Phase transition behavior of single phosphatidylcholine bilayers on a solid spherical support studied by DSC, NMR and FT-IR

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ABSTRACT For the first time, the chain melting transition from the gel phase to the liquid crystalline phase of a single DPPC bilayer on a solid, spherical support (silica beads) is observed by differential scanning calorimetry (DSC). This transition is remarkably cooperative, exhibits a transition temperature T_m which is 2°C lower than usually found for DPPC multilamellar vesicles and its excess enthalpy is about 25% less than in DPPC multilayers. 31P- and 2H-NMR data as well as FT-IR data provide evidence that despite the highly asymmetric characteristic of the model system, the whole single bilayer undergoes the transition at T_m, i.e., there is no decoupling of the two monolayer leaflets at the main phase transition. Furthermore, our results show that the formation of the ripple ($P_{\beta'}$) phase is inhibited in single bilayers on a solid support. This result confirms a conclusion which we reached previously on the basis of neutron scattering data obtained on planar supported bilayers. The most likely reason for this inhibition as well as for the above mentioned thermodynamic differences between multilamellar vesicles and supported membranes is a significantly higher lateral stress in the latter. Moreover, the exchange of lipids between two populations of spherical supported vesicles (DMPC and chain perdeuterated DMPC) is studied by DSC. It is shown that this exchange process is symmetric and its half-time is a factor of 3–4 higher than observed for small sonicated DMPC vesicles.

INTRODUCTION

Phospholipid bilayers on a solid support represent a rather new, geometrically well defined model system with unique features for studies of membrane structure and of protein-membrane interaction. So far, supported bilayers were used in numerous fluorescence studies on lipid lateral diffusion (1) and on protein-bilayer interaction (2–3). Infrared spectroscopy on supported bilayers in the attenuated total reflection mode is another method coming up for studies of protein-membrane-interaction (4).

Recently, we introduced specular reflection of neutrons (5) as well as deuterium and phosphorus NMR (6–7) as powerful tools for investigating the structure and the dynamics of single lipid bilayers on a spherical solid support. These experiments provided evidence that some physical properties of supported bilayers such as the order parameter distribution function along the bilayer normal and certain dynamical parameters are similar to those observed in multilamellar vesicles. However, significant differences are indicated for the phase behavior of supported bilayers. In contrast to multilamellar vesicles, no pretransition (i.e., a transition between the $L_{\beta'}$ and the $P_{\beta'}$ phase) can be observed by neutron reflection on a DMPC planar supported bilayer (5). Moreover, previous NMR experiments indicated that (for spherical supported bilayers) part of the bilayer detaches from the support during the transition from the fluid phase to the gel phase (6). Finally, single bilayers on a solid support represent a highly asymmetric system, since one monolayer interacts with the bulk water whereas the other faces the solid state surface via an ultrathin structured water layer. It is not yet established whether and to what extent such asymmetries modify the phase behavior of such bilayers, in particular the cooperativity and the temperature of the main phase transition. More detailed knowledge on this subject is required in order to assess the usefulness and the potential of this model system for spectroscopic membrane studies.

Differential scanning calorimetry (DSC) is widely established as a unique method for measuring phase transition temperature, excess enthalpy and cooperativity of lipid bilayers (for review, see references 8–9). So far, the study of single bilayers on a solid support by DSC was prevented by the geometrical properties of such samples. However, our recent introduction of spherical supported vesicles (SSV) (6–7) changed this situation. As SSV's we denote nearly monodisperse spherical beads of glass or silica, coated in the average with one lipid bilayer which is separated from the bead surface by a water layer of 15–20 Å thickness. In contrast to planar supported bilayers, SSV's are well suited for DSC studies owing to their adjustable size in the submicrometer range and the comparatively large surface coated with a lipid bilayer per sample volume.

In this work, we apply DSC in combination with the attenuated total reflection (ATR) technique of Fourier-transform infrared spectroscopy (FT-IR) as well as deuterium NMR for the elucidation of the phase behavior of SSV's coated with DPPC.

