

Ultrasound interaction with large unilamellar vesicles at the phospholipid phase transition: perturbation by phospholipid side chain substitution with deuterium

Reef (Philip D., II) Morse ^{a,*}, Loralie D. Ma ^{b,1}, Richard L. Magin ^c,
Floyd Dunn ^b

^a Department of Chemistry, Illinois State University, Normal, IL 61790-4160, USA

^b Biocoustics Research Laboratory, Beckman Institute for Advanced Science and Technology, University of Illinois, 405 North Mathews Street, Urbana, IL 61801, USA

^c Bioengineering Department (MC603) University of Illinois at Chicago, 851 South Morgan Street, Chicago, IL 6060, USA

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Abstract

The ultrasonic absorption, $\alpha\lambda$, as a function of temperature and frequency was determined in large unilamellar vesicles (LUVs) in which specific phospholipid side chains were deuterated. Deuteration significantly altered the temperature and frequency dependence of $\alpha\lambda$. The frequency change was especially marked, with decreased frequency and broadening of the ultrasound relaxation, even with only minor changes in the phase transition temperature. Deuteration decreased the T_m and enthalpy of the lipid phase transition, as shown by differential scanning calorimetry, whereas electron spin resonance showed that at and above the lipid phase transition, no differences in the mobility as a function of temperature were observed. These results show that the observed increase in ultrasonic absorption in LUVs at the phospholipid phase transition arises from the interaction of ultrasound with the hydrophobic side chains, probably coupling with structural reorganization of small domains of molecules, a process which is maximized at the phase transition temperature. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Ultrasound acoustic energy probes the properties of materials and molecular processes (Wada, 1987; Dunn, 1996). The typical frequencies employed (1–500 MHz) overlap those used by magnetic resonance and dielectric spectroscopy. In this paper, we applied ultrasound spectroscopy in

* Corresponding author. Tel.: +1-309-438-5595; fax: +1-309-438-5538.

E-mail addresses: reef@xenon.che.ilstu.edu (R.P.D.I. Morse), rmagin@uic.edu (R.L. Magin)

¹ Present address: Advanced Radiology, P.A., 7253 Ambassador Road, Baltimore, MD 21244, USA.

the megahertz frequency range to obtain a more detailed understanding of the dynamic events at the transition temperature of phospholipid bilayers. Previously we have shown that ultrasound absorptivity is sensitive to the individual molecular configuration (size, shape, molecular weight) of the component phospholipid molecules when dynamic processes occur with transitions in the nanosecond to microsecond time scale. Thus ultrasound can obtain information about both short- and long-range interactions within the phospholipid bilayer.

Ultrasonic absorption is expressed as the ultrasonic absorption per wavelength, $\alpha\lambda$, the exponential reduction in the sound pressure amplitude in traveling one wavelength (Dunn and O'Brien, 1976). For a given system at equilibrium, $\alpha\lambda$ will have maximum amplitude at the relaxation frequency of the chemical reaction, conformational change, or structural change with which it interacts.

To localize, on a molecular level, the site or sites of the interaction of ultrasound with biological membranes, agents with specific partitioning properties can be incorporated into the membrane bilayer structure. These agents alter the physical properties of the membrane and if these alterations are sufficient in amplitude, they can be detected as changes in $\alpha\lambda$ as a function of temperature and/or frequency. Changes in the ultrasonic absorption caused by the addition of perturbing agents to the membrane will occur if: (1) the specific ultrasonic interaction is at the site of membrane perturbation and (2) the event which is altered takes place on a time scale accessible to the specific acoustic interferometer (in the work presented here, this time scale is microseconds or less as described in Section 2.2).

The theoretical basis for ultrasound absorption in liposome suspensions has been analyzed using a simple two-state transition model to characterize the observed single-relaxation spectra (Tata and Dunn, 1992a). This structural relaxation model also accounts for the observed increase in the ultrasonic nonlinearity parameter (B/A) that occurs in the vicinity of the gel to fluid phase transition temperature of the phospholipid (Tata and Dunn, 1992b).

