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Effect of temperature on liposome structures studied using EPR spectroscopy

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Abstract. The effect of temperature on liposome structures has been investigated by means of electron paramagnetic resonance spectroscopy with the use of the spin labelling technique. The EPR spectra were recorded on a Bruker EMX spectrometer at the X band in the temperature range 300–340 K. Liposomes were prepared from L-α-phosphatidylcholine dipalmitoyl (1,2-dihexadecanoyl-sn-glycerol-3-phosphocholine) 99% (DPPC), DL-α-phosphatidylcholine dimyristoyl (1,2-ditetradecanoyl-rac-glycerol-3-phosphocholine) 99% (DMPC) and cholesterol (5-cholesten-3β-ol) 99+% to constitute the membrane. The spin marker, 2-(3carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl free radical (5-DOXYL), placed in a liposome membrane, allows for observation of structural changes in liposomes with temperature increase. The changes of rotational correlation time and order parameter values with increasing temperature result from the motion rise of the spin probe. The intensity of the EPR signal of 5-DOXYL gives us information about membrane fluidity.

Keywords: Liposomes, structures, 5-DOXYL, EPR

1. Introduction

The investigations of liposome application as a drug carrier have been carried out for a number of many years [1–6]. Recently many studies have concentrated on the physical state of the lipid components of biological membranes. Many of these, especially spin-label studies have largely been responsible for the development of membrane fluidity. Water associated with a cell membrane is probably important in modulating and controlling the membrane structure and its function. It is also conceivable that modifications of the membrane-associated water by ions or drugs can result in the modification of the membrane structure [1]. Liposomes are used as a model structure of biological membranes for study of their structure and function. It has been confirmed that only stable liposomes, i.e. remaining in an unchanged form for a long time and capable of delivering drugs into circulatory system, may be used for drug transport [2]. The importance of the delivery of skin applied drugs entrapped in liposomes in comparison with the application of a drug in the conventional delivery system is well known. Liposome stability depends on several parameters e.g. properties of the drugs, size of the drug and liposomes, homogenity and type of lipid, liposome preparation method and pH. Frequently, the incorporation of a drug into liposomes leads to the reduction of its toxicity. Preparation of the liposomes as single- oligo-

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or multilamellar vesicles with different diameter [3] play a fundamental role in obtaining the liposomes exhibiting a long drug release time in the living organism or the ability to migrate to a specific part in the organism (i.e. tumours) [4]. In the structure of liposomes one or many double phospholipid layers separate the water inside from that outside the vesicle. It was found that the inserting of a drug into phospholipids containing hydrophilic long-chain polymers in the liposome membrane leads to the liposome releasing the drug slowly. Depending on their physicochemical properties drugs can exist in the water phase inside the liposome or can assemble into a liposome membrane. The amount of encapsulated hydrophilic therapeutic substance depends on the liposome water phase volume. The requirement of slow drug release needs the protection of the liposome surface from lipoproteins action which lead to a quick destruction of the liposome and uncontrolled drug release. The length of time the liposomes remaind in a system of turned out to be a factor that favours high liposome concentration in quickly metabolized tissues like e.g. tumours.

Taking into account the influence of the preparation and the stability on the liposome properties and on their possible use, the study of the liposome composition and of the temperature effect on the prepared liposome structure was conducted. To determine the correlation time τ, the $2A_{\mathrm{II}}$ parameter and the order parameter S of the 5-DOXYL spin marker placed in liposome membranes, the analysis by means of electron paramagnetic resonance technique was carried out.

2. Materials and methods

L-α-phosphatidylcholine dipalmitoyl (1,2-dihexadecanoyl-sn-glycerol-3-phosphocholine) 99% (DPPC), DL-α-phosphatidylcholine dimyristoyl (1,2-ditetradecanoyl-rac-glycerol-3-phosphocholine) 99% (DMPC) and the spin marker 2-(3carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl free radical (5-DOXYL) were purchased from Sigma Chem. Co., alpha, alpha, alpha-Tris–(hydroxymethyl)-methylamine 99.9% (TRIS) were purchased from Fluka, cholesterol (5-cholesten-3β-ol) 99+%, chloroform, dichloromethane, ethyl ether and hydrochloric acid were purchased from POCH, Gliwice, Poland.

