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FATTY ACID CONTAMINATION AND DIELECTRIC RELAXATION IN PHOSPHOLIPID VESICLE SUSPENSIONS

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Aqueous vesicle or micelle suspensions from various synthetic lecithins or surfactants – most of them purified by a simple ion-exchange procedure in methanol – were investigated, some with ionic admixtures. The dielectric permittivity $\epsilon'(\nu)$ between 5 kHz and 100 MHz was determined by different time- and frequency-domain methods, with attention given to electrode polarization below 1 MHz. Pure ether lecithins (used to reduce hydrolysis during preparation) as well as ester lecithins showed no dielectric dispersion below 10 MHz ($\Delta\epsilon' \leq 3$). In contrast, even dilute colloidal solutions containing about 1 mol% (with respect to solute) ionic amphiphiles normally exhibited large dielectric dispersion ($10 < \Delta\epsilon' < 700$), especially with electrolyte present. This low-frequency dispersion is sensitive to vesicle coagulation or fusion. Underlying relaxation mechanisms are discussed, and the main relaxation is shown to be the same as for other charged colloids. This conclusion suggest a new interpretation of measurements, previously reported by other authors, who gave an interpretation in terms of correlated zwitterionic head group orientation in multilamellar lecithin liposomes. Possible effects from traces of impurities in lipids are discussed.

1. Introduction

Aqueous suspensions of phospholipids have been investigated by dielectric relaxation spectroscopy to determine macroscopic properties, such as the dielectric permittivity of lipid membranes [1,2], and to obtain information about microscopic parameters of the dynamics of the zwitterionic head groups in the polar regions of lecithin bilayers [3–7]. Suspensions with zwitterionic molecules can be studied without introducing artificial labels.

Dielectric spectra of vesicles from crude soybean lecithin in buffer solution have been reported, between 100 Hz and 100 MHz, by Schwan et al. [1]. Two dispersion regions were found: a large one well below 1 MHz, that has been attributed to restricted diffusion of counterions at the surface of charged vesicles, and a small dispersion above 1 MHz, which was treated as a Maxwell-Wagner relaxation. The same interpretation was given for a dispersion step near 40 MHz, found by Redwood et al. [2] with suspensions of uncharged small unilamellar vesicles from purified egg lecithin, investigated between 0.1 and 500 MHz. When about 10 mol% fatty acid had been admixed, an additional low-frequency dispersion was observed, as expected for charged vesicles [8,9].

However, in both publications contributions to the dielectric permittivity from orientational motion of lecithin head group dipoles were not considered. When synthetic lecithins became available, Kaatze, Henze et al. [3–7] used data obtained by time-domain spectroscopy (TDS [10,11]) to determine the microscopic behaviour of lecithin head groups. The vesicles were assumed to be essentially uncharged, and all TDS spectra of lecithin suspen-

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sions showed a vanishing dispersion at about 100 kHz, so no dispersion due to diffusion of counterions was taken into account. The dispersion step, found between 1 and 10 MHz, had a typical magnitude of $\Delta\epsilon' \approx 10-80$ and was treated as a Maxwell-Wagner relaxation combined with highly correlated reorientation of lecithin head groups. Much larger dispersion steps were observed, if the suspensions had been cooled below the temperature T_t of the lipid phase transition [3,5].

The aims of this work are to reconsider the low-frequency part ($\nu \leq 1$ MHz) of Fourier-transformed TDS spectra from lecithin suspensions, to extend the frequency range of the spectra to about 5 kHz by frequency-domain methods, and to demonstrate the influence of small amounts of ionic contamination on the dielectric properties of lecithin suspensions. To this end it was necessary to consider electrode polarization effects below 1 MHz, to discuss possible sources of contamination in lecithin suspensions, and to develop a simple purification procedure, suitable in particular for ether lecithin. Ether lipids are known for their chemical stability and can be found – in their natural forms – for instance, in membranes of acidophilic and halophilic archaebacteria [12]. Synthetic forms are useful as stable references. The importance of assuring the purity of lipids has been stated repeatedly [13–15]. Dielectric measurements turn out to be very sensitive to certain classes of impurities in suspensions, for instance, fatty acids and fusogenic substances, found in commercial lecithin [16]. This impurity sensitivity makes a reinterpretation of earlier works necessary.

2. Experimental

2.1. Materials

Synthetic, chromatographically pure phospholipids and other amphiphiles were used. Dimyristoylphosphatidylcholine (DMPC) was purum (Koch-Light, batch 65508 and 82980), dipalmitoylphosphatidylcholine (DPPC) and myristic acid (both from Fluka), and distearoylphosphatidylcholine (DSPC, Bio-Science) were all puriss grade. The anionic lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl-1′-(3,3′-dimethylbutyl) ester (sodium salt, DPBE) and the ether lipid 1,2-ditetradecyl-*sn*-glycero-3-phosphorylcholine (DTPC) were synthesized by Dr. H. Eibl (Max-Planck-Institut für biophysikalische Chemie, Göttingen) [17], 1,2-dihexadecyl-*sn*-glycero-3-phosphorylcholine (DHPC) was an ether lipid from Serva (specified to be 97–99% pure). The non-ionic surfactant lauric acid diethanolamide (LDEA) was a technical product and contained about 30% myristic acid diethanolamide (specified by the manufacturer, Henkel).

DPPC, DSPC, myristic acid, DPBE and, in some cases, DMPC and DTPC were used without further purification. Ether lipids were chosen to avoid lipid degradation in the course of the purification process and afterwards.