1.1. Previous studies of ultrasound interaction with liposomes

Liposomes can be formed from a variety of lipids and provide a useful model of the cell membrane. Large unilamellar vesicles (LUVs), formed by the reverse phase evaporation method (Szoka and Papahadjopoulos, 1978), with diameters in the range 0.2–0.8 μm , can be produced from natural or synthetic phospholipids (Strom-Jensen et al., 1984). Ultrasonic absorption of LUVs has been used to determine the effect of perturbations on membranes (Hammes and Roberts, 1970; Eggers and Funck, 1976; Gamble and Schimmel, 1978; Harkness and White, 1979; Sano et al., 1982; Maynard, 1984; Strom-Jensen et al., 1984; Maynard et al., 1985; Hianik et al., 1993; Wójtowicz and Gruszecki, 1995; Hianik et al., 1996, 1997; Wójtowicz et al., 1998). Previous ultrasound studies of liposomes showed that in multilamellar vesicles (MLV) and LUV suspensions, $\alpha\lambda$ exhibited a large increase near the phase transition temperature (T_m) of the phospholipids in the vesicle membrane (Harkness and White, 1979; Sano et al., 1982). For the LUV suspensions, this peak is correlated with structural changes in the membrane that also lead to dramatic increases in the permeability of LUV membranes in the vicinity of T_m (Magin and Niesman, 1984).

At the phase transition temperature of LUVs containing dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) (4:1, w/w), a maximum in $\alpha\lambda$ occurs at 2.1 MHz. This identifies a frequency where ultrasound presumably couples to conformational changes of individual phospholipids or groups of phospholipids in the vesicle membrane (Strom-Jensen et al., 1984). Alteration of the head group region of the phospholipid with Ca^{2+} or of the water structure with D_2O did not change the relaxation frequency at which the maximum in $\alpha\lambda$ occurred. However, other characteristics of the phase transition, such as T_m , were altered. This suggests that ultrasound interaction is not at the surface of the bilayer (Ma et al., 1987, 1989).

Addition of membrane-spanning ionophores such as gramicidin (Ma et al., 1987) and A23187

(Ma et al., 1989) lower the relaxation frequency to 0.75 and 1.55 MHz, respectively. This indicates that the interaction of the ultrasound involves the hydrophobic chains of the membrane (Ma et al., 1987, 1989, 1990). This is further supported by ultrasound studies of membrane-soluble lipids related to vitamin A (Rufini et al., 1990; Wójtowicz and Gruszecki, 1995). In these studies, a sharpening of the $\alpha\lambda$ curve by lutein, but not β -carotene nor violaxanthin, was reported. The authors attribute the sharpening to the higher affinity of lutein to membrane lipids, as reflected by the increase in size of the cooperative unit associated with the phase transition. Ultrasound studies, in conjunction with a number of other biophysical techniques (monolayer, calorimetric, and electrochemical) of liposomes and black lipid membranes doped with membrane-soluble are interpreted in a similar fashion proteins (Hianik et al., 1993, 1996, 1997; Wójtowicz et al., 1998).

1.2. The present study

Replacing side chain protons with deuterons results in a phospholipid with slightly increased molecular weight (796.54 compared to 734.05 Da for DPPC in which all fatty acyl side chains are deuterated) and whose ΔH and T_m are slightly decreased compared to protonated DPPC, Guard-Friar et al., 1985). Therefore, an alteration of the mass of the fatty acyl side chains should lead to a change of the relaxation kinetics of the molecular processes which interact with ultrasound. We performed measurements of $\alpha\lambda/c$ versus temperature of LUV composed of DPPG and DPPC with either the 1', 2' or both fatty acid chains perdeuterated. This choice was made to determine the roles of each of the fatty acid chains on ultrasound absorption and thus localize the site of ultrasound interaction within the bilayer. Differential scanning calorimetry (DSC) was used to determine the phase transition temperature and enthalpy for comparison with the ultrasound data. Electron spin resonance using oxazolidine nitroxides located at specific positions on the 2-phospholipid chain was used to detect the mobility of phospholipids at different depths within the membrane in the different LUV preparations.

