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PAPER

Volumetric stability of lipid bilayers

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In agreement with recent reports, a commercial densimeter has yielded a gradual decrease in lipid molecular volume of DPPC multilamellar vesicle dispersions in the gel phase upon repeated thermal cycling between 10 °C and 50 °C. The considerable size of this decrease would have significant implications for the physical chemistry of biomembranes. In contrast, neutral buoyancy measurements performed with similar thermal cycling indicate no gradual change in lipid volume in the gel phase at 20 °C. Remixing the lipid in the densimeter shows that the apparent volume decrease is an artifact. We conclude that gel phase DPPC bilayers exist in a volumetrically stable phase.

1. Introduction

In addition to their interest as biological prototypical-membranes, lipid bilayers have been fascinating objects of physical chemistry studies for many years.[1] An important physical property of lipid bilayers is molecular volume. Measuring molecular volume *versus* temperature reveals phase transitions,[2,3] and comparison of the volume changes at these transitions leads to estimates of the cohesive energy of the hydrocarbon chains.[4,5] Furthermore, accurate volumes are essential to obtain the quantitative structure of bilayers in their different phases.[6]

It has been recently reported that the molecular volume of several common lipid bilayers gradually changes upon repeated thermal cycling through their transitions.[4,7] In particular, the hydrocarbon volume of the benchmark lipid DPPC in its gel phase at 20 °C was reported to decrease from the first thermal cycle to the fifth cycle five days later.[4] Decreases were also reported for the shorter chain lipid DMPC[4] and the mixed chain lipid SMPC.[7] For the reverse mixed chain lipid MSPC, the volume was reported to increase in its gel phase, but by a much smaller magnitude than for the large decrease reported for SMPC.[7] For all lipids the molecular volumes of the fluid phase above the main transition temperature T_M were reported to change by much smaller amounts than for the gel and ripples phases at temperatures below T_M.[4,7] This difference led to the suggestion that the gel phase is relatively unstable compared to the fluid phase.[7] To our knowledge, this new behavior has not been previously observed. As it would have significant implications for both the energetics and the structure of lipid bilayers, we felt that it was important to repeat these measurements. Indeed, using the same commercial densimeter we obtained even larger decreases in DPPC volume upon repeated thermal cycling. However, using our traditional, more laborious, neutral buoyancy method[8] we observed negligible change in volume of DPPC at 20 °C when similarly thermally cycled. In this paper we resolve this difference in favor of the simple neutral buoyancy method and we identify the artifact that occurs in the commercial densimeter.

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2. Experimental

Sample preparation

1,2-Dipalmitoyl-*sn-glycero*-3-phosphocholine (DPPC, Lots 160PC-297 and 160PC-302) was purchased from Avanti Polar Lipids (Alabaster, Alabama). Pure D_2O was purchased from Sigma-Aldrich (St. Louis, Missouri). Multilamellar vesicle (MLV) samples for densimetry were prepared by adding 50 mg DPPC to 1.2 grams of pure milli-Q water and hydrated by thermal cycling three times above and below the main phase transition temperature (T_M = 41.4 °C), vortexing at the high and low temperatures. For the neutral buoyancy experiments, H_2O and D_2O were measured into vials to produce known solution densities. 1–2 mg DPPC was added and hydrated the same way as the densimetry samples by temperature cycling and vortexing. Parafilm was wrapped around the plastic screw tops to prevent evaporation.

Densimetry

A DMA 5000M densimeter (Anton Paar, Ashland, Virginia) was used. The sample was loaded from a plastic syringe, slowly to avoid air bubbles. The temperature of both the sample and the densimeter was 50 °C. After loading, the temperature was lowered to 10 °C and held overnight before initiating thermal cycling the next day. Heating scans were from 10 °C to 50 °C at a rate of ~12 °C h^{-1} and cooling scans were from 50 °C to 10 °C at ~4 °C h^{-1}. Density was recorded every 0.5 °C. The cooling scan was usually started within 10 to 30 minutes after completion of the heating scan. The sample was typically held at 10 °C overnight until the next heat/cool cycle began

the following day. Conversion of raw densities ρ_s into lipid molecular volumes V_L used the equation[9]

$$V_L = \frac{M_L}{0.6022\rho_s}\left[1 + \frac{m_W}{m_L}\left(1 - \frac{\rho_s}{\rho_W}\right)\right], \quad (1)$$

where M_L is the lipid molecular weight, m_w and m_L are the masses of the water and lipid, respectively, and ρ_W is the measured density of water.

