ESR Study of the Liposome Membrane Physical Parameters in the Heating-Cooling Cycles

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Z. Naturforsch. **53c,** 191–196 (1998); received October 13/December 30, 1997

Thermal Hysteresis, Lecithin Liposomes, ESR, Spin Probes

Changes of dynamic and structural parameters of egg yolk lecithin (EYL) liposome membranes in the heating-cooling cycles have been studied using the ESR spin probe method. The investigations were conducted in the range of temperatures from -18 °C to +60 °C. It has been found that in the range of temperatures -15 °C to +45 °C in both the heating and the cooling run the spectroscopic parameters changed practically along the same curve (reversible changes). However, after exceeding this range of temperatures one of the parameters (partition coefficient of the spin probe 2,2,6,6 – tetramethylpiperidine -1-oxyl; TEMPO) changed along a closed curve, showing the phenomenon of thermal hysteresis. In the heating process the TEMPO content in liposome membranes was smaller than this in the cooling process. We assume that during the heating, the lipid molecules of the outer liposome layers dissolve in the aqueous medium. In the cooling process they can aggregate and form new liposomes, what in turn increases the surface of liposome membranes, accessible for the TEMPO probe (active surface).

Introduction

Both the natural and synthetic lipids can spontaneously form different liquid crystalline structures in water solutions (Israelachvili, 1985; Cullis *et al.,* 1979; Gruner *et al.,* 1985; Israelachvili *et al.,* 1976; 1977). During the temperature changes these structures undergo phase transitions. In the case of synthetic lipids the transitions are sharp and for a given lipid take place at a characteristic, precisely defined temperature (Shimshick *et al.,* 1973; Hatta *et al.,* 1993). The phase transitions can be detected and studied using various physical methods: differential scanning calorimetry (DSC), X-ray diffraction, nuclear magnetic resonance (NMR), spectrofluorimetry or electron spin resonance (ESR). Transition temperatures depend on the length and unsaturation degree of lipid molecules fatty acid chains as well as on their head group structure.

Phase transitions of the natural lipids are not sharp and take place in a relatively broad temperature interval. For example in the case of lipids obtained from the *Acholeplasma laidlawii* bacteria this range extends over 15 °C or even 30 °C broad temperature interval (Mc Elhaney, 1974a; 1974b). In similar range of temperatures (from -15 °C to +9 °C) the phase transition of the whole mitochondrium isolated from eukariotic cells takes place (Hackenbrock *et al.,* 1976).

In the last years several works appeared which show that the changes of physical and chemical parameters of the lipid water dispersions, among others the position and shape of phase transitions, depend on the direction of the temperature changes. Investigated samples show the thermal memory (hysteresis). It has been revealed (Tenchov *et al.,* 1989; Yao *et al.,* 1991) that in the dipalmitoylphosphatidylcholine (DPPC) water dispersion phase transition between gel ($L_{\beta'}$) and liquidcrystalline (L_α) phases is irreversible. DPPC heated from $L_{\beta'}$ goes through the intermediate phase (ripple phase – $P_{\beta'}$) to L_α. DPPC cooled from L_α phase goes to $L_{\beta'}$ through the metastable phase $P_{\beta'}$ (mst), other than $P_{\beta'}$. The transition from $P_{\beta'}$ (mst) to $P_{\beta'}$ occurs after a relatively long time (24 hours) or after cooling the sample to the gel phase ($L_{\beta'}$) and renewed heating. Futhermore Tenchov *et al.,* (1989) showed that the water dispersions of DPPC and ethanol undergo a sharp

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0939–5075/98/0300–0191 $ 06.00

phase transition about 41.5 °C during the heating which did not appear in the cooling process. Hatta *et al.*, (1993) demonstrated that during the heating the water dispersions of dielaidoylphosphatidylcholine transform from the L_α phase to the hexagonal II (H_{II}) at temperature of 61 °C and during the cooling return from H_{II} to L_α at temperature of 56 °C. Gershfeld *et al.* (1993) have studied the temperature dependence of the specific heat (C_{PL}) of dimyristoylphosphatidylcholine (DMPC) multilamellar liposomes. The study showed that C_{PL} decreased slowly with increasing the temperature. At temperature T^*=28.96 °C a sudden decrease of C_{PL} has been observed, connected with transition of DMPC from multilamellar structure I (through unilamellar) to multilamellar II. Return of DMPC from the structure II to I requires a cooling of the sample below the main phase transition temperature of DMPC (24 °C).