All three methods presented here provide complementary information about events at the phase transition temperature of the bilayer. EPR provides information about the local molecular orientation and motion while DSC provides information about bulk properties. Ultrasound spectroscopic measurements provide composite information about the interplay between the mechanical properties (density, compressibility) and molecular properties (dynamics, lattice vibrations) and uniquely responds to molecular cooperatively. In combination these three methods provide information about the macroscopic and microscopic properties of the lipid bilayer when perturbed by changes in side-chain mass of the phospholipids in the lipid bilayer.

2. Experimental procedures

Control liposome preparations were made from mixtures of DPPC–DPPG, in a 4:1 (w/w) ratio. All lipids, including deuterated and nitroxide phospholipids, were obtained from Avanti Polar Lipids (Alabaster, AL). DPPG, whose head group has a net negative charge at pH 7.4, was incorporated into the liposomes to inhibit aggregation and fusion by increasing surface repulsion of the liposomes (Maynard et al., 1985). *N*-2-ethanesulfonic acid (Hepes) was purchased from Sigma (St. Louis, MO, USA). Hepes-buffered saline (buffer) was composed of 10 mM Hepes, 139 mM NaCl, 6 mM KCl, and distilled water, using 10 M NaOH to adjust the pH to 7.4 at room temperature.

2.1. Liposome preparation

LUV preparations were made from DPPC–DPPG mixtures in a 4:1 (w/w) ratio, using the reverse phase evaporation method developed by Szoka and Papahadjopoulos (1978). This process results in liposomes with an average diameter of $0.21 \pm 0.01 \mu\text{m}$ that range in size from 0.10 to $0.43 \mu\text{m}$, as determined by electron microscopy (Magin and Niesman, 1984). The average concentration of phospholipid in the preparation was $25.0 \pm 0.2 \text{ mg/ml}$ as determined by the Bartlett phosphorus assay (Bartlett, 1959). Samples of the

original suspensions were diluted to 2 mg/ml phospholipid in buffer for acoustic measurements. This preparation is referred to as a standard LUV preparation. For deuterated phospholipid suspensions, phospholipids were obtained for which specific fractions of the protons of the fatty acyl side chains had been replaced by deuterons. Deuterated phospholipid LUV suspensions were subsequently prepared in the same manner as standard LUV suspensions, except that a portion of the phospholipids were replaced with deuterated phospholipids. The following preparations were studied: 100% perdeuterated DPPC (100% pd DPPC), in which all of the side chain protons were replaced by deuterons; 50% perdeuterated DPPC (50% pd DPPC), in which half the phospholipids were protonated DPPC and half the phospholipids were 100% perdeuterated DPPC; 100% 1' perdeuterated DPPC (1' pd DPPC), in which all protons of the 1' fatty acyl chain were replaced by deuterons; 100% 2' perdeuterated DPPC (2' pd DPPC) in which all protons of the 2' fatty acyl chain were replaced by deuterons. These preparations were chosen such that it could be determined: (1) whether there is a simple direct relationship between the amount of deuterated side chain and change in frequency or amplitude of ultrasonic absorption, (2) whether a difference could be observed in deuteration of the 1' chain versus the 2' chain and (3) whether 50% deuteration of DPPC is equivalent to 100% deuteration of either single side chain (all three preparations have the same average molecular weight). These physical changes should provide direct evidence for determining if ultrasound interaction is sensitive to bulk mass effects or individual molecular modifications.

For electron spin resonance (ESR) experiments, LUV suspensions were prepared with the nitroxide-labeled phospholipids 5-doxyl stearoyl phosphatidylcholine (5-DS-PC), 7-doxyl stearoyl phosphatidylcholine (7-DS-PC), 12-doxyl stearoyl phosphatidylcholine (12-DS-PC), and 16-doxyl stearoyl phosphatidylcholine (16-DS-PC). These spin probes consist of an oxazolidine nitroxide attached to the 5, 7, 12, or 16th carbon of the stearate chain of 1-palmitoyl-2-(*N*-doxylstearoyl) phosphatidylcholine. They provide information

about the membrane structure and mobility at various depths of the LUV membrane (Jost et al., 1971). These liposome preparations were not diluted prior to their ESR measurements. Experiments were begun within 24 h of LUV preparation for all LUV suspensions.

