500 MHz ^{1}H NMR of phospholipid liposomes

Lanthanide shift on glycerol-γ and acyl-chain C2 resonances

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Phosphatidylcholine liposomes *500 MHz ^{1}H NMR* *Lanthanide shift* *Monolamellar vesicles*

1. INTRODUCTION

Phospholipid liposomes are widely used today as membrane models to study structural and dynamic aspects under controlled conditions. Among the techniques available to elucidate phospholipid arrangements in the liposome bilayer, NMR-spectroscopy has become a powerful tool.

Chapman and Penkett [1] observed that suspensions of phospholipids in D_2O yield spectra with low resolution, but that linewidths are considerably narrowed on ultrasonic irradiation of the suspensions. It is now well established that certain ions (lanthanide tri-valent cations being repeatedly investigated) influence the proton resonances in their immediate vicinity [2–4]. Externally added Ln^{3+} affect only outside membrane proton resonances, as they cannot cross the lipid barrier by transverse diffusion.

One drawback of the NMR-technique is its inherent lack of sensitivity. Consequently exceptionally strong magnetic fields are necessary for high resolution spectra of phospholipid liposomes since the energies of the nuclear spin states depend upon the strength of the magnetic field and the sensitivity of a technique is exponentially proportional to the energy changes. Using a 500 MHz ^{1}H-NMR spectrometer it should be possible to obtain more information about the various liposome resonances of the inside and outside bilayer and how deep into the membrane core the lanthanide-induced shift can be observed.

2. MATERIALS AND METHODS

Details of extraction and purification of egg yolk phosphatidyl choline (PC) are given in [5]. Liposomes were prepared by ultrasonic irradiation using a Branson B 12 Sonifier fitted with a Ti-tip. NMR experiments were carried out on an 80 MHz CW-spectrometer (Bruker WH 80) and on a 500.13 MHz FT-spectrometer (Bruker WM 500). All chemical shifts are given in parts per million (ppm) relative to tetramethylsilane (TMS). Homogeneity of the liposome preparations was checked by electron microscopy [6] and by light scattering experiments using an Argon laser at 488 nm [7].

3. RESULTS

Fig.1 shows the autocorrelation function of the Fourier-transformed power spectrum from quasi-

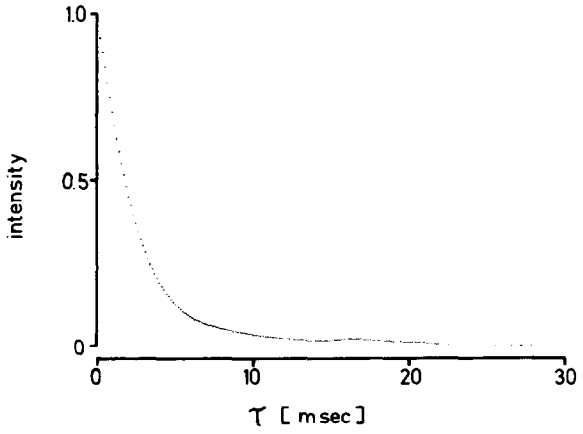

Fig.1. Autocorrelation function from the power spectrum of phosphatidylcholine liposomes in D_2O, detected at an angle of $\vartheta = 30°$.

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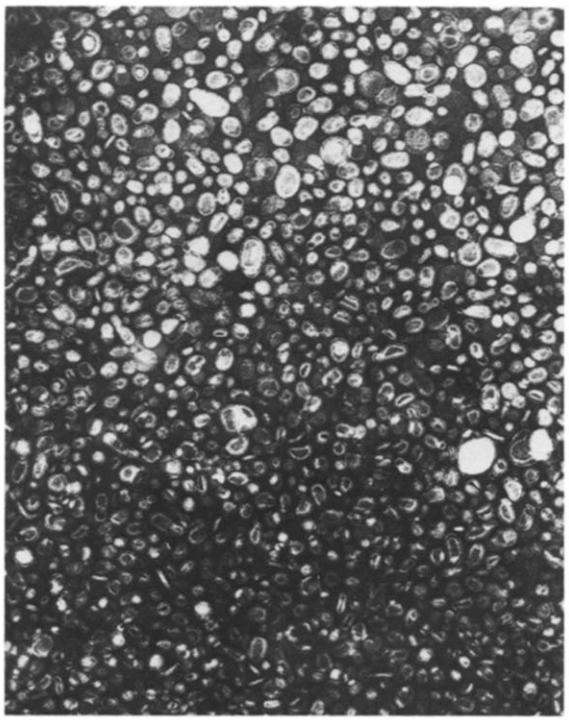

