Biophysical Chemistry, 32 (1988) 153–160
Elsevier

BPC 01323

High-frequency ultrasonic absorption spectroscopy on aqueous suspensions of phospholipid bilayer vesicles

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Received 8 December 1987
Revised manuscript received 3 August 1988
Accepted 15 September 1988

Ultrasonic spectroscopy; Sound absorption coefficient; Acoustic relaxation; Phospholipid vesicle; Rotational isomerization

Between 1 MHz and 3 GHz the ultrasonic absorption coefficient has been precisely measured as a function of frequency for some aqueous suspensions of single-walled phospholipid bilayer vesicles. All solutions of the specially purified phospholipids clearly show excess absorption, reflecting three molecular relaxation processes with discrete relaxation times. Typical values for these times are 50, 3 and 0.5 ns. The attempt is made to relate these relaxation processes to mechanisms of rotational isomerization in the hydrocarbon chains. Some other molecular mechanisms which could also contribute to the ultrasonic excess absorption spectra are also briefly discussed.

1. Introduction

The frequency dependence of the sound absorption coefficient of liquids reflects various molecular relaxation mechanisms resulting from elementary chemical processes of great significance. Accordingly, ultrasonic spectroscopy is an important experimental method for studying such processes and for gaining insight into molecular interactions and motions. This is all the more valid as ultrasonic methods utilize the molar volume and the enthalpy of reaction as naturally present 'marks' and do thus not need artificial labels.

Among the phenomena studied so far in liquid systems are isomerization reactions, conformational changes, mechanisms of dimerization, association, aggregation and complex formation, protolysis and hydrolysis reactions, as well as processes of solvation and hydrogen bonding [1–7].

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Also intensively investigated by ultrasonic spectroscopy and the corresponding time-domain techniques has been the kinetics of micelle formation in aqueous solutions of amphiphiles [8–10]. So far, however, little attention has been given to the ultrasonic absorption spectrum of phospholipid/water mixtures. This is even more surprising as evidence has been obtained that in the range between 0.5 and 100 MHz, fast relaxations occur in aqueous solutions of lecithin vesicles [11].

Phospholipids play an important role not only for their applications such as in drug production. The interest in these molecules springs in particular from their ability to form spontaneously bilayer assemblies which are considered useful models of biological membranes [12–17]. Information on conformations, interactions, molecular motions and kinetics of phospholipid bilayers is assumed to promote substantially our understanding of the structure and function of cell membranes. It seemed to be of interest to us to measure the ultrasonic absorption spectrum of aqueous solutions of phospholipid bilayer vesicles over a wide frequency range.

In this communication we present spectra measured between 1 MHz and 3 GHz using three different methods [18–20]. We used the following highly purified synthetic phospholipids to prepare colloidal solutions of single-walled vesicles: 1,2-dilauroylglycero-L-3-phosphatidylcholine (DLPC, C_{12}-lecithin); 1,2-dimyristoylglycero-L-3-phosphatidylcholine (DMPC, C_{14}-lecithin); 1,2-dipalmitoylglycero-L-3-phosphatidylcholine (DPPC, C_{16}-lecithin), 1,2-dimyristoylglycero-L-3-phosphatidyl-*N*,*N*-dimethylethanolamine (DMDME, C_{14}-dimethylcephalin). By this means we aim at a comparison of results for molecules with different head groups and hydrocarbon chains. To look for changes at the crystalline-liquid crystalline phase transition temperature T_t of the bilayer [21] we recorded the spectrum of a C_{14}-lecithin solution at two different temperatures. Comparison is made with previous ultrasonic absorption measurements of this laboratory [22] in which solutions of C_{14}-lecithin multi-bilayer liposomes had been studied using other experimental techniques. The sound absorption spectra are also compared to dielectric spectra [23–25] of colloidal aqueous solutions of phospholipids.

