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THE PHOSPHOLIPID PACKING ARRANGEMENT IN SMALL BILAYER VESICLES AS REVEALED BY PROTON MAGNETIC RESONANCE STUDIES AT 500 MHz *

JOSEPH R. SCHUH, UTPAL BANERJEE, LUCIANO MÜLLER and SUNNEY I. CHAN **

Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, CA 91125 (U.S.A.)

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Proton magnetic resonance spectra of saturated phospholipids in small unilamellar vesicles have been recorded at 500 MHz on a Bruker WM500 spectrometer. The additional spectral dispersion reveals new structure in the acyl chain resonances. At temperatures near the thermal phase transition, the chain methylene and methyl peaks are split, both showing a broad and a relatively sharp component. Magnetization transfer experiments together with studies in the presence of manganese ions inside or outside the vesicles indicate that the sharp component is to be assigned to the protons from the acyl chains in the inner half of the bilayer and the broad component to chains in the outer monolayer. These experiments demonstrate unambiguously that the extreme surface curvature intrinsic to small unilamellar vesicles induces a profound asymmetry in the packing arrangement of the hydrocarbon chains in the two leaflets of the bilayer and causes the two monolayers to exist in markedly different motional states.

Introduction

Phospholipid molecules in a biological membrane or in an aqueous dispersion undergo highly anisotropic motions [1]. The motional characteristics of the chains are naturally governed by the nature of packing of lipid molecules. It has been argued on geometric grounds [2,3] that the molecular packing in small unilamellar vesicles is dictated by the surface curvature and is highly asymmetric with respect to the inner and outer halves of the bilayer. Thus the motional freedom of chains in sonicated vesicles can be markedly different from that in multilamellar arrays. The first indication that there are motional differences in the chains between vesicles and multilayers came from nuclear magnetic resonance studies [2,4,5]. Unfortunately, the analysis of NMR line shapes in terms of local segmental motion of the hydrocarbon chains and vesicle tumbling has been a controversial subject. It is now known from calorimetric data as well as NMR studies that the thermal phase transition behavior of saturated lipids is dramatically altered when lipid multilayers are sonicated to yield small unilamellar vesicles [2,6,7]. Not only is the thermal transition broadened, the onset of the transition also occurs at a significantly lower temperature. Accompanying these effects is a corresponding decrease in the enthalpy and entropy of the transition as well. Raman studies also suggest [8] a significant difference in the 'intermolecular order' of the chains between multilamellar dispersions and small single-walled vesicles, reflecting a more disordered packing of the lipid molecules in the

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** To whom requests for reprints should be sent.

Abbreviations: NMR, nuclear magnetic resonance; EDTA, ethylenediamine tetraacetic acid; NOE, nuclear Overhauser effect.

vesicles. Thus, surface curvature can have real effects on the structural and dynamical properties of a phospholipid bilayer membrane.

Since the two monolayers of a small bilayer vesicle exhibit curvature which is different in both magnitude and sign, if follows that one should expect structural and motional differences between them. Recent work on small vesicles has in fact emphasized such possible differences between the two halves of the bilayer. ^{19}F-NMR of vesicles comprised of lipids with fluorine substituted on the chain in fact showed chemical shift nonequivalence and resonance width difference between the fluorine labels located on the inside and outside chains of the vesicle [9]. Also choline methyl protons in the two halves of the bilayer exhibit different spin lattice relaxation times [10] in addition to chemical shift nonequivalence [11].

The present paper reports further characterization of small bilayer vesicles by ^{1}H-NMR at 500 MHz. At this frequency, one might expect the additional spectral dispersion to permit resolution of the hydrocarbon chain resonances from the two halves of the bilayer, given the packing arrangements and motional states that have been conjectured. This expectation is clearly borne out in the present study, as, in addition to the split head group resonances, two component peaks are also now observed for both the methylene and the methyl protons. Analysis of these spectra has provided additional insights into the effect of surface curvature on the structural and dynamical properties of bilayer vesicles.

Experimental methods

Materials

Synthetic phosphatidylcholines were purchased from Calbiochem-Boehringer and found to be pure by thin-layer chromatography. Deuterium oxide (99.8 atom%), all salts and EDTA were obtained from Aldrich Chemical Company.

