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Characteristics of Liposomes Prepared by the Modified Reverse-Phase Evaporation Method: ^{31}P-NMR and TEM Study

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Summary: The NMR spectroscopy was used to determine a structure and physicochemical properties of liposomes prepared from L-α-phosphatidylcholine dipalmitoyl (DPPC) by the modified reverse-phase evaporation method (mREV). ^{31}P-NMR study of the liposome in the temperature range from 340K to 350K allowed us to suggest that the single-bilayered liposome has been obtained. Transmission electron micrographs (TEM) showed that the liposome vesicles, obtained with the use of the modified (mREV) method, were spherical in shape and in majority they were less than 100 nm in diameter.

Keywords: drug delivery systems; electron microscopy; membranes; NMR; phospholipids

Introduction

The properties of phospholipids as the major components of biological membranes have been the subject of extensive biochemical and biophysical research for over several decades. Much insight has been gained on their structure, function, and physical properties in lipid bilayers. Most studies of phospholipids have focused on lipids with C_{14} or longer chains, which are the most frequently found species in cell membranes.[1] The length of the hydrophobic chain of phospholipids controls the thickness of the bilayers that are formed from them.[2]

Liposomes are vesicle-like structures basically constituted of phospholipids arranged in the form, of concentrical bilayers containing an aqueous compartment in their interior.[3] Due to their amphipatic characteristics, they can incorporate substances in the aqueous compartment, the lipidic bilayer or even in both compartments simultaneously. Owing to this property, they have been recognized for their great potential as drug delivery systems.[4]

Liposomes are spherical vesicles, whose membranes consist of one (unilamellar) or more (oligolamellar, multilamellar) bilayers of phosphatidylcholine. The vesicles can differ in size (diameter about 15–3500 nm) and shape (single and fused particles). At a given chemical composition these parameters strongly depend on the process of preparation. It is common for the preparation to be metastable. This means that the state of free enthalpy is not in an equlibrium with the environment. As a result the vesicles change their lamellarity, size, size distribution and shape with time. For example, small vesicles tend to form larger ones and large vesicles smaller ones. Nevertheless the stability seems to be the best in range of about 100–300 nm.[5]

The liposome size affects the amount of carrier compound. It also plays a crucial role in the transport of anticancer drugs.

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Many tumors have gaps in their capillaries permitting particles smaller than 120 nm to slowly extravasate into the tumor body. This allows us to choose such a vesicle size that it penetrates the tumor tissue without leaving the circulation elsewhere.[6]

Various analytical methods can be used to characterise the liposome size: freeze-fracture electron microscopy,[7] negative staining electron microscopy,[8] gel chromatography,[9] or photon correlation spectroscopy by means of different apparatus like Malvern,[10] Nicomp[11] or Coulter.[12,13]

To determine the size and number of lipid bilayers (lamellarity) a straightforward application of NMR is sufficient during the quality control of liposomes.

The lamellarity of liposomes influences the encapsulation efficiency and the release kinetics of drugs. Furthermore, when liposomes are processed in the cell the intracellular fate of the drug is affected by the lamellarity.

The analysis of liposome lamellarity is therefore an important parameter to be considered.[14] ^{31}P-NMR in combination with the use of chemical shift reagents has been described for the determination of lamellarity of liposomes. Results were discussed by using transmission electron microscopy as a reference method.[14]

Materials and Methodology

Materials

L-α-phosphatidylcholine dipalmitoyl (DPPC) from Sigma Chem. Co., praseodimium (III) nitrate hexahydrate 99.9% from Aldrich, α,α,α-Tris–(hydroxymethyl)-methylamin 99.9% (TRIS) from Fluka, chloroform, dichloromethane and hydrochloric acid from POCH, Gliwice, Poland. D_2O 99%, chloroform-d 99%, stab. with Ag, and phosphoric acid-d3, 85WT% in D_2O was purchased from ARMAR Chemicals, Switzerland.

