CALORIMETRIC AND EPR STUDIES OF THE THERMOTROPIC PHASE BEHAVIOR OF PHOSPHOLIPID MEMBRANES

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Transmission electron micrographs (TEM) showed that liposome vesicles prepared from *DL*-α-phosphatidylcholine dimyristoyl (1,2-ditetradecanoyl-rac-glycerol-3-phosphocholine) (DMPC) by the modified reverse-phase evaporation method (mREV) were spherical in shape and in majority of them were less than 100 nm in diameter. Differential scanning calorimetry (DSC) method was used to determine the influence of cholesterol content and pH of Tris-HCl buffer used for the preparation of liposomes on the temperature of phase transition T_C of phospholipids which form the investigated liposome vesicles. The use of DSC method made it possible to determine not only the temperature of the main phase transition of phospholipids but also the temperature of the phospholipid phase transition from the tilted gel phase ($L_{\beta'}$) to the ripple gel phase ($P_{\beta'}$). The results were compared with those obtained with EPR study. EPR study was carried out in the temperature range from 284 to 310 K i.e. below and above the phase transition temperature T_C of DMPC. On the basis of EPR spectra of spin marker 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) incorporated into the liposome, the values of parameters *f* were determined. Hence TEMPO can be used to observe the change in partition between aqueous and fluid lipid regions. The change in the relative values of *f* determined for DMPC as a function of temperature shows that this phospholipid undergoes a transition from a 'gel phase' to a lamellar smectic liquid crystalline phase in the presence of excess water. The EPR study of TEMPO allowed us to determine the transition temperature T_C. The results were compared with those obtained with DSC method.

Keywords: DSC, electron paramagnetic resonance, synthetic amphiphile vesicles, thermotropic phase behavior

Introduction

Liposomes are self-assembled structures that occur in nature and can be also easily synthesized in a laboratory. The use of liposomes is of current interest in a number of areas such as biochemistry, molecular biology, cosmetics, food technology and pharmacology as drug delivery systems in vivo [1, 2].

Liposomes are microscopic particles composed of lipids. The most common constituent of a liposome is a phospholipid that spontaneously forms closed structures in an aqueous solution [3]. A typical property of bilayer-forming lipids is their amphiphilic nature i.e. a polar headgroup covalently attached to one or two hydrophobic hydrocarbon tails. When these lipids are exposed to an aqueous environment, interactions between them (hydrophilic interactions between polar headgroups and Van der Waals' interactions between hydrocarbon chains) and water (hydrophilic interactions, hydrophobic effect) lead to spontaneous formation of closed bilayers. Liposomes can differ in size, they can range from the smallest vesicle obtainable on theoretical basis (diameter ~20 nm) to liposomes which are visible under a light microscope, with a diameter of 1 μm or greater, equal to the dimensions of living cells [4].

The size range is a compromise between the loading efficiency of liposomes (increase with increasing size), liposome stability (decrease with increasing size above an optimal 80–200 nm range) and the ability to extravasate (decrease with increasing size). The thickness of the membrane is around 4 nm and it can contain a polymer coating and/or ligands with defined functions, such as specific binding or fusogenic activity [5].

Liposomes can also differ in terms of lipid composition and structural organization, corresponding to uni-, oligo- or multi-lamellar vesicles. They are built in such a way that the solute can be encapsulated in the aqueous compartment (polar solutes) or embedded in the lipid bilayers (lipophilic or amphiphilic solutes) [4].

The determination of physico-chemical properties of liposomes depending on their different size and structure as well as the different methods of their preparation is of great practical importance. For this purpose physico-chemical methods are applied.