The objective of the present study was to investigate the changes of dynamic and structural parameters of the sonicated and multilamellar egg yolk lecithin liposome membranes during the heating-cooling cycles.

Materials and Methods

The liposomes used in the present study were obtained from fresh hen egg yolks lecithin (EYL) prepared in our laboratory by the method described in the paper of Singleton *et al.*, (1965). EYL was stored in the chloroform solution at -20 °C. The liposomes were formed by sonicating (liposomes LS) or mechanically shaking (multilamellar liposomes LM) of the EYL dispersion in distillated water. The study was conducted by means of the ESR spin probe method. Three spin probes were used in the study: 2,2,6,6 – tetramethylpiperidine – 1 – oxyl (TEMPO) of chemical formula $C_9H_{18}NO$ prepared in the Biophysical Department of the Łódź University, 5-doxyl stearic acid (FA 10,3) of chemical formula $C_{22}H_{42}NO_4$ and 16-doxyl stearic acid (FA 1,14) of chemical formula $C_{22}H_{42}NO_4$ from SIGMA CHEMICAL CO. (St.Louis MO, USA).

The spin probe dissolved in methanol was added to the definite volume of EYL chloroform solution. After thorough mixing the organic solvents were removed from the sample (for an hour) by means of the vacuum pump. Then the distilled water (1.5 ml) was added to the sample and the liposomes LS (by 5 min of sonicating) or multilamellar liposomes LM (by 15 min of mechanical shaking) were formed. EYL concentration in a sample was 0.04 M, and the spin probe to EYL had a molar ratio of 0.01. ESR spectra of the studied samples were recorded by means of the SE X/25 spectrometer (Technical University Wrocław, Poland). Measurements were performed at temperatures ranging from -18 °C to +60 °C. In this range a broad phase transition of EYL bilayers with the middle at about -5 °C takes place (Mason and Huang, 1978). On the basis of the ESR spectra of the FA(1,14) spin probe, localized in the middle of the lipid bilayer of the liposome membranes, the τ parameter (quantity inversely proportional to the rotational velocity of the probe) was determined. This parameter was calculated by using the formula for rotational correlation time (Hemminga, 1983; Schreier *et al.*, 1978) (Fig. 1). The value of parameter τ for the sample at temperature T= 20 °C is denoted by τ_k. Based on the ESR spectra of the spin probe FA(10,3), localized in the hydrophobic part of the membranes (near the lipid bilayer surface), the parameter $2A$ was determined (Fig. 2). The value of parameter $2A$ for the sample at T=20 °C is denoted by $2A_k$. The parameter $2A$ is proportional to the order parameter (Hemminga, 1983; Schreier *et al.*, 1978). The TEMPO spin probe dissolves both in the liposome membranes (lipid phase) and in the aqueous medium. On the basis of ESR spectra of this probe the partition parameter F was determined (Fig. 3a). The value of parameter F for the sample at T=20 °C is denoted by F_k. The parameter F value is connected, among others, with membrane fluidity. Increase of membrane fluidity causes an increase of the parameter F (Shimshick and Mc Connell, 1973).

For each of the spin probes used, the measurements were conducted for dozen samples (measurement series). The points on figures represent the mean value of at least three measurements (for the same temperature) obtained within one of the selected serie. The deviations from the mean value (mean error of a measurement series) are given in the figures, too.

Results and Discussion

Fig. 1 shows the temperature dependence of the relative value of parameter τ (τ/τ_k) of the FA(1,14) probe embedded in liposome LS membranes. As it follows from Fig. 1, in temperatures between -18 °C and +50 °C parameter τ decreased with increasing the temperature, thus indicating an increase both the FA(1,14) probe mobility and LS membranes fluidity. During the temperature decreasing the parameter τ values decreased. Changes of the parameter τ in the heating and cooling processes went almost through the same curve (Fig. 1) (reversible changes). Heating and cooling curves were also almost the same for the parameter $2A$ of the FA(10,3) probe studied in the temperature interval -18 °C to +50 °C (Fig. 2). Increase of temperature caused decreasing of $2A$ parameter values, thus indicating a decrease of the lipids ordering degree in the liposome LS membranes. In the cooling process the values of the $2A$ parameter increased.