Fig.2. Electron micrograph (99 000 ×) of sonicated phosphatidylcholine liposomes (1 mM), negatively stained (0.5% ammonium molybdate solution) on a carbon-coated copper grid, continuous sonication for 25 min, sonifier power level 1, $T = 25°C$.

elastic light scattering of phosphatidylcholine liposomes. The exponential decay indicates a homogeneous population of liposomes with an average hydrodynamic diameter of $d = 836$ Å. The electron micrograph (fig.2) reveals an average liposome diameter of 500–600 Å. Integration of the choline methyl NMR peaks of fig.5 (outside and inside membrane proton resonances) and calculations according to [8] result in a liposome diameter of 600 Å.

Fig.3 and 4 are the 500 MHz ^1H NMR spectra of egg yolk phosphatidylcholine in deuterated chloroform and of liposomes in D_2O which were obtained at 27°C. Assignments of the ^1H resonances are based on [9–11]. A typical lanthanide titration experiment of externally added Pr^{3+} at

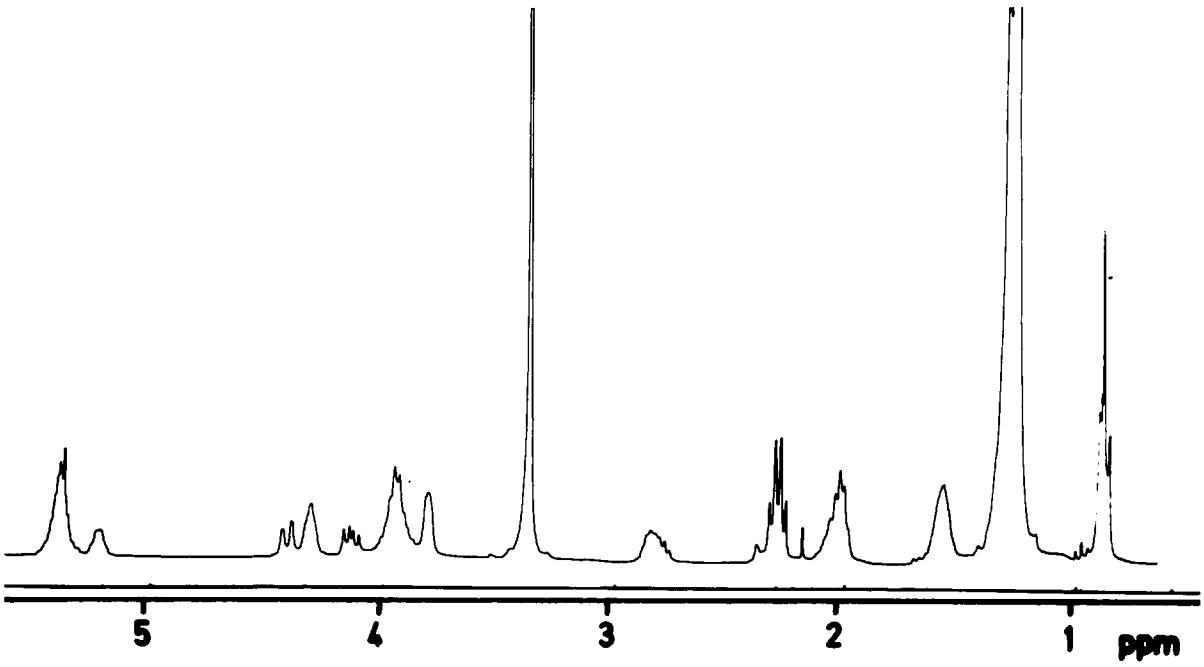

Fig.3. 500 MHz spectrum of egg yolk phosphatidylcholine in deuterated chloroform (20 mM), $T = 27°C$, 25 transients. Assignment of ^1H-resonances: terminal methyl, 0.88; $(CH_2)_n$ of β- and γ-chains–highfield component, 1.27; lowfield component, 1.30; $-CH_2C=C$, 2.02; protons on C3 of acyl chains, 1.57; protons on C2 of acyl chains, 2.37; $-CH_2-(C=C)_2$, 2.82; choline methyl, 3.36; CH_2N, 3.80 (choline 1); glycerol-α, 3.94; glycerol-γ, 4.14/4.42; $POCH_2-$, 4.31 (choline 2); glycerol-β, 5.21; $CH=CH$ (vinyl), 5.39.