2. Experimental

2.1. Measurements

Besides that part which is due to the chemical relaxation processes of interest, the ultrasonic absorption coefficient α of liquids always contains a so-called classical contribution. It results from thermal conductivity and viscous friction and increases as ν^2 with the frequency ν. In the lower part of the frequency range of measurement ($\nu \leq$ 10 MHz) the attenuation coefficient of the phospholipid solutions was low enough to utilize a resonator technique [18]. At higher ν ultrasonic (10–400 MHz [19]) and hypersonic (300 MHz–3 GHz [20]) pulse transmission methods have been applied. Using these methods the signal is transmitted through a sample of variable thickness.

Applying the resonator technique the α value is derived from the change in the quality factor which results if the solution under test is replaced by an appropriate reference liquid. We have chosen aqueous solutions of NaCl as reference. Their concentrations were carefully adjusted so that the sound velocities of the samples and respective reference liquids agreed.

The pulse transmission methods were both based on a comparator technique in which a computer controls the direct substitution of the variable path length cell by an RF reference attenuator [26]. The use of pulsed signals allows the desired waveforms to be separated from electrical cross-talk and multi-reflected waves. At lower frequencies ($\nu < 400$ MHz) piezoelectric lithium niobate discs, operated at odd harmonics of the fundamental frequency of their thickness vibrations, were used as transducers. In the higher frequency range ($\nu > 300$ MHz) surface vibrations of small rod-shaped lithium niobate transducer crystals were excited.

The experimental error in the α values is $\pm 5\%$ for the data obtained by resonator measurements and $\pm 1\%$ for those measured with the variable path length methods. Errors in the determination of the frequency were negligibly small. The temperature of the liquids was controlled to within ± 0.1 K. The intensity of the sound field within the liquid was sufficiently small to avoid heating of the sample by absorption of ultrasonic energy.

Failing to notice systematic errors is largely prevented by the fact that three different methods are used to cover the frequency range. In addition, as already briefly mentioned above, we will also refer to some further spectra of C_{14}-lecithin solutions. These spectra had been measured previously in this laboratory [22] by use of two other methods. Between 4 and 150 MHz a fixed path length cell had been utilized to yield the absorption coefficient of the sample relative to that of a suitable reference liquid [19,27]. Between 280 MHz and 3 GHz absolute measurements had been performed by directly recording the signal transmitted through a cell at continuously varying sample length [28]. The error in these previous measurements was $\Delta\alpha/\alpha = \pm 5\%$.

2.2. Samples

The phospholipids were purchased from Sigma, Fluka, Koch-Light and Bio-Science. All compo-

nents were specified by the purity grade 'puriss' and were additionally purified according to a recently developed special method [29]. For this purpose, the phospholipids were dissolved in methanol. In this solvent the amphiphilic substances are molecularly dispersed or form very small aggregates only [30]. The alcoholic solutions were treated twice according to a mixed-bed ion-exchange procedure using Amberlite MB-2 (Serva, p.a.). With both runs the first 10% of the eluate was discarded. The main fraction of the methanolic solution was evaporated at 40 °C under dry nitrogen (Messer, 99.99%). To remove any residues of dipolar solvent the lipids were repeatedly recrystallized in *n*-pentane (Merck, p.a.) afterwards. The white powder obtained by this procedure was dried for about 4 h at 50 °C under reduced pressure and stored, if necessary, at 3 °C.

The ion-exchange resins may catalyse hydrolysis of the phospholipids. The purified compounds were thus checked by thin-layer chromatography using different solvent mixtures. No indications of the decomposition of the molecules have been found.

The aqueous suspensions were prepared in a manner that is expected to yield predominantly unilamellar vesicles [31,32]. Doubly distilled, degassed, and ultraviolet-sterilized water was added to a preweighed amount of lipid. For reasons of swelling the solutions were stored for some hours at a temperature above the crystalline-liquid crystalline phase transition temperature T_t. Also above T_t the solutions were subsequently sonicated in a bath-type homogenizer (Bransonic 12) until macroscopically homogeneous, clear suspensions of low viscosity were obtained. Normally, less than 2 h of sonication were necessary. The 0.069 M solution of DMPC, however, required nearly 6 h acoustic irradiation.