Neutral buoyancy

For each neutral buoyancy experiment, 6 samples were prepared. Fig. 1 shows the samples prepared for the first neutral buoyancy experiment.

The different densities ρ_{sol} were obtained by mixing mass m_D of D_2O and mass m_H of H_2O with mass fraction $f = m_D/(m_D + m_H)$. Assuming that D_2O and H_2O mix ideally,

$$1/\rho_{sol} = f/\rho_D + (1 - f)/\rho_H \quad (2)$$

where ρ_D and ρ_H are the known densities of D_2O and H_2O at the mixing temperature. The samples were placed in an Incufridge (Revolutionary Science, Shafer, Minnesota) which heated the sample at a rate of $\sim$16 °C h^{-1} and cooled the sample at a rate of $\sim$13 °C h^{-1}. For the first neutral buoyancy experiment, a thermal cycle consisted of the samples being heated from 21.2 °C to 41.7 °C and held for 10 minutes before being cooled to 21.2 °C. There were usually 2 to 3 days between cycles in order to allow the lipid to gravitationally equilibrate. Pictures were taken after equilibration and it was observed whether the MLVs in each sample floated or sank. For the second neutral buoyancy experiment the samples were hydrated as before and held at 21.4 °C for 6 days before the first cycle. Thermal cycling consisted of heating to 51.2 °C, holding for $\sim$30 minutes, and then cooling to 21.4 °C. The time between thermal cycles varied. It may be noted that previous practice in our lab used centrifugation to reduce the gravitational equilibration time to the order of an hour,[8,10] but pictures like Fig. 1 are more difficult to obtain in centrifuge tubes.

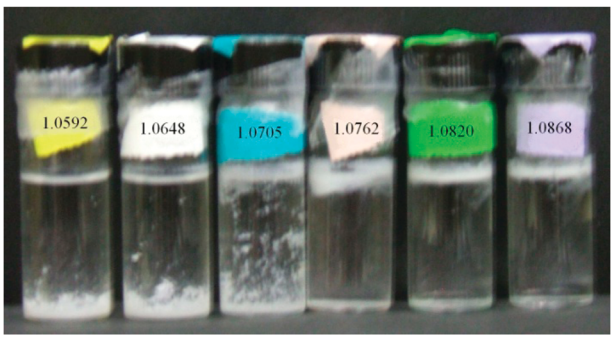

Fig. 1 Neutral buoyancy samples. The solvent densities ρ_{sol} (g ml^{-1}) in the vials are shown. The white MLV dispersions of DPPC lipid sank in the two less dense solutions on the left and floated in the three more dense solutions on the right. Neutral buoyancy was achieved in the vial with density 1.0705 g ml^{-1}. Temperature was 21.2 °C.

Thin layer chromatography (TLC)

TLCs performed on samples after the densimetry experiments and the neutral buoyancy experiments showed less than 1% degradation.

3. Results

Densimetry

Fig. 2 plots lipid molecular volumes obtained from the densimeter. Remarkably, after nine thermal cycles the lipid volume apparently decreased by 3.5% at 10 °C. This is as large a change as occurs at the main transition in the first heating scan. Each scan clearly shows the large main transition and the lower, so-called pretransition that exhibits the usual hysteresis between heating and cooling scans.[2,11] Although a subgel transition occurs in DPPC bilayers at 14 °C,[12,13] transformation into the subgel phase is very slow above 6 °C.[12] We chose not to go to lower temperatures in order to avoid the complications of forming incomplete subgel phases.[14]

Although it is customary to report molecular volume[6] or methylene volume,[4,5,7,15] for this study it is important to show the raw data for the density of the dispersions as in Fig. 3 to allow a comparison to water. The reason that the percentage changes in Fig. 3 are so much smaller than those in Fig. 2 is that the density in Fig. 3 includes 96% water.