2.2. Apparatus

The acoustic interferometer used in this study is based on the design of Labhardt and Schwarz and has been described in detail elsewhere (Labhardt and Schwarz, 1976). Briefly, it consists of two identical X-cut quartz transducers (diameter, 2.54 mm; fundamental resonance frequency, $f_0 = 4.0$ MHz), positioned coaxially and parallel 5.5 mm apart forming the end walls of the measuring cell. One of the transducers, the transmitter, is excited electrically (continuous wave) at a predetermined frequency and transmits ultrasonic waves through the fluid medium. The other transducer receives the sound wave and converts it into an electrical signal. The electrical input to the transmitting transducer is obtained from a synthesized signal generator (HP 8660B, Hewlett-Packard, Palo Alto, CA, USA), and the stable power drive is maintained by the HP 86601A RF section. The electrical output from the receiving transducer is monitored by a spectrum analyzer (either an HP 85521 or HP 8553B). Ultrasonic intensities of less than $1 \mu\text{W}/\text{cm}^2$ are used, which is many orders of magnitude below that needed to produce cavitation which would disrupt the liposomes or cause a temperature increase (Maynard et al., 1985). The entire system resonates acoustically at certain input signal frequencies. The mechanical quality factor Q of this resonance is related to the acoustic absorption per wavelength by (Maynard, 1984):

$$\pi/Q = \pi\Delta f/f_0 = \alpha\lambda$$

where α is the amplitude absorption coefficient per unit path length, $\alpha\lambda$ is the absorption per wavelength λ , Δf is the 3 dB bandwidth of the resonance (viz. the difference in the two frequencies for which the output power of the signal is one-half that at the resonance frequency, f_0), and Q is the quality factor $f_0/\Delta f$.

The excess absorption due to the presence of LUVs in the suspension is obtained by subtracting the absorption coefficient of the reference buffer from that of the sample under study (reference buffer plus LUVs). For this situation, in which the acoustic velocity and impedance of the suspension are virtually the same as that of the reference buffer, correction for diffraction is unnecessary (Maynard et al., 1985). The excess absorption is:

$$(\alpha\lambda)_{\text{excess}} = \pi(\Delta f - \Delta f_{\text{ref}})/f_0$$

2.3. Ultrasound measurement procedure

The excess absorption coefficient, $\alpha\lambda/c$, (c is the concentration of phospholipid in g/ml) was determined as a function of both temperature and frequency. The $\alpha\lambda/c$ measurement error was estimated by Ma to be $\pm 1\%$ of $\alpha\lambda/c$ for this system (Ma, 1988). The temperature was increased from 35 to 45°C, while at each temperature the measurement frequency was increased from 0.58 to 5.2 MHz, which provides information on relaxation events on the time scale of 0.27–0.03 μs , respectively.

The temperature of the interferometer was maintained to within $\pm 0.05^\circ\text{C}$ during data collection by immersion in a temperature-controlled water bath (Exacal 500 with Endocal 350 refrigeration unit, and a DCR-4 temperature digital controller, Neslab, Portsmouth, NH, USA). At least 30 min was allowed for the thermal stabilization of the interferometer after each temperature change. Remixing was performed after each temperature change to ensure that the LUV suspension was not experiencing aggregation or fusion. Settling was not observed, as evidenced by the lack of change in $\alpha\lambda/c$ over time at any one temperature, and the lack of change in $\alpha\lambda/c$ after remixing of the suspension.

2.4. DSC procedure

A DSC-2 (Perkin-Elmer, St. Louis, MO) was used to obtain the specific heat at constant pressure (C_p) versus temperature on 50 μl samples of LUV suspensions. The LUV suspensions (25.0

mg/ml phospholipid) were studied over the temperature range 30–50°C. The DSC measurements were performed to compare the different deuterated DPPC LUV suspensions with standard LUV suspensions, with respect to both T_m and ΔH .