To determine the fluidity of liposomes the electron paramagnetic resonance (EPR) [6–10], deuterium nuclear magnetic resonance (^{2}H NMR) with the use of deuterium labeled phospholipids [11–14] and fluorescence depolarization [15, 16] spectroscopies are the most often used. To study the interaction be-

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tween liposome vesicles and their fusion many different techniques were used i.e. NMR [17, 18] and EPR [19] spectroscopy, differential scanning calorimetry (DSC) [20], electron microscopy [21], gel fraction [21] and fluorescence spectroscopy [21–26].

The use of spin markers in EPR spectroscopy in many cases is not sufficient to determine the absolute values of many physico-chemical parameters characterizing the state of liposome membranes. However it allows us to assess by comparison the influence of the modification of liposome structure on its properties. The modification processes are carried out by various physical and chemical agents on liposome membranes.

The aim of our investigation of liposomes with the use of spin markers was to estimate the adequacy of liposomes for the transport of hydrophilic drugs.

Experimental

Materials

DL-α-phosphatidylcholine dimyristoyl (1,2-ditetradecanoyl-rac-glycerol-3-phosphocholine) 99% (DMPC) and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) were purchased from Sigma-Aldrich Chem. Co., α,α,α-Tris–(hydroxymethyl)-methylamin 99.9% (TRIS) from Fluka, cholesterol (5-cholesten-3β-ol) 99+% (Chol), chloroform, dichloromethane and hydrochloric acid from POCH, Gliwice, Poland.

Liposomes preparation and characteristics

We obtained small liposomes (DMPC/Chol) by the modified reverse-phase evaporation method (mREV) [27–31] using the DMPC:Chol of molar ratio: 4.00:1.00, carried out at 303 K. Tris-HCl buffer (pH=1.9, 5.0, 7.4, 8.0 and 8.4) was applied. For EPR study 0.096 mL of the spin marker (TEMPO) (0.78 mg mL^{-1} in chloroform) was added to the preparation mixture. Liposomes not containing cholesterol were also obtained. The average time of their preparation did not exceed 12 min. The excess of spin marker was separated by treble centrifugation using High Speed Brushless Centrifuge MPW – 350, with the speed of 4500 revs. min^{-1}, for a period of 10 min. Liposomes obtained by mREV method were extruded through a filter of 100 nm in diameter using LiposoFast Basic apparatus produced by Avestin [32].

Methods

Transmission electron microscopy

Electron microscopy micrographs of liposome before their filtration were obtained by a transmission electron microscope (TEM) Philips EM400 with accelerating

voltage 100 keV. The solution of liposome bilayer was placed on Sigma-Aldrich Chemie GmbH copper and carbon-coated grids of 300 mesh. The micrographs were made after total drying of the liposome layer. The wide range of electron beam of weak intensity (low electron density) was used during the measurements to prevent the overheating of the sample.

Differential scanning calorimetry

The DSC studies of obtained liposomes were done to determine the temperature of the phase transition of the phospholipids. DSC measurements were carried out on differential scanning calorimeter Setaram Micro DSC III with heat flow of Calvet type and cylindrical measurement system in the temperature range 278–333 K. The 'batch' type cuvettes of 1 cm^3 were used. The heating rate was 1.2 K min^{-1}.

Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) spectroscopy was used to monitor the molecular dynamics of lipids. Molecular formula and typical EPR spectra of the spin marker TEMPO are given in Fig. 1.
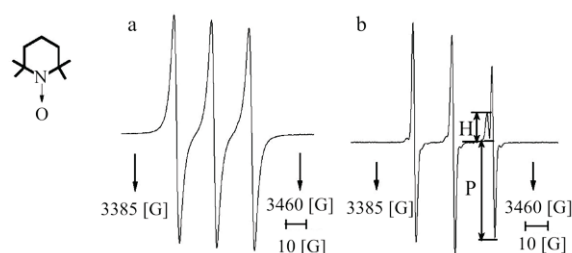

Fig. 1 Structural formula and typical EPR spectra of spin marker TEMPO at 310 K: a – in solution, b – after incorporation into the liposomes