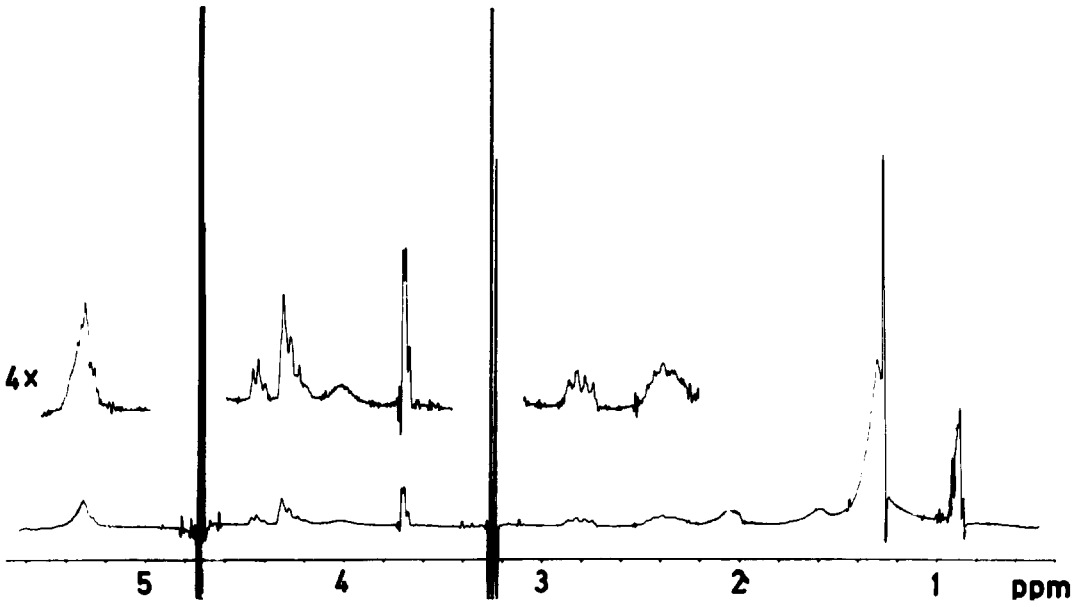

Fig.4. 500 MHz spectrum of egg yolk phosphatidylcholine liposomes in D_2O (34.8 mM), T = 27°C, 512 transients. Assignment of ^1H-resonances: terminal methyl, 0.88; $(CH_2)_n$ of β- and γ-acyl chains–highfield component, 1.267; lowfield component, 1.30; protons on C3 of acyl chains, 1.59; $-CH_2C=C$, 2.01; protons on C2 of acyl chains, 2.37; $-CH_2-(C=C)_2$, 2.80; choline methyl highfield component, 3.23; lowfield component, 3.25; CH_2N, 3.70 (choline 1); glycerol-α, 4.01; glycerol-γ, 4.23/4.44; $POCH_2-$, 4.30 (choline 2); glycerol-β and CH=CH (vinyl), 5.30.