In Fig. 4 the densimeter results are expressed as the volume of the CH_2 group *versus* temperature. The data were converted from densities to CH_2 volumes using the equations in Jones *et al.*[4] in order to be able to directly compare our results to theirs. After nine cycles, the CH_2 volume had decreased even more than reported by Jones *et al.*[4]

Details of the sequence of density changes at 12 °C in the gel phase are shown in Fig. 5a. The main increase in the density occurred between a heating scan and the following cooling scan and there was little change in density between the previous cooling scan and the next heating scan even though the total elapsed time was greater between the end of the cooling scan and the beginning of the subsequent heating scan. Details of the sequence of density changes at 49 °C in the fluid phase are shown in Fig. 5b. The density increased between the cooling scan and the subsequent heating scan, whereas it slightly decreased between the heating scan and the rapidly ensuing cooling scan. Note, however, that the scale for the changes in density in Fig. 5b is much smaller than the scale in Fig. 5a, so the overall changes in density at 49 °C are much smaller than at 12 °C, consistent with Fig. 3.

Fig. 2–5 show a most important result. After the ninth cycle, the sample was remixed within the densimeter by withdrawing the dispersion into the syringe used for loading and then reinjecting for a total of ten times. This was done gently, as with initial loading, to avoid introducing air bubbles into the vibrating tube. The density read by the densimeter upon the first heating scan after mixing was close to the initial value of freshly loaded DPPC dispersions. The density change upon the subsequent cooling scan followed the previous pattern shown in Fig. 5.

The results in Fig. 2–5 were confirmed with a second sample that was run for 10 heat/cool scan cycles following the same