KOH, methanol, *n*-pentane (all p.a., Merck), and the ion-exchange resin (Amberlite MB-2, p.a., Serva) were used without further purification. Nepton and Permion ion-exchange membranes were from a membrane kit (Serva). Water was quartz-bidistilled and ultraviolet sterilized. The specific electric conductivity was smaller than 2×10^{-4} S/m. Nitrogen was 99.99% pure (Messer).

2.2. Purification procedures

Three purification methods were compared in order to find a procedure, which is suitable to remove ionic contaminants from at least 0.2 g zwitterionic lipid, and which does not require fractionation or a special detector for the purified substance.

First, electrodialysis of 0.1 M aqueous lipid suspensions through ion-exchange membranes was carried out in a thermostated three-chamber perspex cell. Small coils from platinum wire served as electrodes in the outer chambers. They were separated by one of two cylindrical inner chambers, 2 or 5 ml, each 14 mm in diameter. The membranes in H^+ and OH^- forms were inserted between the chambers, and the suspension to be purified was stirred by a PTFE-coated magnetic bar in the inner chamber. 5–20 V d.c. were applied, but the current never exceeded 0.1 A (current density < 0.07 A/cm^2). The outer chambers initially contained distilled water. During electrodialysis both

chambers were intermittently diluted with distilled water.

The second procedure was mixed-bed ion exchange of aqueous suspensions at $T \approx T_t$, and is essentially the same as the third one, described below, except that the lipid was not crystallized from methanol after purification. Lipid concentration was determined by phosphate analysis as described by Eibl and Lands [18].

Most lipids were purified by the third method, in which water has been replaced by methanol as solvent during ion exchange. The column (300 mm long, 3 mm inner diameter, Duran glass, Schott; glass or quartz wool wad, polyethylene stopcock) was filled, using methanol, without demixing the two resin components, and then washed with 5 ml methanol. About 1 g lipid or surfactant was dissolved in about 10 ml methanol, in which the amphiphilic substances were contained as molecular solutes, or as very small aggregates [19]. For this reason no encapsulation of contaminants can occur.

The flow rate was 0.25–0.5 ml/min at 20–25 °C, and the first and last 0.5–1 ml of the eluate were discarded. At least 90% of the main fraction was evaporated under nitrogen at 40 °C in a water bath. Methanol was removed by shaking with *n*-pentane and evaporating as above (two to three cycles). Finally, the white powder was dried at 40 °C in an incubator for about 3 h, and thereafter stored like all our lipids at 3 °C.

The ion-exchange resin catalyses hydrolysis of ester lecithin. Most of the resulting fatty acid will be retained within the ion exchanger, but not lysolecithin as the byproduct. Even small amounts of lysolecithin influence the properties of lecithin vesicle suspensions [13,14], although the dielectric properties are not directly affected. The problem was avoided by the use of ether lecithin instead of ester lecithin.

2.3. Preparation of samples

The suspensions were prepared in a manner that is expected to give predominantly small, probably unilamellar vesicles [13,14,20] of about 15–20 nm radius.

Weighed amounts of lipid or surfactant were sonicated with a bath-type homogenizer (Bransonic) in 2 ml freshly bidistilled water or in dilute aqueous solutions of KCl, NaCl or potassium myristate until a macroscopic homogeneous, almost clear suspension was obtained, normally with relative low viscosity. During sonication the probe was kept at about 45 °C, i.e., above the lipid phase transition temperature.

Potassium myristate was obtained by neutralization of myristic acid with KOH and subsequent recrystallization. The amount of potassium myristate added to the lipid suspensions was always less than 0.7 wt.% lipid, a value which had been specified by Koch-Light as the possible fatty acid contamination concentration of their lecithin. Such quantities are not easily detected by thin layer chromatography.

2.4. Dielectric measurements

The frequency-dependent complex permittivities, $\epsilon(\nu) = \epsilon'(\nu) - i\epsilon''(\nu)$, were normally obtained by frequency-domain methods. In some cases they could be compared with data from TDS [10,11].

At seven fixed frequencies between 1 and 100 MHz we used a Boonton 33 D/1 admittance bridge, together with a coaxial cut-off cell [21]. The cell constants were determined by calibration with air and pure water.

Below about 1 MHz one has to correct for the electrode polarization [22,23]. A plane parallel plate guard ring capacitor with variable electrode spacing, d, was constructed and used in combination with a three-terminal capacitance bridge (Boonton 75C) between 5 and 500 kHz. A General Radio model 1654 impedance comparator (with 1433-F decade resistor and 1412-BC decade capacitor) could be used at 0.1, 1, 10 and 100 kHz, as long as the capacitance value C_{exp} was larger than 50 pF and the specific electric conductivity was small enough. Both bridges allow one to measure the impedance

$$Z_{exp} = (G_{exp} + i\omega C_{exp})^{-1} \quad (1)$$

of the cell without being influenced by the leak impedance to ground. Conductance-dependent errors $\Delta C(G_{exp})$ and $\Delta G(G_{exp})$ of the Boonton 75C

bridge were reduced by calibration with a set of 96 precision resistors, designed for negligible parallel capacitance $C < 50 \times 10^{-15}$ F (O. Göttmann, personal communication). Using short coaxial cables, lead length could be ignored.

The capacitor cell for liquids with $\epsilon' \gg 1$ has been constructed to combine advantages of different cells described in the literature [22,24–27]. A detailed description of the cell is found in the appendix to ref. 28.