When the sonication procedure had been finished the suspension was immediately placed in a cell. Measurements with a cell were completed within 12 h. The first measurements were repeated afterwards to ensure that the sample did not change its properties during this period of time.

3. Results

As an example, the dependence on the frequency ν of the α/ν^2 values is displayed in fig. 1 for 0.01 M DPPC solution at 25 °C. The α/ν^2 data, measured between 1 MHz and 3 GHz, decrease monotonically with ν to reach asymptotically the classical contribution B' at the highest frequencies. This behaviour indicates the presence

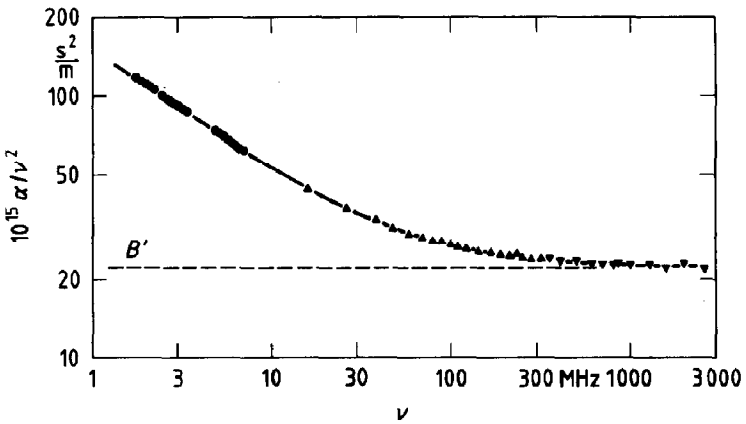

Fig. 1. Bilogarithmic plot of the sound absorption spectrum of 0.01 M aqueous solutions of C_{16}-lecithin at 25 °C. Shown as a function of frequency ν is the quantity α/ν^2. Different symbols indicate different methods of measurement: (●) resonator technique [18]; (▲) ultrasonic pulse transmission method [19]; (▼) hypersonic comparator technique [20]. The dashed line shows the classical contribution B' to the α/ν^2 values. The full curve represents the description of the excess absorption $\alpha/\nu^2 - B'$ by a sum of three Debye-type relaxation spectral terms (eq. 1).

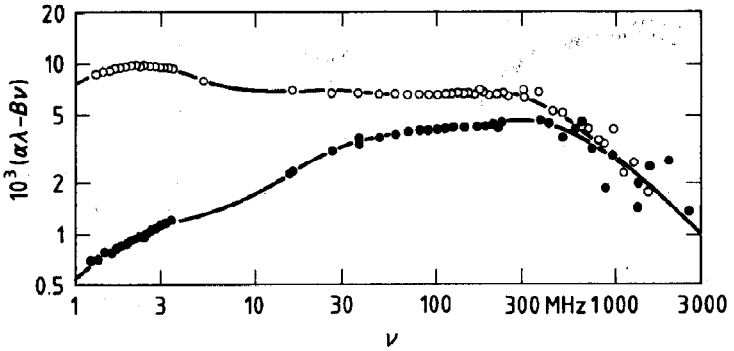

Fig. 2. Dependence on frequency ν of ultrasonic excess absorption per wavelength, $\alpha\lambda - B\nu$, for 0.069 M aqueous C_{14}-lecithin solution at 20 °C (○) and 30 °C (●). $B = B'v_s$ where v_s denotes the velocity of sound.

of relaxation processes in the whole frequency range under consideration.

The finding of a very broad relaxation region is also demonstrated in fig. 2, where spectra for the 0.069 M DMPC solution are shown in an alternative representation of data. Plotted as a function of frequency is the excess absorption $\alpha\lambda - B\nu$ per wavelength λ ($B = B'v_s$, $v_s = \lambda\nu$). In particular, the data at 20 °C show a broad plateau region, indicating that the excess absorption spectra of bilayer systems (other than those of micellar solutions of zwitterionic amphiphiles [33]) cannot be described by one or two relaxation terms each with a discrete relaxation time (Debye relaxation terms [34]).