Methods

Vesicles were prepared by ultrasonic irradiation of lipids dispersed in ^2H_2O. The concentration of lipids used was 10 mg/ml in most experiments. Sonication was done with a model W-225R cell disrupter from Ultrasonics Inc. using a microtip at a power setting of 5–6 and a 50% duty cycle for 20–30 min. During this period the sample tube was immersed in a glycerol bath to prevent overheating. The resulting clear solution was then centrifuged for 5 min to remove any large particles.

The samples containing manganese ions were prepared by dilution of sonicated vesicles with appropriate solutions to maintain osmotic balance across the bilayer membrane (see figure legend for details).

Proton NMR spectra were recorded at various temperatures on a Bruker WM500 spectrometer operating at a field strength of 11.74 tesla (500.13 MHz proton frequency). The magnetization transfer experiments were performed by irradiating the acyl resonances until a steady state was reached. The amplitude of irradiation (γH_2) was kept at approx. 30 Hz to prevent spillovers. H_2 was then turned off and a strong nonselective 90° pulse was applied to generate a free induction decay. The sequence was then repeated for signal enhancement.

Results

The 500 MHz proton NMR spectra of dipalmitoylphosphatidylcholine vesicles, recorded at various temperatures, are shown in Fig. 1. Throughout the temperature range examined, the choline *N*-methyl resonance (3.2 ppm) is resolved into two peaks. The larger, downfield component has been shown to arise from lipids located in the outer half of the bilayer, while the smaller peak is assigned to the inner-facing lipids [2]. A similar inside-outside distribution with similar chemical shift differences is also seen for both the choline *N*-methylene signal (3.65 ppm) and the choline PO-methylene peak (4.25 ppm).

In a recent NMR study of distearoylphosphatidylcholine vesicles it was reported that the inner monolayer choline methyl resonance undergoes an upfield shift of approx. 0.04 ppm as the temperature is lowered through the thermal phase transition (T_m) [11]. We have observed the same phenomenon for dipalmitoylphosphatidylcholine with an identical upfield shift of the inner choline methyl resonance of 0.04 ppm, except that the shift begins at about 38°C instead of 47°C in the

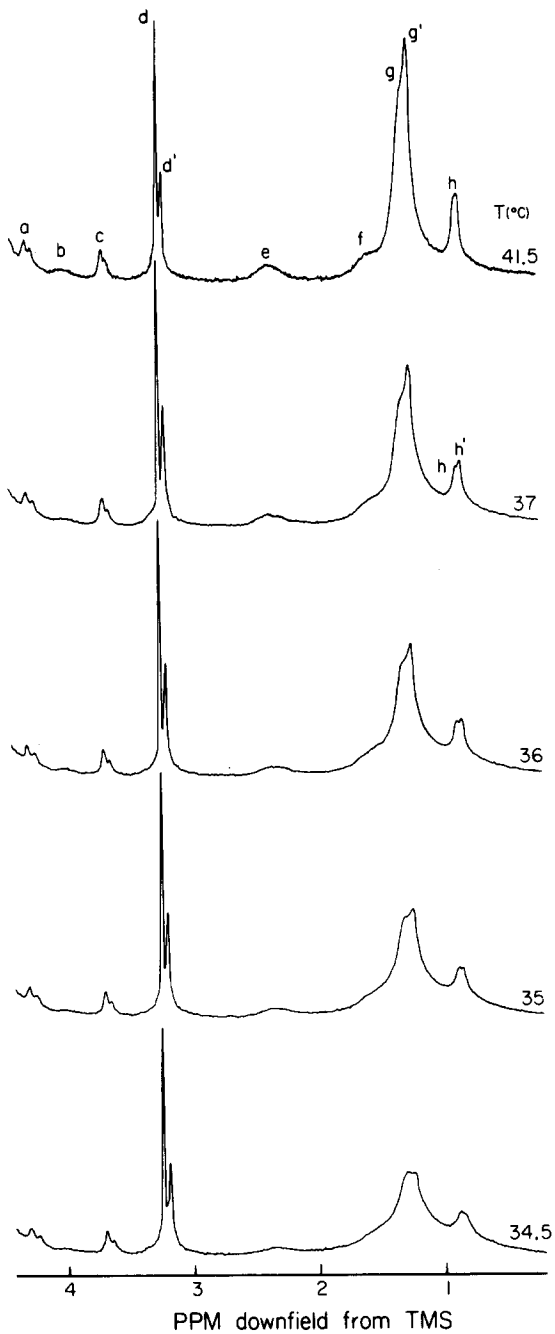

Fig. 1. 500 MHz proton NMR spectrum of dipalmitoylphosphatidylcholine vesicles at various temperatures. Spectral assignments: a, choline PO-methylene; b, glycerol CH; c, choline *N*-methylene; d, choline methyls; e, α-methylenes of the hydrocarbon chains; f, β-methylenes; g, bulk methylenes; h, terminal methyls.