A plot of the apparent permittivity

$$\epsilon'_a(d, \nu) = \left[C_{exp}(d, \nu) + \Delta C(G_{exp})\right] / C_{0,th}(d) \quad (2)$$

vs. d^{-1} gives a straight line. $C_{0,th}(d)$ is the theoretical capacitance of an empty guard ring capacitor [24]. Linear regression allows calculation of the true permittivity $\epsilon'(\nu)$ by extrapolation to $d^{-1} = 0$ [28]. This was sufficiently accurate for all conclusions drawn here. Errors larger than 1% at low frequencies are indicated in the figures. In some cases an increased electrical conductivity of the suspension prevented measurements in the lower frequency range due to large electrode polarization.

For time-domain measurements we used the same apparatus as Henze et al. [3–7,11]. For different time windows between 200 and 1300 ns the step responses were digitally stored and numerically transformed into the frequency domain [7,11], to obtain the spectra for frequencies from about 0.5 to 30 MHz. Special problems at lower frequencies will be mentioned below.

3. Results

Because of their importance for the interpretation of the dielectric measurements on lecithin, two results concerning peculiarities of the methods shall be presented first. Subsequently the dielectric spectra of purified lecithins, and of lecithins with small amounts of fatty acid salt and/or low molecular weight electrolyte are given.

3.1. Electrode polarization and time-window length in TDS

TDS is known as a very convenient method to obtain dielectric spectra up to several gigahertz [10,29]. However, when electrically conducting lipid suspensions are investigated, the low-frequency limit of the time-domain reflectometer and sample cell, both used in refs. 3–7 and in this work, is found to be markedly higher than had been assumed in the past [11].

If there is any dispersion at about 1 MHz, then a short time-window ($t \lesssim 1$ μs) leads to truncation errors, which diminish the apparent dispersion step and give misleading stationary ϵ' values for $\nu \approx 100$ kHz. Namely, for all suspensions of DMPC (Koch-Light) considerable truncation errors for $t \lesssim 1$ μs were revealed by calculating spectra from measurements with time windows up to $t \approx 1.3$ μs or by checks against spot frequency measurements.

Further extension of the TDS window is not useful with our method for several reasons, which are discussed in refs. 7 and 11. The main argument against time windows $t > 1.3$ μs is, however, that dielectric relaxations within the bulk suspension occur on the same time scale as electrode polarization effects. In principle, these effects can only be separated with a four-terminal cell or a cell (similar to our parallel plate capacitor) with variable dimensions. It would be difficult to utilize either cell with our time-domain reflectometer. To avoid truncation errors and ϵ' enhancement by electrode polarization as well, for $\nu \lesssim 1$ MHz only corrected results from frequency-domain measurements (see section 2.4 and ref. 28) are presented here.

3.2. Purification

Since indications are found, as described below, that ionic impurities, even in thin-layer chromatographically 'pure' lipids, can affect some physical properties to a large extent, purification proved to be essential.

First, electrodialysis of aqueous lipid suspensions through ion-exchange membranes was found to be very time-consuming due to membrane fouling. Furthermore, it led only to incomplete ion removal, since the contaminants partly remained encapsulated in the vesicle interior.

The latter objection also applies to mixed-bed ion exchange of aqueous lipid suspensions. We tried to overcome this problem by holding the

temperature close to the lipid phase transition temperature T_t during the purification process, since vesicles were reported to show an increased permeability for many different substances near T_t [30]. This gave suspensions of lower conductivity and smaller dielectric dispersion. However, by both procedures the lipid concentration was altered, and had to be determined by phosphate analysis after purification.

Best results were obtained by ion exchange with a mixed-bed ion exchanger in methanolic solution, as described in section 2.2.

The zwitterionic surfactant 3-[*N*-(*n*-hexadecyl)-*N*,*N*-dimethylamino]propane-1-sulfonate (crystallized technical product, Henkel) could not be purified in this manner, since it totally blocked the column. This indicates that the described method removes surfactants and lipids, both ionic and zwitterionic (if the latter contained a strong acidic or basic group), and small ions.

It should be noted, that the purified nonionic surfactant LDEA was not soluble in water to a perceptible degree, in contrast to its behavior before ion exchange. Afterwards it behaved rather like, e.g., hexadecanol/water in the range 0–70 °C.

The specific electric conductivity of the solutions of purified ether lecithins was, in spite of the ability of the ion-exchange purification, about 10-times larger than was expected from the conductivity of the distilled water. This may be due to small amounts of phosphorylcholine, which would increase conductivity, but not be detectable by dielectric measurements.

In table 1 parameters of the different suspensions are compiled. Several suspensions were measured twice, both before (b) and after being cooled (c) for a few seconds below the lipid phase transition temperature T_t. This cooling process triggers an irreversible change in the structure of the lipid suspension, as reported by other authors (e.g., refs. 3–7). The differences in temperature T are immaterial.

3.3. Purified lecithin

As a remarkable result, the aqueous pure DTPC and DHPC vesicle suspensions (nos. 9, 15 in fig. 1; see also table 1) show no significant dielectric dispersion from about 10 MHz down to 5 kHz. These ether lecithins should be the best defined pure lecithins that have been investigated by dielectric relaxation spectroscopy until now. In fig. 1 the low-frequency spectra for different purified lecithins (nos. 7c, 9b, 9c, 10c, 15) can be compared to the static dielectric permittivity of pure water at 30 °C, and to a typical example of an unpurified DMPC suspension (no. 2c). Due to partial dielectric screening of the water in the trapped volume of vesicles, pure lecithin suspensions have a lower ϵ' value with respect to pure water. The spectrum of DTPC is not significantly altered by addition of KCl (no. 10), which will not bind to pure lecithin [31].