The EPR measurements were carried out on a Bruker EMX spectrometer at the X-band (9 GHz), equipped with Bruker N$_2$ temperature controller in the temperature range 284–310 K and the constant temperature at ±0.5 K during the experiment. All measurements were performed in a triplicate. The spectra were recorded on microwave power 20.070 mW, with 4.48·10^4 signal amplification, 0.80 G modulation amplitude, sweep time 20.973 s and number of scans –10. For the EPR experiment 0.1 mL of liposome sample solutions were kept in closed quartz capillaries.
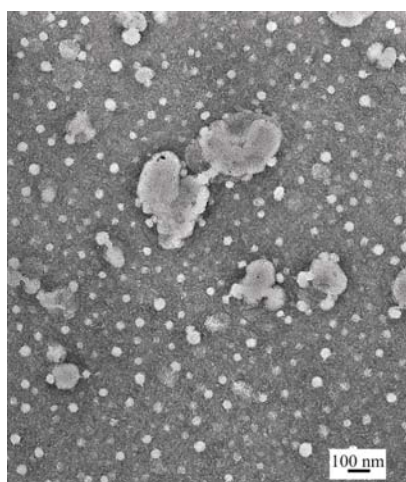
Fig. 2 Transmission electron micrographs of small liposomes obtained by mREV method, enlargement 50.000 times; pH 7.4; room temperature

Results and discussion

Transmission electron microscopy study

Transmission electron micrographs showed that the liposome vesicle, obtained by using 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 ≤100 nm and of regular spherical structure.

Phase transitions of DMPC bilayer membrane

Differential scanning calorimetry method was used to determine the influence of cholesterol content and pH of Tris-HCl buffer used for the preparation of liposomes on the temperature of phase transition T_C of phospholipids which form the investigated liposome vesicles. Investigations were carried out for liposomes prepared with the use of Tris-HCl buffer of pH 5.0, 7.4, 8.0, 8.4.

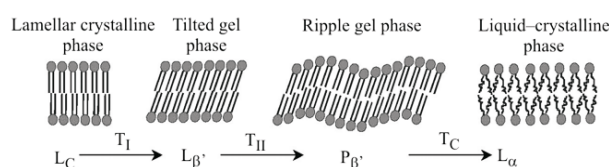With the increase of temperature the thermotropic transitions should take place in phospholipid

Fig. 3 Thermotropic phase transitions in phospholipid bilayer

membranes [33, 34]. Three temperatures of phase transition are usually i.e. subtransition, pretransition and the main phase transition [33, 35]. These three temperatures of phase transition separate four phases i.e. the lamellar crystalline phase (L_C), the tilted gel phase ($L_{\beta'}$), the ripple gel phase ($P_{\beta'}$) and the liquid-crystalline phase (L_α) (Fig. 3).

For the liposome formed from DMPC prepared by mREV method (Fig. 4, Table 1) subtransition is not observed. One can observe the phase transition from tilted gel phase ($L_{\beta'}$) to the ripple gel phase ($P_{\beta'}$) at temperature range from 286.4±0.1 to 288.9±0.1 K and the main phase transition at temperature range from 298.3±0.1 to 298.7±0.1 K. There is no dependence between the temperature of the phase transition and pH of buffer used for the preparation of the liposome vesicles.

After adding 1 mole of cholesterol per 4 moles of phospholipids to the liposome the phase transition temperatures disappear for the majority of studied liposome vesicles (Fig. 4, Table 1).

According to the literature data a high concentration of cholesterol causes that the phase transition in the bilayer becomes less sharp [6, 27, 36–38]. The similar effect is observed in the natural membranes which are also rich in cholesterol. There the decay of the phase transition occurs. Probably the observation of the phase transition at DMPC:cholesterol molar ratio above 4:1 is not possible.