In fig. 3 the results obtained on two equimolar DMPC solutions at 30 °C are compared with one another in order to show that the unusually broad ultrasonic excess absorption spectra of vesicle solutions do not result from an experimental artefact. The previously measured $\alpha\lambda - B\nu$ data are also nearly constant over a wide range of frequencies, namely, between 10 MHz and 2 GHz. Altogether, however, the previous solution exhibits

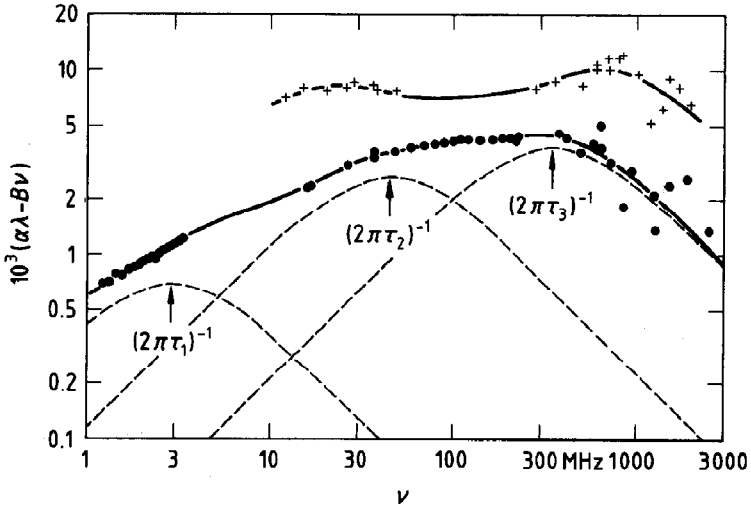

Fig. 3. Ultrasonic excess absorption per wavelength, $\alpha\lambda - B\nu$, displayed as a function of frequency ν for the present (●) and a previously measured (+[22]) 0.069 M aqueous C_{14}-lecithin solution at 30 °C. The previous data refer to a solution of multi-bilayer liposomes instead of single-walled vesicles. The dashed curves show the subdivision of the present spectrum into three Debye-type relaxation spectral terms (eq. 1).

Table 1

Sound velocity (v_s) and parameters of the relaxation spectral function (eq. 1) for colloidal aqueous solutions of phospholipid bilayer vesicles

Phospho-lipid	c (mol/l) (±0.5%)	T (°C) (±0.1 K)	v_s (m/s) (±1%)	A_1 ($\times 10^{-3}$) (±5%)	τ_1 (ns) (±5%)	A_2 ($\times 10^{-3}$) (±0.1)	τ_2 (ns) (±0.03	A_3 ($\times 10^{-3}$) (±0.2)	τ_3 (ns) (±0.01)	B ($\times 10^{-12}$) (s) (±0.01)
DLPC	0.079	30	1521	0.7	5	3.8	0.69	10.7	0.13	33.59
DMPC	0.069	20	1494	18.1	78	8.5	5.96	10.9	0.67	44.73
	0.069	30	1511	1.4	53	5.3	3.59	7.6	0.45	36.19
	0.01	20	1486	2.7	27	1.3	2.57	2.6	0.14	38.10
DPPC	0.01	25	1497	0.4	63	0.8	8.63	1.4	0.95	33.48
DMDME	0.01	25	1497	1.4	19	1.1	2.22	1.4	0.31	33.47

higher values of its sound absorption coefficient than the present solution of identical C_{14}-lecithin concentration. This fact is assumed to be due to the presence of different types of bilayer aggregates in the suspensions. In the previous study, the samples had been prepared in a manner which preferably yields extended onion-like structured multi-bilayer liposomes [35–37] instead of small, single-walled vesicles.