All purified lecithin suspensions have a small dielectric dispersion near 80 MHz (30 °C), which is identical for both types of lecithin. This dispersion had long been overlooked, as it is very difficult to see with unpurified ester lecithin, but Göpel [32], who used the purification procedure described here, has now investigated it in detail.

Even when purified as described, ester lecithin (fig. 2, no. 4) shows a second variable dispersion at lower frequencies, which is small compared to that of an unpurified sample (fig. 1, no. 2).

3.4. Fatty acid added

The incorporation of only 1 mol% fatty acid anions into the aggregates influences the suspension and the dielectric spectrum in several ways.

First, fatty acid anions stabilize the suspensions and act as solubilizers. This is seen from shorter sonication times needed to suspend the amphiphiles, DPPC from Fluka (suspension no. 14, not shown here; see ref. 28), and even more impressively with purified LDEA. With 1 mol% potassium myristate, DPPC gave a homogeneous suspension after about 90 min of weak sonication, whereas formerly – without admixture – high-intensity sonication had been unavoidable [4] (DPPC analogs mentioned in ref. 4 could be dispersed by weak sonication due to a small content of phosphatidic acid [33]).

Second, a low-frequency dielectric dispersion is seen only in those cases where the ether lecithin suspensions contain admixed fatty acid anions

Table 1

Composition of aqueous lipid or surfactant suspensions and their electric conductivity before and after conversion (suspension numbers as in ref. 28 and in figs. 1–3)

m, w: ion exchange in methanol or water, respectively; x, X: possible small or large contamination with ionic surfactant or low molecular weight electrolyte; b, c: ϵ' spectrum and σ measured before or after conversion by cooling below T_t.

No.	Substance	T_t (°C)	Ion exchange	Concentration (mol/l)	Addition (mol%)		T (°C)	Conductivity σ(mS/m)		Comments
					Potassium myristate	KCl		b	c	
1	DMPC	23.7	–	0.050	X	X	20	–	9.9	
2	DMPC		–	0.10	X	X	30	77.0	70.0	2c+ = 2c with 2.5 mol% KCl
4	DMPC		m	0.10	x	–	30	2.4	1.7	settles at 20 °C
7	DTPC	≈ 27	w	0.090	–	≈ 1	30	10.5	–	phosphate determined (Eibl/Lands)
9	DTPC		m	0.10	–	–	30	2.75	2.79	
10	DTPC		m	0.10	–	0.5	30	–	7.7	
11	DTPC		m	0.10	1.0	–	30	9 ± 1		b (= c?)
12	DTPC		m	0.10	1.0	0.5	30	16.4	15.8	
13	DTPC		m	0.010	1.0	0.5	30	2.6		
15	DHPC	≈ 49	m	0.10	–	–	30	2.4		
16	DHPC		m	0.10	1.0	–	30	8.0		Conversion at 30 °C very slow
17	DSPC	54.9	–	0.0010	X	x	25	0.35		ϵ' (5 MHz) = 77.4 ± 2.5
18	DSPC		–	0.0010	X	100.	25	12.0		NaCl instead of KCl added
20	LDEA		m	0.10	1.0	–	30	7.6		high viscosity, ϵ' (11 kHz) ≈ 205
21	DPBE	≈ 40	–	0.059	X	X	20	≈ 32		TDS (see section 2.4), ϵ' (500 kHz) ≈ 300

(nos. 11, 12, 16). The spectrum then can be very similar to that of unpurified DMPC.

Orientation polarization of small permanent dipoles – as are the lecithin head groups – is detectable by dielectric relaxation spectroscopy, if the dipole concentration is not much below 0.1 mol/l. As is seen from figs. 1 and 2, this concentration of zwitterionic groups is neither required (nos. 20, 21), nor it is sufficient (nos. 9, 10, 15) for a low-frequency dispersion. Even in comparatively dilute suspensions (fig. 3; nos. 1, 17, 18) a large dispersion is possible.

Incorporated fatty acid anions plus low molecular weight electrolyte are still not sufficient for a dielectric low-frequency dispersion (cf. nos. 12 and 13 in fig. 3). Using a simple concentration proportionality, the 10-fold dilution of sample 12c with pure water (sample 13c) should show a dispersion of $\Delta\epsilon' \geqslant 20$. The vanishing dispersion after dilution indicates a sharp concentration dependence of the dispersion.

In no case could the low-frequency dielectric dispersion region be completely covered by the available measuring techniques, a problem also