Liposome Preparation and Characteristics

We obtained small liposomes from DPPC by the modified reverse-phase evaporation method (mREV).[15,16] Besides the Tris-HCl buffer (pH = 1.9, 5.0, 7.4, 8.0 or 8.4) was applied. The preparation was carried out at 318 K. The average time of liposome preparation did not exceed 12 minutes. Liposomes obtained by mREV method were extruded through a filter of 100 nm in diameter using LiposoFast Basic apparatus produced by Avestin.[17]

Transmission Electron Microscopy (TEM)

Electron microscopy micrographs of liposome prior to their filtration were carried out on a transmission electron microscope (TEM) Philips EM400 with accelerating voltage 100 keV. Samples were deposited on carbon film coated Cu grids of 300 mesh. Negative staining with 2% uranyl acetate was performed to enhance image quality.

NMR Experiments

All spectra were obtained on 9.4 Tesla Bruker Avance Ultra Shield (400.00 MHz for ^{1}H and 161.92 MHz for ^{31}P) using a 5-mm broad band inverse probe. NMR spectra were recorded at the temperature range 297–350 K. Temperature of the studied samples was controlled by air and monitored by Bruker thermal control system. The samples were heated at a rate of about 1K/min up and were left at this temperature for approximately 20 min to attain the equilibrium condition which was monitored by the invariability of the free induction decay (FID) signal. The temperature was maintained at ±0.1 K. Spectra processing was performed with TOPSPIN 1.3b Bruker software.

In order to determine the number of bilayers in the vesicles the effect of the chemical shift reagent was investigated. Measurements were performed with and without external shift reagent Pr^{3+}, which was added to the liposomes prior to the ^{31}P-NMR analysis. $Pr(NO_3)_3 \cdot 6H_2O$ was added to the selected samples of liposomes to yield final concentrations: 5 mM, 10 mM, 50 mM, and 100 mM. Peak areas were analyzed by PeakFit v4, AISN software Inc (Fig. 1).

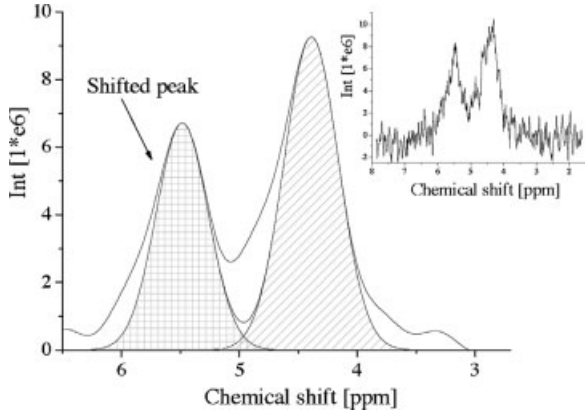

Figure 1.
Method of peak area determination from ^{31}P-NMR spectra. In the figure ^{31}P-NMR spectrum of liposomes at pH = 1.9 is presented; $Pr(NO_3)_3{}^*6H_2O$ concentration is 5 mM.

The analysis of liposome lamellarity (L) is an important parameter to be considered. There are numerous methods for the determination of liposome lamellarity such as electron microscopic techniques (freeze fracture),[18] cryo-EM,[14] small angle X-ray scattering,[18] detergent induced liposome loading[19] and spin-labeled lipids.[20] Transitional ions Ln^{3+} such as: Pr^{3+}, Dy^{3+}, Eu^{3+} and Yb^{3+} have a very short electron relaxation time which affects the chemical shifts of resonances. The downfield (Pr^{3+}, Dy^{3+}) or upfield (Eu^{3+}, Yb^{3+}) shift with negligible line broadening is then observed.[21] Liposome lamellarity was calculated as reported earlier.[14] The number of bilayers L was calculated by the formula:

$$L = \frac{\text{peak area of both peaks}}{2 \times \text{peak area of shifted peak}}$$

Results and Discussion

Transmission Electron Microscopy – Liposome Size Analysis

For liposome size analysis TEM was used. Transmission electron micrographs showed that the liposome vesicle, obtained by the modified mREV method, were spherical in shape (Fig. 2) and in majority they were less than 100 nm in diameter before filtration. The method applied allowed us to obtain liposomes of diameter $\leq$100 nm (Fig. 3) and of regular spherical structure.