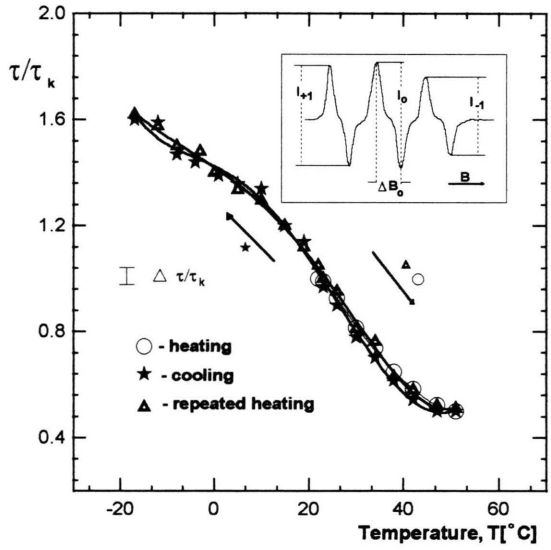

Fig. 1. Dependence of the relative value of the parameter τ (τ/τ_k) of 16-doxyl stearic acid (FA 1,14) spin probe embedded in sonicated egg yolk lecithin (EYL) liposome membranes (LS) on the temperature (T) in heating-cooling process. The figure presents the mean error of a measurement series and an ESR spectrum of FA (1,14) probe in the samples on inset. Parameter τ=5.59 ΔB_0 ($\sqrt{I_0/I_{+1}} + \sqrt{I_0/I_{-1}} - 2) \cdot 10^{-10}$s (Hemminga, 1983), where ΔB_0 means the peak – to – peak linewidth of the center line of ESR spectrum (in Gauss); I_{+1}, I_0, I_{-1} – amplitudes of the low, center and high magnetic field lines respectively (inset). The arrow on the inset denotes direction of magnetic field (B) increase. Symbol τ_k denotes the parameter τ of the sample at T=20 °C (τ_k= 5.7 ns).

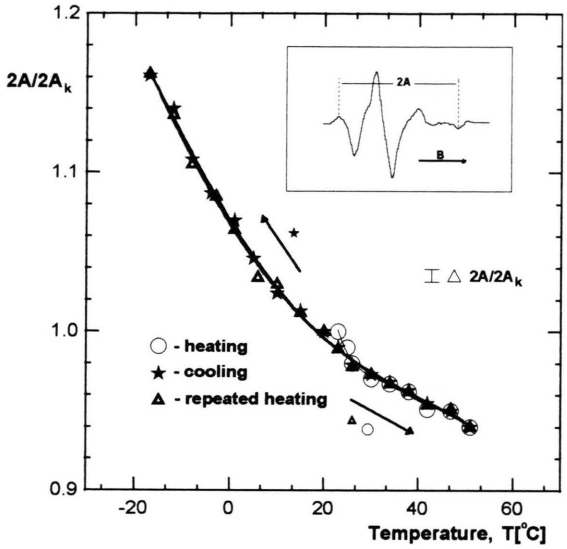

Fig. 2. Dependence of the relative value of the parameter $2A$ ($2A/2A_k$) of 5-doxyl stearic acid (FA 10,3) spin probe embedded in sonicated EYL liposome membranes (LS) on the temperature (T) in heating-cooling process. The figure presents the mean error of measurement series and an ESR spectrum of FA(10,3) probe in the samples on inset. The arrow in the inset means direction of magnetic field (B) increase. Parameter $2A$ denotes the distance between utmost lines of the FA(10,3) spin probe spectrum (inset). Symbol $2A_k$ denotes parameter $2A$ of the sample at T=20 °C ($2A_k$=24.9 Gauss).