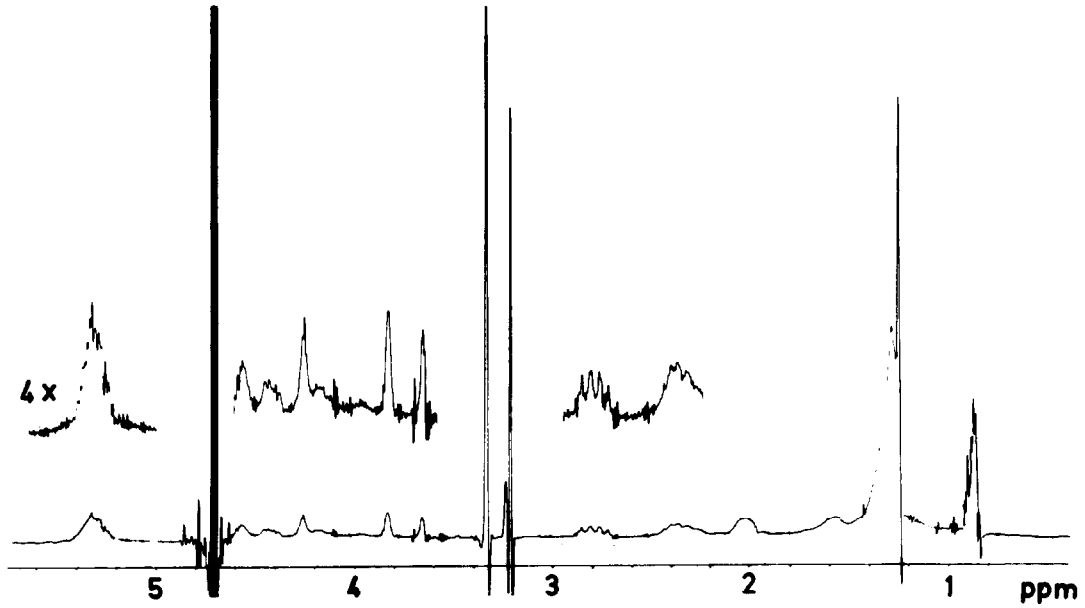

Fig.5. 500 MHz spectrum of egg yolk phosphatidylcholine liposomes (34.2 mM) in D_2O, plus Pr^{3+} (0.82 mM) Rp = 0.024, T = 27°C, transients, 168. Assignment of ^1H resonances: terminal methyl, 0.88; $(CH_2)_n$ of acyl chains–highfield component, 1.267; lowfield component, 1.30; protons on C3 of acyl chains, 1.59; $-CH_2C=C$, 2.01; protons on C2 of acyl chains, 2.37; $-CH_2(C-C)_2$, 2.80; choline methyl–inside membrane, 3.23; outside membrane, 3.35; choline 1 ($-CH_2N$)–inside membrane, 3.66; outside membrane, 3.74; glycerol-α1 or -α2, 4.97; choline 2 ($-POCH_2-$)–inside membrane, 4.26; outside membrane, 4.58; glycerol-γ, 4.18 and 4.44; glycerol-β and CH=CH (vinyl), 5.32.

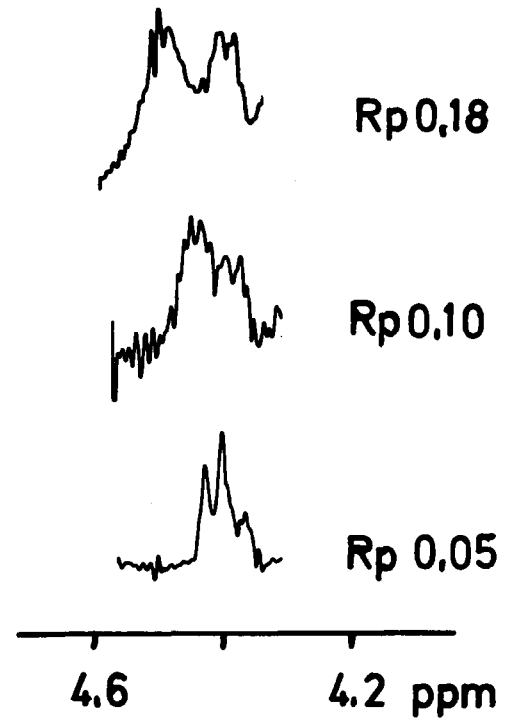

Fig.6. Lanthanide (Pr^{3+})-induced split of one glycerol resonance into an inside (highfield) and outside (lowfield) membrane component of egg yolk phosphatidylcholine liposomes (34.2 mM) in D_2O.

an *Rp*-value of 0.08 is shown in fig.5. The lanthanide-induced shift on 1 glycerol γ membrane outside resonance for 3 different *Rp*-values is demonstrated in fig.6. From the same series of experiments fig.7 was derived. Three different [Pr^{3+}] have been chosen to visualize the influence of the cation on the acyl-chain C2 resonances. Experiments were also carried out with other ions (Eu^{3+}, Yb^{3+}, Dy^{3+}) but results are principally the same, although size and direction of the shift may be different.