The pictures obtained by the use of TEM confirm that the liposomes of diameter $\leq$100 nm were obtained using the modified reverse-phase evaporation method. It was found that liposomes of diameter below

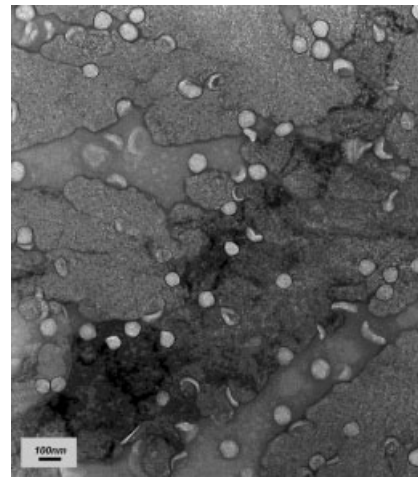

Figure 2.
Electron micrograph (50 000×) of liposomes at pH = 8.4, obtained by the modified reverse-phase evaporation method, negatively stained (2% uranyl acetate solution) on a carbon-coated copper grid.

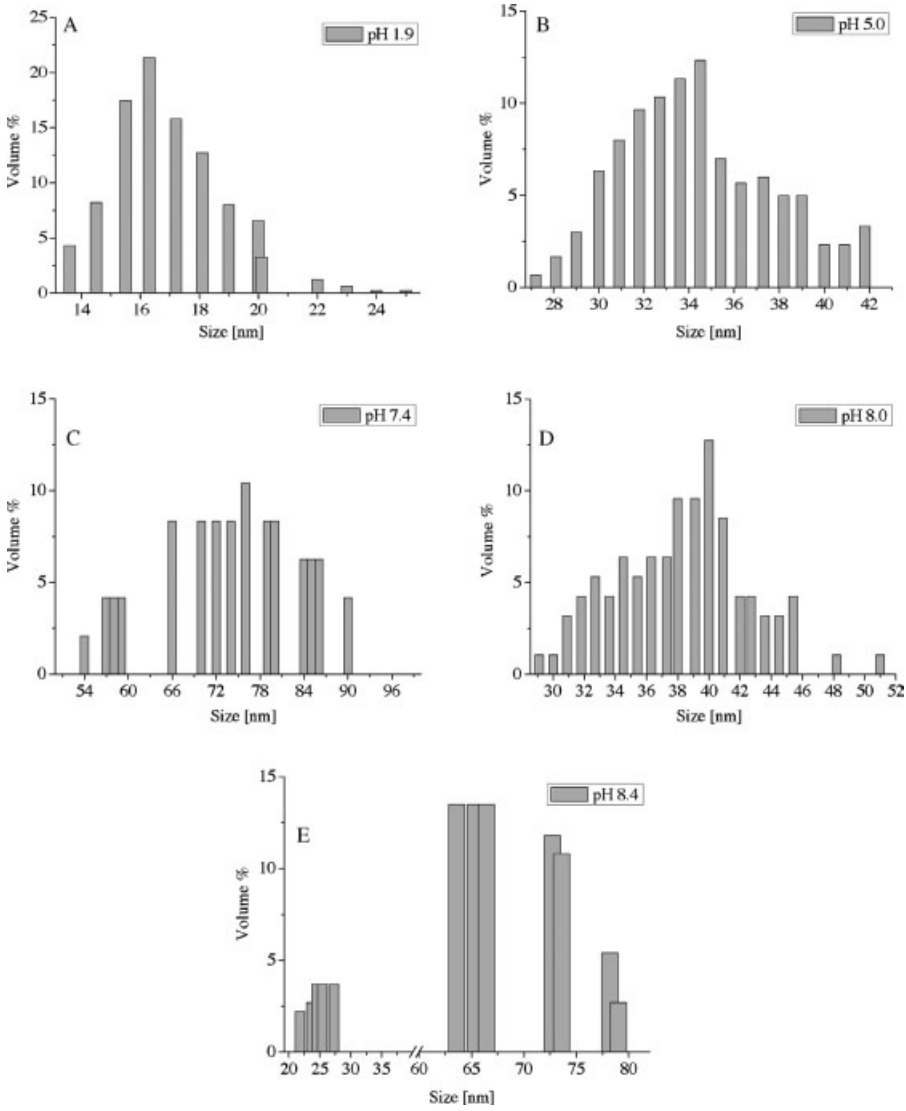

Figure 3.
Analysis of size distribution of liposomes formed from DPPC; pH of Tris-HCl buffer: A) 1.9, B) 5.0, C) 7.4, D) 8.0, E) 8.4).