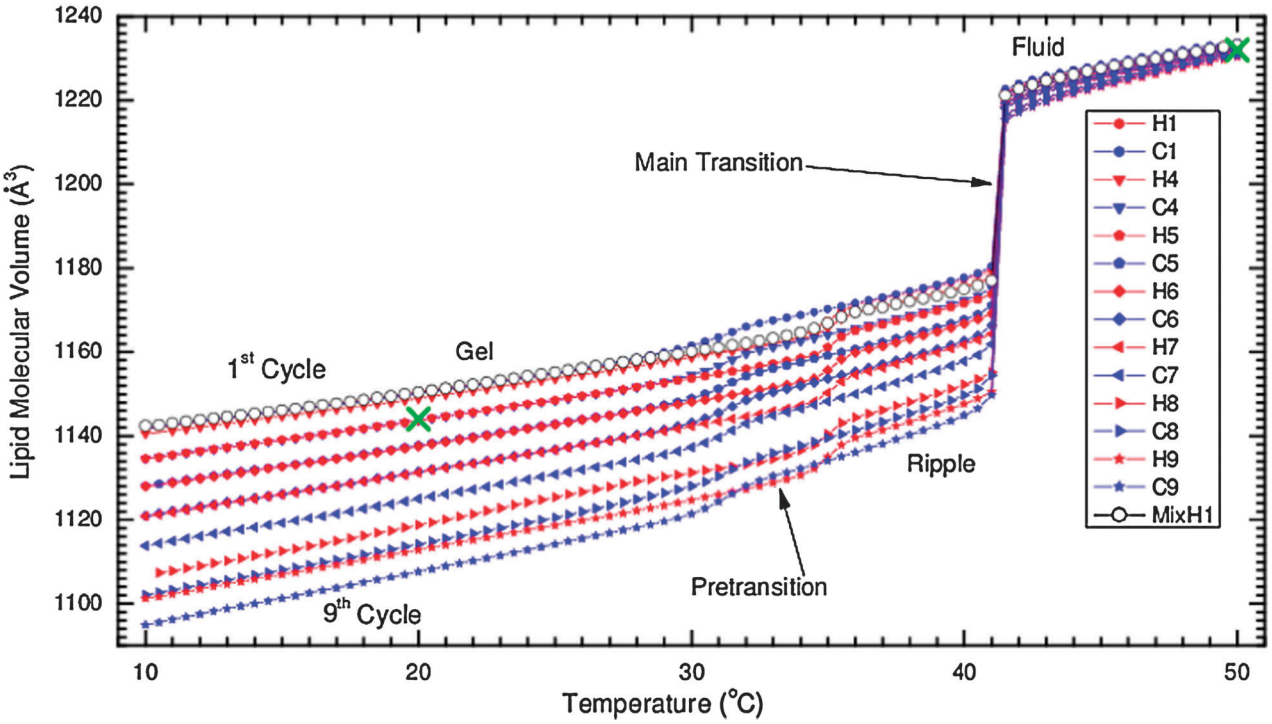

Fig. 2 Apparent molecular volume of a DPPC dispersion *vs.* temperature from repeated densimeter heating (red symbols) and cooling (blue symbols) scans. Heating and cooling scans with the same symbol constitute one thermal cycle. Cycles 2 and 3 are not shown for clarity. The white circles show the first heating scan after remixing. The two green X's compare literature values at T = 20 °C and 50 °C.[6]

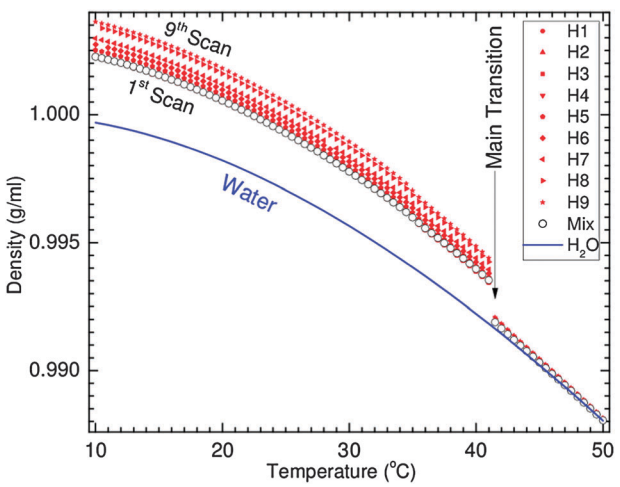

Fig. 3 Apparent density *vs.* temperature obtained from sequential densimeter heating scans of a dispersion of 50 mg of DPPC in 1.2 g of H_2O. The legend indicates the sequence. The blue line shows the measured density of water. The white circles show the heating scan after remixing. Fig. 2 used these raw data.

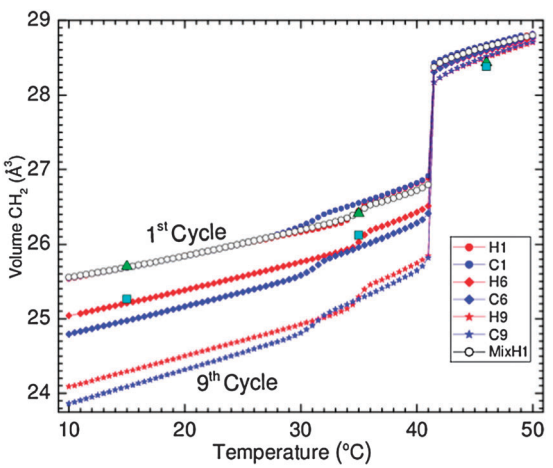

Fig. 4 Volume of CH_2 Group *versus* temperature. Only 3 cycles, and the heating scan after mixing are shown for clarity. The volumes decreased until the mixing scan. The green triangles show the results at 3 temperatures from the first heating scan done by Jones *et al.* and the cyan squares are the results from their final scan.[4]

protocol. Density also increased, although the total increase was only about half as large as for sample 1. Furthermore, when we mixed the sample and ran it through one more heat/cool cycle, we again saw the density revert close to its original value. At the specific temperatures in the gel and fluid phase, we also saw the same patterns as in Fig. 4. However, the pattern appeared more quickly than for the first sample; a clear increase in density at 12 °C occurred between the first and second heating scans. Both samples also had larger apparent density increases between the later scans than between the earlier scans.