Our results are consistent with other study on liposomes formed from DPPC [27, 36–38]. It was stated that the use of 1 mole of cholesterol on 4 moles of DPPC during preparation of liposomal membranes by mREV is sufficient for the maximal stiffening of the

Table 1 The influence of cholesterol content and pH of Tris-HCl buffer used for preparation of the liposome vesicles on the temperature of DMPC phase transitions

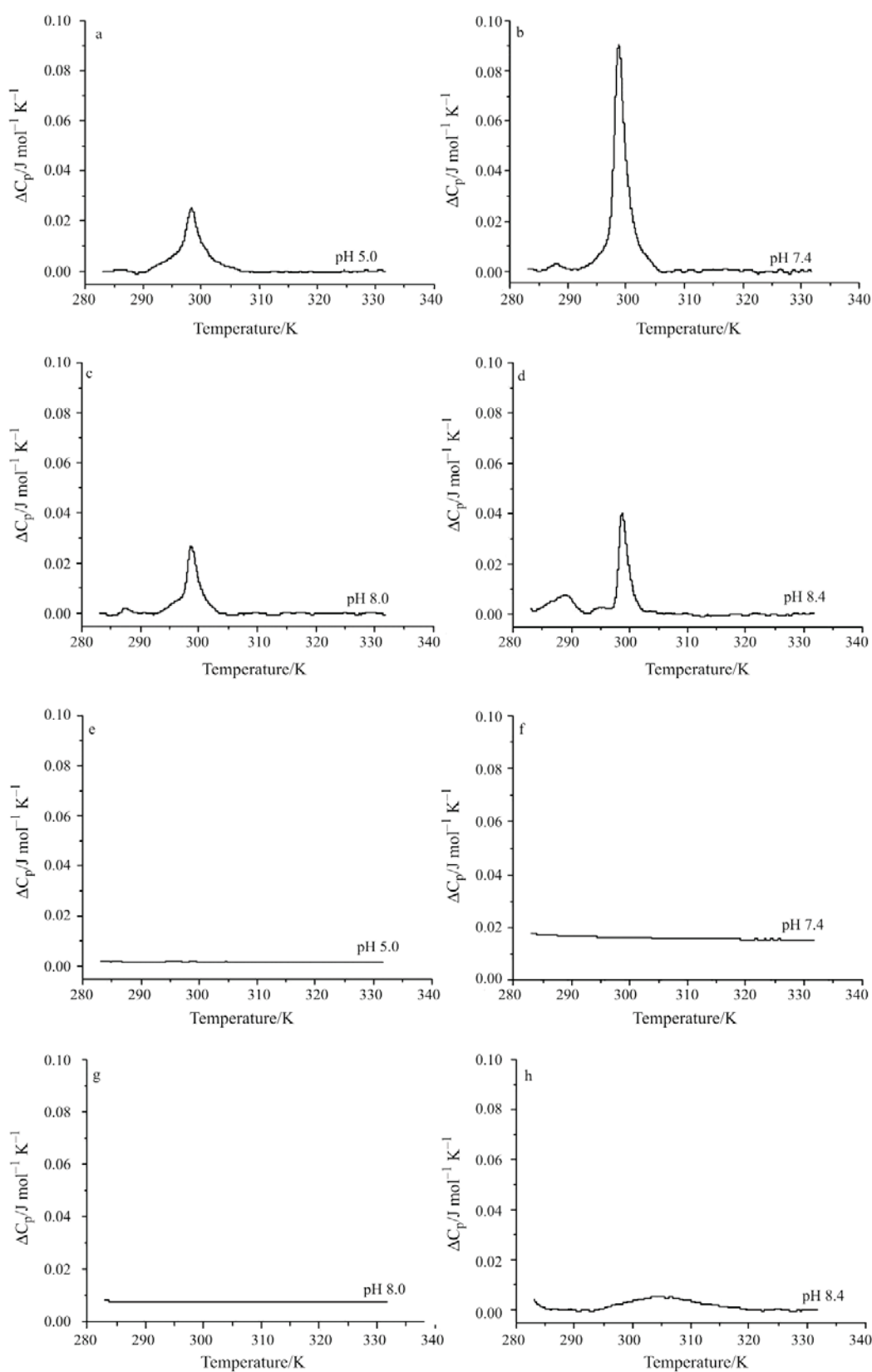| pH of buffer | Type of liposome membrane | | | |
| | DMPC | | | DMPC/Chol |
| | Temperature of phase transition/K | | | |
	T_I	T_{II}	T_C	T_C
5.0	–	286.4±0.1	298.3±0.1	–
7.4	–	287.9±0.1	298.7±0.1	–
8.0	–	287.1±0.1	298.7±0.1	–
8.4	–	288.9±0.1	298.8±0.1	303.9±0.1