Within the limits of experimental error, all spectra reported in this paper can be analytically represented by the function ($\omega = 2\pi\nu$)

$$\alpha\lambda = \sum_{i=1}^{3} \frac{\omega\tau_i A_i}{1+\omega^2\tau_i^2} + B\nu. \qquad (1)$$

Using this function the excess absorption per wavelength is considered by three Debye terms (fig. 3) with relaxation amplitudes A_i and relaxation times τ_i ($i = 1$–3). Eq. 1 has been fitted to the measured spectra using a nonlinear least-squares regression analysis. The results for the parameters determined by this procedure are collected in table 1. Also included in this compilation of data are the values of the sound velocity v_s. These have been derived either from the resonance frequencies of the resonator used at low frequencies or from the distance dependence of the receiver signal in the hypersonic cell. Due to multiple reflections this signal shows ripples at small transducer spacing.

We also fitted the function

$$\alpha/\nu^2 = \sum_{i=1}^{3} \frac{A_i'}{1+\omega^2\tau_i^2} + B' \qquad (2)$$

to the absorption spectra. This relation is indeed another version of the relaxation spectral function defined by eq. 1 ($A_i' = 2\pi\tau_i A_i/v_s$; $B' = B/v_s$) but it considers the experimental points with different weighing factors. Differences in the parameter values obtained by use of the corresponding functions are included in the error given in table 1.

4. Discussion

A most striking and unexpected result of our measurements on solutions of phospholipid vesicles is the existence of ultrasonic excess absorption ($\alpha\lambda - B\nu > 0$) in an unusually broad frequency band. This finding seems to be characteristic for bilayer solutions with a comparatively high degree of order in the hydrocarbon chains. In the frequency range under consideration the sound absorption spectra of solutions of more labile zwitterionic micelles exhibit at most two well-separated Debye-type relaxation processes [33].

Unfortunately, however, flat spectra (figs. 2 and 3) can be represented by a great variety of model relaxation functions. In particular, relaxation terms reflecting a relaxation time distribution instead of a discrete relaxation time are also appropriate. The relation defined by eq. 1 has been preferred here for the reason that it allows the measured spectra to be described analytically by a minimum of adjustable parameters. Despite the precise measurements on solutions of highly purified phospholipids, the 'true' model relaxation

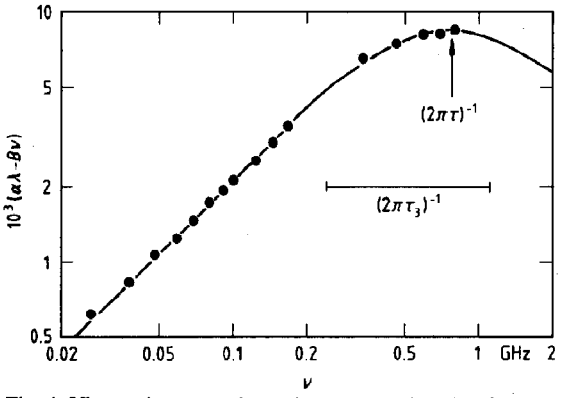

Fig. 4. Ultrasonic excess absorption per wavelength, $\alpha\lambda - B\nu$, bilogarithmically plotted vs. frequency ν for *n*-tetradecane at 25 °C [38]. The full curve is the graph of a Debye-type relaxation term with the relaxation time $\tau = 0.2$ ns. Also indicated is the range of relaxation frequencies $(2\pi\tau_3)^{-1}$ of the present C_{14}-lecithin solutions (table 1).

function thus remains unknown at present. We are therefore cautious in discussing the parameter values compiled in table 1 and shall restrict ourselves rather to attempting to relate the different relaxation terms of eq. 1 to relevant molecular mechanisms.

The three fast relaxation processes observed in this study can be explained in a rather uniform manner by consideration of the modes of rotational isomerization in the hydrocarbon interior of the bilayers. Let us begin with the fastest process (denoted by '3' in eq. 1). For purposes of comparison, the ultrasonic excess absorption spectra of pure *n*-tetradecane is shown in fig. 4. A relaxation region emerges in the frequency range around $(2\pi\tau_3)^{-1}$. Such a region is found not only with this liquid but also with other *n*-alkanes [38] and with micellar solutions of zwitterionic [33] or ionic [39] amphiphiles. We assume this high-frequency relaxation term (subscript 3) to reflect the same molecular mechanism in the pure *n*-alkanes and in the solutions of micelles and vesicles, namely, segmental modes of motions in the hydrocarbon chains. It is well known that the mobility of chain segments in phospholipid bilayers increases when going from the head group to the middle of the membrane [40,42]. The terminal hydrocarbon groups of the membrane molecules are thus likely to undergo motions similar to those of the less ordered chains in the core of micelles or of pure *n*-alkanes.

