Dielectric Analysis of Phosphorylcholine Head Group Mobility in Egg Lecithin Liposomes

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Purpose. A knowledge of the interfacial properties of lecithin underpins our understanding of many of the physicochemical characteristics of drug delivery systems such as liposomes and lecithin stabilized microemulsions. In order to further this understanding, a high frequency dielectric study of the interfacial properties of egg lecithin liposomes was undertaken.

Methods. The effect of temperature, lecithin concentration and probe sonication on the interfacial dielectric properties of liposomal suspensions was investigated by high frequency dielectric relaxation spectroscopy between 0.2–6 GHz.

Results. The frequency dependent permittivity of each suspension exhibited a dielectric dispersion centred around 100 MHz, corresponding to the relaxation of zwitterionic head groups. The activation energy for head group reorientation was estimated as $\Delta H = 6.3$ kJ mol^{-1}. There was an increase in extent of inter-head group interactions on increasing the liposome volume fraction, whereas the effect of probe sonication showed that: (i) head groups in both the outer and inner lamellae contribute to the dielectric response; (ii) the head groups may be less restricted in liposomes of high surface curvature with few lamellae; (iii) the high frequency permittivity of the suspension increased on sonication, as a result of a reduction in the amount of (depolarized) interlamellar water following a reduction in the number of lamellae per liposome.

Conclusions. Dielectric analysis of the zwitterionic head groups of lecithin therefore provides a means for investigating the surface of lecithin liposomes, and may be used to investigate the effect of drugs and other solutes on membranes.

KEY WORDS: lecithin; phosphorylcholine; polarization; dielectric-relaxation; activation-energy; liposome.

INTRODUCTION

Dielectric relaxation spectroscopy (DRS) determines the magnitude and time dependency of electric polarization processes (i.e., the separation of localized molecular charge distributions) by measuring the polarizability of a material as a function of the frequency of an applied oscillating electric field (1,2). At high frequency, the reorientation polarization of dipolar molecules can no longer keep pace with the alternating field and therefore the molecules relax to their random orientation. The frequency ranges over which molecules relax depend, in part, on the rotational freedom of the molecule.

The sensitivity of DRS to changes in molecular mobility has pharmaceutical applications in the investigation of the mobility of water in hydrated polymers (3,4) and the interactions of electrolytes with liposome surfaces (5). In the high frequency dielectric analysis of lecithin liposomes, the zwitterionic head groups polarize by rotating in a manner describing a cone with an axis normal to the membrane. The kinetics of reorientation depend on the environment of the head group, and therefore the dielectric relaxation time is sensitive to membrane fluidity, molecular packing and the extent of interactions with neighbouring molecules. Relaxation times between 0.5–8.0 ns (corresponding to a frequency range of 20–300 MHz) have been observed (2).

The aim of this study was to investigate the effect of temperature, lecithin concentration and probe sonication on: (i) the relaxation behavior/mobility of the head groups of lecithin liposomes; and, (ii) the contributions to the high frequency relative permittivity (otherwise known as the dielectric constant) from the dipole polarization of water.

MATERIALS AND METHODS

Preparation Liposome Suspensions

Multilamellar liposomes (30% w/w lipid) were prepared from egg lecithin by hydrating a dry film of lipid in distilled water at 50°C for 20 min, followed by bath sonication for 20 min. Dilutions of liposomes between 5–25% w/w were prepared from the 30% w/w suspension. Small vesicles were then prepared from the multilamellar liposomes by probe sonicating the lipid suspension (for 20 min) until almost clear. Throughout probe sonication the temperature of the suspension was maintained below 20°C by immersing the lipid suspension in a mixture of ice and water.

