. Some of the observed liposomes are multilamellar and contain lipid which will itself change volume at the transition temperature by about 4% [16]. More important, however, is the fact that changes in internal volume require flow of water from one side of the bilayer to the other. This process can be fairly slow for such large liposomes (for example, see Ref. 8). We found some changes in the apparent volume of the liposomes to take place over a few minutes when the temperature was kept constant and above the chain melting temperature.

## Discussion

Although the phospholipid bilayer is only about 45 Å thick, we have shown that optical microscopy, with resolution of no better than about 2500 Å, can be used to accurately monitor area changes of the molecules in liposomes. While microscopy is not the only technique which can measure liposome size, in that photon correlation spectroscopy can also be used for such purposes, this technique is simple, rapid, and uses instrumentation widely available in most research institutions. The temperature-controlled stage we used for the microscopy is not strictly speaking necessary, as the temperature can be easily raised above ambient with a simple heat lamp, a technique which we employed in earlier studies of this nature [17].

While it need not be a limitation of the applicability of the technique, it should be emphasized that we have dispersed our lipids in distilled water, not a buffer or salt solution. The phospholipid bilayer is relatively impermeable to small ions as compared to water, so that while water is capable

of diffusing across the bilayer in response to a change in the surface area of a liposome, the ions will not. A spherical liposome above the main transition temperature which we suddenly dropped below the transition temperature would have to lose contents in order to be able to shrink its surface area. In the presence of pure water only this is accomplished by diffusion of water through the bilayer, but in the presence of salts or other non-permeant species, the membrane would shrink only until an osmotic gradient built up to sufficient strength to prevent further size changes. At that point the only route for further size changes would be the much slower diffusion of ions through the membrane, or catastrophic rupture of the membrane, followed by resealing. Use of pure water for the aqueous phase avoids these complications.

The changes in bilayer dimensions which we have measured by this technique are slightly larger than those measured by X-ray diffraction. The area per molecule as measured by crystallography is calculated from the inter-chain spacing. The bilayer area in the phase between the main and pretransitions is reduced from the area one would expect from the X-ray values by periodic rippling. This ripple disappears above the main transition, so the total change seen in area might be larger than expected from crystallographic predictions. Estimations of area change obtained from monolayer experiments may also be inaccurate for bilayers as there is no evidence of a rippled phase in monolayers on water. It is tempting to speculate that such microscopic details are responsible for the discrepancy between our area changes and others in the literature, but the accuracy of those measurements of liposomes by microscopy is not sufficiently high to resolve the question. In light of the current controversy in the field concerning the change in size of liposomes of various types at the chain melting transition we find it reassuring that the area changes we see here are so close to those predicted from X-ray data.

It has been reported from microscopic observations that the number of opposed lamellae determines the melting temperature of the lipid bilayer [6]. We have not noted such a dependence, but have consistently seen the afore-mentioned difference in phase transition rates between small and large liposomes. It is possible that a kinetic effect may be mistaken for a thermodynamic one if the rate of temperature change is too high. Since we have seen that the large liposomes spend substantially more time in transition between their high and low temperature forms than to small ones, we are certain that the kinetic difference exists.

The slower rates of conversion of the large liposomes from a fluid to a solid state are not unexpected in light of kinetic measurements on the phase transition by other techniques [1]. Among the possible reasons for the difference are the diffusion of water across the bilayers, and the constraints imposed upon multilamellar liposomes by the close apposition of bilayers which must change their area (and phase state) in unison. In the case of melting, at least, there is no reason to believe that water flow across the bilayer is indeed a rate-limiting factor, as the liposome increasing its surface area may do so indefinitely without any requirement for water flow, the liposome can simply ruffle, wrinkle, or increase its ellipticity. Such liposomes may in time relax to a more spherical shape after flow of water across the bilayer, but there would be little or no resistance to the conversion of the bilayer from frozen to fluid because of this. In the freezing direction, the flow of water might have an effect on restricting the possible rate of conversion, but as there is evidence that the rates of freezing and melting in multilamellar liposomes are comparable [1] it is unlikely that the water flow is a controlling factor in either direction. The requirement for cooperative melting of the bilayers in a multilamellar structure is almost certainly the limiting factor, as it applies equally for melting and freezing. A related factor is the topological complexity of the liposomes, as the liposomes with irregular shapes are clearly seen to relax to new shapes more slowly than those with more regular appearance. Such irregular liposomes can only occur in preparations with large heterogeneous populations of liposomes with a high degree of multilamellarity. This dependence of the rates of phase transition on shape and size must be taken into account in future studies of the kinetics of the transitions.

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