MATERIALS AND METHODS

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-(perdeuterio)-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC-d54) were obtained from Avanti Polar Lipids (Alabaster, AL) and were used for all prepara-

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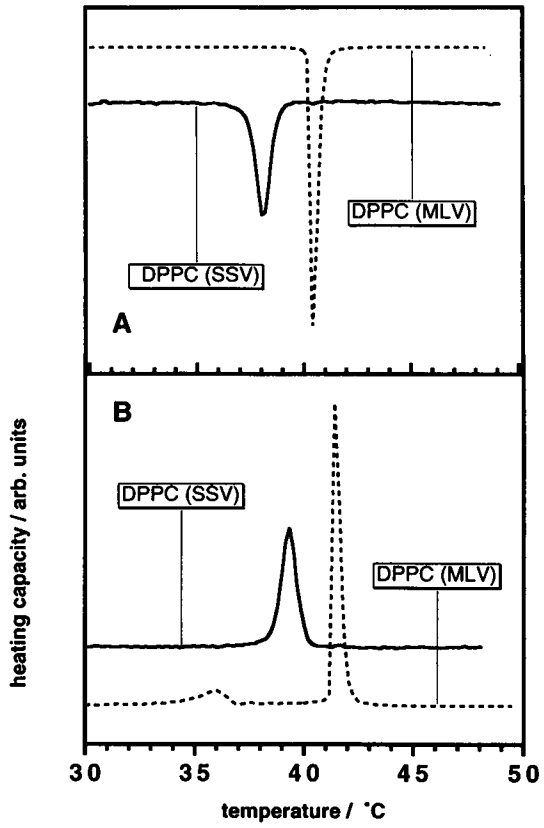

FIGURE 1 DSC exotherms (*top*) and endotherms (*bottom*) of DPPC in two types of vesicles: multilamellar vesicles (*dotted line*); spherical supported vesicles with a diameter of 640 nm (*full line*).

tions of supported and nonsupported vesicles without further purification. Silica beads of highest purity were kindly provided as a gift by Dr. Meyer from the Degussa AG (Hanau, Germany). The size of the beads is $R = 320 \pm 20$ nm. The size distribution of the beads was determined by electron microscopy and was found to be nearly symmetric about the average.

Vesicles preparation

Multilamellar vesicles (MLV) were prepared by weighting the appropriate amount of dry phospholipid (DPPC or DMPC) into a test tube and adding ultrapure water (Millipore Corp., Bedford, MA) to give a concentration of 25 mg/ml. After mild vortexing, the sample was incubated under gentle shaking for 15 min at a temperature which was 5°C above the phase transition temperature of the lipid. Spherical supported vesicles were prepared by condensation of small unilamellar vesicles on the silica beads according to procedures described in detail previously (6). The small unilamellar vesicles were obtained by treatment of the MLV with a titanium rod sonifier under a nitrogen atmosphere in a thermostated water bath, where the latter kept the sample at 45°C (for DPPC) and at 25°C (for DMPC) during the sonication. For details see reference 6.

DSC

The DSC measurements were performed using two instruments: a Microcal MC-2 (Microcal Inc., North Hampton, MA) and a Privalow DASM-4 (Privalow, Russian Academy Workshops, Pascino, CIS). Both calorimeters are interfaced to IBM AT computers and the data acquisition is controlled by these devices. Only the DASM-4 was used for measurements in the descending temperature mode (scan rate 30°C/h). The scan rate for all measurements in the ascending temperature mode was 30°C/h. Control measurements were done at 10°C/h (MC-2) but no significant changes of the endotherms of supported systems were observed. In particular, we observed no reduction of the HWHM of the endotherms at the lower scan rate. Nevertheless, this does not rule out that the MLV endotherms are slightly broadened is due to a calorimetric lag. Data analysis was performed using either the MC-2 software (for MC-2 data) or a home written software for the DASM-4 data. The transition enthalpy ΔH was calculated after baseline subtraction according to criteria previously described (10).

The lipid concentration in the samples was 0.6 mg/ml. For spherical supported vesicles, the lipid concentration was calculated from the number of beads in the sample volume according to a method described previously (6).

NMR

Deuterium NMR and phosphorus NMR experiments were performed at 61 and 161 MHz, respectively, using a Varian VXR-400 spectrometer with a high power equipment. For further experimental details see reference 11.