Measurement of Frequency-Dependent Dielectric Properties

The frequency-dependent dielectric properties of liposome suspensions were determined between 10 MHz and 6 GHz using a Hewlett-Packard HP85070M materials measurement system, comprising a HP8753D network analyzer, HP85070B dielectric probe and HP85071B materials measurement software. The measurement software calculates the relative permittivity (ϵ') and imaginary permittivity (i.e. dielectric loss, ϵ'') of the material from the transmission/reflection characteristics of the probe/sample interface. The instrument was calibrated using air, a calibrated short circuit and distilled water as a measurement standard of defined dielectric properties. Refresh calibrations using distilled water were performed prior to each sample measurement. The sample (or water, as the calibration standard) was contained in a glass vial attached to the surface of the probe using a silicon rubber adapter. Sample volumes

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of 15 mL were used routinely in order to avoid errors associated with reflection of the incident energy from the top surface of the liquid. The probe, with the sample vial attached, was then inverted to bring the sample into intimate contact with the surface of the probe, and the dielectric properties (ϵ' and ϵ'') determined from the network analyzer measurements as a function of frequency.

RESULTS AND DISCUSSION

The frequency dependent relative permittivity (ϵ') of aqueous suspensions of egg lecithin liposomes (20°C) exhibits (i) a dielectric relaxation centred around 100 MHz (Figure 1) corresponding to the relaxation of the phosphorylcholine head groups (6), and (ii) the beginning of a high frequency relaxation resulting from the dipole polarization of water. The solutions were not buffered as the addition of electrolyte (0.01 M phosphate buffer) caused the appearance of a pronounced low frequency dispersion that effectively swamped the dispersion associated with head group reorientation. This low frequency dispersion results from interfacial/RC charging of the phosphatidylcholine membrane (otherwise known as Maxwell-Wagner charging) and counter-ion polarization over the surface of the liposomes. Electrolyte increases the conductivity of the aqueous phase such that the relaxation time for membrane charging ($\tau = RC$[8]) shifts the dispersion to higher frequencies (7), whereas an increased counter-ion concentration significantly increases the permittivity over the low frequency part of the head group dispersion (8). The net effect is to mask the small dispersion due to head group polarization, of which the increment in permittivity is a mere 0.3 per 1% w/w lecithin. Further studies with lipids of high purity, however, will significantly reduce the interference associated with counter-ion polarization by reducing the fatty acid content of the lipid. The use of pure lipid may then allow studies on solutions containing nominal concentrations of electrolyte/buffer.

Treatment of the Results

The frequency dependent relative permittivity (ϵ') was modelled by regression analysis of a function (Equation 1) comprising a Cole-Cole dispersion equation (9), which characterizes the relaxation of the head groups (permittivity increment $\Delta\epsilon_1$), a Debye dispersion equation (10), which characterizes the dipole relaxation of water (permittivity increment $\Delta\epsilon_2$) and a constant, ϵ_∞, known as the high frequency limiting permittivity. In each case, τ_m is the macroscopic relaxation time (otherwise known as the exponential decay function, and equal to the reciprocal of the exponential rate constant, k), ω is the angular frequency of the applied electric field and α_1 (in the Cole-Cole equation modelling head group relaxation) is a parameter reflecting the distribution of relaxation times and/or the extent of co-operativity characterizing that dispersion. The distribution of relaxation times broadens and the co-operativity increases as α_1 tends to 1. If the relaxation of the head groups shows a non-Debye-like response (i.e., $\alpha_1 > 0$) then τ_{m1} is translated as the mean relaxation time of the dispersion.

$$\varepsilon' = \Delta\varepsilon_1 \left[\frac{1 + [(\omega\tau_{m1})^{1-\alpha_1} \sin(\alpha_1\pi/2)]}{1 + 2(\omega\tau_{m1})^{1-\alpha_1} \sin(\alpha_1\pi/2) + (\omega\tau_{m1})^{2(1-\alpha_1)}} \right] + \frac{\Delta\varepsilon_2}{1 + (\omega\tau_{m2})^2} + \varepsilon_\infty \quad (1)$$