2.5. ESR Procedure

All ESR spectra were taken at X-band (9.5 GHz) on a Varian E-4 spectrometer equipped with a Varian temperature controller (variation in sample temperature $\pm 0.3^\circ\text{C}$). Sample temperatures were measured by placing a copper-constantan thermocouple next to the sample.

Liposomes were taken up in 1-mm (i.d.) glass capillaries, sealed at both ends, and placed in an ESR tube that was subsequently placed in the ESR cavity Dewar. The spectra were recorded in standard first derivative mode with 100 kHz modulation and a microwave power of 5 mW. Data were collected and stored as arrays of 1024 points per spectrum with a personal computer (Morse, 1987). Data collection and evaluation were performed using commercial EPR software (EPRWare, Scientific Software Services, Bloomington, IL). The order parameter is indicative of the motion of phospholipids within membrane bilayer; decreasing values of the order parameter indicate more isotropic motion of the nitroxide probe (Jost et al., 1971). The order parameter was calculated according to the following formula (Jost et al., 1971):

$$S = A_{\parallel} - A_{\perp} / (A_{zz} - 1/2(A_{xx} + A_{yy}))$$

where S is the order parameter, A_{\parallel} is the value of the outer hyperfine splittings, A_{\perp} is the value of the inner hyperfine splittings, and A_{xx} , A_{yy} , and A_{zz} , are the values of the hyperfine tensor of the oxazolidine nitroxide (5.9, 5.4, and 32.9 G, respectively, Jost et al. (1971)).

3. Results

The T_m , $\Delta T_{1/2}$ and $\alpha\lambda_{\text{max}}$ for the different liposome preparations are tabulated in Tables 1 and 2. Specific trends with increasing deuteration of the phospholipid side chains and differences for 1' versus 2' perdeuteration are given below.

Table 1

Characteristics of the phase transition from differential scanning calorimetry measurements in LUV suspensions with varying proportions of phospholipid side chain deuteration^a

LUV perdeuteration	T_m (°C)	ΔH (kcal/mol)	Mol. wt. difference (%)	Difference in ΔT_m	Difference in ΔH
DPPC/DPPG control (ave. mol. wt. 736.23)	42.0 ± 0.5	7.5 ± 0.2	–	0	0
100% 1'2'pd DPPC (ave. mol. wt. 786.22)	38.7 ± 0.5	4.9 ± 0.1	+6.8	–3.3	–35
50% 1'2'pd DPPC (ave. mol. wt. 761.23)	40.0 ± 0.5	6.3 ± 0.2	+3.4	–2.0	–16
100% 1' pd DPPC (ave. mol. wt. 761.23)	39.6 ± 0.5	6.0 ± 0.2	+3.4	–2.4	–20
100% 2' pd DPPC (ave. mol. wt. 761.23)	39.4 ± 0.5	5.5 ± 0.2	+3.4	–2.6	–27

^a ΔH (kcal/mol) was obtained versus T (°C) using differential scanning calorimetry. Estimates of error are given for each value. The difference values are calculated relative to the DPPC/DPPG control.

3.1. DSC results

As the deuterium content, and therefore the molecular weight, increased, the phase transition temperature decreased (Table 1). A slight decrease in ΔH was observed with increasing deuteration of the phospholipid preparations. These small changes in ΔH and T_m scale approximately with the percent change in the overall molecular weight of the perdeuterated samples. Within samples of equivalent average molecular weight, deuteration at the 2' position of the DPPC produced a statistically significant change in ΔH but not T_m .