FT-IR

A Nicolet 60 SXR spectrometer equipped with a liquid nitrogen cooled MCT detector was used. The interferograms were acquired at a resolution of 2 cm^{-1} using a home built horizontal ATR unit which contained a $30 \times 10 \times 2$ mm silicon crystal. The aqueous SSV sample (same lipid concentration as used for the DSC measurements) was deposited as a $\approx$2 mm thick layer of beads on the top side (large face) of the crystal. The bottom side of the crystal as well as the crystal holder were thermostated by a computer controlled circulating Julabo FC 10 water bath (Julabo, Seelbach, Germany). The temperature was measured on line by a Pt-100 thermocouple at the top surface of the crystal and was held constant within ±0.1°C. To minimize temperature gradients, the whole ATR assembly is thermally insulated by a 1 cm thick polystyrene foam layer. The equilibration time at each temperature step was 15 min. before the starting of the data acquisition. 1,000 transients were accumulated, apodized by a Happ-Genzel function before the Fourier transform. The difference spectra were obtained by dividing sample and reference spectrum by the instrumental background and afterwards subtracting the reference spectrum (silica beads without lipid in water, measured at the corresponding temperature) from the sample spectrum.

RESULTS AND DISCUSSION

Fig. 1 shows the DSC endotherms exhibited by DPPC in two types of vesicles: (*a*) the well known multilamellar vesicles (MLV) and (*b*) spherical supported vesicles (SSV) of 640 nm diameter. The thermodynamic parameters obtained from these data are listed in Table 1. The

TABLE 1 Thermodynamic parameters of DPPC in two types of vesicles (multilamellar vesicles, MLV and spherical supported vesicles, SSV), extracted from the DSC endotherms shown in Fig. 1

	T_p	T_m	ΔH	$\Delta T_{1/2}$
	°C	°C	kcal/mol	K
MLV (heating scan)	36.0	41.4	7.5	0.2
MLV (cooling scan)	—	40.4	—	0.2
SSV (heating scan)	—	39.3	5.5	0.75
SSV (cooling scan)	—	38.0	—	0.8

The error of the temperature is ±0.05°C, that of the enthalpy is ±0.5 kcal/mol.

two systems show significant differences regarding their phase behavior, most noteworthy are the following features: (*a*) The pretransition in the SSV's has disappeared and the main phase transition from the gel to the liquid-crystalline phase takes place at a temperature which is 2°C lower for the supported vesicles. (*b*) The half-width of the main phase transition is a factor of about 4 broader for SSV's than for MLV's. (*c*) The enthalpy of the main phase transition is ~25% lower for the SSV's. (*d*) The hysteresis observed between the T_m values of heating and cooling scans (i.e., ascending and descending temperature scans) is a factor of 1.3 larger for SSV's, when compared to MLV's.

In spite of the observed broadening of the endotherm by a factor of 4, the phase transition exhibited by the SSV's is still surprisingly sharp (i.e., highly cooperative). After all, the supported system consists of a single bilayer only and thus, can be considered as a quasi two-dimensional system. In MLV's, however, interbilayer interactions such as van der Waals- or electrostatic interactions in general cannot be neglected and they can be expected to increase the cooperativity of a phase transition.

Another very interesting feature of the SSV phase transition is the finding that no splitting of the endotherm can be observed although the SSV single bilayer represents an extremely asymmetric system. The outer monolayer is in contact with the bulk water only while the inner monolayer is separated from the solid state silica surface by an ultrathin water layer of 15 ± 4 Å (5, 6). Thus, the attractive interaction force which keeps the single bilayer tightly attached to the silica surface, even upon traversing a phase transition or during the washing of the sample in buffer, can be expected to excert a different effect on the two monolayer leaflets. Moreover, the trapped ultrathin water layer is likely to consist of highly structurated water molecules which can modify the headgroup hydration of the inner monolayer. Our finding of a single step phase transition of the SSV single bilayer in both heating and cooling mode can be explained in two ways: first, we could assume that the inner monolayer is completely withdrawn from the phase transition due to the interaction with the silica surface via a structurated water layer. Second, we can interpret the data in terms of a complete coupling between the two monolayers.

The first hypothesis is supported by the finding that the enthalpy ΔH for SSV's is significantly lower than observed in MLV's. It should be emphasized, however, that such a reduction of ΔH can also be caused by a modulation of the intermolecular interaction between the phospholipid tails, such as due to the addition of steroid molecules like cholesterol or bile salts (12–14).