Discussing temperature-jump and pressure-jump measurements together with previous ultrasonic absorption data, Holzwarth et al. [43] designed a model in which the formation of kinks [44,45] in the hydrocarbon phase of bilayers with a comparatively high degree of order leads to at least two further relaxation process. In conformity with the ideas of Gamble and Schimmel [46], the faster process is assumed to reflect a single kink coming into being. It can be identified with term

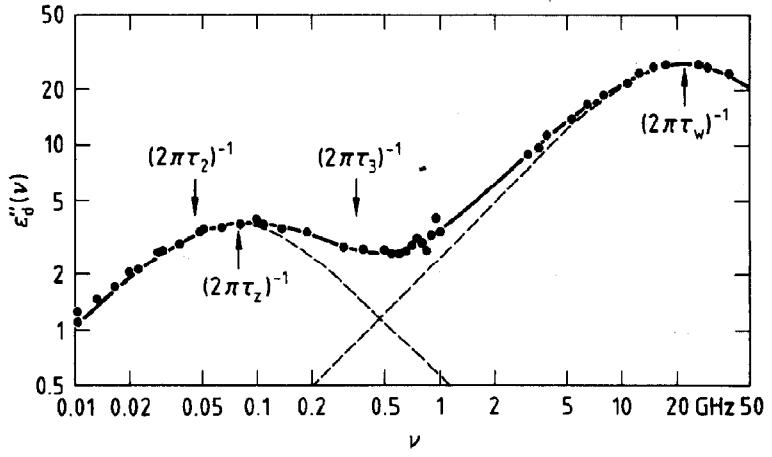

Fig. 5. Dielectric contribution $\epsilon_d''(\nu) = \epsilon''(\nu) - \sigma/(2\pi\epsilon_0\nu)$ to the negative imaginary part of the complex permittivity plotted vs. frequency ν for a 0.15 M aqueous solution of C_{14}-lecithin at 30 °C [25] (σ, specific electric d.c. conductivity; ϵ_0, electrical field constant).

'2' in eq. 1. The time ($\tau_2 \lesssim 10$ ns, table 1) for this initial 'kink-step' [47–49] probably does not suffice to complete the new state of equilibrium of the bilayer. Cooperative processes on a longer time scale are assumed to be necessary to allow for the changes in bilayer structure which obviously have to follow the formation of a kink. In particular, an expansion of the membrane to a larger surface area per molecule is required. We assume term '1' in eq. 1 (4.8 ns $\leq \tau_1 \leq$ 78.3 ns) to reflect these slow cooperative mechanisms.

A completely different process, however, may also add Debye-type contributions to the ultrasonic absorption spectrum in the frequency range of interest, namely, the thermally driven reorientation of the zwitterionic head groups. As illustrated by fig. 5, dielectric spectra of aqueous suspensions of single-walled phospholipid vesicles clearly reflect these head group motions [25] by a relaxation process. Its relaxation time τ_z adopts values between 1.2 and 2.4 ns ($30\,^\circ\mathrm{C} \leq T \leq 45\,^\circ\mathrm{C}$). It is likely that these zwitterionic reorientational motions are accompanied by volume changes in the hydration water. The mechanism will thus also couple to sound waves with the relevant frequency. Additional contributions to the ultrasonic excess absorption spectra have therefore to be expected. The present spectra, however, do not allow for an unambiguous analysis in terms of four Debye-type relaxations. Definite knowledge of the underlying molecular mechanisms requires additional studies in which properties of the bilayers are systematically varied by steps.

Acknowledgements

We thank Professor R. Pottel for stimulating and critical discussions, Mrs. H. Thomas and M. Oberdiek for purification of the phospholipids. Financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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