case of distearoylphosphatidylcholine. In addition, both the inner *N*-methylene and the PO-methylene resonances display similar upfield shifts as the temperature is lowered through T_m.

The chain methylene protons alpha to the carbonyl give rise to a broad peak centered at 2.35 ppm which is made up of overlapping components. Below T_m, this signal as well as the β-position methylene centered at 1.55 ppm becomes extremely broad and is difficult to monitor. The remaining methylene protons give rise to a large, asymmetric peak centered at approx. 1.25 ppm. The composite peak appears to be made up of at least two overlapping components: a broad downfield component and a narrower upfield component. Attempts to deconvolute this signal into two resonances yielded an apparent linewidth for the broad component that was two to 3-times larger than the narrow component. Both methylene components broaden and lose apparent intensity as the system is cooled below T_m. These spectral changes are accompanied by a downfield shift of the broad component with the lowering of temperature just above T_m, as illustrated for distearoylphosphatidylcholine vesicles in Fig. 2. Only the broad methylene component appears to have shifted (approx. 30 Hz) over the range of temperature examined.

An analogous behavior is observed for the methyl resonance at 0.85 ppm. This peak is also split into two components. While the sharper component shows no detectable change in its chemical shift when the temperature is lowered, the broad resonance moves downfield by 19 Hz over the range of temperature investigated in the case of dipalmitoylphosphatidylcholine.

Somewhat similar changes were observed for resonances of distearoylphosphatidylcholine and dimyristoylphosphatidylcholine (Fig. 3). In distearoylphosphatidylcholine, the bulk methylenes are better resolved and exhibit a temperature dependence in their chemical shifts similar to that for dipalmitoylphosphatidylcholine. The methyl signal for distearoylphosphatidylcholine, however, seems to be more symmetrical and shows no discernible chemical shift dispersion. In contrast, the methyl and bulk methylene signals of dimyristoylphosphatidylcholine are asymmetric over the full range of temperatures studied.

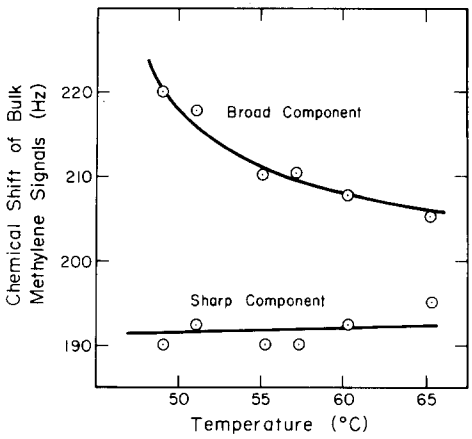

Fig. 2. Variation of the resonance positions of the broad and sharp components of the chain methylene protons from distearoylphosphatidylcholine vesicles vs. temperature. The chemical shifts have been corrected for the effect of temperature on the bulk magnetic susceptibility of the sample by referring them to the sharp component of the terminal methyl signal. Owing to the strong overlap of the two components, only the apparent positions of these resonances are plotted here. The experimental uncertainty in these measurements is ±1 Hz.

Assignment of acyl resonances

On the basis of the available data on lipid organization in vesicles, we have considered three alternative assignments for the split acyl resonances recorded in this work. The possibilities are that the broad downfield and the narrow upfield components correspond to: (A) a rigid upper segment and a more fluid lower segment of the hydrocarbon chain, (B) the outside facing and inside facing lipid populations of the vesicle bilayer, or (C) the acyl chains esterified to the 2-carbon and 3-carbon position of the lipid glycerol moiety.