Striking evidence against the validity of the first hypothesis comes from NMR measurements of the SSV bilayers. Fig. 2 shows 31P- and 2H-NMR spectra of a SSV bilayer in the liquid crystalline phase state. Since the 2H-NMR spectra were obtained using chain perdeuterated DPPC, this spectrum provides unique information about the orientational order in the tail region while the 31P-NMR spectrum gives this information about the headgroup region of the bilayer. Both spectra are characteristic for an axially symmetric motion and a spherical distribution of the molecular director axies. They clearly indicate that all phospholipids are in the liquid crystalline phase state. Moreover, it has been previously demonstrated that the orientational order parameter profile along the tails of SSV bilayers is in good agreement with that obtained for MLV's at the same temperature above T_m (6).

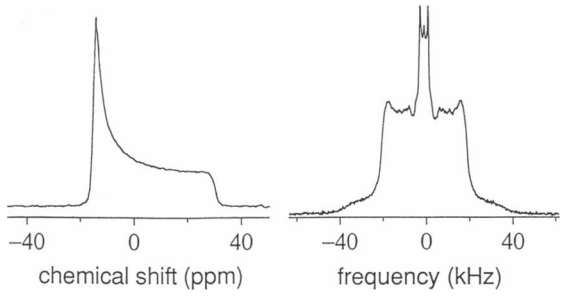

FIGURE 2 2H-NMR (*right*) and 31P-NMR (*left*) spectra of spherical supported vesicles (640 nm diameter) in the liquid crystalline phase state (T = 45°C). For 2H-NMR, chain perdeuterated DPPC-d62 was used.

The NMR data effectively rule out that the presumed withdrawal of lipids from the phase transition in SSV's can be accomplished by keeping the inner monolayer in the highly ordered gel phase state above T_m. Nevertheless, one might still speculate about the opposite, namely that the observed reduction in ΔH is caused by inhibition of the transition of the inner monolayer from the liquid crystalline to the gel state, i.e., that the fluid phase state is retained in the inner monolayer at temperatures below T_m. Evidence against this presumption is provided by the data shown in Fig. 3. The 2H-NMR first moment of a SSV and a MLV sample as shown in Fig. 3 *A* gives the average quadrupolar splitting $\langle \Delta\nu_Q \rangle$ of the two samples as a function of temperature (cf. reference 15 for a detailed discussion of moments). The value of $\langle \Delta\nu_Q \rangle$ and thus, the average degree of orientational order in the two phase states is clearly similar for both samples, whereas the only significant difference is the transition width. This broadening of the SSV phase transition was observed also by DSC as reported above. A similar result is obtained by FT-IR measurements (Fig. 3 *B*). The frequency shift of the symmetric CD_2 stretching vibration with temperature depends on the avergage number of gauche conformers per fatty acyl chain. These frequencies are in good agreement with those obtained for MLV's (denoted by a + in Fig. 3 *B*) in both phase states, i.e., the average number of gauche conformers per lipid molecule is similar for both systems. The suspected presence of a measurable fluid lipid component in SSV's