The curve fitting package (KaleidaGraph 3.0™) uses a Levenberg-Marquardt algorithm (11) and provides estimates and error values for the parameters $\Delta\epsilon_1$, τ_{m1}, α_1, $\Delta\epsilon_2$, τ_{m2} and ϵ_∞. The error in the determination of $\Delta\epsilon_1$, τ_{m1} and α_1 was of the order of 4–10%, 4–12% and 12–30% respectively, and depended on lecithin concentration. Much of the error in the determination of the relaxation parameters for the low frequency dispersion result from measurement errors below 45 MHz. However, it is the incomplete spectrum for water dipole relaxation that results in a significant error (>40%) in the estimates for $\Delta\epsilon_2$, τ_{m2} and ϵ_∞. Information derived from the relaxation time (τ_{m2}) and dielectric increment ($\Delta\epsilon_2$), while invaluable to the characterization of liposomal suspensions, would therefore be unreliable. However, the contribution of water-polarization to the high frequency permittivity can be determined from the sum ($\Delta\epsilon_2 + \epsilon_\infty$), though this parameter also includes contributions from the fast librational and vibrational polarizations of lecithin, as well as the reorientation polarization of water. Since ($\Delta\epsilon_2 + \epsilon_\infty$) lies in that part of the spectrum corresponding to the frequency window of the probe, then the error in the determination of this parameter was assumed to equal that given in the manufacture's specification for the probe (i.e., ~1–2%).

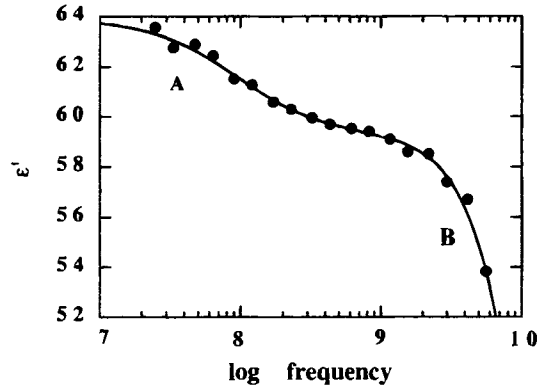

Fig. 1. Frequency dependent relative permittivity (20°C) of a 15% w/w suspension of multilamellar egg lecithin liposomes in distilled water.

[8] R is a function of the conductivity of the interanal and external phases of the liposomes, and C is a function of the membrane capacitance.

The Dielectric Response of Multilamellar Lecithin Liposomes as a Function of Temperature

According to Eyring's equation (Equation 2) (12),

$$\tau_m = \frac{1}{k} = \frac{h}{kT} exp^{-\Delta S/R} exp^{\Delta H/RT} \quad (2)$$

where R is the Gas constant (8.314 J K^{-1} mol^{-1}), the macroscopic relaxation time, and therefore exponential rate constant ($k = 1/\tau_m$), is dependent on three parameters:

(i) The frequency of thermal fluctuations, as given by kT/h, where k is the Boltzmann constant, T is absolute temperature and h is the Planck constant.

(ii) The configurational entropy change (ΔS) associated with the transition from the unpolarized to the polarized state.

(iii) The activation energy (ΔH) associated with the transition from the unpolarized to the polarized state (This activation energy is equal to the energy required to break the intermolecular bonds that are restricting the reorientation of the dipole, and thus indicates the strength of the PO_4^- ... $CH_3{}^+N$) bond.

A physical interpretation of this equation, is that the macroscopic relaxation time decreases on increasing the temperature, because of an increase in the frequency of thermal fluctuations and the associated increase in the number of head groups that have sufficient energy to overcome the activation energy barrier opposing reorientation (ΔH). Also, the smaller the activation energy barrier the shorter the relaxation time.

The relaxation behavior of a 25% w/w suspension of lecithin in water was investigated, and the logarithm of the mean dielectric relaxation time ($ln\ \tau_{ml}$) plotted against the inverse of temperature (Equation 3, Figure 2) (13).

$$ln\ \tau_{ml} = ln\frac{h}{kT} - \Delta S/R + \Delta H/RT \qquad (3)$$

The linear relationship indicates that the relaxation behavior follows the Arrhenius law, and therefore the activation energy for head group reorientation under the influence of the external field can be calculated from the slope of the line, $\Delta H/R$. The configurational entropy change, ΔS, does not vary with temperature and cannot be extracted from the Arrhenius plot.

