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ESR as a valuable tool for the investigation of the dynamics of EPC and EPC/cholesterol liposomes containing a carboranyl-nucleoside intended for BNCT

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Abstract

Electron Spin Resonance (ESR) spectroscopy of long-chain nitroxides (5-, 7-, and 16-doxyl stearic acid) has been used to evaluate the perturbations induced by β-5-*o*-carboranyl-2′-deoxyuridine (CDU) on the structure and dynamics of egg phosphatidylcholine (EPC) and EPC/cholesterol liposomes. Loaded liposomes are intended for the use in Boron Neutron Capture Therapy (BNCT). From a detailed analysis of the motional and order parameters determining the ESR line shape as a function of temperature and of CDU content in liposomes, an increased order and a hindered motion of the phospholipid membranes resulted in the presence of increasing CDU concentration. This occurred particularly at the liposome surface level. Both higher ordering and increased rigidity of the membrane lipids were the result of the constraints exerted by the embedded carboranyl-nucleoside.

Keywords: Spectroscopy; Phosphatidylcholine; Cholesterol liposome

1. Introduction

Boron neutron capture therapy (BNCT) is based on the $^{10}B(n,\alpha)/^{7}Li$ nuclear reaction which occurs when ^{10}B nuclei, which have a large capture cross section relative to the more abundant endogenous nuclei (^{1}H, ^{12}C, ^{31}P, ^{14}N), are exposed to thermal neutrons [1–3]. BNCT is referred to as a binary therapy because the individual components (i.e., the boron atoms and the neutrons) unto themselves are not efficacious. However, in combination, they have the potential to create a highly selective damage to cancerous cells and associated biological macromolecules because alpha particle and lithium ion, the nuclear fragments produced in the nuclear reaction, have very short diffusion distances (5 μm for ^{7}Li and 9 μm for α particles; approximately one cell diameter) within tissues. As a result, those cells that have bound or taken up a ^{10}B-containing agent are selectively destroyed.

In order to achieve successful cell killing, BNCT agents must be able to selectively deliver a considerable amount of boron to the tumor cells. It is generally accepted that 5–30 μg ^{10}B per g of tumor are required for successful therapy [4]. This amount will be substantially reduced if the boron is concentrated in or near the cell nucleus [5–7]. Another important requirement is that the boron delivery vehicle is non-toxic and preferably carries more then one boron atom.

One of the major efforts in developing boron-containing drug, therefore, is focused on the link of stable boron clusters to various biomolecules. Higher boron percentages in such molecular adducts may be beneficial in achieving the needed cellular concentration without affecting the compound toxicity. The first cluster that has been used for this purpose is the *o*-carboranyl moiety (or carborane) which contains 10

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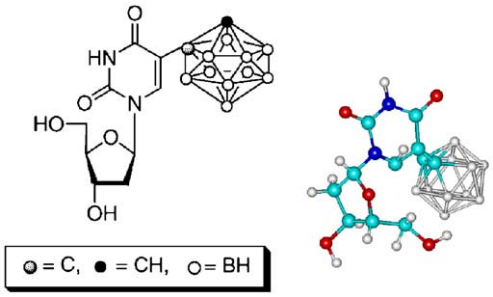

Chart 1. 5-*o*-carboranyl-2′-deoxyuridine (CDU).

boron atoms [8]. Details on the compounds containing carborane moieties are found in a recent review [9].

Among the many low molecular weight compounds synthesized for BNCT, there are carborane-containing amino acids, peptides, phospholipids, carbohydrates, porphyrins, DNA intercalator, antibodies, hormones [10].

Several nucleosides modified with a carboranyl group have been developed in the last decade [11–13]. β-5-*o*-carboranyl-2′-deoxyuridine (CDU, Chart 1) is a non-toxic pyrimidine nucleoside analogue designed for BNCT of brain tumors, which has been studied extensively for its cytotoxicity, anticancer, antiviral activity, and cellular uptake in the laboratory of one of the authors [14–17].

This pyrimidine nucleoside has a high boron content and intact 3′- and 5′-hydroxyl functions. Schinazi et al. [14] have proved that D-CDU is taken up by human lymphocytic cells (CEM), primary human peripheral blood mononuclear (PBM) cells, and brain tumor cells. It is phosphorylated intracellularly by cellular kinases. This finding suggests that the 5-*o*-carboranyl moiety is well tolerated and can mimic a pyrimidine nucleoside. These results suggest that the delivery and trapping in cells of large amounts of a boron-containing nucleoside is possible.

One strategy for the amplification of drug delivery is represented by using liposomes, which received special attention within BNCT community.

Liposomes have the ability to carry large amounts of both hydrophilic and lipophilic molecules and, if an appropriate lipid composition is used, a significant increase in the circulation time of the encapsulated species can be achieved. Moreover, many studies have shown that liposomes passively accumulate at tumor sites or sites of infection [18–24]. Liposomes loaded with boronated compounds have been investigated for boron delivery in animal models [18–22].

The physico-chemical characterization of liposomes containing boronated drugs has been performed by several techniques [25–29].

Edwards et al. [30,31] have demonstrated the occurrence of unilamellar liposomes with high monodispersity in both egg phosphatidylcholine (EPC) and EPC/Cholesterol (55–45 mol%) liposomal dispersions. Most lipid mixtures used to produce liposomes for drug delivery contain cholesterol, typically in concentrations of 30–50 mol%. This is because cholesterol has the ability to increase the cohesive strength and reduce the membrane permeability of phospholipid bilayers. Recently, interesting structural effects of carboranyl-carbohydrate containing EPC liposomes have been found by employing cryogenic Transmission Electron Microscopy (cryo-TEM) and Electron Spin Resonance (ESR) spectroscopy of lipophilic spin probes [32]. In the present work, liposomes of egg phosphatidylcholine and its mixtures with cholesterol (Chart 2) were used as model structures to obtain information on the potentiality of liposomes as a drug carrier for carboranyl-nucleoside.