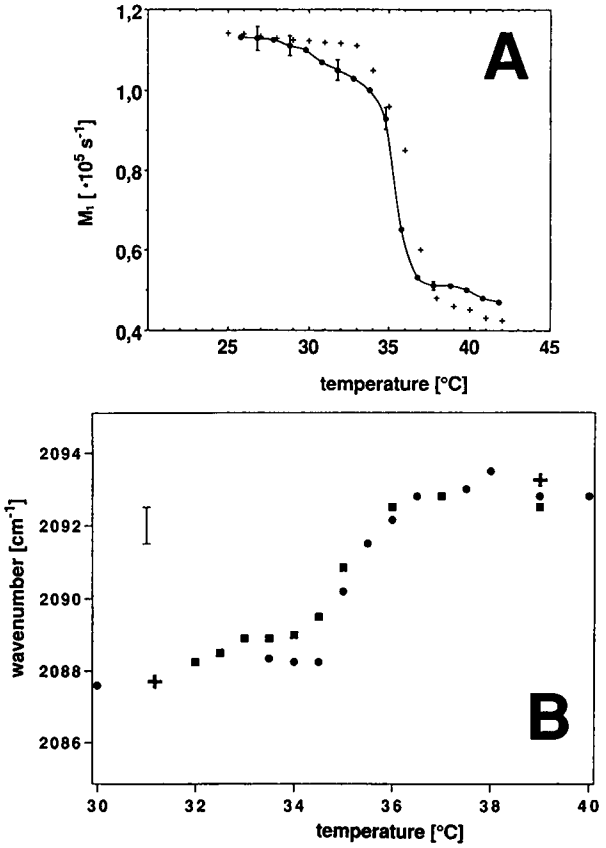

FIGURE 3 (*A*) 2H-NMR first moment M_1 as a function of temperature for spherical supported vesicles (●) with a diameter of 640 nm and for a multilamellar vesicles sample (+) of chain perdeuterated DPPC-d62. (*B*) Symmetric CD_2 stretching vibration versus temperature for spherical supported vesicles (640-nm diameter) of DPPC-d62 obtained in the ascending temperature mode (●) and in the descending temperature mode (■). For comparison, the vibration frequency obtained for multilamellar vesicles of DPPC-d62 in the gel phase state and in the liquid crystalline phase state, respectively, are shown (+). Note that the main phase transition temperature of chain perdeuterated DPPC-d62 is 4.2 ± 0.2°C lower than for protonated DPPC.

at temperatures below T_m would be revealed by a higher vibration frequency as compared to MLV's at the same temperature, which is clearly not the case. A similar consideration rules out that a significant amount of SSV lipids remains in the gel phase state above T_m.

Summing up the results presented in Fig. 3, the lower excess enthalpy ΔH observed for SSV's by DSC cannot be explained by a partial withdrawal of gel phase lipids from the phase transition or by partial prevention of the transition into the gel phase state. We conclude that there is indeed a coupling between the outer and the inner monolayer in SSV's of DPPC. However, we have no conclusive explanation yet for the reduced ΔH but we suspect that lateral stress along the bilayer plane, caused by the abrupt change of the molecular area at T_m might be the origin of it.

That lateral stress plays an important role for the SSV phase behavior can be concluded also from the finding that no pretransition can be observed. We obtained a similar result recently for a planar supported DMPC bilayer employing specular reflection of neutrons (5). Hence, the missing pretransition in SSV's is not due to the spherical shape of the solid support in particular, but is a general feature of solid supported membranes. We conclude that the supported bilayer does not form the ripple phase $P_{\beta'}$, but forms a phase analogous to the low temperature $L_{\beta'}$ phase instead. This conclusion is in good agreement with results obtained by Needham and Evans (16), who observed a nonrippled phase (denoted as $L^*_{\beta'}$) in DMPC at intermediate temperatures using micromechanical techniques. They demonstrated that the formation of this phase is possible only under lateral stress. The lateral stress that the SSV bilayer acquires at the transition from the fluid to the gel phase (due to the 5–8% reduction of the volume per molecule) is obviously sufficient to prevent the formation of the $P_{\beta'}$ phase.

One of the most intriguing features of SSV's is their potential to cause a mismatch between bilayer and bead surface by variation of the temperature. Since the volume expansion of the silica bead is negligible compared to the thermal expansion coefficient (in two dimensions) of the lipid bilayer, a temperature change by 10°C off the coating temperature causes an area mismatch of $\approx \pm 5\%$. Hence, by coating the SSV's at a temperature T_{coat} sufficiently high above T_m, one should be able to control the lateral stress of the membrane by adjusting the temperature of the SSV's at a level $T_m < T < T_{coat}$. At the transition to the gel state at the temperature T_m, there is an additional significant reduction of the bilayer area. Since the lateral stress the bilayer can take is limited (a shrinking by more than 10% of the initial area usually leads to the collapse of a large unilamellar vesicle), a breakup and a detachment of the disintegrated bilayer from the beads surface at the transition to the gel phase would be expected.

However, the SSV's used in this study do not exhibit any detachment of bilayers at all. Even washing the SSV's in water with consecutive centrifugation (all done in the gel phase state) does not remove measurable amounts of lipids from the SSV's. This finding is in contrast to our previous work with SSV's, where we observed a detachment of the bilayer due to incubation of the sample at temperatures below T_m using deuterium NMR (6). The reason for this discrepancy is probably that the previous experiments were performed using beads of glass, which contain a number of different ions, whereas for the experiments presented here, silica beads of highest purity were used. The latter exhibit a negatively charged surface whereas the surface charge of glass is rather ill defined. Although the exact nature of the attractive interaction force between bilayer and solid surface is not yet understood, the presence of a continuous negatively charged surface seems to be a prerequisite for a tight adsorption of the bilayer. This conclusion is further supported by the finding that a coating of silica