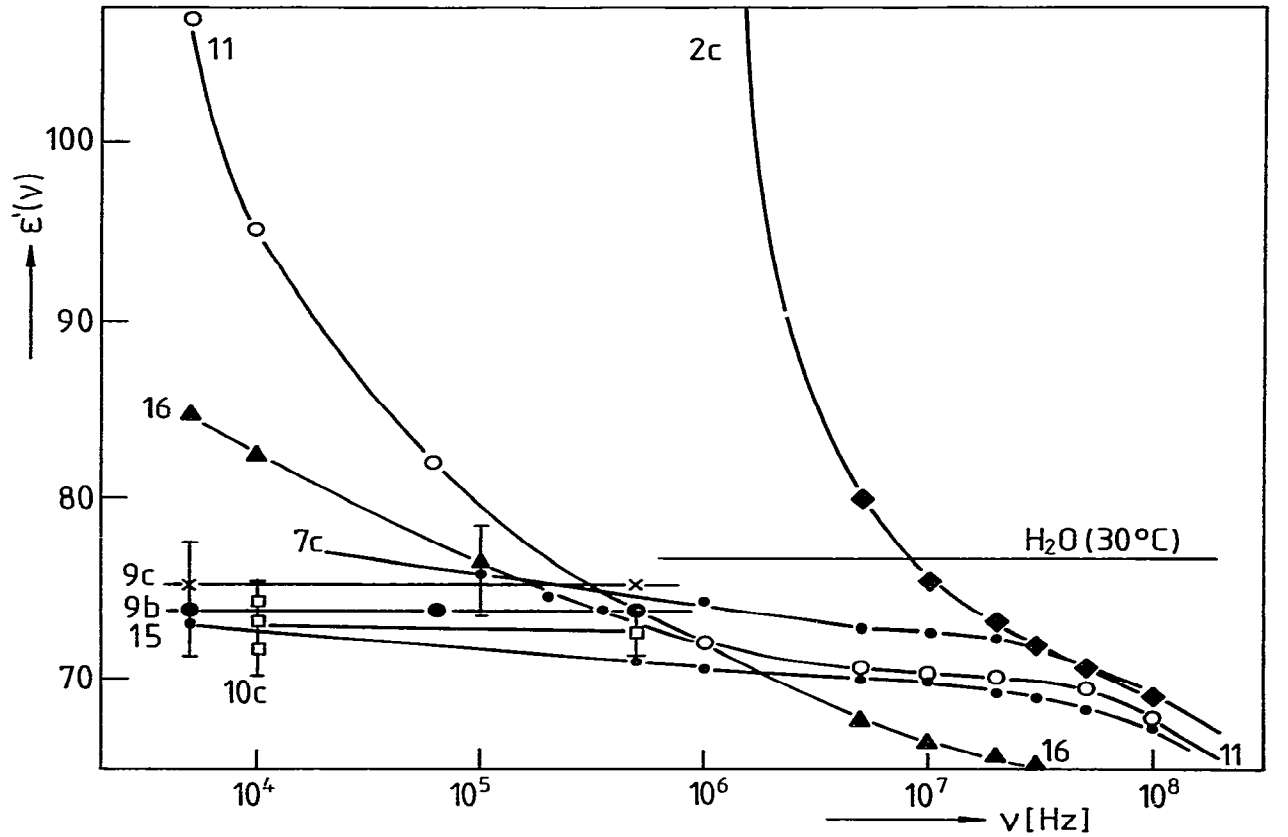

Fig. 1. Frequency dependence of the dielectric permittivity ϵ' of concentrated aqueous lecithin suspensions at 30 °C, all 0.10 M except no. 7 (0.09 M), before (b) or after conversion (c) by cooling below T_t. Nos. 9b, 9c, 15: pure ether lecithins sonicated in H_2O; and nos. 7c, 10c: pure ether lecithin in dilute aqueous KCl. Nos. 11, 16: ether lecithins with 1 mol% potassium myristate, suspended in H_2O. For comparison $\epsilon'(\nu)$ of pure H_2O and of unpurified ester lecithin (DMPC, no. 2c) in H_2O are shown.