Since CDU has high hydrophobicity because of its ortho-carborane domain, its localization is expected to be inside the hydrophobic region of the membrane. In the present

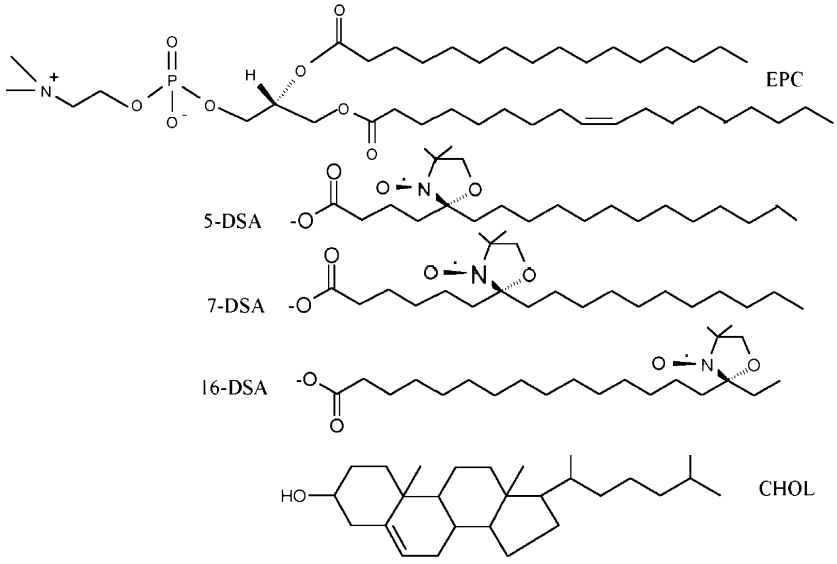

Chart 2. Structures of the relevant chemicals in this work.

work, the ESR of lipophilic spin labels was used to investigate the effect of this nucleoside on both the structure and dynamics of EPC and EPC/cholesterol liposomes. ESR spectroscopy was also applied to bring out the influence of CDU on the main phase transitions of phospholipid membrane as a function of CDU mole fraction.

ESR spin labeling has, in fact, proved to be a powerful technique for studying liposome bilayers and drug–lipid interactions [27,33–39]. To evaluate the perturbations induced by carboranyl-nucleoside on the dynamic structure of the liposome membrane, we used spin probes with the nitroxide moiety in different positions of the stearic acid chain (5-, 7- and 16- doxyl stearic acid; 5-, 7-, and 16-DSA, respectively, Chart 2).

2. Materials and methods

The liposomes examined were built up with egg-yolk lecithin, EPC, mixed PCs containing both saturated and unsaturated alkyl chains (approximately 70% 1-palmitoyl-2-oleoyl-PC) of grade 1, purchased from Lipid Products (Nutfield, England) and a mixed system composed by EPC/Cholesterol in a molar ratio 55–45 mol% (Chart 2). Cholesterol (CHOL) was purchased from Avanti Polar Lipids (Alabaster, AL). The boronated drug was synthesized by Schinazi et al. [14] and it was more than 98% pure as determined by HPLC [14].

The spin-probes used in this work were 5-, 7-, and 16-DSA (Chart 2). They were purchased from Sigma Chemicals, München, Germany, and used without further purification.

All other salts were of analytical grade and used as received.

2.1. Sample preparation

The required amounts of phospholipid, cholesterol, spin label (~1 mol%) and boronated compound (in the case of loaded liposomes) were dissolved in a 2:1 (v/v) mixture of chloroform and methanol in a round bottom flask. The spin probe concentration was low enough to prevent ESR line width broadening by spin–spin interactions. The solvent was evaporated under a stream of nitrogen gas to form a thin film of the solute on the bottom. This film was dried overnight under vacuum to remove any trace of the solvent. The lipid multilayers were formed by hydrating the mixture with HEPES buffer (0.15 M NaCl, 0.02 M HEPES, pH=7.4), freeze–thawing the dispersion eight times. Then, liposomes were downsized by extrusion (Liposofast apparatus, Avestin, Ottawa, Canada) with 15 passages through a polycarbonate membrane of 100 nm pore diameter. Unloaded liposomes prepared with this technique were unilamellar as shown by previously published results [30–32]. CDU containing liposomes were unilamellar as shown by cryo-TEM measurements (unpublished results). The total lipid concentration used in this work was 5 mM. The dispersions were transferred to 100 μl capillaries which were used as sample cells for the ESR spectrometer.

2.2. UV spectroscopy

A Lambda-5 UV-visible spectrophotometer connected to a HAAKE KV20 thermostat set to 298 K was used for the UV measurements. Quartz 1 mm length cuvettes were used and turned upside down a couple of times before measurement. The absorbance of each dispersion was measured against a blank of HEPES buffer solution.

2.3. ESR spectroscopy

ESR spectra were obtained on a Bruker spectrometer model 200D operating at X-band (~9.5 GHz) with a typical rectangular cavity. Data acquisition was performed with the ESR software commercialized by STELAR Meda, Italy. Temperature was controlled with Bruker VTB 3000 accessory (accuracy=±0.5 K) and was monitored by means of a thermocouple placed directly in the proximity of the sample cavity. Typical value of the microwave power was 2.1 mW. All temperature scans were performed by heating samples from temperatures well below any transition.

Samples were incubated for at least 20 min at the starting temperature and for at least 10 min before scanning at each new temperature. The microwave frequency was about 9.51 GHz while the central magnetic field was set at 3390 G with sweep widths of 100 G. DPPH (g=2.0036) was used as internal standard for g value determination. In order to prevent inhomogeneous line broadening, the modulation amplitude was usually kept less than one tenth, and never more than one third, of the peak-to-peak line width of the narrowest line.

2.3.1. Spectral line shape analysis

Suitable spectral parameter that characterized the ESR spectra of randomly oriented, not immobilized spin labels, is the half distance between the extreme peaks of the total spectra, A'_{II}. It is the value of the parallel component of the coupling constant partially modulated by the nitroxide motion dominated by the so-called correlation time relevant for the nitroxide motion, $<\tau>$ [33,38]. Therefore, this quantity is directly correlated with the motional rates. The latters were calculated according to the procedure given by Freed et al. in terms of non-linear least-squares (NLLS) analysis of the spectra based on the stochastic Liouville equation [40–42]. From the calculation, the rotational diffusion rates $R_{\perp}$ and R_{II} and the order parameter S_{00} (see below) were obtained. $R_{\perp}$ and R_{II} are the rotational diffusion rates of the nitroxide radicals around the axis perpendicular and parallel to the mean symmetry axis for the rotation. The value of $\tau_{\perp}$, the perpendicular component of the correlation time, was calculated from $R_{\perp}$ from the relationship: $\tau_{\perp}=1/6R_{\perp}$. The microscopic orientational

ordering of the spin label is characterized by the order parameter S_{00}, which is related to the amplitude of the rotational motion. S_{00} is defined as follows:

$$S_{00} = \langle 1/2(3\cos^2\theta - 1) \rangle$$

It is a measure of the extent of alignment of z' molecular axis with respect to the z'' local director frame, taken as the normal to the bilayer. The results reported in this work were analyzed in terms of both A'_{II} and $<\tau>$. We were more interested to the bulk variations of the mobility in the different domains of the liposome bilayer, rather than to the finest details of the membrane dynamics; the discussion carried out mainly in terms of A'_{II} gained therefore in clarity. A'_{II} reflected, indeed, both the mobility and the amplitude of motion of the segmental lipid chains. The A'_{II} values measured from spectra of independently prepared samples were averaged over at least three determinations. The reproducibility was ±0.1 G.