Fig. 4 The influence of cholesterol and pH of Tris-HCl buffer used for the preparation of phospholipids on the temperature of DMPC phase transitions. The measurements were done for liposomes prepared from DMPC in Tris-HCl buffer of pH: a – 5.0, b – 7.4, c – 8.0, d – 8.4 and liposomes prepared from DMPC and cholesterol at molar ratio 4.00:1.00 in Tris-HCl buffer of pH: e – 5.0, f – 7.4, g – 8.0, h – 8.4

membrane. Therefore a study of the influence of cholesterol on the properties of the obtained liposomal membranes was conducted mainly for this molar ratio.

Electron paramagnetic resonance study – partitioning of TEMPO between the hydrocarbon and aqueous phases

The EPR study of liposomes with the spin marker TEMPO has been carried out in order to assess the usefulness of the obtained liposomes to transport hydrophilic drugs. This spin marker was chosen due to the similarity of its properties to those of hydrophilic drugs. The EPR study of liposomes labeled by the spin marker TEMPO allows us to determine the content of the marker in both the hydrophobic (H) and hydrophilic (P) parts of the liposomal membrane basing on the estimation of parameter f (Eq. (1)) (Fig. 5).

$$f = \frac{H}{H + P} \qquad (1)$$

The EPR spectrum of spin marker TEMPO incorporated into liposomes comprises with two superposed spectra, i.e. spectrum of the marker located in the hydrophobic part of the membrane and of the marker located in the hydrophilic one. Studies were carried out for liposomes formed with DMPC/TEMPO, DMPC/Chol/TEMPO in Tris-HCl buffer of pH 1.9, 5.0, 7.4, 8.0 and 8.4.

The content of the spin marker located in the hydrophobic part of the membrane (H) increases with the rise of temperature from 284 to 310 K (Fig. 5). The changes in the EPR spectrum of the spin marker TEMPO can be explained by the developing penetration of the spin marker between the hydrophilic and hydrophobic parts of the liposome. The increase of value H causes the rise of parameter f (Fig. 6).

This is probably connected with the loosening of the membrane and with the penetration of water between the hydrocarbon chains. The obtained results are in accordance with literature data [39, 40]. The presence of cholesterol in the liposomal membrane causes the decrease of the value of the partition coefficient f. This in turn may be due to the stiffening effect of sterol and the inhibition of the extend of the spin marker penetration between the parts of the membrane. The partition coefficient f changes with temperature for all liposomes (Fig. 6). These dependences allow us to determine the phase transition temperature T_C of phospholipids (DMPC) (Table 2).

We determined T_C as the first derivative from the sigmoid curve, presenting the change of f parameter with temperature. The maximum of this Gauss`s curve shows the T_C value.