3.2. Ultrasound results

Ultrasonic absorption as a function of temperature and frequency are shown in Figs. 1 and 2 and Table 2. Deuterated phospholipids in LUV suspensions exhibit a decrease in phase transition temperature (T_m , as measured by the maximum in $\alpha\lambda/c$) compared to the nondeuterated control. However T_m of the LUV's containing 1' and 2' perdeuterated phospholipids were different (Table 2). While T_m of 2' pd LUVs showed only a 1.5°C temperature change and some broadening, 1' pd LUVs had a 2.5°C decrease in T_m and considerable broadening as evidenced by $\Delta T_{1/2}$, and a decrease in peak amplitude of ultrasound absorption. T_m of 100% pd LUVs was decreased even further, and the amplitude of ultrasound absorp-

tion within the frequency range of the interferometer was decreased by at least 80%. The 1:1 mixture of native DPPC and 100% pd DPPC LUVs showed a mixture of these characteristics, with a probable combination of two peaks; T_m for these preparations were 41.2 and 40.0°C, respectively (data not shown).

The ultrasound relaxation frequency at T_m is also significantly changed by the deuteration of phospholipid side chains. 2' pd LUVs showed the least perturbation, with a broadened relaxation centered around 1.3–1.4 MHz. However, the $\alpha\lambda_{\max}$ of 1' pd LUVs was changed markedly, with a peak apparently below the low frequency limit of the acoustic interferometer. The 100% pd LUVs showed no peak frequency of relaxation. However, this may have been due either to a relaxation frequency below that of the range of measurement of the interferometer, or a frequency peak significantly below that detectable by the interferometer due to low absorption. The 50% mixture of native and 100% pd LUVs showed a combination of the characteristics of the three aforementioned suspensions (Fig. 2).

3.3. ESR results

The results of all EPR experiments are shown in Fig. 3. At and above the phase transition temperatures of the phospholipids, no changes in order parameter were seen for 5-, 7-, 12-, or

Table 2

Characteristics of the lipid phase transition determined from ultrasound absorption coefficient measurements in LUV suspensions with varying proportions of phospholipid side chain deuteration

LUV perdeuteration	T_m (°C)	$\Delta T_{1/2}$ (°C)	$\alpha\lambda_{\max}$ (MHz)	Difference in ΔT (%)	Difference in $\Delta T_{1/2}$ (%)
DPPC/DPPG control	42.0 ± 0.1	1.7 ± 0.2	2.1	0	0
100% 1'2' pd DPPC	39.0 ± 0.1	3.4 ± 0.3	None ^a	-7.1	+100.0
50% 1'2' pd DPPC	40.8 ± 0.1	1.6 ± 0.2	<1 ^b , 1.7	-2.9	-5.9
100% 1' pd DPPC	39.5 ± 0.1	2.4 ± 0.2	<1	-6.0	+41.2
100% 2' pd DPPC	40.5 ± 0.1	1.9 ± 0.2	1.3–1.4	-3.6	+11.8

^a No relaxation peak was visualized, possibly due to low amplitude of absorption versus a relaxation peak below that measurable by the acoustic interferometer.

^b The lowest relaxation peak was below that measurable by the acoustic interferometer.

16-DS-PC. This suggests that deuterating the side chains does not significantly affect the mobility of the phospholipids either at or above the phase transition. However, below the phase transition, deuteration of side chains shows complex effects on order parameter. The 1' perdeuterated phospholipids appear to decrease order approximately 1/3 of the way into the phospholipid membrane hydrocarbon region (as reported by 5-DS PC), while further down in the membrane hydrocarbon region as reported by the 7- and 12-DS PC labels, deuteration increases membrane order. No significant effect is seen for the 16-DS PC label for any of the deuterated lipids. This suggests a change in the fluidity of the membrane near the membrane-water in the solid phase of the bilayer, perhaps due to a mismatch in the mass of the 1' perdeuterated DPPC side chains.