3. Results and discussions

3.1. UV measurements

The 298 K electronic absorption spectra of CDU and 2′-deoxyuridine in HEPES buffer solution are shown in Fig. 1A and B. The maximum wavelength of the transition of deoxyuridine base was 260 nm. The maximum absorption of CDU was shifted to higher wavelength, that is, ~274 nm. The occurrence of an electron-deficient moiety, such as carborane covalently bound to the uracil, produced this red-shift effect. The extinction coefficient of CDU in HEPES buffer was calculated to be 8.6×10^3 l/mol × cm. The spectrum of EPC liposomes containing CDU is also shown in Fig. 1A.

This spectrum was registered a few minutes after the extrusion of the mixed dispersion in the spectrophotometer cell (1 mm, optic length). As mentioned above CDU has high hydrophobicity and for this reason we expected its localization within the phospholipid bilayer. A valuable parameter which strengthened this theory is represented by the CDU partition coefficient. Jarugula et al. [43] have determined the average octanol/phosphate buffer, pH 7.4, partition coefficient (P) of CDU which is 3050±215, corresponding to a log P value of 3.48. This evidence proved that the partition of CDU in the lipidic phase is therefore complete. The insertion of the boronated compound in EPC liposomes did not affect either the position or the width of the absorption at 274 nm (in the limit of experimental error), whereas the 228-nm peak was shifted to 222 nm. This is clearly evident in Fig. 1B, where the spectrum of CDU-loaded EPC liposomes, opportunely subtracted by the pure liposome absorption, is reported in comparison with the CDU water solution. The same result was obtained with EPC/CHOL liposome dispersions.

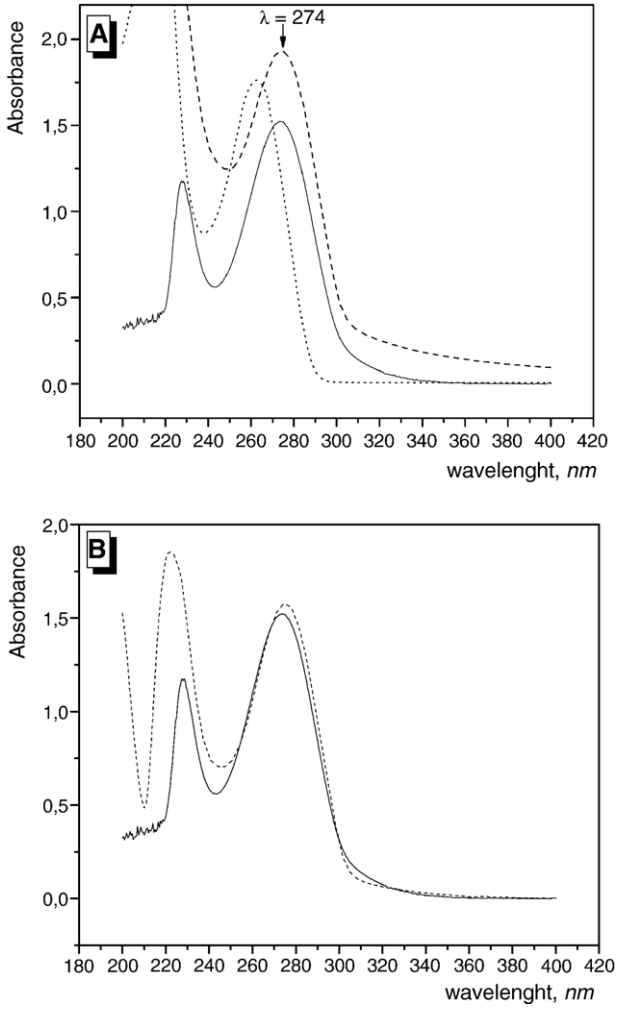

Fig. 1. (A) UV spectra at 298 K of CDU (full line) and of 2′-deoxyuridine (dotted line) in HEPES buffer ([CDU]=0.175 mM; [2′-deoxy-U]=0.151 mM) as compared with the 298 K UV spectrum of CDU-loaded EPC liposomes in HEPES buffer (total lipid concentration=5 mM; dashed line). (B) UV spectrum obtained by subtraction of the spectrum of CDU-loaded EPC liposomes from that one of pure EPC dispersion at the same phospholipids concentration and temperature (dashed line) as compared with UV absorption of CDU in HEPES buffer solution (full line).

3.2. ESR measurements

Because of the structural analogies of membrane lipids and n-DSA, the stearic spin probes are suitable probes for the alkyl chain mobility of phospholipids. The use of stearic acids with the nitroxide moiety in different positions along the lipid chain allowed us to investigate different segmental hydrocarbon domains: the hydrophilic glycerol moiety and the deepest interior of the hydrocarbon regions were probed with 5-, 7-DSA, and 16-DSA, respectively.

Spectral measurements were made at different temperatures and the choice of the temperature range from 253 to 330 K for EPC and EPC-Cholesterol liposomes was dictated by the gel-to-liquid crystal phase transition temperatures of such phospholipid.

3.2.1. 5-DSA spin probe

5-DSA inserted in EPC membranes gave the typical slow-motion spectrum as it is very often observed when this radical is introduced in ordered molecular assemblies. This was observed in micelles, cationic and neutral liposomes, lamellar phases [27,32,33,37,44–49].

The temperature dependence of the ESR line shape of 5-DSA in EPC membranes with and without CDU 35 mol% is shown in Fig. 2.

At the lowest investigated temperature (253 K), the spectra of 5-DSA in unilamellar vesicles of both CDU-free and CDU-containing EPC liposomes dispersed in HEPES buffer approximated to the ESR powder pattern and were characteristic of a rigid phase. A'_{II} was ~33 G for both samples at 253 K as expected for phospholipid membranes in the gel phase. The spectral anisotropy variations with temperature were different in CDU-free and in CDU-loaded liposomes. The values of A'_{II} for 5-DSA in EPC/CDU system were larger than in pure EPC liposomes at the same temperature and this finding was particularly marked in the temperature range 263 K to 283 K. The ESR data suggested that addition of CDU to EPC liposomes reduced the degree of the segmental lipid chain motion in the proximity of the interfacial region with a significant influence on the temperature-dependent phase behavior.