It was confirmed that T_C depends not only on the phospholipid used for the liposome membrane prepa-
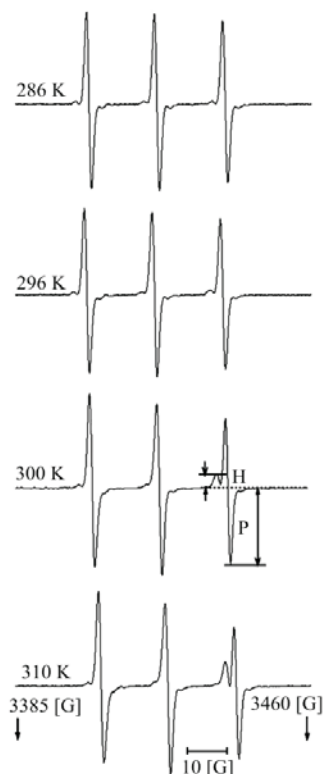

Fig. 5 Superposition of two spectra: spin label TEMPO located in the hydrophobic part of the membrane (H) and spin label located in the hydrophilic part of the liposome (P). Liposomes were obtained from DMPC/TEMPO Tris-HCl 7.4 pH; temperature 286, 296, 300 and 310 K

ration but also on the pH of the buffer used for its preparation and on the cholesterol content. All these parameters determine the thermotropic properties of phospholipids forming liposomes.

The addition of cholesterol induces the increase of T_C value. The highest values of phase transition temperature T_C were determined for liposomes obtained in buffer of pH 1.9 (Table 2).

For the majority of the studied liposomes the decrease of T_C value with increase of pH of the buffer used for liposome membrane preparation from 1.9 to 8.0 was observed.

The aim of DSC and EPR study was the estimation of temperature effect on the liposome membranes

Table 2 The effect of pH of the buffer on the temperature of phase transition T_C

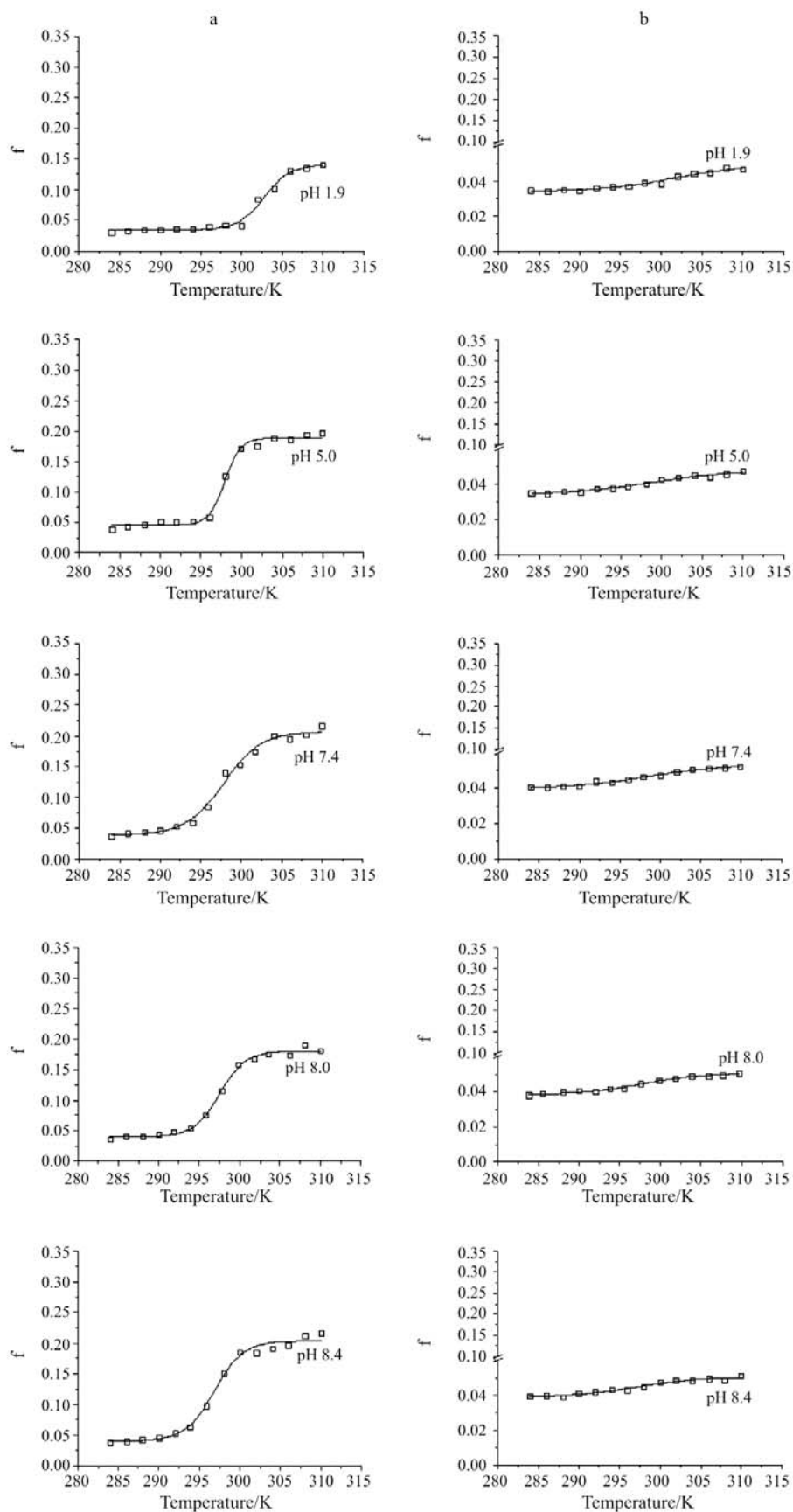| pH	T_C/K	
	DMPC/TEMPO	DMPC/Chol/TEMPO
1.9	302.0	302.0
5.0	296.0	298.0
7.4	296.0	296.0
8.0	295.8	298.0
8.4	296.0	300.0