4. DISCUSSION

From laser light scattering, electron microscopy and calculations of the choline methyl membrane inside and outside NMR resonances, it can be concluded that the liposome preparations are homogeneous in size with an average diameter of 600 Å. Although 'smallest possible' [12] liposomes and larger ones were found in all preparations, ≥80%

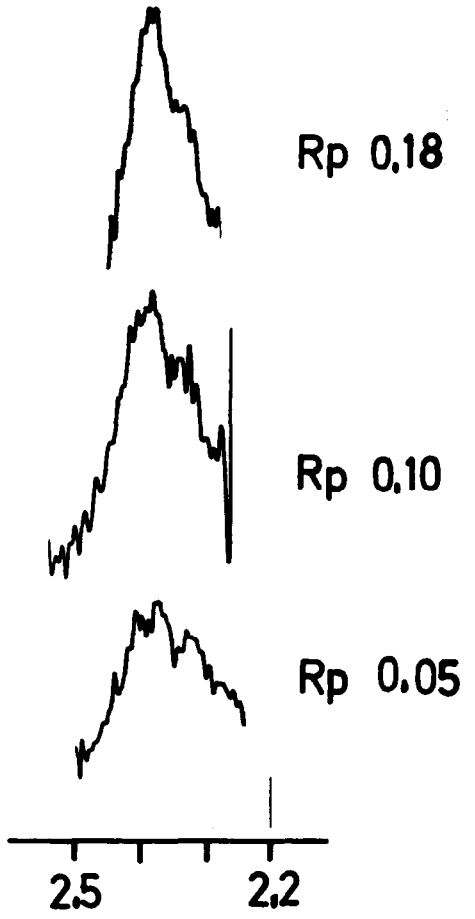

Fig.7. Lanthanide (Pr^{3+})-induced shift of acyl chains C2 resonance of egg yolk phosphatidylcholine liposomes (34.2 mM) in D_2O.

are of average diameter. Reproducibility of our preparations is within an average diameter of 500–700 Å provided all parameters are kept constant (sonifier power level, sonication time, PC concentration and volume, temperature). A comparison of the 500 MHz spectra of PC in $DCCl_3$ and of sonicated PC liposomes in D_2O shows differences for the glycerol and choline region of the molecule. The acyl-chain resonances are identical with the exception of the C2 quartet at 2.37 ppm, which is substantially broadened in liposomes. The split of the $(CH_2)_n$-acyl-chain resonance at 1.27/1.30 ppm is also observed in the liposome spectrum. In [13], the acyl-chain highfield resonance at 1.27 ppm was shown to be assigned to the protons from the inner half of the bilayer and the lowfield

(broad) component to the outer monolayer. It was found that the ratio of the 2 peaks alters when titrating the liposome suspensions with Ln^{3+}. The reason for this is not entirely clear.

The fact that all glycerol resonances are shifted and broadened supports the view that in bilayers the strongest hydrophobic interactions between adjacent lipid molecules occur in the region of the glycerol backbone.

When comparing the choline peaks it is found that they are well resolved also in the liposome spectrum where the choline methyl resonance is split into a doublet. Externally added Ln^{3+} shift the entire lowfield resonance relative to its highfield component either upfield (Eu^{3+}, Yb^{3+}) or downfield (Pr^{3+}, Dy^{3+}) [14]. This is shown for Pr^{3+} in fig.5 and allows the conclusion that the highfield peak originates from the inside and the lowfield from the outside choline methyl protons. This finding is also supported by the view that the inside lipid headgroups are more tightly packed, favouring a stronger interaction of adjacent molecules which causes different resonance conditions. A split of the two remaining choline resonances ($-POCH_2$; $-CH_2N$) into a membrane inside/outside component when Ln^{3+} are added externally can also be seen in fig.5. All these shifts are reversible when retitrating the liposome suspension with tetracaine as this compound displaces Ln^{3+} [15].

Figures 6 and 7 were taken from the same series of experiments. It is quite surprising to see an influence of the Ln^{3+} on protons beyond the glycerol 'backbone' of the outer monolayer. According to conventional view Ln^{3+} are located near or adjacent to the 'choline-oxygen' ($-P-O-CH_2CH_2N(CH_3)_3$) and influence protons in their immediate vicinity only (and hence the strongest shift on the choline 2 protons). The 'binding' of the Ln^{3+} to the outer headgroups of the liposomes can set up an additional magnetic field inside the vesicle which causes the observed shift.

Any influence on resonances in the immediate vicinity of the C2 protons should be detectable provided a strong magnetic field is used, the $[Ln^{3+}]$ is adequate and liposome size is sufficiently reproducible.

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