The $1/T$ dependence of ln A'_{II} of 5-DSA in pure EPC liposomes and in EPC liposomes loaded at two different CDU concentrations are shown in Fig. 3A. The discontinuity observed in CDU free liposomes at ~273 K ($1/T = 3.66 \times 10^{-3}$ K^{-1}), corresponding to chain melting phase transition, practically disappeared when 35 mol% CDU was loaded. As a contrast, the transition at ~310 K ($1/T = 3.23 \times 10^{-3}$ K^{-1}) was maintained whose physical meaning is not clear at the moment. The behavior with temperature of the correlation time obtained from the simulation of 5-DSA in CDU loaded liposomes was similar to that of A'_{II} (Fig. 3B).

As anticipated, the 5-DSA correlation time $<\tau>$, which monitored the lipid mobility close to the bilayer surface, displayed a marked temperature dependence. It is interesting to note that, opposite to the A'_{II} values, $<\tau>$ showed a marked sharp variation of slope at 273 K in plain EPC liposomes, which shifted to $T = 279$ K ($1/T = 3.58 \times 10^{-3}$ K^{-1}) in CDU-containing liposomes.

There were other remarkable features to be observed in Fig. 3A and B. First, there was an increase of A'_{II} and $<\tau>$ even at the lowest temperatures that became more marked at 273 K. Second, at increasing temperature, both A'_{II} and $<\tau>$ in the two systems tended to converge. This happened at $T > 310$ K. Two different explanations could be given to interpret these results. Because of the increasing water solubility of CDU at the higher temperatures, CDU embedded in the double layer could come out externally in the water matrix and we suspect that the structural differences of lipids in the gel and liquid-crystalline phases allowed for the accommodation and for the interaction of

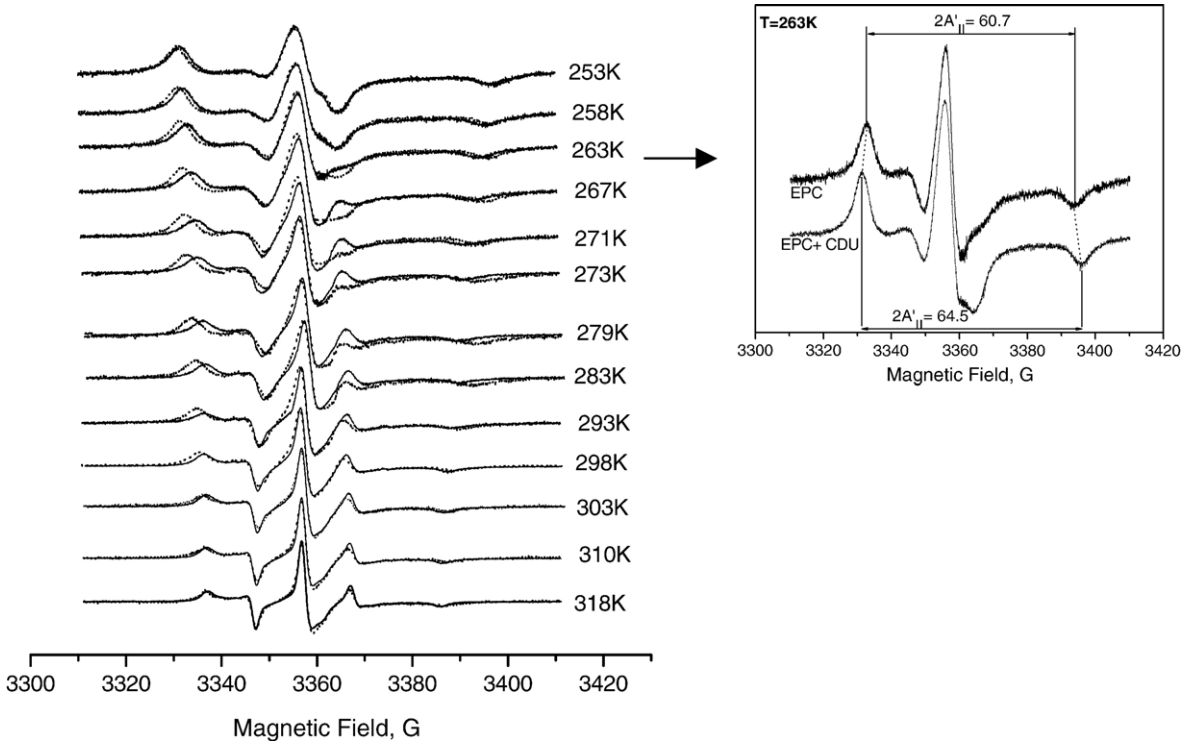

Fig. 2. ESR spectra of 5-DSA with the nitroxide moiety at the C-5 position of the acyl chain, in plain EPC liposomes (solid line) and in EPC liposomes containing CDU 35 mol% (dotted line) ([EPC] = 5 mM). The temperature at which the spectra were recorded is indicated in the figure. Inset: 2 A'_{II} as it is defined in the ESR spectra of 5-DSA in the two systems at 263 K.

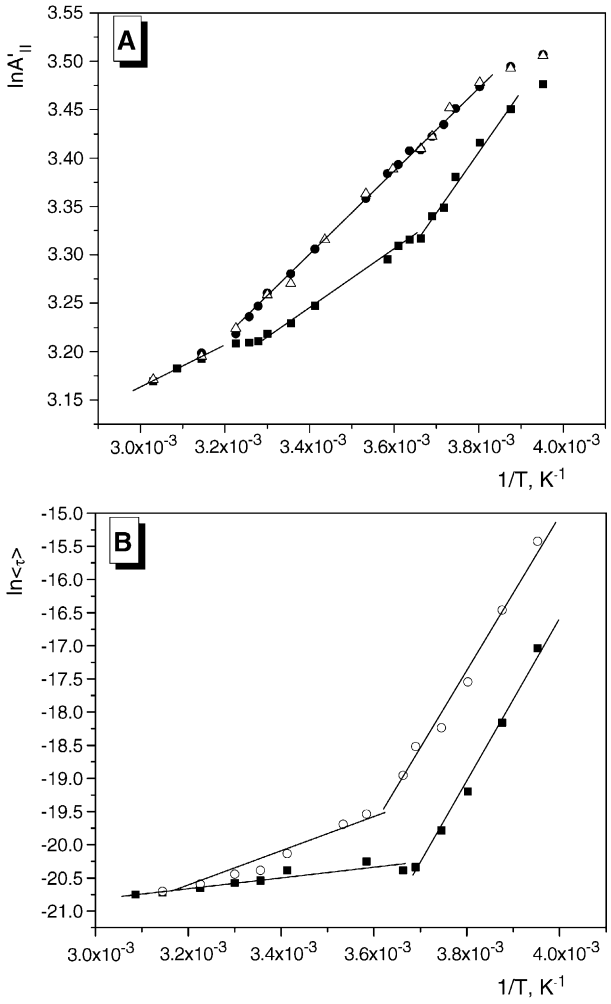

Fig. 3. (A) Dependence of the hyperfine splitting, A'_{II}, on the reciprocal temperature; 5-DSA in 5 mM EPC liposomes in the absence (■), and in the presence of CDU 35 mol% (●) and 43 mol% (△). (B) dependence of $<\tau>$ of 5-DSA on reciprocal temperature in pure EPC liposomes (■), and in EPC dispersions containing 35 mol% of CDU (○).