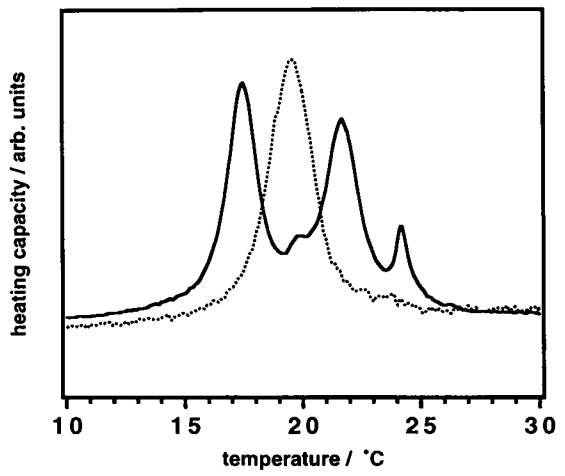

FIGURE 4 DSC mixing endotherms of two populations of spherical supported vesicles (one is coated with DMPC-d54 giving the endotherm at lower temperature and one with DMPC) obtained right after mixing the two populations (*full line*) and after a 24 h incubation of the same sample at 30°C (*dotted line*). See Results and Discussion for details.

beads with a DPPC bilayer containing more than 20 mol% DPPG cannot be achieved in a buffer of low ionic strength (T. M. Bayerl, unpublished results).

In a previous DSC work we established that small unilamellar DMPC vesicles in the liquid crystalline phase can readily exchange lipids by monomeric transfer via the bulk water (10). An interesting question is now, whether SSV's exhibit a similar behavior. In Fig. 4, we present preliminary results on the symmetric lipid transfer between two equimolar SSV populations. One is coated with a DMPC bilayer while the other consists of a DMPC-d54 bilayer. The 4.2 ± 0.2°C lower T_m of DMPC-d54 as compared to the protonated analog enables the observation of the phase transition of both populations separately in one DSC scan. In Fig. 4, the initial and final state of the transfer experiment is presented. Since the mixing behavior of DMPC and DMPC-d54 is nearly ideal, the initial T_m values of each population should undergo a symmetric shift toward the center with increasing incubation time and finally coalesce there into one peak due to the lipid transfer (see reference 10 for details). This is confirmed by the results shown in Fig. 4. However, in contrast to the small unilamellar DMPC vesicles studied previously, the lipid transfer rate between SSV's is lower, the half time (defined as the time after which 50% of the lipids are exchanged) being a factor of 3–4 longer at the same temperature. The small peaks which can be observed in Fig. 4 at 2°C to the right of the large features arising from the two isotopically distinguishable supported DMPC bilayers are caused by a small amount (5–8% of the total lipid) of free sonicated vesicles in the sample. The latter were added in order to monitor the lipid transfer between SSV's and between sonicated vesicles in one scan. We found that their presence has no effect on the lipid transfer between the SSV's, since a sample without any sonicated vesicles gave similar results regarding the SSV's lipid transfer characteristics as reported above.

An interesting question is about the asymmetric lipid transfer between SSV's. Suppose that one SSV population in the above exchange experiment is replaced by SSV's coated with DPPC. Under such conditions, small unilamellar vesicles exhibit a complete asymmetric lipid transfer, i.e., the lower melting lipid (DMPC) is transferred into the vesicles of the higher melting lipid (DPPC) until the DMPC vesicles population ceases to exist. For SSV's, such asymmetric transfer would result in contious "peeling off" of the single DMPC supported bilayer which is unlikely for energetic reasons. Moreover, the DPPC acceptor SSV's are saturated with DPPC and could accommodate DMPC molecules only by increasing the lateral pressure of the single bilayer. Hence, it seems more feasible that in the case of SSV's asymmetric lipid transfer changes into a forced symmetric transfer with a significantly lower transfer rate. This subject is presently under investigation in our laboratory.

CONCLUSIONS

We have shown that a single phospholipid bilayer on a solid spherical support exhibits a cooperative main phase transition similar to that observed in the commonly used multilamellar vesicles while the formation of the ripple ($P_{\beta'}$) phase is suppressed, most likely due to lateral stress in the membrane. The finding that the main phase transition in SSV's is observed at a lower T_m and exhibits a 25% lower excess enthalpy ΔH (as compared to MLV's) in conjunction with an increase of the transition halfwidth by a factor of 4 is a further indication that lateral stress can significantly influence the phase behavior. It also demonstrates the potential of SSV's for spectroscopic studies of single bilayers under well defined lateral stress conditions.

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