In order to discriminate between these possibilities, we took advantage of the enhanced relaxation that a paramagnetic ion can have on a proton in its proximity. In one series of experients, isotonic vesicles with manganese ions in the external solution were prepared. In another, vesicles were prepared in manganese chloride and the potential effect of the broadening agent on the lipid molecules in the outer monolayer was quenched by chelating the manganese ions with EDTA. Since the vesicle bilayer is impermeable to the manganese ions under these conditions, the enhanced relaxation brought on by the presence of the paramagnetic ions result in preferential broadening of resonances arising from lipids in the monolayer directly in contact with them [2]. As was expected, the downfield choline resonance is eliminated in the first series of experiments. In the second series of experiments, where both the inner and outer choline resonances were initially broadened beyond detection by the manganese, the outer choline resonance reappears when the external manganese is chelated with EDTA.

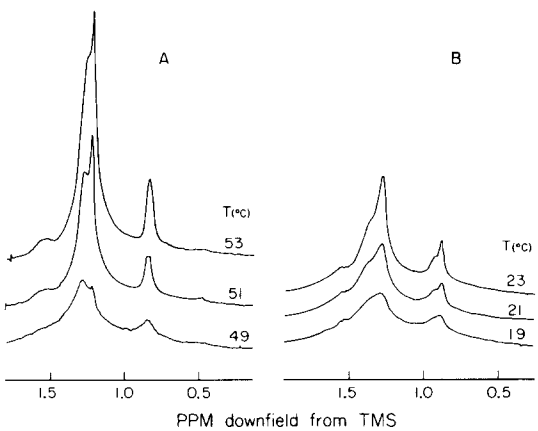

Fig. 3. 500 MHz proton NMR spectrum of distearoylphosphatidylcholine (A) and dimyristoylphosphatidylcholine (B) vesicles at various temperatures.

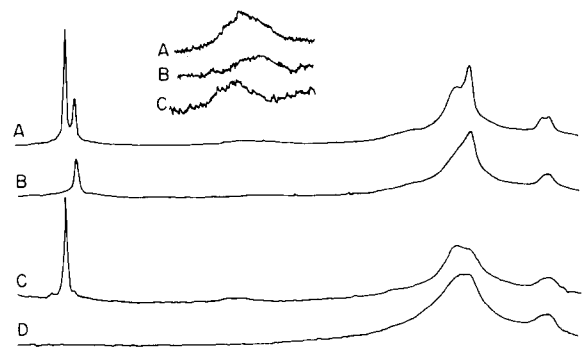

Fig. 4. 500 MHz proton NMR spectrum of dipalmitoylphosphatidylcholine vesicles at 36°C. (A) Vesicles prepared in 90 mM NaCl, diluted 1 : 1 (v/v) with 90 mM NaCl (control). (B) Vesicles prepared as in (A) but diluted 1 : 1 with 90 mM NaCl+0.5 mM $MnCl_2$. (C) Vesicles prepared in 90 mM NaCl+0.5 mM $MnCl_2$, diluted 1 : 1 with 60 mM EDTA. (D) Vesicles prepared as in (C) but diluted 1 : 1 with 90 mM NaCl+0.5 mM $MnCl_2$. Insert is a vertical expansion of the spectra in the region of the α-methylene resonances.

The effects of manganese, present only in the extravesicular medium, on the bulk methylene signal are shown in Fig. 4. External manganese ions reduce, preferentially, the intensity of the broad downfield component. A similar preferential intensity reduction is also seen for the downfield component of the α-position methylene. When vesicles are prepared in $MnCl_2$ and the extravesicular ions are scavenged by EDTA, the sharp component in the methylene signal and the upfield component of the α-methylene are eliminated. Taken together, these observations clearly indicate that the splitting in the bulk methylene resonance arises from differences in the two leaflets of the bilayer rather than the two esterified fatty acid chains within the same lipid molecule.

