Biochimica et Biophysica Acta, 684 (1982) 277–281
Elsevier Biomedical Press

BBA Report

BBA 70006

TRANSITION OF A LIQUID CRYSTALLINE PHOSPHATIDYLCHOLINE BILAYER TO THE GEL PHASE IN A VESICLE REDUCES THE INTERNAL AQUEOUS VOLUME

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(Received September 1st, 1981)

Key words: Phosphatidylcholine bilayer; Phase transition; Trapped volume; Membrane vesicle

The liquid crystalline to gel phase transition in phospholipid bialyers is associated with a marked reduction in the area per phospholipid molecule. Geometric considerations based on published data suggest that this decrease in molecular area is accompanied by a reduction in the internal aqueous volume trapped within a unilamellar bilayer vesicle. This volume reduction, which depends upon the shape of the vesicle, is shown to be between 23 and 60 percent. We have observed a 25 to 30 percent reduction in the internal aqueous volume of unilamellar vesicles about 700 Å in diameter formed from dipalmitoylphosphatidylcholine using the self-quenching of 6-carboxyfluorescein trapped within this compartment.

Phospholipid bilayers in the form of vesicles and liposomes have been extensively studied as a model of biological membranes [1]. Aqueous dispersions of vesicles are also potentially useful as drug delivery systems [2]. Understanding the dependence of bilayer properties on various factors, such as vesicle size, composition and temperature, is therefore of importance. Special efforts have been devoted in the past several years to investigating the effect of the gel to liquid crystalline phase transition on the structure and motional state of the phospholipids within the bilayer [3]. In these studies, vesicles of various sizes and composition have been examined using a variety of techniques [4]. One important factor which has received relatively little attention, however, is the effect of the phase transition on the internal aqueous volume trapped within each vesicle. This situation is due largely to difficulties in measuring the trapped aqueous volume of vesicles in their gel phase.

In general, there is no method for determining directly the amount of water trapped within vesicles. It is, however, possible to measure the amount of a water soluble marker molecule trapped within the vesicles. If it is assumed that the concentration of the marker is the same within the vesicles as it was in the external medium when it was trapped, the internal aqueous volume can be easily calculated. Introduction of the marker can be achieved by forming the vesicles in the presence of the marker or by incubating pre-formed vesicles in an aqueous solution of the marker until equilibrium between the external medium and the internal aqueous vesicle volume is achieved [5]. Neither of these procedures however, can be used with confidence to measure the volume trapped within liposomes in the gel phase, because adequately sealed bilayers are very difficult to form below the phase transition temperature (T_m) [6]. In

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Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

addition, the permeability of gel phase vesicles to the various indicators is limited [7].

In the work described below a new method is introduced for measuring the phase transition-induced change in the aqueous volume trapped with vesicles. This method is based on determination of changes in the concentration of trapped 6-carboxyfluorescein. If this molecule is trapped at a partially self-quenched concentration during the course of vesicle preparation above T_m [5,8], changes in its concentration due to changes in the volume of the internal aqueous vesicle compartment will be reflected in fluorescence intensity changes.

Fig. 1 gives the temperature dependence of the fluorescence intensity measured at 520 nm of carboxyfluorescein trapped in unilamellar vesicles about 700 Å in diameter formed from dipalmitoylphosphatidylcholine (DPPC) according to the method of Schullery and co-workers [28]. It is apparent that as the temperature is varied across the phase transition region, a large change in the fluorescence of the probe occurs. The fluorescence is markedly lower in the gel phase than in the liquid crystalline phase. The ordinate in fig. 1 is the percent of the unquenched intensity which is defined by the fluorescence intensity obtained after the addition of the detergent Triton X-100. Addition of the detergent disrupts the vesicles and releases all trapped carboxyfluorescein into the ambient aqueous phase. The resulting dilution completely abolishes self-quenching of the fluorophore. Control experiments indicate that Triton X-100 has no detectable effect on either the excitation or emission spectrum of carboxyfluorescein. Based on the calibration curve shown as an insert in Fig. 1, the unquenched intensity of 35% exhibited below T_m in Fig. 1 corresponds to a concentration of trapped carboxyfluorescein of 16 mM. Heating the system (solid line) to a temperatur above T_m (44°C) causes the unquenched fluorescence intensity to increase to 51%. If the observed change in fluorescence intensity is due only to a change in volume of the internal aqueous compartment, the data in Fig. 1 lead to a volume change of 25 to 30 percent.