The DMA 5000M allows capturing a picture of the U-tube. Fig. 6a shows a sample freshly loaded into the densimeter. Fig. 6b shows the sample that gave the results in Fig. 2–5 after nine thermal cycles and Fig. 6c shows the second sample after 10 thermal cycles. Both have a visible meniscus and the dispersion appeared less homogeneous than upon first loading in Fig. 6a.

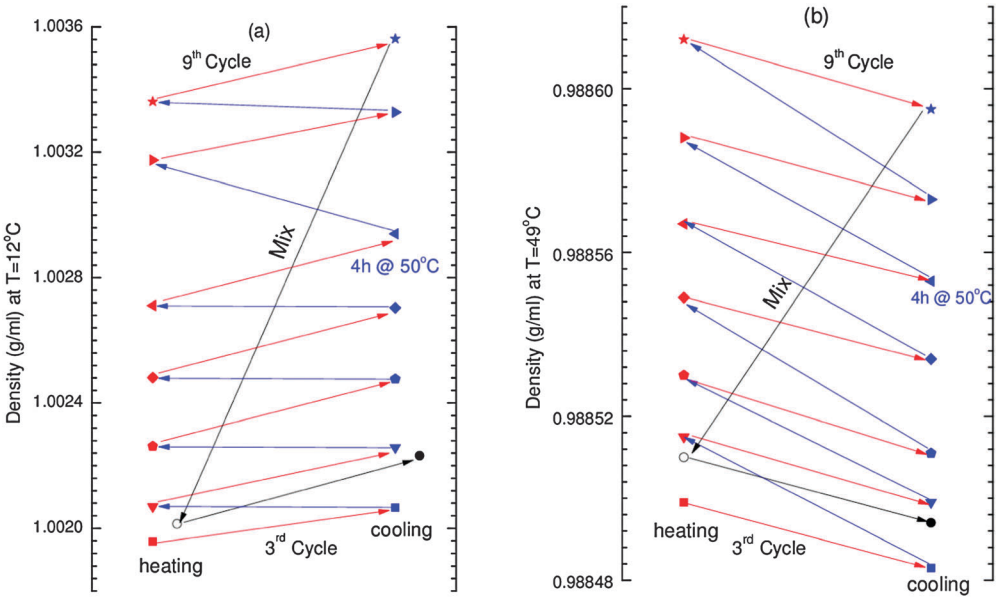

Fig. 5 Densities of a DPPC dispersion (a) for $T = 12$ °C and (b) for $T = 49$ °C obtained during heating scans (red symbols) and cooling scans (blue symbols), using the same symbols as in the Fig. 2 legend to indicate the time sequence, which is also indicated by the arrows here. The first two scans had negligible density change and are not shown. Wait times between the end of heating scans and the beginning of cooling scans were typically 10–30 minutes unless noted otherwise. The white and black circles show the densities of the heating and cooling scans, respectively, after mixing.

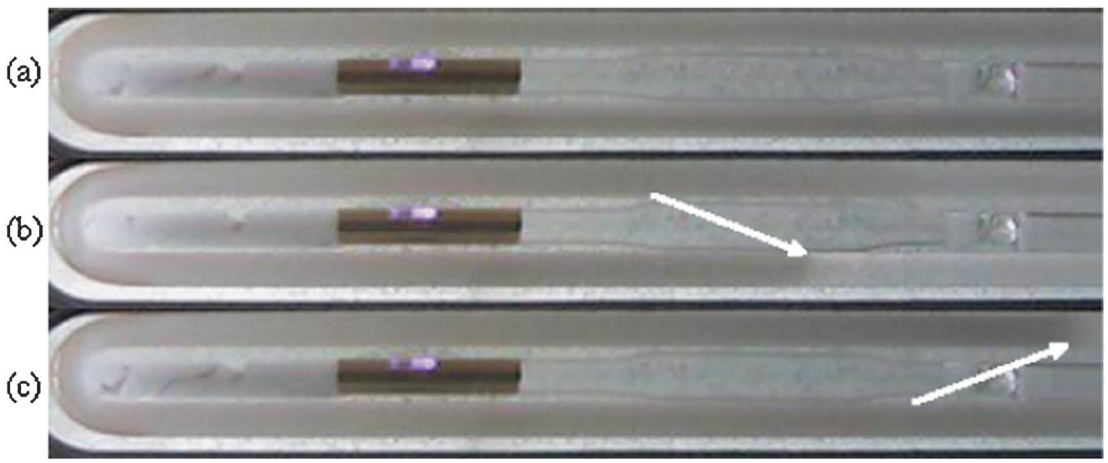

Fig. 6 Pictures of the densimeter U-tube when (a) a sample was initially placed in the densimeter, (b) after the first nine thermal cycles for sample 1, and (c) after ten thermal cycles for sample 2. Arrows point to visible menisci. All pictures appear brighter at the left due to the light in the densimeter.