Fig. 6 Effect of temperature and cholesterol on the *f* parameter values for liposomes formed from a – DMPC/TEMPO,
b – DMPC/Chol/TEMPO (DMPC:Cholesterol molar ratio 4.00:1.00); temperature range 284–310 K; pH 1.9, 5.0, 7.4, 8.0 and 8.4

formed from DMPC. A slight difference in the obtained results originates probably from the way of preparation of samples for analysis. DSC study was conducted in the wider range of temperature (278–333 K; 1.2 K min^{-1}). This allows us to observe at least 2-phase transitions. The influence of cholesterol on the phase transition is known [6, 27, 36–38]. The presence of cholesterol causes the decay or decrease of the phase transition sharpness. In a different way from the EPR study that was conducted for the TEMPO incorporated liposomes. Each intervention into the liposome membrane (change of its composition) causes changes of temperature of the phase transition. Our previous results reflect this situation [27].

The differences of T_C values are of order of 2–3 K. EPR spectroscopy is more sensitive than the DSC technique. Therefore for the samples with cholesterol we can observe the main phase transition with EPR spectroscopy and not with DSC.

Conclusions

The physico-chemical properties of liposomes obtained by mREV method have been determined with the use of the electron paramagnetic resonance (EPR) spectroscopy, differential scanning calorimetry (DSC) and transmission electron microscopy (TEM).

The results of the study with transmission electron microscopy show that the diameter of the liposome vesicles obtained by mREV method is less than 100 nm.

The spin marker TEMPO has been used since its properties are similar to those of hydrophilic drugs.

The above study confirms that TEMPO can be used to observe the changes in the partition between aqueous and fluid lipid regions. The change in the relative value of f as a function of temperature for DMPC shows that in the presence of water excess, this phospholipid undergoes a transition from the 'gel phase' to the lamellar smectic liquid crystalline phase.

The use of DSC method made it possible to determine not only the temperature of the main phase transition of phospholipids but also the temperature of the phospholipid phase transition from the tilted gel phase ($L_{\beta'}$) to the ripple gel phase ($P_{\beta'}$).

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