In addition to the putative volume change in the aqueous compartment, several other factors

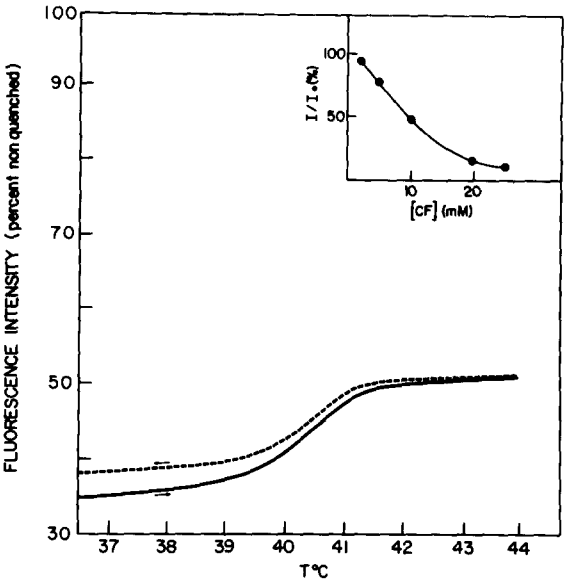

Fig. 1. Temperature dependence of the fluorescence intensity of 6-carboxylfluorescein trapped in dipalmitoylphosphatidylcholine vesicles. 700 Å diameter unilamellar phospholipid vesicles (10 mM in lipid phosphorous), prepared and characterized as previously described [28], were incubated at 42°C for 18 h with 50 mM 6-carboxyfluorescein, pH 7.4. The dispersion was then passed, at room temperature, through a Sephadex G-25 column equilibrated with 100 mM KCl, 20 mM sodium phosphate buffer, pH 8.2. The vesicles containing trapped carboxyfluorescein appeared in the void volume. They were diluted with 1.5 volume of column buffer and passed six times through the phase transition by incubating at 45°C for three 5-min periods, followed by room temperature incubation. The vesicles were then passed a second time through the Sephadex column to remove carboxyfluorescein which had leaked into the ambient aqueous phase during the successive incubations. Fluorescence intensity was measured at 520 nm. The exciting wave length was 490 nm. Fluorescence measurements were carried out in a stirred cell as a function of temperature. The scanning rate was 1 K/min and the temperature was continuously monitored in the cell. The 100% fluorescence intensity was obtained by addition of 0.1% Triton X-100 after completion of the scan. The solid line is a scan of the fluorescence while heating the vesicle dispersion whereas the dashed line is a cooling scan. The precision of the data is ±0.5%. The insert shows the relation between relative fluorescence intensity and the concentration of carboxyfluorescein [5].

may contribute to the observed fluorescence intensity change with temperature shown in Fig. 1. (i) Leakage of trapped carboxyfluorescein, due to passage through the phase transition temperature range, certainly contributes to the increase in fluorescence [10–12]. However, the change in the fluo-