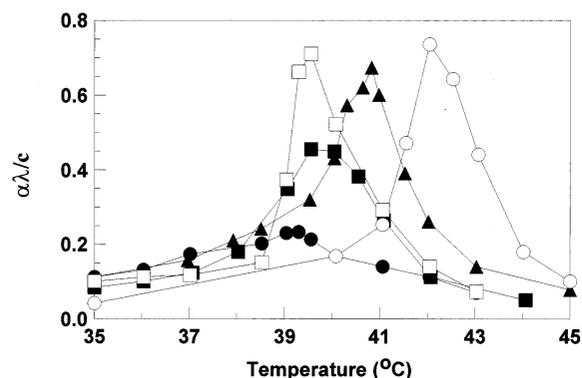


Fig. 1. $\alpha\lambda/c$ Vs. temperature at 2 MHz in: standard LUVs in suspension (\circ), 50% 1'2' pd DPPC LUVs at 40.8°C (\blacktriangle), 100% 1' pd DPPC LUVs at 39.5°C (\blacksquare), 100% 2' pd DPPC LUVs (\square) and 100% 1'2' pd DPPC LUVs at 40.5°C (\bullet). Errors are calculated (Ma, 1988) to be $\pm 1\%$ of the $\alpha\lambda/c$ values.

4. Discussion

The overall goal of this study was to obtain a more detailed understanding of the dynamic events at the transition temperature of phospholipid bilayers. This work obtained site-specific details by studying changes in ultrasound absorption by LUV membranes near the phase transition temperature induced by deuteration of the phospholipid side chains, which changed their molecular weight and phase transition temperature.

DSC demonstrates a decrease in the phase transition temperature, as well as a slightly decreased enthalpy of the transition, with increasing deuteration of the fatty acid side chains. Ultrasound results also showed a decrease in phase transition temperature with increased deuteration of the

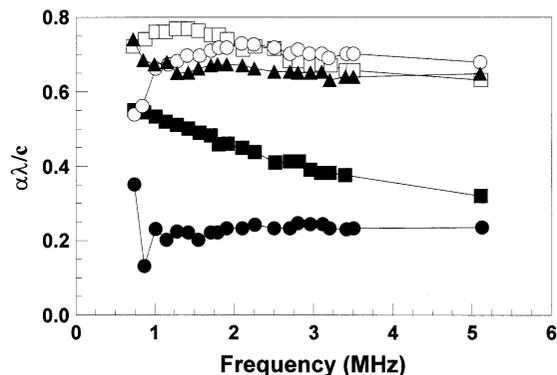


Fig. 2. $\alpha\lambda/c$ Vs. frequency at the T_m for each preparation in: standard LUVs in suspension (\circ), 50% 1'2' pd DPPC LUVs at 40.8°C (\blacktriangle), 100% 1' pd DPPC LUVs at 39.5°C (\blacksquare), 100% 2' pd DPPC LUVs at 40.5°C (\square) and 100% 1'2' pd DPPC LUVs at 40.5°C (\bullet) and 100% 1'2' pd DPPC LUVs at 39.0°C (\bullet). Errors are calculated (Ma, 1988) to be $\pm 1\%$ of the $\alpha\lambda/c$ values.

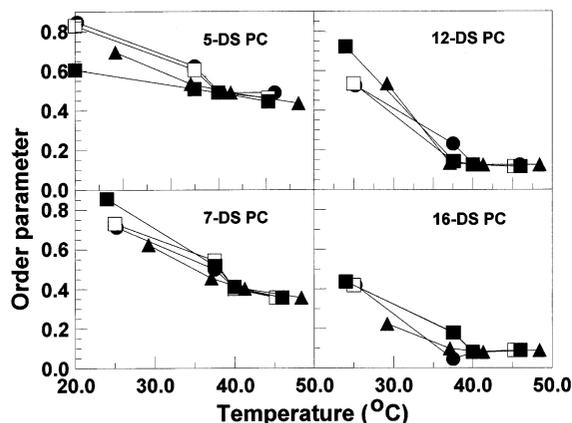


Fig. 3. Order parameter vs. temperature for: 50% 1'2' pd DPPC LUVs (▲), 100% 1' pd DPPC LUVs (■), 100% 2' pd DPPC LUVs (□) and 100% 1'2' pd DPPC LUVs (●), with addition of 1 mol% 5-, 7-, 12-, and 16-doxyl stearoyl phosphatidylcholine. Errors in values are less than the size of the symbols.

fatty acid side chain. Of note, 1' and 2' perdeuterated DPPC LUVs showed no difference in phase transition temperature by DSC, while ultrasound did show a small but significant difference in the phase transition temperature and large differences in the relaxation curves between the two preparations. This suggests that ultrasound interacts with molecular events that take place as the phospholipids enter the phase transition, but that the ultrasound is not responding simply to enthalpic events brought on by the phase transition.