The estimated activation enthalpy of 6.3 kJ mol^{-1} is approximately a third of the bonding enthalpy of a hydrogen bond of liquid water (18.8 kJ mol^{-1}) (14), and comparable to ΔH = 8.4 kJ mol^{-1} calculated from a theoretical model of phosphatidylcholine head groups (15).

Dielectric Response of Multilamellar Liposomes as a Function of Lecithin Concentration

The increment in relative permittivity ($\Delta\epsilon_1$) associated with zwitterion polarization is proportional to the concentration of lecithin, though a disproportionate increase towards higher concentrations is apparent (Figure 3A). The mean relaxation time and distribution of relaxation times also increases with increased lecithin concentration (Figures 3B and 3C), whereas the high frequency limiting permittivity ($\Delta\epsilon_2 + \epsilon_\infty$) decreases predictably as the lecithin volume fraction (of low permittivity) increases and the water volume fraction of water (of high permittivity) decreases (Figure 3D). Extrapolation of ($\Delta\epsilon_2 + \epsilon_\infty$) to 0% w/w lecithin gave an estimate for the real part of the relative permittivity of pure water ($\epsilon \sim 77 \pm 5\%$; typical accuracy of the probe), in agreement with the literature value of ϵ = 80.4 at 20°C (16).

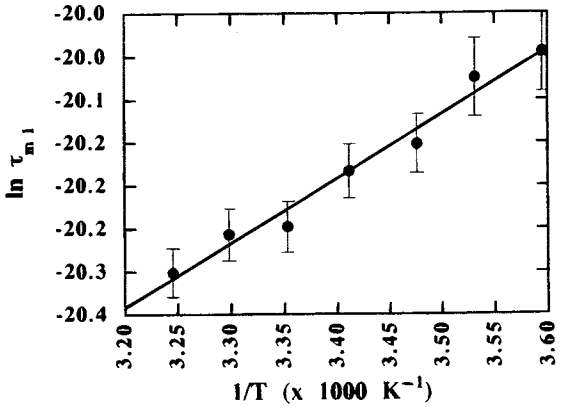

Fig. 2. Arrhenius plot for the mean relaxation time (τ_{ml}) of head group reorientation polarization in a 25% w/w suspension of multilamellar egg lecithin liposomes in distilled water. The activation energy calculated from the slope ($\Delta H/R$) is 6.3 kJ mol^{-1}. Error bars reflect the uncertainty in the Levenberg-Marquardt curve fit of the experimental data (11).

Progressively higher volume fractions of liposomes increases the interactions between head groups, and results in a population of head groups that must wait a longer period until they have sufficient energy to overcome an increased activation barrier opposing head group reorientation. The extended intermolecular network and the associated diversification of the head group environments therefore increases both the mean relaxation time (τ_{ml}) and the distribution of relaxation times (as reflected by the magnitude of α_l). Extrapolation of τ_{ml} and α_l to 0% w/w lecithin then gives the mean intrinsic relaxation time and Cole-Cole function ($\alpha_l{}^{0\%}$) for liposomes in dilute solution. At 0% w/w lecithin the Cole-Cole function may be non-zero ($\alpha_l{}^{0\%}$ = 0.05), which could point to co-operativity of head group polarization in multilamellar liposomes. However, given the error in determining α_l, it is difficult to predict with any accuracy the degree of co-operativity at infinite dilution. Further, more precise measurements are required.

The extent of cooperativity (given by $\alpha_l{}^{0\%}$) is another parameter that can be used to characterize the liposome membrane, just as the width of thermal analysis peaks indicates the cooperativity of phase transitions.