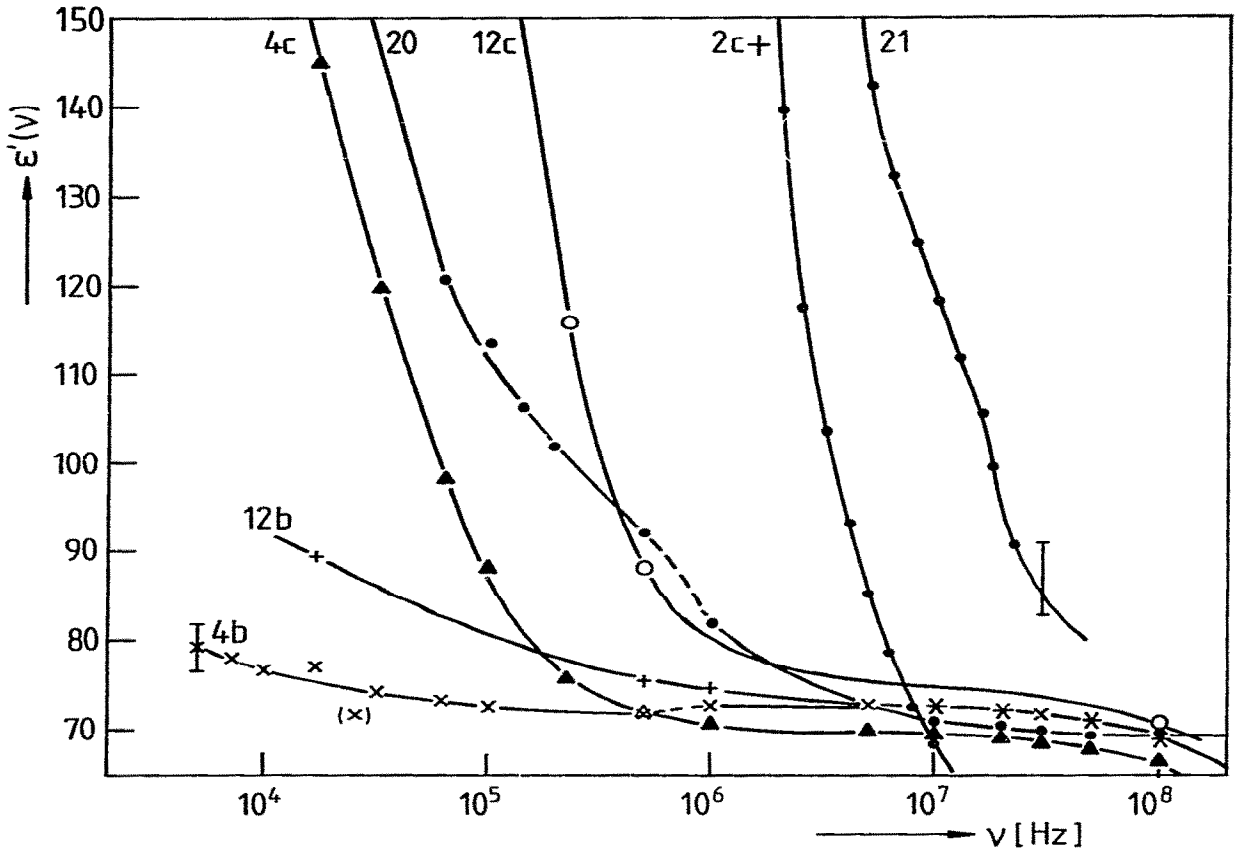

Fig. 2. Dielectric permittivity $\epsilon'(\nu)$ of concentrated aqueous suspensions containing charged aggregates. No. 21: anionic lipid DPBE, 0.059 M in H_2O at 20 °C; the error bar indicates uncertainty in the absolute values of TDS data. All other suspensions are 0.10 M and measured at 30 °C by frequency-domain methods. Nos. 4b, 4c: purified DMPC sonicated in H_2O, before and after conversion; nos. 12b, 12c: pure DTPC with 1 mol% potassium myristate sonicated in 5×10^{-4} M aqueous KCl (identical ϵ' for nos. 4b and 12b at $\nu > 10$ MHz); nos. 2c+: unpurified DMPC in 2.5×10^{-3} M aqueous KCl, after conversion; no. 20: nonionic surfactant (LDEA) with 1 mol% potassium myristate in H_2O (note the constant ϵ' at $\nu > 10$ MHz).

faced in earlier investigations (ref. 1, p. 1111). As a consequence, the magnitude of the dispersion step and the relaxation time (distribution) cannot be determined without assumptions. Yet, limiting values can be estimated. In any case the low-frequency dispersion data could only be fitted by relaxation spectral functions with broad relaxation time distributions.

Finally, fatty acid anions and low molecular weight (1-1) electrolytes are involved in some phenomena, seen near the lipid phase transition temperature T_t.

Upon cooling a sonicated suspension (a few seconds are enough) for the first time somewhat below T_t we found, as described by Henze [7], an irreversible change in the character of the suspensions of both unpurified and purified ester lecithin. Typically, the specific electric conductivity de-

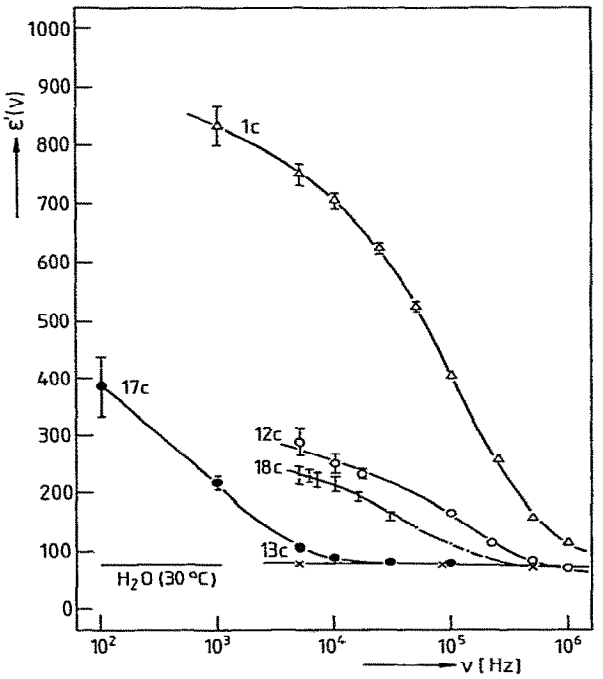

Fig. 3. Low-frequency dielectric dispersion in concentrated and dilute aqueous lecithin suspensions after conversion. No. 1c: 0.05 M unpurified DMPC in H_2O; no. 12c: 0.1 M pure DTPC with 1 mol% potassium myristate in 5×10^{-4} M aqueous KCl; no. 13c: same as 12c, but 10-fold diluted with H_2O; no. 17c: 0.001 M unpurified DSPC in H_2O; no. 18c: same as no. 17c, but in 0.001 M aqueous NaCl.

creases by some percent, whereas the viscosity and turbidity increase. The low-frequency dielectric dispersion increases several-fold. The latter effect increased additionally up to 8-fold, whereas the conductivity after conversion was nearly unaltered, if the suspension had been prepared with $1-2.6 \times 10^{-3}$ mol/l KCl or NaCl, or when using DMPC batch 82980 unpurified.

With ether lecithin unequivocal indications for a similar irreversible change (affecting mainly conductivity and dielectric dispersion) were found only if potassium-myristate and low molecular weight electrolyte simultaneously were present (no. 12; nos. 11 and 16 are unclear).

4. Discussion

4.1. Low-frequency spectra of uncharged and charged vesicles

The results with purified ether lecithin show that aqueous suspensions from uncharged vesicles have a constant $\epsilon'(\nu)$ spectrum below about 10 MHz. Vesicles formed from pure lecithin must be uncharged, whether or not a (1-1) electrolyte is present, since lecithin is zwitterionic near pH 7 and does not bind monovalent ions at low or moderate ion concentrations [31]. If the results on DMPC containing fatty acid are extrapolated to pure lecithin, the same constant spectrum as that of ether lecithin is expected. This is consistent with data, obtained only at frequencies above 100 kHz, on purified egg lecithin [2], but contrasts with a previously reported dielectric dispersion near 1 MHz, which had been found with not additionally purified commercial synthetic ester lecithins [1,3–7].