Saturation transfer experiments

Further evidence in support of the inside-outside assignment of the split acyl chain signal was obtained by investigating the nuclear Overhauser effect (NOE) on the acyl chain signal intensities when specific resonances from different parts of the chain are saturated with a second rf field. The NOE method has been successfully applied in the past to detect geometrical arrangements of nuclear spins by exploiting the distance dependence of the effect [12]. However, in multinuclear systems with overlapping resonances, the NOE may spread spatially by spin diffusion, an energy conserving polarization transfer process involving the mutual flip-flop of nuclear spins [13]. For the system under investigation, the α-methylenes at the esterified end of the acyl chain and the terminal methyl groups show up as well resolved signals, whilst the resonances of the intervening methylene segments are strongly overlapping. We therefore expect magnetization transfer along these hydrocarbon chains to occur chiefly by spin diffusion, particularly when the chain is sufficiently rigid that the dipolar coupling between CH_2 segments favors cross relaxation over T_1 relaxation at the various sites. Only at the ends of the hydrocarbon chains will magnetization exchange occur via the nuclear Overhauser mechanism.

The results of the saturation transfer experiments are presented in Fig. 5, where the effect of selective saturation of either the α-methylenes or the terminal methyls on the bulk methylene signal

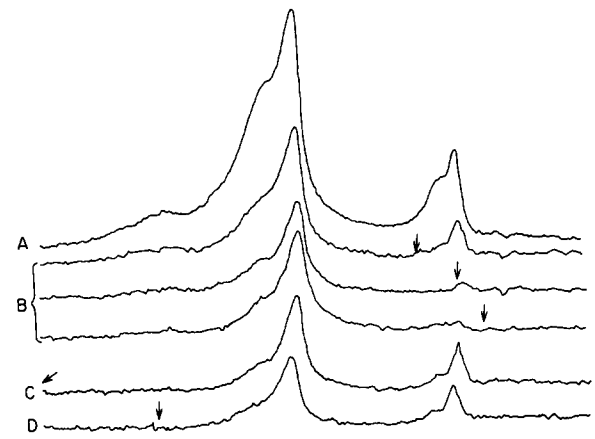

Fig. 5. Saturation transfer experiments on dipalmitoylphosphatidylcholine vesicles at 37°C. (A) Normal proton spectrum of acyl chain resonances at 500 MHz. (B) Spectra obtained upon irradiation near the chain methyl resonances. (C) Resultant spectrum when the α-position methylene resonance is selectively saturated. (D) Resultant spectrum when the β-position methylene resonance is selectively saturated. Arrows indicate the frequency at which saturating signals were applied.

is seen. In both cases, we observe a negative NOE for the downfield component of the split bulk methylene signal. The narrower, upfield component is affected to a significantly lesser extent. The preferential saturation of the downfield component of the bulk methylene and the methyl signals, when the α-methylene protons are irradiated, as well as the preferential saturation of the downfield components of the bulk methylenes when the terminal methyls are saturated indicates clearly that the broader bulk methylene and terminal methyl components of the signals arise from one complete lipid chain along which transfer of saturation is quite efficient, while the sharp components arise from lipid chains that are less rigid and have a lower efficiency for saturation transfer. These results are consistent with our assignment of the two component peaks of the acyl resonances to the chains of the two leaflets of the bilayer. Inasmuch as we have assigned the broad downfield resonance to the protons on the outer chains and the sharper upfield resonances to the protons on the inside chains, we infer from the apparently broader resonance widths and the preferential negative NOE observed for the lipids in the outer

monolayer, that there is a greater degree of motional restriction for these chains in bilayer vesicles.

Discussion

The present proton NMR studies of small unilamellar phosphatidylcholine vesicles at 500 MHz have revealed separate partially resolved resonance peaks for the protons on the chains of the inner and outer leaflets of the bilayer membrane. These results augment similar findings on the head group resonances of phosphatidylcholines previously reported [2,11,14,15]. We assert that, taken together, these observations argue for a significant difference in the packing of the lipid molecules between the two monolayers of a highly curved bilayer vesicle.

Spatial variations in the local magnetic susceptibility across the bilayer membrane have been mentioned as a possible cause for the chemical shift difference observed between the inside and outside head group resonances. However, Kostelnik and Castellano [15] have shown that this effect would only manifest itself for non-spherical vesicles. There is, at this time, no evidence in support of a large deviation from sphericity for small bilayer vesicles. In any case, this mechanism cannot account for the observed chemical shift nonequivalence between the inside and outside terminal methyls, as these moieties occupy essentially the same part of the bilayer membrane. Similarly, the pattern of temperature shifts observed for the various resonances near T_m is not consistent with this interpretation. Whereas in the case of the head groups, it is only the inside resonances that show a significant upfield shift as the temperature is lowered through T_m [11], for the chain resonances, it is the outside chain protons that move as the temperature is varied near T_m. Unfortunately, the methylene peaks become very broad near T_m and it was not possible to monitor them over the entire phase transition region. Nevertheless, we were able to ascertain that the bulk methylene signal arising from the chains of the outer leaflet undergoes a displacement towards lower fields as the temperature is decreased towards T_m. These observations could only be rationalized in terms of intrinsic packing differences between the lipid molecules located in the two leaflets of the bilayer and concomitant variations in the packing characteristics that must necessarily accompany the thermal phase transition under the geometrical constraints imposed by the extreme surface curvature.