50 nm were obtained when Tris-HCl buffer of pH 1.9, 5.0 and 8.0 was used during the preparation (Fig. 3). However, liposomes of diameter between 50 nm and 100 nm were obtained with the use of Tris- HCl buffer of pH 7.4 and 8.4 (Fig. 3).

^{1}H-NMR Study

To estimate the effect of pH and temperature on liposome membranes, the ^{1}H-NMR studies were carried out. ^{1}H-NMR spectra were recorded in the temperature range from 297 K to 340 K. The analysis of chemical shifts of resonances assigned to the protons of methyl groups of ammonium of phospholipids $-N^+(CH_3)_3$ was conducted.

The downfield shift of these resonances was observed. It was accompanied by a splitting of two components (Figs. 4 and 5).

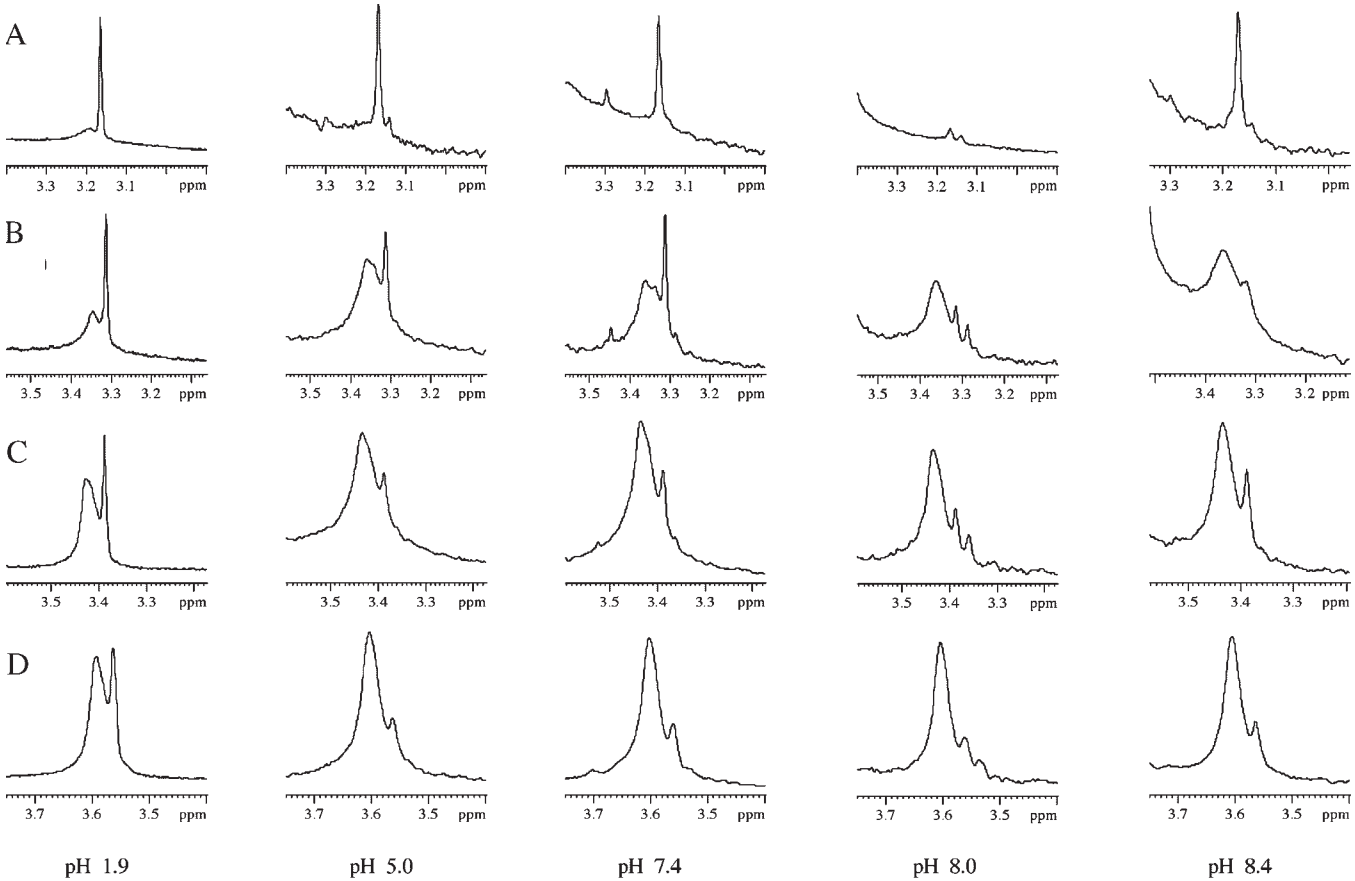

Figure 4.