the boronated compound in different ways inside the membrane. In the fluid phase, the spacing between the acyl chains was large enough to accommodate CDU molecules without disrupting the membrane. Finally, the intensity of the ESR overall spectra decreased markedly at higher temperatures and the ESR signal disappeared at $T>318$ K. This was simply explained assuming that the 2′-deoxyribose unit in CDU acted as a reducing agent toward the nitroxide group of the spin probe with formation of the corresponding diamagnetic hydroxylamine.

Another interesting feature of the mixed system is that at high CDU concentration (up to 43 mol%), the A'_{II} values did not change significantly and the spectra obtained from 5-DSA were simulated with the same parameters used for the sample containing 35 mol% of carboranyl-nucleoside (Fig. 3A). It is noteworthy that all of the spectra from EPC and EPC/CDU dispersions were simulated as a single component with the following best-fit parameters $g_{xx}=2.0083$, $g_{yy}=2.0070$, $g_{zz}=2.0029$, $<\tau>=2.0061$; $A_{xx}=A_{yy}=5.5$ G, $A_{zz}=32.8$ G, $<A>=14.6$ G.

However, the spectra from 5-DSA in EPC/CDU liposomes at $T=263-267$ K showed an additional feature of a broadened component which was a clear indication of spin–spin interactions obtained from some 5-DSA molecules clustering.

The changes of the order parameter S_{00} with temperature for loaded and unloaded EPC liposomes are shown in Fig. 4. As expected, pure EPC liposome dispersion showed a decrease of the order parameter in the proximity of polar head region as temperature was increased. This result was consistent with the changes in membrane fluidity induced by the increase of chain motions.

The accuracy of the simulations of the experimental spectra at selected temperatures, obtained from 5-DSA in the free and loaded systems, is shown in Fig. 5. The 298 K ESR line shapes of 5-DSA in pure EPC (full line) and in EPC/CHOL (dotted line) liposomes are compared in Fig. 6 with the ESR line shape of 5-DSA in EPC liposomes containing CDU in 35 mol% (total lipid concentration is 5 mM). The ESR spectra of 5-DSA in EPC bilayer at 298 K, above its gel to liquid-crystalline phase transition temperature, were affected by the occurrence of CDU in a similar way as in presence of cholesterol in molar ratio 55–45 mol%.

The most apparent feature in the spectra shown in Fig. 6 was the A'_{II} value which was appreciably higher in plain EPC/CHOL liposomes than in plain EPC liposomes. The EPC/CHOL ESR spectra looked very similar to the ESR spectra from CDU-containing EPC liposomes. This unambiguously suggested an increased rigidity of the liposome surface induced by the presence of cholesterol. This was not a new observation since many cases are reported in which the occurrence of cholesterol reduce the surface mobility of liposomes [44,50,51]. The rigid tetracyclic ring structure of cholesterol and the rigid angle at the *cis* double bonds in the

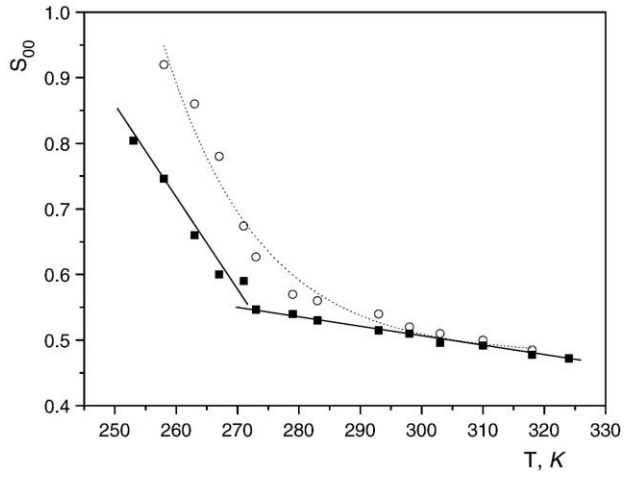

Fig. 4. The order parameter, S_{00}, of the 5-DSA spin label, as a function of temperature T in pure EPC liposomes (■) and in EPC dispersions containing 35 mol% of CDU (○).

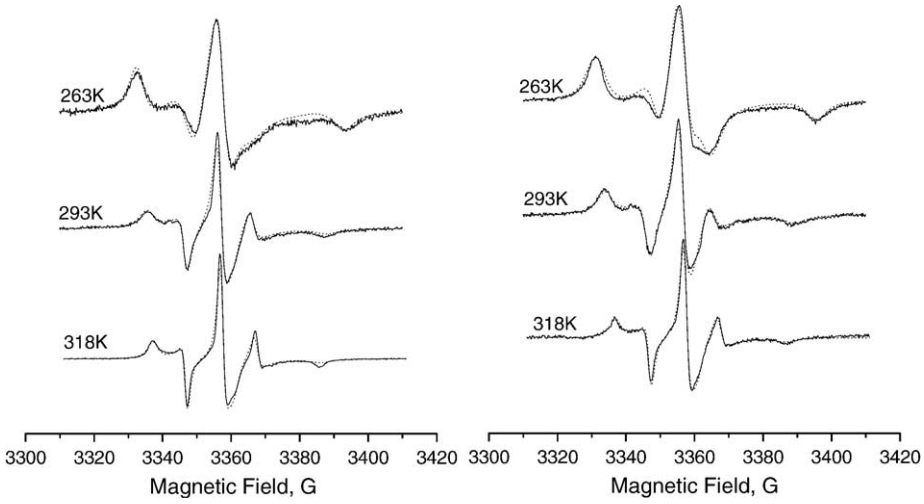

Fig. 5. ESR spectra of 5-DSA (1 mol%) in EPC liposomes dispersions in the absence (left) and in the presence (right) of CDU 35 mol% at T=263, 293, and 318 K. Solid lines, experimental spectra; dotted lines, simulated spectra; [EPC]=5 mM.

oleoyl chains in EPC did not fit to each other when they were in direct contact in the membrane. Due to this structural non-conformability: (1) cholesterol molecules tended to be excluded from unsaturated PC domains and they segregated out; (2) the ordering effect of cholesterol was much weaker in unsaturated PC bilayers compared to the saturated PC membranes. It is well known that the cholesterol favors the *tutto trans* conformation of PC alkyl chain next to cholesterol. The ordering effect of cholesterol could be defined as a physical interaction of cholesterol itself with saturated acyl chains.