Parameter F of TEMPO probe introduced to the water dispersion of LS liposomes behaved differently. Approximately reversible changes of the parameter F (in the heating and cooling process) were observed only in the range of temperatures from -15 °C to +45 °C (Fig. 3a). In the range -18 °C to +50 °C and higher these changes were irreversible. In these cases the F (T) dependence had form of a hysteresis loop (Fig. 3b). With increasing the temperature the parameter F also increased.

In the cooling process the F-parameter initially increased and then decreased along the curve placed above that determined in the first heating process (original curve). During repeated heating of the sample, after cooling it to -18 °C, parameter F increased and its values were located (approximately) on the original curve. When the sample was cooled to the temperature lower than -15 °C,

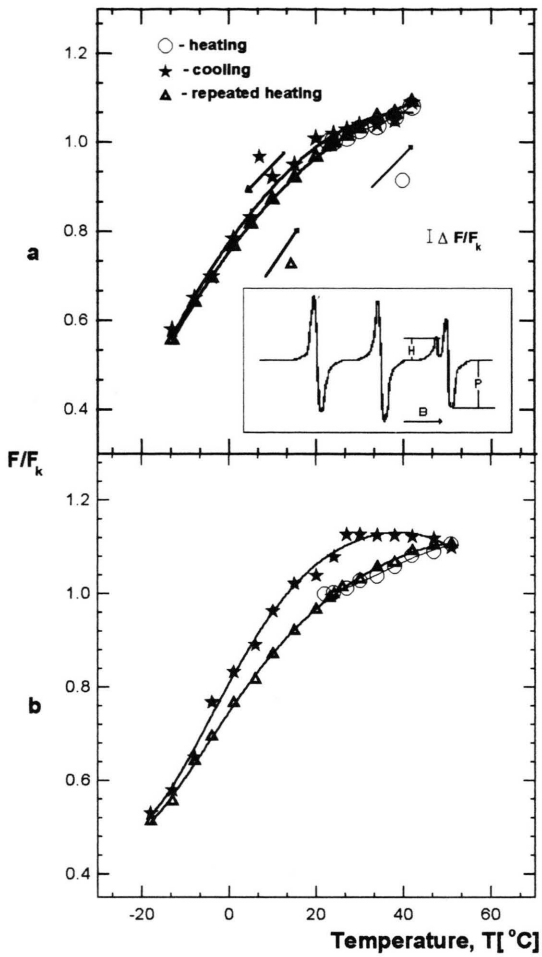

Fig. 3. Dependence of the relative value of the partition parameter F (F/F_k) of a 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) spin probe dissolved in water suspension of sonicated EYL liposomes (LS) on the temperature (T) in heating-cooling process. The figure presents the mean error of a measurements series and an ESR spectrum of TEMPO spin probe in the inset. Partition parameter $F=H/H+P$, where H and P denote high field line amplitudes of TEMPO probe placed to the lipid medium (membrane) (H) and to the aqueous medium (P) respectively (inset). The arrow in the inset denotes direction of magnetic field (B) increase. Symbol F_k denotes the partition parameter of the sample at T=20 °C (heating process) (F_k=0.52). Measurements were carried out in the temperatures range −15 °C to +45 °C (a) and −18 °C to +50 °C (b).

the F-parameter values measured in the repeated heating run followed not the original curve but the curve determined in the cooling run (upper curve on the Fig. 3b). The effect of thermal hysteresis of the parameter F in the range of temperatures -18 °C to +55 °C was also observed for the multulamellar liposomes (LM). In this case (Fig. 4) it was even stronger (greater area of the hysteresis loop).

Fig. 5 shows changes of the parameter F of the TEMPO probe introduced to the water dispersion of the liposomes LS in the initial period of the cooling process after the first heating to the temperatures of 50 °C, 55 °C and 60 °C. From the figure it follows that cooling of the sample caused an increase of F values above those obtained in the heating process. The F parameter values increased with increasing the highest temperature reached in the preceding heating run.