For 2' pd DPPC LUVs, T_m is decreased to 40.5°C, with slight broadening of the phase transition. $\alpha\lambda$ as a function of frequency is broadened and $\alpha\lambda_{\max}$ is shifted down to 1.3–1.4 MHz. This suggests that the site (and event) of ultrasound interaction with the membrane was affected, the relaxation slowed, and the relaxation curve broadened as a result of the heavier 2' side chain. For 1' pd DPPC LUVs, T_m is decreased to 39.5°C, lower than that for 2' pd DPPC LUVs and $\alpha\lambda_{\max}$ is shifted below 1 MHz all of which indicate an even greater influence on ultrasound interaction by the 1' perdeuterated side chain.

These results may be interpreted in terms of the structure of DPPC; the longitudinal axis of the molecule consists of the charged head group, glycerol backbone and the 2' fatty acyl side chain,

while the 1' side chain is off the central axis (Stryer, 1988). Therefore, a change in the molecular weight of the 1' side chain may have an effect on the local phospholipid structure of the membrane which is greater than the proportional change in molecular weight. This could be a result of a rotational effect whereby the orientation and alignment of the 1' side chain has a larger influence on neighboring molecules compared to either 100% pd or 2' pd DPPC LUVs.

When both phospholipid side chains are perdeuterated, DSC reports a shift in T_m to 39.0°C. However, the ultrasonic absorption over the frequency range of the acoustic interferometer is decreased by at least 80% in amplitude which results in a smaller area under the $\alpha\lambda$ versus temperature curve. The $\alpha\lambda$ versus frequency curve shows no definitive peak, indicating either a decoupling of the ultrasound and the molecular events with which ultrasound interacts, or the presence of a frequency limit outside the frequency range of the acoustic interferometer. These results again indicate that ultrasound interaction with the bilayer is with the hydrophobic side chains and that even a small change in molecular weight of the side chains causes a significant alteration in the character of ultrasound absorption.

ESR results indicate that at and above the phase transition temperature, there is little or no significant change in the mobility of phospholipids (other than would be expected for the decrease in T_m for the deuterated phospholipids). Below the phase transition temperature, the deuteration of the 1' side chain changes the mobility of the phospholipids much more so near the membrane–water interface (Fig. 3).

Deuteration of DPPC phospholipid side chains markedly affects the relaxation frequency of ultrasound absorption at the phase transition, more so than would be predicted only on the basis of the change in transition temperature. Ma and co-workers (Ma et al., 1987, 1989) have shown that changing the phase transition temperature by the addition of Ca^{2+} or Mg^{2+} ions or by changing the solvent from H_2O to D_2O did not change the relaxation frequency even in the presence of large (> 5°C) changes in T_m . It has also been shown

previously (Strom-Jensen et al., 1984; Ma et al., 1990) that the addition of substances that may alter the configuration or kinetics of the hydrophobic portion of the side chain, for example, gramicidin and A23187, could cause a large change in $\alpha\lambda_{\max}$ even without appreciable change in T_m . However, it may be argued that the physical properties of the membrane are changed by the addition of such molecules (although A23187 is a very small molecule, which even in very small fractions in the membrane, without appreciable change in DSC from normal LUVs, showed large changes in $\alpha\lambda_{\max}$). Thus, deuteration of side chains changes the mass of the individual phospholipids, but not the intrinsic structure of the membrane bilayer. These finds are consistent with ultrasound spectroscopic studies of β -carotene and other membrane-associated lipids (Rufini et al., 1990; Wójtowicz and Gruszecki, 1995).

In conclusion, these data support the premise that ultrasound interacts most probably with small domains of phospholipid molecules and specifically couples with molecular interactions occurring within the outer regions of the bilayer.

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