Obviously, the low-frequency dielectric spectrum is extremely sensitive to a net charge on the colloidal particles. Furthermore, suspensions from charged vesicles depend in their properties on the ionic strength and the preliminary treatment of the individual sample.

Assuming that lecithin suspensions contain only zwitterions but no charged aggregates, a low-frequency dielectric dispersion had been previously interpreted in terms of highly correlated reorientation of zwitterionic head groups [3–7].

For the low-frequency dielectric dispersion in vesicle (and micelle) suspensions a different interpretation is now suggested. The results presented above indicate that this dispersion is independent of zwitterionic groups and only occurs if the colloidal particles are charged. A similar dispersion is observed with many different colloidal solutions, provided the particles carry a net charge [34,35]. This interpretation seems to be at least in qualitative accordance with theoretical models, as discussed below.

Further support comes from investigation of the small dispersion step near 80 MHz, which we observed with all purified lecithin suspensions and which probably was only masked in unpurified

samples. Göpel [32] could attribute this dielectric dispersion to the reorientation of zwitterionic head groups. This is consistent with findings of Müller [21,36–38] on lysolecithin and other molecules with zwitterionic groups in aqueous solution, and also consistent with data on lecithin bilayer stacks, published by Shepherd and Büldt [39].

4.2. Origin of contaminants and effects on lecithin vesicle suspension stability

Vesicles from lecithin can acquire a net charge in several ways: (i) by means of charged impurities, e.g., phosphatidic or fatty acids from synthesis; in the case of DMPC (batch 65508, Koch-Light) fatty acids amount to about 1 mol%. Myristic acid contaminants in batch 65508 were reported by Kremer et al. [16]. (ii) Ester lecithins can be hydrolyzed during sonication, in particular at higher temperatures and in the presence of acidic or basic centers. Degradation products of lecithin have been studied by Hauser [15]. In our view, the effectiveness of sonication in suspending lipids seems to result not only from fine mixing, but sometimes from degradation of lipids. In the course of sonication charged amphiphiles may accumulate until they eventually stabilize even a concentrated suspension. Stabilization against coagulation, according to the DLVO theory of colloid stability [40], is expected in the presence of fatty acid anions. Stabilization seems to be enhanced by small amounts of charges (about 1 elementary charge $e_0/200$ hydrocarbon chains, or even less, as was seen from DPPC or LDEA; section 3.4). Similar results for DMPC and a cationic surfactant were recently reported [41].

With ether lecithins it is possible to bypass both problems i and ii, since they are easily purified of original ionic contaminants in a simple ion-exchange procedure. Unlike ester lecithins (see section 2.2) they will not hydrolyze to fatty acid plus lysolecithin, either in contact with the ion exchange resin or during sonication.

Both lysolecithin and fatty acid are likely to be present in ester lecithin in small quantities, and both are reported to be involved in processes leading to vesicle fusion near T_t (conversion) [16,42–46]. A reasonable mechanism for these processes could not be found, but the dielectric spectra can supply some additional information about changes in aggregate radius (a) and volume fraction (v_v) of vesicles.

Fusion will alter the ϵ' spectrum at high frequencies if v_v changes. An increasing v_v at about 500 MHz ($\approx \epsilon_{sw}$ with aqueous suspension [33]) gives smaller ϵ' values, which indicate dielectrically screened water. During conversion the dispersion step near 80 MHz decreases in magnitude only slightly [32], and ϵ' (500 MHz) changes by nearly the same amount as ϵ' (10–100 MHz). So the latter can be used as a qualitative measure of v_v, if a low-frequency dispersion is subtracted (quantitative evaluation of v_v from ϵ' data requires knowledge of the vesicle size distribution).

As is seen from fig. 2, mere coagulation occurs during conversion with ether lecithin (nos. 12b, c), whereas ester lecithins undergo fusion with increasing v_v (nos. 4b, c; see also no. 2c+ for $\nu > 10^7$ Hz). This difference may be explained by various amounts of lysolecithin, present even after purification, in our DMPC but not in DTPC. Coagulation is often considered as a preliminary step to fusion [43–46]. Probably lysolecithin leads to fusion of our DMPC vesicles in a second step following coagulation [42,46].

Coagulation requires a decrease of the Debye screening radius

$$\kappa^{-1} = \left[\epsilon_0 \epsilon_w kT / \left(e_0^2 \Sigma c_i z_i^2\right)\right]^{1/2} \qquad (3)$$

(where ϵ_0 is the permittivity of a vacuum, ϵ_w the relative permittivity of the solvent, c_i and z_i the concentration and valency, respectively, of low molecular weight ion species i in the solvent, and e_0, k and T have their usual meaning) to a value where the attractive long-range van der Waals forces [40,47] among the vesicles can overcome the electrostatic repulsion. The decrease of κ^{-1} must be related to specific properties of the lipid vesicles at T_t. Increased ion permeability in the phase transition region [30] may play a role. An asymmetric distribution [48,49] of different amphiphiles (in this case fatty acid anions) between the inside and outside of mixed vesicles, as well as an electrolyte concentration gradient between the trapped volume and the outer volume of charged vesicles

would be important, if the distribution of those ions were, for some reason, not initially in equilibrium after sonication. Additional complications arise due to the existence of two modes of coagulation [50] depending on vesicle charge and size.