Thermal hysteresis of the F-parameter observed in this paper could be explained as follows. In the temperature interval from -18 °C to +45 °C the liposome structure does not change. Increase of temperature in this range causes only an increase of the liposome membranes fluidity registered as an increase of the parameter F value. In this range of temperatures the changes of the parameter F of the studied sample in the heating and cooling processes are reversible (lack of the hysteresis effect). Heating of the sample above 45 °C may cause dissolution of the EYL molecules forming

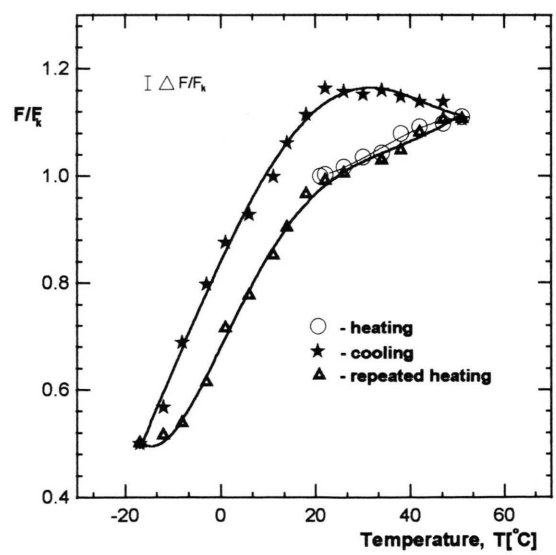

Fig. 4. Dependence of the relative value of the partition parameter F (F/F_k) of a TEMPO probe dissolved in water suspension of multilamellar EYL liposomes (LM) on the temperature (T) in heating-cooling process. Symbol F_k denotes partition parameter of the sample at T= 20 °C (heating process) (F_k=0.58). The measurements were carried out in the temperature range from −18 °C to +50 °C.

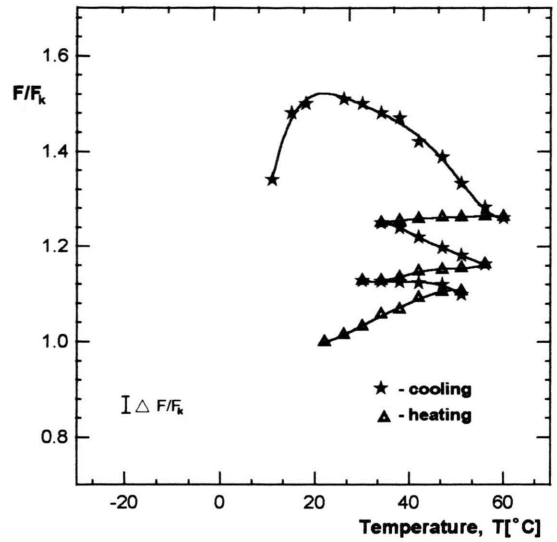

Fig. 5. Dependence of the relative value of the partition parameter F (F/F_k) of a TEMPO probe dissolved in water suspension of sonicated EYL liposomes (LS) on the temperature (T) in heating-cooling process. The sample was heated three times to the three different temperatures 50 °C, 55 °C and 60 °C respectively. Symbol F_k denotes partition parameter of the sample at T= 20 °C (first heating process) (F_k=0.52).

the external liposome membrane layers and decrease of the total surface of liposomes accessible for the TEMPO probe (active surface). Increase of the parameter F in these temperatures can be result of predominant influence of the increase of the liposome membranes fluidity. In the begining of the cooling process the EYL molecules dissolved in water can form the new liposomes what in turn causes an increase of their total active surface and an increase of the parameter F above the values obtained in the heating process. Further cooling of the sample may cause a predominant decrease of the obtained liposome membranes fluidity. Return of the parameter F values to the original curve requires cooling of the sample to the temperature lower than -15 °C. This result may mean that in these temperatures the liposomes aggregate what causes that their number and active surface decreases to the origin state.

Increase of the hysteresis loop area in the case of LM liposomes (Fig. 4) (compare to LS liposomes, Fig. 3b) may mean that more EYL molecules dissolved in water while heating and more new liposomes are formed during the cooling process. For the LM liposomes the changes of the active surface and of the F-parameter value in the heating-cooling cycle can be greater than for LS liposome membranes. Spectroscopic parameters τ and $2A$ studied by means of FA(1,14) and FA(10,3) probes did not show the hysteresis effect in the temperature range -18 °C to +50 °C. This result may mean that the changes of the liposome EYL dynamic properties are reversible in the studied range of temperatures.

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