We obtained two type of small liposomes (DPPC/Chol/5-DOXYL and DMPC/Chol/5-DOXYL) with the use of modified reverse-phase evaporation method (mREV) by applying 2 ml TRIS buffer (pH $= 7.4$) and 4 ml of organic solution prepared from methylene chloride and ethyl ether 1 : 1 v/v and the suitable phospholipide (DPPC or DMPC), chlesterol and spin maker (5-DOXYL) [3,7]. Mass fractions of phospholipide were: DPPC 17.7 mg/ml and DMPC 17.5 mg/ml. The average time of liposome preparation was about 12 minutes.

The EPR measurements were carried out in the temperature range 300–340 K with maintained constant $\pm$0.5 K during the experiment, on a Bruker EMX spectrometer at the X-band (9 GHz), equipped with Bruker N_2 temperature controller. The hyperfine splitting of the labelled liposome samples was determined with 150.000 G sweep width, 3420.610 G of field intensity, 20.120 mW microwave power, 4.48×10^4 signal amplification, 0.80 G modulation amplitude and 20.973 s sweep time for 10 scans.

3. Results and discussion

The EPR spectrum of 5-DOXYL, incorporated into the liposome membranes, shows an anisotropic motion and the fluidity of the membrane can be estimated from the maximum separation between the spectral extrema and the maximum hyperfine splitting ($2A_{\mathrm{II}}$). The value of $2A_{\mathrm{II}}$ reflects the rotational motional freedom of the phospholipids close to the polar head groups in the bilayer. This value increased

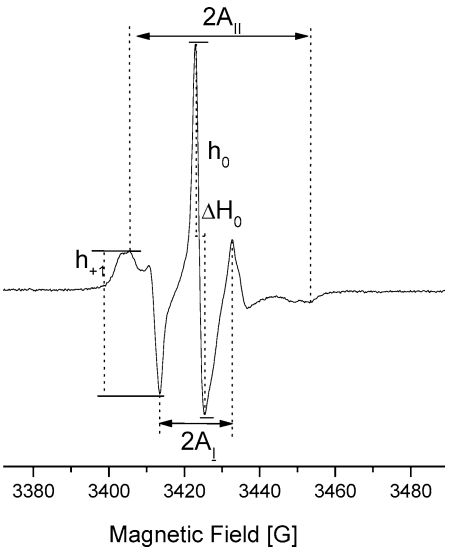

Fig. 1. A typical EPR spectrum of 5-DOXYL located in the liposome membrane, where: ΔH_0 – width of the mid-field line, h_0 – height of the mid-field line, h_{+1} – height of the low-field peak of the inner hyperfine doublet, $A_\perp$ – the peak to peak distance of the outside lines of triplet and $2A_{\rm II}$ – the maximum hyperfine splitting is shown.

with the decrease in fluidity. Line widths $2A_{\rm II}$ and ΔH_0, in Gauss [G], and heights of the mid- and low-field lines, $h_{0/}$ and h_{+1}, respectively, were obtained from the first-derivative of each absorption spectrum (Fig. 1).

The properties of the obtained small liposome of 100 nm in diameter were investigated in the temperature range from 300–340 K (Fig. 2 left and right). The addition of the spin marker allows us to make assumptions as to the structural changes in the obtained liposomes by the observation of the changes in the correlation time parameter. According to literature data [8] the correlation time parameter τ, order parameter S and parameter $2A_{\rm II}$ were calculated (Table 1).

$$\tau = 6.5 \cdot 10^{-10} \cdot \Delta H_0 \cdot \left(\sqrt{\frac{h_0}{h_{+1}}} - 1 \right), \tag{1}$$

$$\mathrm{S} = \frac{A_{\rm II} - A_\perp}{A_{ZZ} - A_{XX}} \cdot \frac{a_N}{a'_N}, \tag{2}$$

$$a_N = \frac{1}{3} \cdot (A_{XX} + A_{YY} + A_{ZZ}), \tag{3}$$

$$A_{XX} \approx A_{YY}, \qquad a_N = \frac{1}{3} \cdot (A_{ZZ} + 2A_{XX}), \tag{4}$$

$$a'_N = \frac{1}{3}(A_{\rm II} + 2A_\perp). \tag{5}$$

The τ, S and $2A_{\rm II}$ values presented in Table 1 are the average of four measurements for samples taken from three different liposome preparation. Calculated values of the liposome DPPC/Chol/5-DOXYL