rescence is essentially reversible. Upon cooling the system (dashed line), the relative fluorescence intensity returns to a value of 38%. (ii) The quantum yield of carboxyfluorescein is temperature dependent. However, this linear temperature dependence of the fluorescence intensity is less than 0.4% per °C. Furthermore, the fluorescence intensity change due to this cause decreases with increasing temperature. (iii) Changes in the so-called inner filter effects are negligibly small for the concentration of vesicles used in this experiment (absorbance less than 0.1) [9]. (iv) In principle, a temperature dependent binding of carboxyfluorescein which alters the quantum yield could give rise to the observed change. This possibility is unlikely, however, since control studies carried out on carboxyfluorescein dissolved in buffer solutions both in the presence and absence of added phosphatidylcholine vesicles which contained no trapped carboxyfluorescein showed no change in either the excitation or emission spectrum under the experimental conditions used to obtain the data in Fig. 1. Furthermore in previous studies it was found that the trapped volume of liposomes obtained in the conventional manner was the same using either carboxyfluorescein or [^{14}C]glucose as marker [5]. This observation argues strongly against absorption or partition of carboxyfluorescein on or in the bilayer. (v) The fluorescence intensity of carboxyfluorescein is dependent on pH [29]. However at pH 8.2, the condition under which the data in Fig. 1 were obtained, the fluorescence intensity is very insensitive to pH. At pH values below 8 the relative decrease in fluorescence with decreasing pH is approx. 20% per pH unit [29]. The vesicle dispersion used to obtain the data in Fig. 1 was buffered at pH 8.2 with a sodium phosphate buffer which had been equilibrated with the internal aqueous vesicle volume (see legend of Fig. 1 for details). As noted above, control experiments in this buffer containing carboxyfluorescein in both the absence and presence of added phosphatidylcholine vesicles which did not contain trapped carboxyfluorescein showed no change in fluorescence characteristics with temperature. (vi) A fraction of the internal aqueous vesicle volume is in the form of water bound to the bilayer surface. This bound water excludes solute molecules such as carboxyfluorescein [18]. A change in the fraction of bound water caused by the gel to liquid crystalline phase transition could contribute to the fluorescence intensity change shown in Fig. 1. Taylor and co-workers have in fact shown that the hydration of phosphatidylcholine bilayers in the gel phase is about 58% of the value in the liquid-crystalline phase [19]. Thus in passing from the gel to liquid crystalline phase there should be a decrease in fluorescence intensity of trapped carboxyfluorescein due to the increase in bilayer hydration. This is opposite to the effect shown in Fig. 1. Studies of preferential hydration in small unilamellar vesicles of egg phosphatidylcholine indicate that the hydration layer is about 6 Å thick [14,18]. In vesicles with an inner bilayer radius of 300 Å, similar to those used to obtain the data in Fig. 1, the fraction of the total internal aqueous volume accounted for by bound water is 6%. It is clear that a 58% change in this fraction is at the limit of detectability of the 6-carboxyfluorescein fluorescence method and can make no significant contribution to the data in Fig. 1.

On the basis of the considerations discussed above, we conclude that the fluorescence intensity change with temperature recorded in Fig. 1 is most probably due to the phase transition-induced volume change in the internal aqueous compartment of the phosphatidylcholine vesicles. This conclusion is further strengthened by the following argument. It has been shown for planar dipalmitoylphosphatidylcholine bilayers by X-ray diffraction [13] that the transition from liquid crystalline to the gel phase is accompanied by a reduction in the area per molecule from about 70 to 47.5 Å^2. In spherical vesicles, the surface area per molecule depends on the radius of the bilayer surface. Using hydrodynamic methods and NMR spectroscopy, it has been concluded for both egg [14] and dipalmitoylphosphatidylcholine [15] that in the liquid crystalline state, the area per molecule of the inner monolayer is smaller than that of outer monolayer. However, in a recent report, Cornell and co-workers [16] have suggested that the surface area per molecule, if defined at the interface between the hydrophobic and polar hydrated regions of the component molecules, is the same for the inner and outer monolayers of the

vesicle bilayer. In the liquid crystalline phase of egg phosphatidylcholine it is equal to 72 Å^2, a value similar to the value obtained for planar bilayers. If it is assumed that at this interface the area per molecule in the gel phase is similar to that observed in gel phase planar bilayers, the surface area per molecule in the gel phase of DPPC should be smaller by the fraction $(47.5/70) = 0.68$ than the value in the liquid crystalline phase. This assumption is certainly reasonable for large radius vesicles. If on passing from liquid crystalline to gel phase the number of phospholipid molecules in both inner and outer monolayers of a spherical vesicle bilayer remains constant [17], the surface area of the inner monolayer, which determines the trapped volume, must then decrease by the same ratio. As a consequence, the radius of the inner monolayer will decrease by a factor of $(47.5/70)^{1/2} = 0.82$ and thus the trapped aqueous volume will decrease by a factor of $(47.5/70)^{3/2} = 0.56$. These factors would, of course, be different for non-spherical vesicles.