Neutral buoyancy

Fig. 7a shows the lipid dispersions in six mixtures of D_2O/H_2O before thermal cycling and Fig. 7b shows the same dispersions after ten thermal cycles. The lipid MLVs were clearly denser than the solution for the two less dense solutions on the left and less dense than the two denser solutions on the right. In Fig. 7b it appears that neutral buoyancy occurred in the solvent with density 1.0681 g ml^{-1}. In Fig. 7a, it appears that neutral buoyancy density would have occurred between 1.0681 g ml^{-1} and 1.0730 g ml^{-1}, but closer to the former value.

From these results, we estimate that the percentage decrease in density after ten thermal cycles was less than 0.12%. This is also an upper estimate for the percentage increase in lipid volume; it is far smaller than the percentage decrease in lipid volume of 3% at 20 °C shown in Fig. 2. It may also be noted that the volume per lipid corresponding to 1.0681 g ml^{-1} is 1141 $Å^3$, compared to the range 1141–1145 $Å^3$ reported in the literature.[6,16]

It should be noted that the pictures after each thermal cycle were not as clear as those in Fig. 7 because less time was allowed for gravitational equilibration after all dispersions floated at the higher temperatures during thermal cycling. It seemed possible that some of the dispersion could be getting stuck to the vials or the meniscus or that there was too much lipid in the vials, so one third of the lipid was removed from

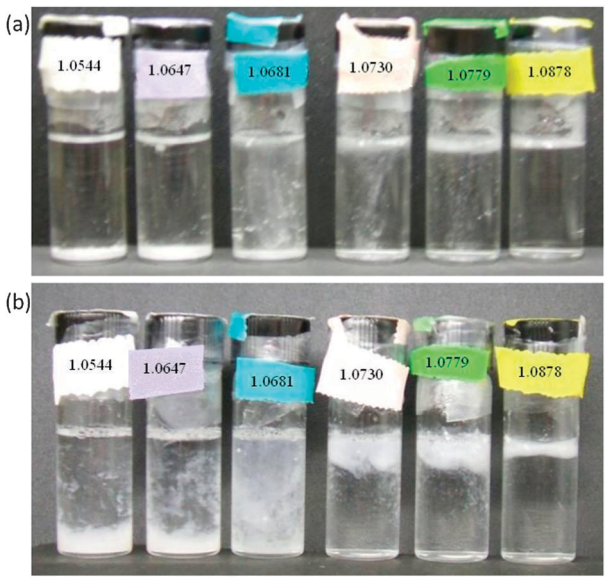

Fig. 7 DPPC dispersions in six vials of D_2O/H_2O solvent with densities indicated, (a) before thermal cycling and (b) after ten thermal cycles. Before (a) the samples were kept at 21.4 °C for six days to allow gravitational equilibration. The pictures in (b) were taken after the samples were shaken and then allowed to settle for seven days.

each vial and the samples were shaken after the tenth thermal cycle. The samples were then kept at 21.4 °C for 7 days with no more thermal cycling. Shaking also simulated the remixing of the densimeter samples after thermal cycling. However, the results from intermediate scans were consistent with the results shown in Fig. 7.

Small differences occurred in another neutral buoyancy experiment. A vial with solution density 1.0705 g ml^{-1} achieved neutral buoyancy after five thermal cycles, but some of the MLVs floated at the beginning and some sank after eight cycles (see Fig. 1). The lipid always floated in a vial with solvent density 1.0762 g ml^{-1} and always sank in a vial with density 1.0648 g ml^{-1}. We estimate that the density increased by less than 0.2% in that experiment.