Effect of temperature and pH on the splitting of proton resonances of methyl groups of phospholipid ammonium. Spectra were recorded at A) 300 K, B) 314 K, C) 322 K, D) 340 K for liposomes formed from DPPC, prepared in Tris-HCl buffer of pH: 1.9; 5.0; 7.4; 8.0; 8.4.

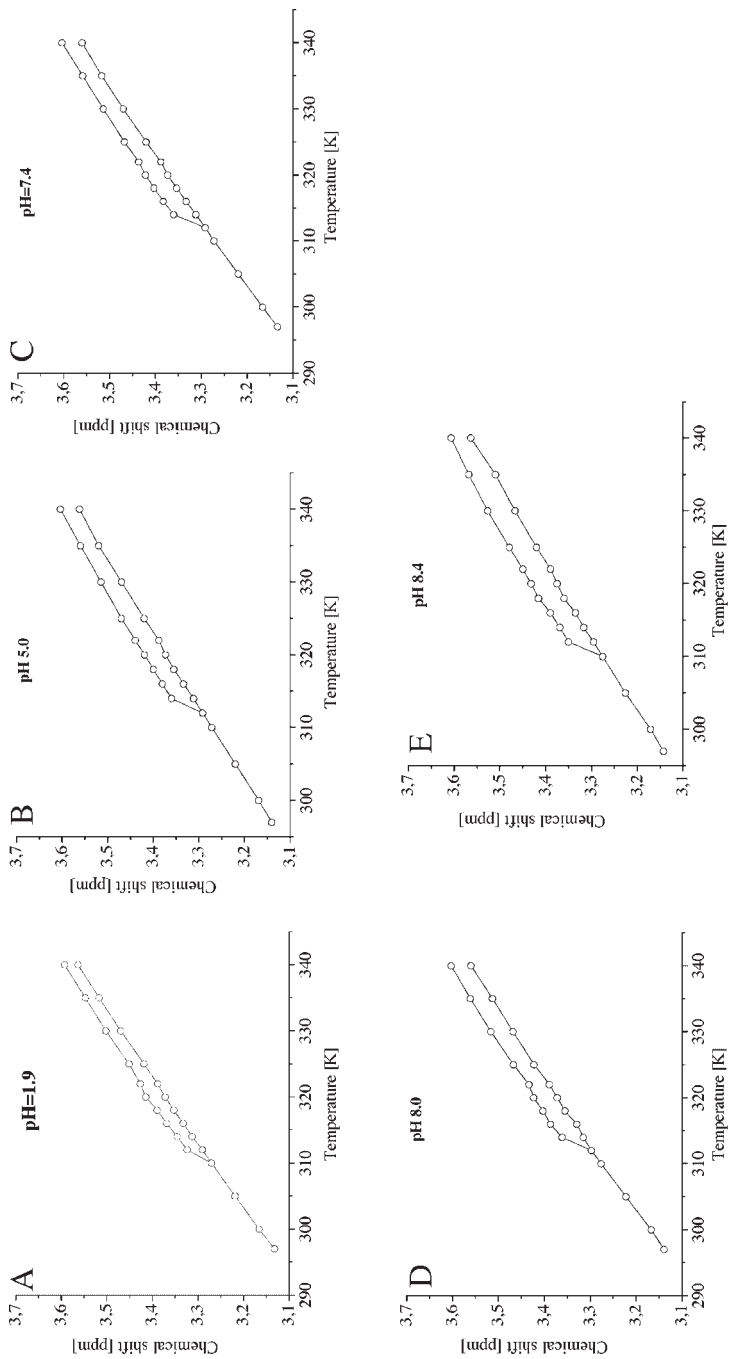

Figure 5.
Effect of temperature and pH on chemical shifts of proton resonances of methyl groups of phospholipid ammonium. Spectra were recorded at A) 300 K, B) 314 K, C) 322 K, D) 340 K for liposomes formed from DPPC, prepared in Tris-HCl buffer of pH: 1.9; 5.0; 7.4; 8.0; 8.4.