ESR measurements with 5-DSA were also conducted on EPC/CHOL liposomes containing CDU even if the maximum amount of boronated compound soluble into the dispersion was lower than 35 mol%. In fact, samples containing EPC/CHOL (55–45 mol%) and CDU 35 mol% gave white aggregates after addition of HEPES buffer to the EPC/CHOL/CDU film mixture. The freeze–thaw process did not induce the formation of a transparent dispersion, even after keeping the solution at 338 K for several hours. The simultaneous occurrence of cholesterol and CDU 35 mol% with their bulk structure constituted chemical stress and promoted the disruption of the aggregates.

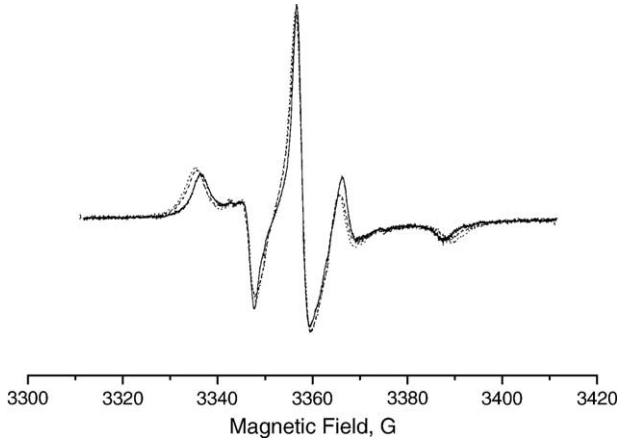

Fig. 6. ESR spectra of 5-DSA in EPC/CHOL liposomes (dotted line), in unloaded EPC liposomes dispersion (full line), and in EPC liposomes containing CDU 35 mol% (dashed line) at 298 K; [lipids]=5 mM.

Fig. 7A and B compare the behavior of A'_{II} of 5-DSA in EPC and in EPC/CHOL liposomes without and with the boronated compound, respectively, in the temperature range increasing from 253 K to 318 K. At each temperature investigated, the A'_{II} values were higher in EPC/CHOL liposomes than in EPC liposomes. This was a further proof that cholesterol induced a decrease in the bilayer mobility. Moreover, the discontinuity, observed at 273 K ($1/T \leq 3.66 \times 10^{-3}$ K^{-1}), in EPC bilayers was also found in EPC/CHOL liposomes. Above 273 K, a marked difference between loaded EPC/CHOL and unloaded liposomes appeared exactly as observed in EPC liposomes (Figs. 3 and 7A). These differences decreased with increasing temperature and above 310 K, the A'_{II} values in EPC/CHOL liposomes were independent on the presence of CDU (compare the ▽ curve in Fig. 7A with the corresponding ▼ curve in Fig. 7B). An interesting observation can be deduced from Fig. 7B. In loaded liposome bilayers, both made of EPC and EPC/CHOL, A'_{II} increased linearly up to 273 K for EPC/CDU system and to 263 K for EPC/CHOL/CDU dispersions; then, the slopes changed. In loaded EPC/CHOL liposomes, the local variations of mobility sensed by the probe decreased below the main transition temperature.

The variation of $<\tau>$ with reciprocal temperature in EPC/CHOL liposomes had the same trend of that one observed in EPC liposomes, although the $<\tau>$ values were higher at each temperature (Fig. 7C).

As already observed in EPC liposomes above the transition temperature, the lipid matrices can accommodate rather high concentrations of the carborane-derivative with significantly altering the motions of the lipid chains. Most probably, the polar head region of the neutral bilayers