4. Discussion and conclusions

The disagreement between the results of the neutral buoyancy method and the densimeter method for the gel phase of DPPC bilayers means that one of the methods has an artifact. Although the neutral buoyancy method can only give results at one temperature at a time, it is difficult to see how it can have an artifact.[8] Our data strongly lead to the conclusion that the densimeter is subject to an artifact, namely, that the MLV dispersion is redistributed within the vibrating U-tube during repeated thermal cycles.

The first clue to this artifact is seen in Fig. 5. For temperatures at which the apparent density of the lipid is close to that of water, there is little change during repeated thermal cycling compared to the change that occurs at temperatures at which the density of the lipid is greater than that of water. The resonant frequency of the U-tube is determined primarily by the density in the free end of the tube. If the lipid becomes redistributed toward that end, then the density would increase there at lower temperatures but not at higher temperatures, consistent with the changes in Fig. 5. Such a redistribution of lipid is indicated by Fig. 6 that shows a meniscus appearing after many cycles with the denser side closer to the free end of the U-tube. This redistribution hypothesis is supported by our result that the density returns to its initial value after remixing the dispersion back to its more uniform state.

We were surprised that this artifact occurs because the densimeter U-tube is oriented horizontally to avoid the gravitational effects that are exploited by the neutral buoyancy method. However, when the tube vibrates with one end pinned and the other end free, there is a centrifugal driving force in the direction of redistribution of denser material to the free end. The speed and uniformity of such a redistribution appears to be variable, beginning only after two thermal cycles for sample 1 upon initial loading, and then undergoing a large change between the seventh and eighth cycles. The final change for sample 1 was almost twice as large as for sample 2, and the meniscus for sample 1 had moved further into the tube in Fig. 6. The result that the changes didn't seem to slow down over time also weighs against the hypothesis that they are due to slow equilibration. Instead, they appear to be due to gross mass redistribution that is subject to complex tectonics.

To investigate if the increases in densities were due to the vibrations of the tube, or to the lipid going through one or other of the phase transitions, we studied a third densimetry sample. Temperature was held constant at 20 °C for 18 days and then at 41.2 °C for 14 days, followed by seven cycles between 38 to 44 °C through the main transition, followed by seven cycles between 20 to 38 °C through the lower transition, finally followed by seven of the original cycles between 10 to 50 °C. Interestingly, this sample showed negligible change in density during any of these procedures. Although this third sample failed to provide new information as to what factors facilitated the tectonic shifts, it reinforced the results from the first two samples that the amount of densification is quite variable. This is also consistent with the published results that there was no densification for MSPC, in contrast to SMPC, DMPC and DPPC.[4,7]

The Anton Paar DMA 5000M densimeter is a very convenient instrument to use. Unlike the more laborious neutral buoyancy method, it quickly obtains densities as a function of temperature. An older differential device also obtained lipid volume as a function of temperature,[17] but it was also difficult to use and it obtained only relative, not absolute volumes, so the neutral buoyancy method was used to pin the absolute scale.[5] However, based on our experience in this study, we suggest that, if anomalous results are obtained with a densimeter, then the neutral buoyancy method should be considered for substantiation. It also appears that the first densimeter scans of dispersions are likely to be more accurate than subsequent scans provided loading does not introduce air bubbles. Of course, it is valuable to be able to scan both with increasing and decreasing temperature in order to examine hysteresis, such as occurs in the lower transition of DPPC. We suggest a scan sequence of heat, cool, heat, mix, cool, heat, cool; this protocol would give first and second scans for both heating and cooling.

Volume compressibility in condensed matter phases is usually quite small corresponding to large energy changes for compression.

One would expect changes in volume to even more strongly affect other properties, such as the enthalpy of the phase transitions, X-ray diffraction, and NMR order parameters, but those properties do not change after thermal cycling.[4,7] Consistent with this and our demonstration of the densimetry artifact, we find that there is also no gradual decrease in the volume of gel phase bilayers. We conclude that the gel phases of saturated, same chain phosphatidylcholine lipid bilayers are stable, robust, and well characterized.[6]

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