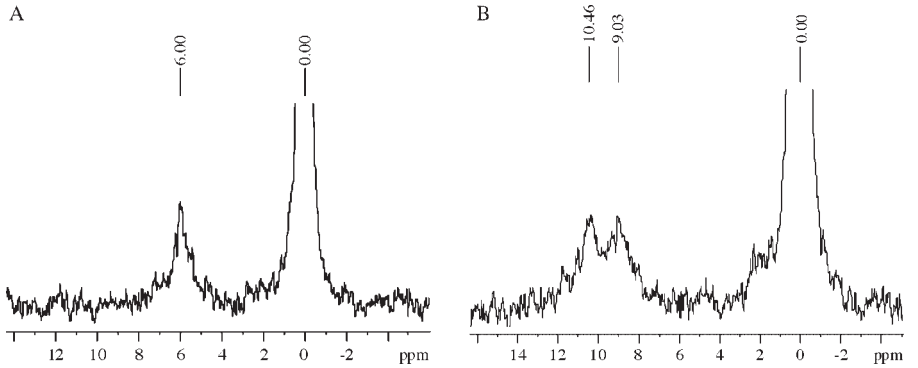

Figure 6.
Effect of $Pr(NO_3)_3 \cdot 6H_2O$ on splitting of resonance originating from phosphate groups of phospholipids in liposomes. ^{31}P-NMR spectra were recorded at 345 K for liposomes formed from DPPC in Tris-HCl buffer of pH 1.9, A) before, B) after adding praseodymium salt.

The splitting allowed us to assign two resonances appearing at the lower and higher field to phospholipid methyl groups located in the outer and inner membrane layer, respectively.[21] The observed splitting does not depend on the pH of the buffer used for the preparation of liposomes from DPPC. This proves that all obtained liposomes were unilamellar. After the addition of praseodymium salt the downfield shift of only one resonance is observed i.e. of the one located at the higher field. Since praseodymium salt was added to the liposomes after their preparation, we assumed that the Pr^{3+} ions only affect the protons in the outer layer of membrane. The analysis of ^{1}H-NMR spectra of liposomes with the use of praseodymium salt $Pr(NO_3)_3{}^*6H_2O$ confirms our suggestion that the resonance at the higher chemical shift originates from the phospholipids in the outer layer of the liposomal membrane.

^{31}P-NMR as a Method for the Determination of Liposome Lamellarity

The ^{31}P-NMR spectra analysis were used to estimate the number of phospholipid bilayers forming liposomes.

It was supposed that the resonance at $\delta = 6.00$ ppm to originate from phospholipids of both outer and inner layer of the membrane (Fig. 6). The addition of the praseodymium salt $Pr(NO_3)_3 \cdot 6H_2O$ to the liposomes after their preparation caused the downfield shift of this resonance and its splitting into two signals (Fig. 6B). This confirms the bilayer structure of the liposome membranes obtained by using the mREV method.

^{31}P-NMR spectra were recorded for liposomes formed from DPPC in Tris-HCl buffer of pH 1.9. Measurements were carried out at 340 K, 345 K, 350 K (Fig. 7) above temperature of phase transition of phospholipids (DPPC – 314 K).[22] The concentration of praseodymium salt $Pr(NO_3)_3 \cdot 6H_2O$ were consecutively: 5 mM, 10 mM, 50 mM, 100 mM.

It was ascertained that the $Pr(NO_3)_3 \cdot 6H_2O$ concentration affects the shape of the spectra, the chemical shifts (Fig. 7) as well as the number of bilayers calculated on the basis of the spectra. Similar results were obtained by Fromlich[14] for liposomes formed from PC and by Kumar.[23] The latter studied the effect of $Pr(NO_3)_3 \cdot 6H_2O$ concentration on the chemical shift of resonances.

The number of phospholipid bilayers (L) (Table 1) determined on the basis of ^{31}P-NMR spectra (Fig. 7) is of the range of 1.03–1.16. This points to a unilamellar structure of the studied liposomes.