The temperature shifts observed for the chain protons on the outer leaflet and the inner head group resonances are readily understood on the basis of well documented chemical shift mechanisms [16]. With respect to chain packing, it is now generally accepted that the major forces of interaction between two lipid chains at short ranges (approx. 5 Å) are those of the van der Waals type. This interaction causes expansion of the electronic cloud around the protons of the methylene segments, which in turn leads to a reduction in the contribution of these electrons to the magnetic shielding of the protons from the applied magnetic field. It then follows that the more tightly packed the hydrocarbon chains become, the greater the deshielding of the protons associated with these chains. For the problem at hand, since the two leaflets exhibit surface curvature differing in both magnitude and sign, the outer hydrocarbon chains are necessarily on the average more tightly packed than the inner chains [3,18]. This packing difference would explain their intrinsically lower field position in the proton nmr spectrum as well as the additional shift toward lower field as the bilayer is condensed into the gel phase. In contrast, electrostatic effects must dominate the intermolecular shifts of the resonances from the zwitterionic head groups. Again, because of the 'wedge' effect, the packing of the inner head groups must more closely resemble that in a flat bilayer, whereas the head groups located at the vesicle surface must be quite loosely packed. Accordingly, we expect a more substantial electrostatic contribution to the chemical shift of the inner head groups as well as a greater sensitivity of these resonances to the packing changes brought about by the thermal phase transition of the lipid.

The intrinsic asymmetry of a highly curved bilayer vesicle is manifested not only as a chemical shift nonequivalence between the protons from the two halves of the bilayer but also differences in resonance widths. While it is not clear to what extent the different linewidths of the inner and outer bulk methylene peaks reflect different chem-

ical shift dispersion of the various methylene segments along the hydrocarbon chains between the two leaflets rather than different relaxation rates arising from motional differences, the observed linewidth nonequivalence observed for the inner and outer terminal methyl resonances most certainly arises from different degrees of motional restriction and rates of segmental motions between the two types of chains. It is evident from our saturation transfer experiments that the hydrocarbon chains located on the outer leaflets are indeed motionally more restricted than their counterparts in the opposing leaflet.

The motional state of the lipid molecules in a small vesicle is highly complex not only because of the intrinsic bilayer asymmetry, but also because of the multiplicity of motions that can occur. On the local level, in addition to fast bond isomerizations along the hydrocarbon chains, there exist bulk motions corresponding to fluctuations of the hydrocarbon chains about the bilayer normal. On the more global scale, the lipid molecules can undergo lateral diffusion along the plane of the vesicle surface, flip-flop exchanges between the two leaflets and in addition, the entire bilayer unit can experience slow Brownian type overall tumbling motions in the medium it is suspended. Generally speaking, the local segmental motions are rapid whereas the global motions are slow. Those specific motions that are primarily effective in averaging the magnetic dipolar interactions among the spins in the vesicle may be inferred from the results of our saturation transfer experiments. According to the theory of Balaram et al. [17], the NOE's obtained under the conditions of our experiment (11.7 tesla) should be negative if all the above motional processes contribute to the motional averaging. In fact, if the dipolar averaging is dominated by vesicle tumbling and lateral diffusion of the lipid molecules, as has been assumed in a number of treatments, the NOE factor would be -1, i.e., the resonance peaks would be completely saturated. In actuality, we observe only an incomplete transfer of saturation even in the steady state irradiation experiments.

In conclusion, the present work confirms the general picture on vesicle structure and lipid chain packing and dynamics that was put forward originally by Sheetz and Chan [2] and elaborated by others [3,18]. It is evident that surface curvature can affect the packing of the lipid molecules in bilayer membranes in subtle and important ways.

Acknowledgements

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