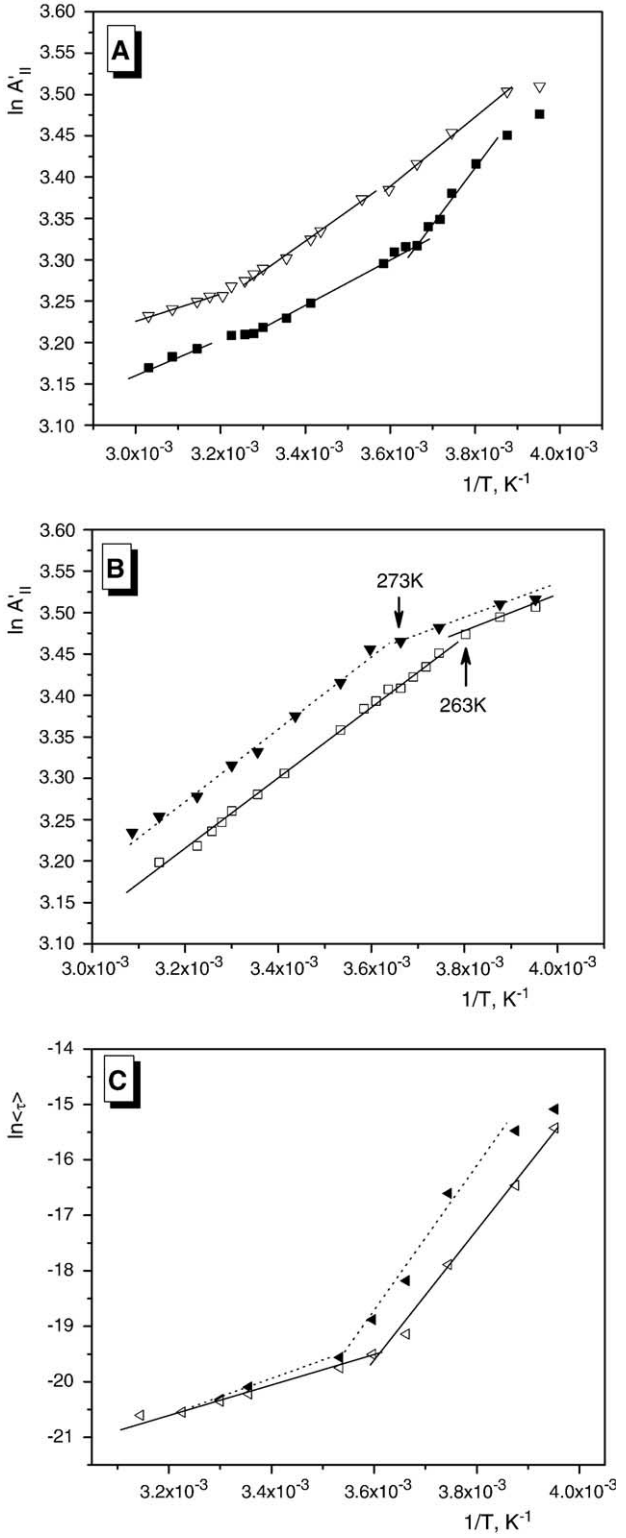

Fig. 7. (A) Temperature dependence of A'_{II} measured on ESR spectra of 5-DSA in EPC (■) and in EPC/CHOL (▽) liposomes. (B) Temperature dependence of A'_{II} for 5-DSA in mixed dispersions containing EPC/CDU 35 mol% (□) and EPC/CHOL/CDU 30 mol% (▼). The lipid concentration in all samples was 5 mM. (C) Variation of ln<τ> of 5-DSA with $1/T$ in pure EPC/CHOL liposomes (◁) and in EPC/CHOL dispersions containing CDU 30 mol% (◀).

became more packed in the presence of CDU. This effect was stronger in EPC liposomes than in EPC/CHOL liposomes, where it occurred at lower CDU concentration and it was apparent in the gel phase, where the area available for each polar head was lower. The fluidity changes observed in our experiments was a result of a higher ordering due to the constraints exerted by the carboranyl nucleosides and an increase in the rotational correlation time which has the same origin.

3.2.2. 7-DSA spin probe

To get further insight into the dynamic changes induced in the lipid backbone by the carboranyl derivative, 7-DSA was used (Chart 2). In this case, the doxyl ring was two carbon atoms deeper inside the liposome than with 5-doxyl ring. Thus, according to the fluidity map along the hydrocarbon chain of the lipid molecules, a slightly higher fluidity could be expected in pure EPC liposomes [52].

The temperature profiles of 7-DSA A'_{II} in pure EPC liposomes as compared with A'_{II} values obtained from 5-DSA in the same dispersion are shown in Fig. 8. In EPC/CHOL liposomes, the same trend was also observed, although the A'_{II} values were higher than those calculated in EPC liposomes, according with the considerations made for 5-DSA. There is a remarkable feature in this figure: the phase transition, indicated by a clear change of the slope of the ln A'_{II} values from 5-DSA in EPC bilayer, was not so clear when 7-DSA was inserted in the same liposome dispersions.

It is noteworthy to observe that the molecular mobility sensed by 7-DSA in the EPC bilayer was higher above 307 K ($1/T = 3.26 \times 10^{-3}$ K^{-1}) than the mobility sensed by 5-DSA even if the slope of the two trends was the same. Below the main phase transition temperature ($1/T = 3.66 \times 10^{-3}$ K^{-1}), the mobility monitored by 7-DSA was slightly lower (if not the same) than that one sensed by 5-DSA. As contrasted, in the temperatures range 273 K to 307 K the behavior of A'_{II}

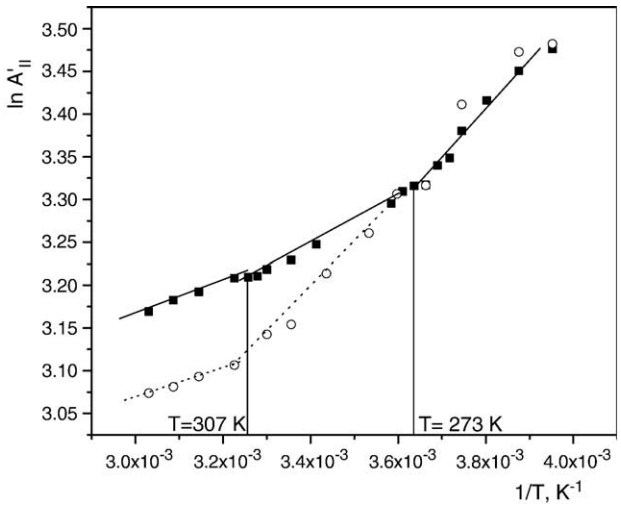

Fig. 8. Variation of ln A'_{II} of 7-DSA in EPC (○) as compared with the values obtained from the spectra of 5-DSA in the same liposomal dispersion (■).

for the two radicals was completely different. The temperature curves of the two kinds of liposomes converged at the main phase transition temperature. This finding was interpreted considering that the two spin-probes 5- and 7-DSA monitored bilayer domains with different viscous processes and different activation energies at temperature between 273 and 307 K. This was an expected result if we considered that 7-DSA is located in region with higher mobility and higher temperature dependence.

When the 310K spectra of 5 and 7-DSA in EPC and EPC/CHOL liposome dispersions were compared, 7-DSA sensed a change in the lipid mobility stronger in EPC bilayer than in EPC/CHOL liposomes, where the difference between the A'_{II} values of 5- and 7-DSA was rather small (Fig. 9).

The A'_{II} values of 7-DSA, as a function of $1/T$, in pure EPC liposomes and in the same liposome dispersion containing 35 mol% of CDU are displayed in Fig. 10. The same behavior was observed by EPC/CHOL in the presence and absence of CDU. As previously observed for 5-DSA in the examined temperature range, A'_{II} values measured in the spectra of loaded liposomes were significantly larger than those for pure systems in both gel and liquid phases. The values of A'_{II} either in the absence or in the presence of CDU converged as previously observed for 5-DSA. However, the temperature at which the A'_{II} overlapped for 7-DSA was appreciably higher.

3.2.3. 16-DSA spin probe

The ESR spectra of 16-DSA in plain EPC liposomes and in CDU-loaded (35 mol%) liposomes are reported in Fig. 11. Above 310 K, the 16-DSA spectra either in plain EPC liposomes or in CDU-loaded EPC closely resembled the usual three-line derivative spectrum of a spin label in almost fast motion as it typically happens when the paramagnetic unit is located in depth into the mobile network of lipid

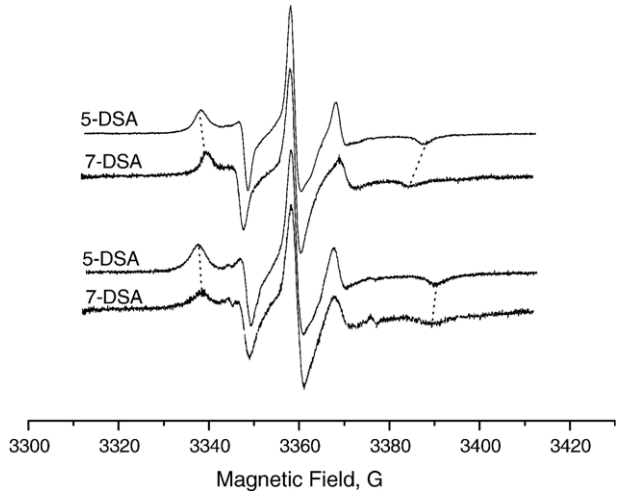

Fig. 9. ESR spectra at 310 K of 5- and 7-DSA in pure EPC liposomes (upper spectra) and in EPC/CHOL (55–45 mol%) liposomes (lower spectra); [lipid]=5 mM.