The use of Pr^{3+} has to be carefully reconsidered. This study shows that increasing concentration of shift reagent leads to errors in calculating lamellarity. It

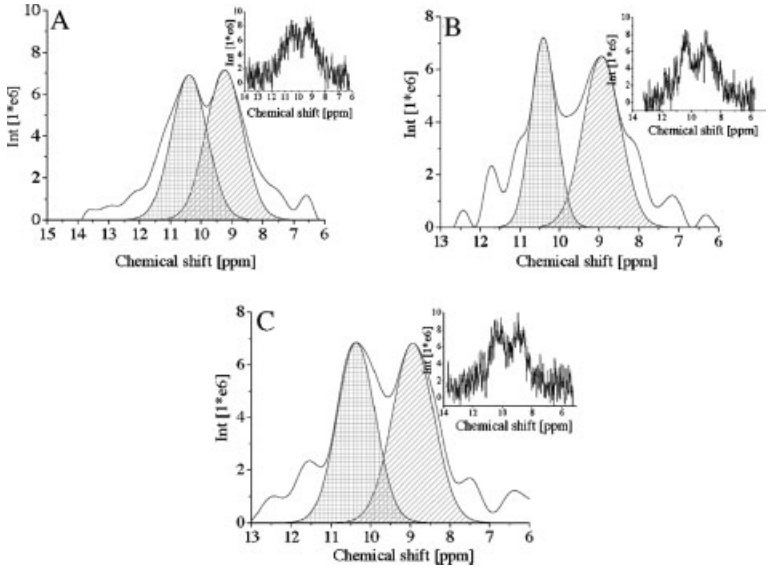

Figure 7.
Effect of temperature and concentration of $Pr(NO_3)_3 \cdot 6H_2O$ on splitting of resonance originating from the phosphate groups of phospholipids in liposomes. ^{31}P-NMR spectra were recorded for liposomes formed from DPPC in Tris-HCl buffer of pH 1.9; temperature A) 340 K, B) 345 K, C) 350 K; concentration of $Pr(NO_3)_3 \cdot 6H_2O$ 10 mM. The fitting of the simulating spectra to the experimental ones were made with the use of PeakFit v4 programme.

Table 1.
Effect of concentration of $Pr(NO_3)_3 \cdot 6H_2O$ and temperature on the number of the lipid bilayers (*L*) calculated on the basis of ^{31}P-NMR spectra of liposomes formed from DPPC.

Concentration of $Pr(NO_3)_3 \cdot 6H_2O$	Number of the phospholipid bilayers calculated on the basis of ^{31}P-NMR spectra (*L*)		
	340 K	345 K	350 K
5 mM	–	–	–
10 mM	1.03 ± 0.33	1.16 ± 0.06	1.04 ± 0.05
50 mM	2.43 ± 0.15	2.26 ± 0.09	2.20 ± 0.15
100 mM	–	–	–

Error was calculated with the use of the logarithmic method.

is shown that under well-defined conditions the effect of Pr^{3+} penetration can be prevented. We suggest that Pr^{3+} is capable of penetrating the liposome bilayer as $Pr(OH)_3$.

Conclusions

The liposomes, used as biological membrane models, are used today in various fields of science and technology as carriers for a broad spectrum of agents in medical, pharmaceutical, cosmetic, and food industry.

The physicochemical properties of liposomes obtained by mREV method have been determined by the use of the transmission electron micrograph technique (TEM) and NMR spectroscopy.

Transmission electron microscopy (TEM) method allows us to confirm that small liposomes, less than 100 nm in diameter, were obtained by the use of the modified reverse-phase evaporation method (mREV).

NMR is a powerful tool for investigating the dynamics and structural organization of liposome membranes.

^{1}H and ^{31}P-NMR spectroscopy was used to estimate the number of phospholipid bilayers in the liposome membranes. Nitrate salt of Pr^{3+} was used as a chemical shift reagent for ^{31}P-NMR analysis of liposomes prepared in a buffer of various pH. The splitting of the ^{31}P-NMR resonances seems to be dependent of the concentration of the shift reagent. Our study shows that for the liposome membranes prepared by mREV method from DPPC, the concentration of $Pr(NO_3)_3 \cdot 6H_2O$ 10 mM proved to be suitable for the correct determination of parameter *L*.

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