The Effect of Probe Sonication on the Dielectric Properties of Multilamellar Liposomes

The increment in permittivity associated with head group polarization is largely independent of liposome size, as probe sonication of an opaque/white multilamellar suspension to a translucent suspension of smaller vesicles only had a small effect on the relaxation parameter, $\Delta\epsilon_l$ (Table 1). This result indicates that $\Delta\epsilon_l$ is independent of the external surface area of the liposomes, and therefore all the head groups (whether they are located at the external surface of the liposomes or at the interlamellar interfaces) contribute to the permittivity increment from head group polarization.

The possibility of a slight decrease in τ_{ml} on probe sonication (Table 1), may indicate that the head group mobility in small vesicles is less restricted than that in multilamellar liposomes. The curvature of the small vesicles may be sufficient to alter the packing of the head groups such that they respond to the external field (i.e. polarize) more rapidly than head groups located in multilamellar liposomes. Alternatively, the slightly larger time constant for head group polarization in multilamellar liposomes may be due to an increased microviscosity of the interlamellar space compared to the microviscosity of the liposome surface. However, as with determination of α_l, more accuracte measurements are required in order to determine whether the difference in the relaxation time for multilamellar liposomes and unilamellar vesicles is significant. Further studies

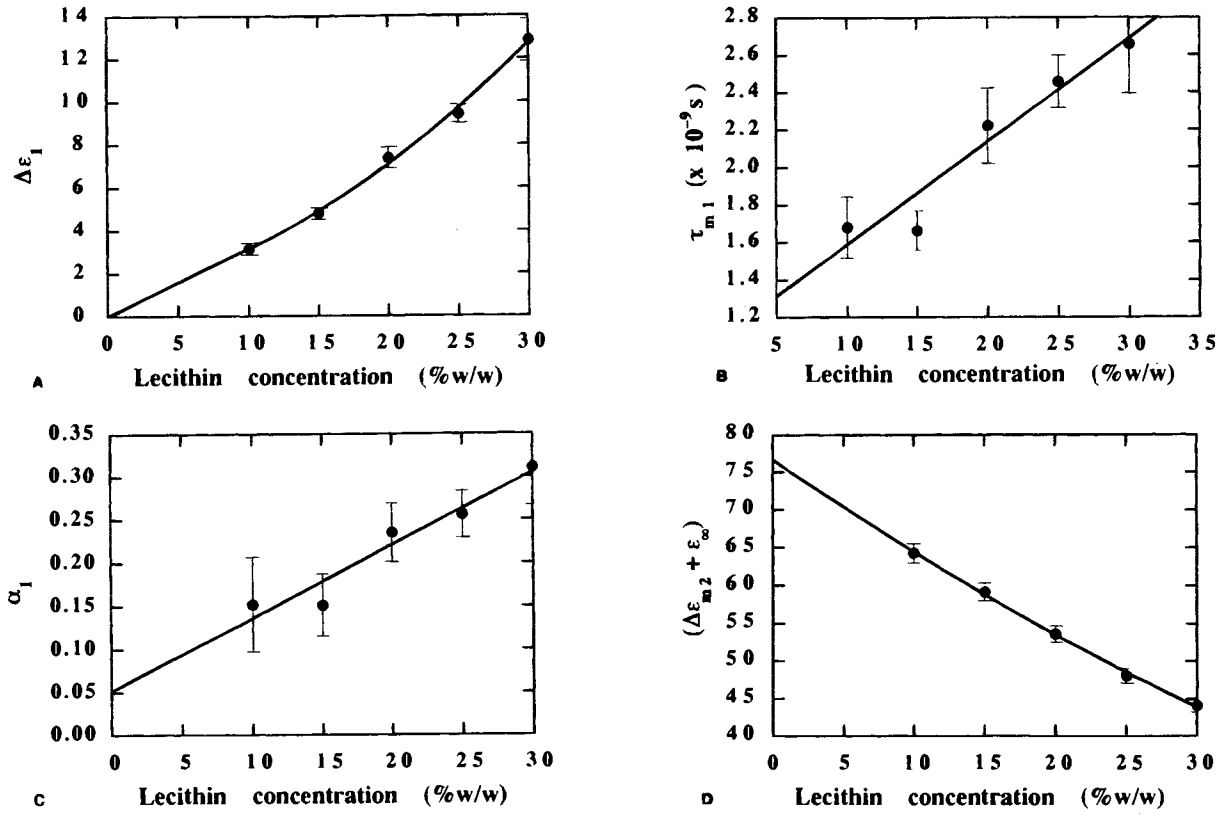

Fig. 3. The relationship between the dielectric relaxation parameters ($\Delta\epsilon_1$, τ_{m1}, α_1 and $\Delta\epsilon_2 + \epsilon_\infty$) of multilamellar egg lecithin liposomes (in distilled water) and lecithin concentration. Error bars reflect the uncertainty in the Levenberg-Marquardt curve fit of the experimental data (11).

are planned to reduce measurement error, thereby enabling the analysis of packing constraints by this method.

Although probe sonication had no influence on the magnitude of the dielectric response resulting from head group polarization ($\Delta\epsilon_1$), this was not the case for the increment in permittivity due to the dipole reorientation polarization of water (as reflected by the sum $\Delta\epsilon_2 + \epsilon_\infty$, Table 1). This observation is explained by the loss of interlamellar water that accompanies the transition from multilamellar liposomes to vesicles with fewer lamellae. The water in the interlamellar space is depolarized by the local field created by the head groups of adjacent lamellar (i.e. vector dipole moment of interlamellar water is effectively reduced) and therefore the transition from multilamellar liposomes to structures containing less interlamellar water, results in an increase in the permittivity ($\Delta\epsilon_2 + \epsilon_\infty$) of the suspension.