4.3. Mechanisms of dielectric low-frequency dispersion

An exact theory describing the studied suspensions is not yet available, but the low-frequency dispersion, although complicated by processes, which depend on the nature of the lipids, seems to be explicable by restricted ion migration at or near charged particles (not necessary vesicles).

In recent years advanced theories of low-frequency dielectric dispersion in colloidal suspensions have been developed by Dukhin et al. [35,51,52] and by Fixman [8,53]. They are limited to dilute solutions and counterion atmospheres, where κ^{-1} is thin compared to the particle radius a (mean distance between neighboring particles $D \gg a$, $\kappa a \gg 1$). Therefore, they cannot be applied to the 0.1 M suspensions before conversion (solutions marked 'b', and nos. 11, 16, 20), where a_b, D_b and κ_b^{-1} all are of the same magnitude ($a_b >$ 15–20 nm, $D_b \approx 30$ nm for $v_v = 0.1$, $\kappa_b^{-1} \approx 20$–60 nm depending on electrolyte concentration). In these cases, due to the complete overlapping of counterion atmospheres, the counterion diffusion is not restricted and does not give rise to dielectric dispersion, as pointed out and discussed by Pottel et al. [33]. However, the lateral diffusion of fatty acid anions is confined to the vesicle shells or to individual micelles and can lead to dielectric dispersion. Since diffusion normal to the particle surface is much slower than lateral diffusion, it can be neglected and the theory of Schwarz [9] can be applied [33]. The relaxation time τ_0 is essentially given by the same relation as in the above-mentioned theories:

$$\tau_0 \propto a^2/ukT, \tag{4}$$

but now u represents the lateral mobility of fatty acid anions within the aggregates, instead of the electrolyte ion mobility. Consequently, very low relaxation frequencies ν_0 are observed with lipid vesicles (nos. 11, 16; distribution of radii very likely) and reasonably higher values for ν_0 are found with micelles (no. 20; smaller particle radius a, narrower distribution of radii and somewhat higher mobility u expected).

In converted suspensions (marked 'c') the vesicles are coagulated to clusters (distance D_c) without change of v_v and/or they have fused to larger liposomes. In any case $D_c \gg D_b$, $a_c > a_b$ and $\kappa_c^{-1} \leq \kappa_b^{-1}$ must be expected and the assumption of the Dukhin/Fixman theories (ideally $D_c \gg a_c \gg \kappa_c^{-1}$) are much better approximated. For charged particles, then, a low-frequency dispersion due to the so-called 'concentration polarization' of electrolyte is predicted by these theories [8,35]. Theoretically expected relaxation times τ_0 and dispersion steps as $\Delta\epsilon' \propto a^2$ are similar to those of the Schwarz theory [9] and depend on several not exactly known parameter values. Estimates of τ_0 and $\Delta\epsilon'$, calculated by means of a modified Schwarz theory, and typical values for our suspensions after fusion are given in ref. 33.

Spherical clusters of coagulated vesicles will have a volume conductivity and must be treated separately, for instance, similar to the treatment of 'ionite' particles in ref. 52. Clusters of this type, or chains of vesicles, are expected in suspension no. 12c ($\Delta\epsilon'$ (5 kHz) $\approx$ 230, fig. 3). 10-fold dilution by distilled water (no. 13c) will increase κ^{-1} by a factor of $10^{1/2}$ and disperse the clusters or chains (not possible if fusion has taken place). Since D_c by this dilution increases only by a factor of $10^{1/3}$ compared to suspensions no. 11 or 12b, overlapping of counterion atmospheres must occur. Thus, counterion relaxation is no longer possible ($\Delta\epsilon'$ (5 kHz) $\ll$ 23), and the dispersion due to diffusion of fatty acid anions will be 10-times smaller than with suspension no. 11 ($\Delta\epsilon'$ (5 kHz) $<$ 40, fig. 1). This is observed: no. 13c (fig. 3) shows no significant dielectric dispersion. This further supports the conclusion that the low-frequency dispersion is not caused by correlated reorientation of zwitterionic head groups, but reflects diffusion of electrolyte in the region between charged vesicles (concentration polarization).

4.4. Conclusions

Various amounts of lysolecithin, fatty acid, and other contaminants in lipid preparations – even if

thin-laer chromatographically 'pure' – may have been responsible for problems with reproducibility, sometimes reported in the past. A number of lipid bilayer (or vesicle) properties depend on lipid packing irregularities and on surface charges. In this way by changes in ion concentrations (H^+, Ca^{2+}, K^+, etc.), lipid properties – for instance, near T_t – can be coupled to other processes within or even outside the ionic double layer at the lipid/water interfaces. This may be sensitively traced by dielectric measurements. The attainable sensitivity is nearly equal to that of the equilibrium spreading pressure method near T_t [54]. If for a suspension most of the parameters are known and the approximations of existing theories of low-frequency dispersion are valid, then the accurate determination of some additional parameters from dielectric measurements will be possible.

Results of other techniques of investigation may be affected by small amounts of impurities as well. As an example, we want to discuss briefly a technique for preparing vesicles, developed in order to avoid lipid (and protein) degradation during sonication [55]. Here the ionic detergents are removed by dialysis to a ratio of about 1 detergent molecule per 100 lipid molecules. We expect the dielectric spectra at low frequencies of these suspensions to be different from those of pure lecithins. Whether improved detergent removal techniques [56] yield sufficient pure suspensions still has to be checked. Our objection does not apply, if vesicles prepared in this way are used as drug carriers, but only applies if a surface charge due to detergent molecules were not taken into account in studies of properties of uncharged lipids.

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