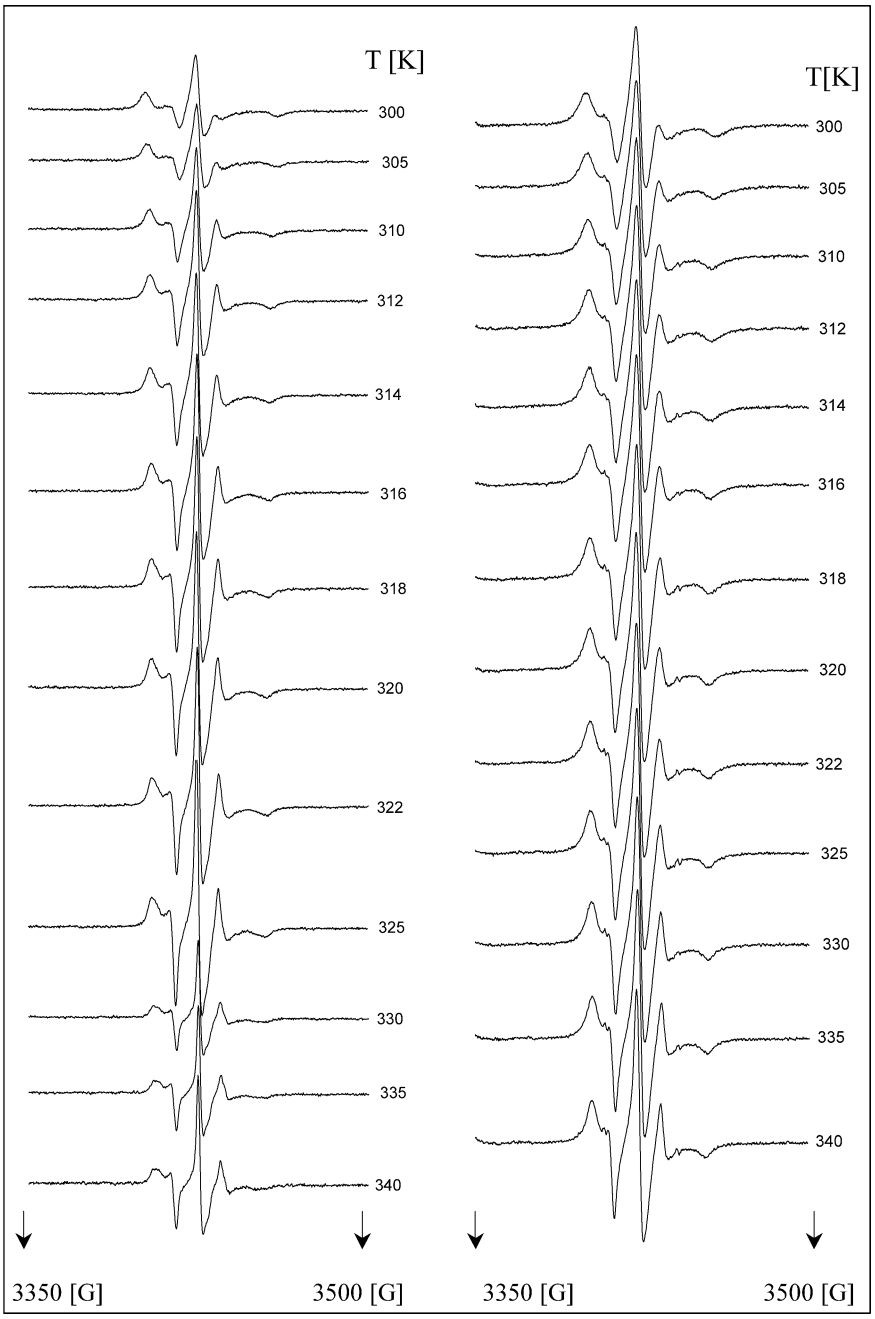

Fig. 2. Changes of the signal intensity of the spin marker 5-DOXYL located in the liposome membrane (left) DPPC/Chol/5-DOXYL, (right) DMPC/Chol/5-DOXYL in the temperature range 300–340 K.

order parameter S change from 0.75 ± 0.15 to 0.47 ± 0.03 in the temperature range from 300–340 K and are similar to those of liposome DMPC/Chol/5-DOXYL (the change from 0.70 ± 0.03 to 0.55 ± 0.02) and their values drop with increase of temperature. This points to the fluidity of the prepared liposome membranes increases with the rise in temperature. When the value of the order parameter S is 0 the

Table 1
Calculated data $\pm\delta$ of the correlation time parameter τ, order parameter S and $2A_{\mathrm{II}}$ parameter of two types of small liposomes