CONCLUSIONS

The dielectric response of multilamellar lecithin liposomes (as given by α_1 and τ_{m1}) is sensitive to lecithin concentration owing to: (i) increased interactions between neighbouring head groups on increasing the lecithin concentration; and, (ii) local field effects that result from the dipole polarizing in an inhomogeneous environment/electric field. The magnitude of $\alpha_1^{0\%}$ may indicate that zwitterionic motion is a co-operative phenomena in multilamellar structures, though more precise measurements are required to confirm this. The occurence of co-operativity, however, would contrast with the Debye-like (non-co-operative) response of head group polarization in small unilamellar vesicles (6). Probe sonication of lecithin liposomes showed that head groups in both the outer and inner lamellae contribute to the dielectric response and that the head groups are possibly

Table 1. Effect of Probe Sonication on the Dielectric Relaxation Parameters of a 5% w/w Suspension of Multilamellar Lecithin Liposomes in Distilled Water. Error Estimates in Brackets Reflect the Uncertainty in the Levenberg-Marquardt Curve Fit of the Experimental Data (11)

Liposome formulation	Relaxation parameter		
	$\Delta\epsilon_1$	τ_{m1}	$\Delta\epsilon_2 + \epsilon_\infty$
Multilamellar liposomes	2.11 (±0.08)	2.3×10^{-9} ($\pm 0.3 \times 10^{-9}$)	71.6 (±1.4)
Probe sonicated liposomes	2.19 (±0.07)	1.9×10^{-9} ($\pm 0.3 \times 10^{-9}$)	74.0 (±1.5)

less restricted in liposomes of high surface curvature with few lamellae. A more distinct effect of probe sonication is that the high frequency permittivity of the suspension increased on sonication, as a result of a reduction in the amount of (depolarized) interlamellar water on reducing the number of lamellae per liposome. The high frequency dielectric response is therefore sensitive to liposome structure and could be used to support particle sizing methods, by enabling the characterization of the number of lamellae per liposome. Other potential applications for this technique are detailed below.

Applications for Dielectric Analysis in the Pharmaceutical Sciences

The results of this work have implications for further areas of study, including: (i) the development and structural characterization of liposomal drug delivery systems; (ii) the analysis of the distribution and dynamics of lecithin in oil-in-water emulsions; (iii) investigations into the effect of drugs and other solutes (e.g., cryoprotectants and penetration enhancers) on the interfacial properties of membranes and liposomes; and, (iv) the investigation of the states of water in pharmaceutical materials.

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