The magnitude of the calculated reduction in volume is dependent upon the choice of molecular areas below and above T_m. Reasonable limits can be set for these values based on the following considerations. The molecular areas for various phospholipids have been reported to lie between 62 and 72 Å^2 for the liquid crystalline state and between 40 and 52 Å^2 for the gel phase. Therefore the largest volume reduction that can reasonably be expected will be 60 percent. This upper limit will be attained only if the molecular area in the inner monolayer is reduced from 72 Å^2 to 40 Å^2. A reasonable lower limit to the reduction of trapped volume of 23% is obtained for a change in molecular area of the inner monolayer from 62 Å^2 in the liquid-crystalline phase to 52 Å^2 in the gel phase. On the basis of this analysis it is reasonable to conclude that the transition of a liquid crystalline bilayer to the gel state should result in a 23 to 60 percent decrease in the volume of the trapped aqueous phase. The volume reduction of 25 to 30% obtained from the data in Fig. 1 is within this range.

Watts and coworkers attempted to obtain the internal volume of both liquid crystalline and gel phase vesicles of dimyristoylphosphatidylcholine made by sonication above T_m using TEMPO choline as the trapped marker [7]. An attempt to solve the problem of introducing the marker in gel phase vesicles was made by briefly resonicating vesicles below T_m in the presence of TEMPO choline. These workers concluded that vesicles treated in this way retained their spherical shape but that the trapped internal volume was reduced by 83% compared to the same vesicles in the liquid crystalline state. This large reduction in volume, which is well outside the range established by our theoretical analysis, was attributed to a 2-fold decrease in the radius of the inner monolayer of the vesicle bilayer upon going from liquid crystalline to gel state. It seems possible, however, that the small gel phase trapped volume determined by these workers was an underestimate of the true internal volume. This would be the case if resonication caused disruption of the original vesicles, followed by reformation below T_m of vesicles leaky to TEMPO choline [6], or if resonication did not result in complete equilibration of TEMPO choline.

Based on photon correlation spectroscopy, Cornell and co-workers [30] have recently reported that the external radius (103 Å) of small unilamellar vesicles prepared by sonication from dimyristoylphosphatidylcholine does not change with temperature over the interval from 10 to 30°C. The gel to liquid crystalline phase transition occurs within this interval. Similar results have been reported for dipalmitoyl- and dimyristoylphosphatidylcholine vesicles by Ceuterick and coworkers [31]. If the outer radius of these vesicles remains constant, the mandatory increase in thickness of the bilayer associated with the phase transition from liquid crystalline to gel phase requires a decrease in the internal aqueous volume of the vesicles. This would be qualitatively consistent with our interpretation of the data in Fig. 1 obtained with larger unilamellar vesicles, 700 Å in diameter, and with the arguments based on the transition-associated change in area per phospholipid molecule.

There are two implications associated with a phase transition-induced change in the aqueous volume bounded by a spherical bilayer. The first of these concerns the osmotic gradient generated by the volume change if impermeant solutes are

present. This osmotic gradient acts in opposition to the volume change and may reduce its magnitude. It is also possible that a phase transition-induced osmotic gradient may contribute to the anomalously high permeability of some bilayers to small water soluble molecules in the transition range [10–12]. The second implication concerns the possibility of intracellular volume changes which may occur when cells are brought to a temperature below the phase transition of their plasma membrane lipids. The bilayers of most biological membranes are believed to be for the most part in the liquid crystalline state under normal growth conditions [20]. The lipid components of the membranes of *Escherichia coli* [21,22] *Halobacterium halobium* [23] and *Acholeplasma laidlawii* [24–26] as well as the membranes themselves, however, exhibit a well defined gel-liquid crystalline phase transition at temperatures below the growth temperature but well above 0°C. It is possible that when these organisms are taken through the phase transitions region, a cytoplasmic volume change occurs which may account in part for the membrane fragility exhibited by these organisms and by certain *E. coli* fatty acid auxotrophs under similar conditons [32–35].

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