T [K]	DPPC/Chol/5-DOXYL			DMPC/Chol/5-DOXYL		
	S [G]*	$2A_{\mathrm{II}}$ [G]**	$\tau \cdot E10$ [s]*	S [G]*	$2A_{\mathrm{II}}$ [G]**	$\tau \cdot E10$ [s]*
300	0.75 ± 0.15	58.9 ± 0.3	12.90 ± 9.63	0.70 ± 0.03	55.9 ± 0.8	14.9 ± 2.92
305	0.77 ± 0.07	57.4 ± 1.3	12.80 ± 12.2	0.67 ± 0.02	53.9 ± 0.4	12.8 ± 3.95
310	0.71 ± 0.03	53.9 ± 0.7	11.40 ± 9.44	0.65 ± 0.02	53.4 ± 0.4	13.1 ± 2.78
312	0.68 ± 0.02	53.1 ± 0.4	10.90 ± 6.64	0.65 ± 0.01	52.7 ± 0.2	12.5 ± 3.05
314	0.67 ± 0.03	52.5 ± 0.6	10.90 ± 6.72	0.64 ± 0.03	52.6 ± 0.6	11.4 ± 3.54
316	0.65 ± 0.05	51.9 ± 0.8	9.97 ± 10.50	0.63 ± 0.02	52.5 ± 0.4	12.0 ± 3.62
318	0.64 ± 0.03	51.5 ± 0.6	9.84 ± 10.40	0.61 ± 0.02	51.8 ± 0.2	11.3 ± 3.33
320	0.62 ± 0.03	50.9 ± 0.6	9.72 ± 10.70	0.61 ± 0.02	51.6 ± 0.5	11.3 ± 3.84
322	0.62 ± 0.02	50.8 ± 0.4	9.35 ± 9.39	0.61 ± 0.01	51.2 ± 0.3	10.7 ± 3.99
325	0.59 ± 0.06	48.8 ± 1.4	9.25 ± 9.17	0.60 ± 0.02	51.1 ± 0.4	10.8 ± 4.21
330	0.55 ± 0.11	47.7 ± 2.4	9.15 ± 15.20	0.58 ± 0.02	49.9 ± 0.4	10.1 ± 4.75
335	0.52 ± 0.14	45.6 ± 3.0	8.86 ± 15.40	0.57 ± 0.01	49.7 ± 0.2	9.60 ± 4.85
340	0.47 ± 0.03	42.9 ± 0.7	8.73 ± 15.50	0.55 ± 0.02	48.9 ± 0.4	9.10 ± 5.72

δ is the error calculated from: *maximum deviation of the mean, **standard deviation.

membrane is disordered. When it is equal 1 the membrane has a crystal structure [8]. According to results presented the liposome membranes are more ordered at 340 K than at 300 K. We also observed that the changes of the order parameter S of DPPC/Chol/5-DOXYL membranes are greater than those observed for DMPC/Chol/5-DOXYL membranes (increase of the value of parameter S by 37.4% and by 21.5% from the starting value at 300 K, respectively).

The values of parameter $2A_{\mathrm{II}}$ are similar for both type of the prepared liposomes and decrease with temperature. Since the maximum of hyperfine splitting $2A_{\mathrm{II}}$ is inversely related to the fluidity, the reversal temperature dependence for $2A_{\mathrm{II}}$, similarly as for correlation time τ, confirms the suggestion that the liposome membrane fluidity changes. This means that the mobility of acyl chains of phosphlipides forming the liposome membranes also changes.

The correlation time τ of spin maker 5-DOXYL located in the liposome membranes decrease with the rise of temperature and for liposome DMPC/Chol/5-DOXYL this value is slightly higher then for liposome DPPC/Chol/5-DOXYL.

When the temperature rises from 300 K to 340 K, the increase of the intensity of EPR frequency of the spin maker 5-DOXYL located in both types of liposome membranes was observed. It is probable that with increasing temperature the fluidity of liposome membranes rises and in consequence the spin maker, located prior to the experiment the liposome membranes, can be released into the water.

4. Conclusions

EPR study of the spin-labelled liposomes was carried out. The correlation time τ, order parameter S and parameter $2A_{\mathrm{II}}$ of these membranes were calculated and their changes in the temperature range 300–340 K were observed. A gradual increase in fluidity can be seen when the temperature rises, proven by a decrease of the correlation time τ, order parameter S and parameter $2A_{\mathrm{II}}$ of the prepared membranes with the increase of temperature from 300 K to 340 K.

The obtained results show that the DPPC/Chol/5-DOXYL membranes are more sensitive to the change of temperature than the DMPC/Chol/5-DOXYL membranes.

Acknowledgement

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