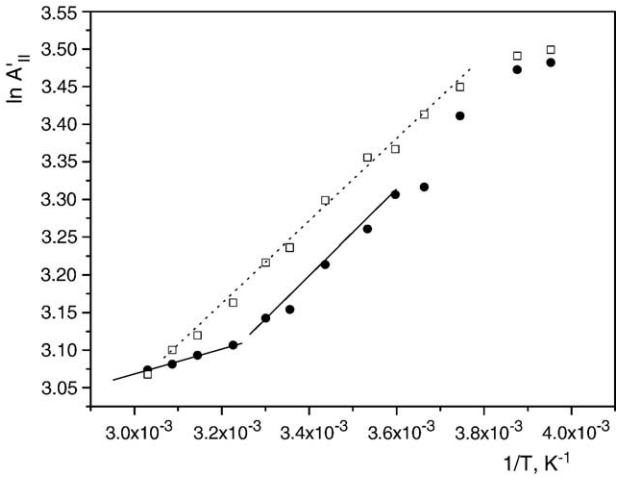

Fig. 10. Dependence of ln A'_{II}, of 7-DSA in pure EPC liposomes (●) and in loaded EPC (35 mol% CDU) liposomes (□) on the reciprocal of temperature. [EPC]=5 mM, [7-DSA]=5×10^{-2} mM.

chains. At temperature ≤267 K, the ESR spectrum was clearly dominated by species in slow motion conditions. In the temperature range 267 K to 298 K, the spectra were attributed to radicals in an intermediate motional region that led to complex line shapes.

In particular, the line shape of these spectra consisted of two component with different motional properties: (i) a larger anisotropic component and (ii) a narrowed isotropic component, which appeared at about 267 K and disappeared above 298 K, indicated by the arrow in Fig. 11. This second signal was due to radicals free to move in the liquid system.

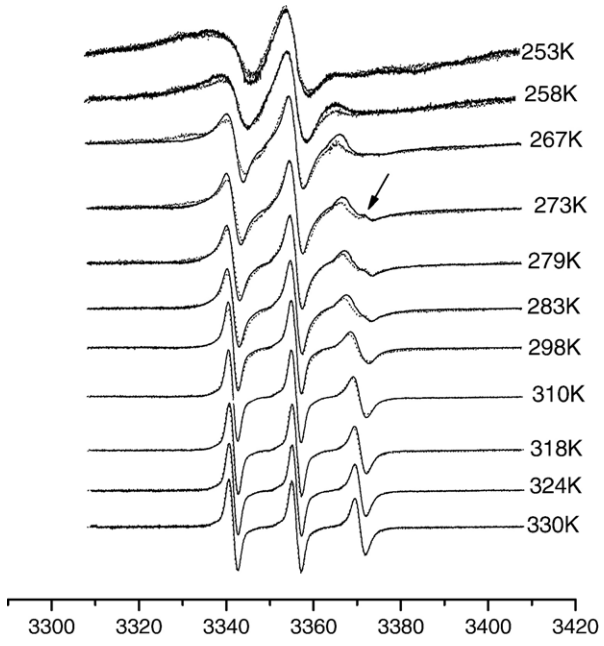

Fig. 11. ESR spectra at different temperatures of 16-DSA in EPC liposomes (solid lines) and CDU-loaded EPC liposomes (CDU=35 mol%; dotted lines). The arrow indicates the sharp peak in the spectra from fast-moving 16-DSA in a different domain. In all of the samples, the concentration of spin probes was 5×10^{-2} mM ([EPC]=5 mM).

This finding was in agreement with other cases reported in literature [37,46].

A further observation regarded the intensity of the spectra which decreased significantly at higher temperature and this finding was explained as it was done with 5- and 7-DSA spectra.

4. Conclusions

We have reported a set of ESR experimental data that allow to characterize EPC and EPC/Cholesterol liposomes membrane loaded with the highly hydrophobic drug CDU. Results show evidences that the carboranyl nucleoside interact with the closest environment of the polar head group of EPC bilayer. CDU modulating effect on both the degree of packing and motional features of lipid phase in EPC unilamellar liposomes. In liquid crystal EPC bilayer, CDU was found to decrease the reorientational motion of lipid chains and slightly increase alkyl chains order at regions monitored by 5- and 7-DSA. Ordering effect of CDU at the C5-position decreases very quickly with increasing the temperature. More marked difference in segmental lipid chain motion and order degree is clearly evident for temperatures below the main phase transition. Spin labels incorporated in 5 mM EPC/Cholesterol indicate that the bilayer has lost a well-defined gel-to-liquid crystalline phase transition. The occurrence of cholesterol in EPC membrane induces dynamic changes by an increased rigidity of the lipid chains in a similar way as CDU molecules produces in EPC liposomes. The simultaneous occurrence of the carboranyl nucleoside and cholesterol avoids the formation of aggregates at concentrations above 30 mol%, whereas for EPC liposomes 43 mol% of CDU has been reached. CDU produces the same dynamic effect into the lipid bilayer of EPC/cholesterol membrane as compared to EPC liposomes, even though this effect is less pronounced.

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References

[1] M.F. Hawthorne, New horizons for therapy based on the boron neutron capture reaction, Mol. Med. Today 4 (1998) 174–181.
[2] B. Larsson, I. Crawford, Advances in Neutron Capture Therapy, vol. 1, Elsevier, Amsterdam, 1997.
[3] R.F. Bart, A critical assessment of boron neutron capture therapy: an overview, J. Neuro-Oncol. 62 (2003) 1–5.
[4] T. LaHann, D.R. Lu, G. Daniell, C. Sills, S. Craft, P. Gavin, W.F. Bauer, Advances in Neutron Capture Therapy, Plenum Press, New York, 1993.
[5] T. Hartman, J. Carlsson, Radiation dose heterogeneity in receptor and antigen mediated boron neutron capture therapy, Radiother. Oncol. 31 (1994) 61–75.
[6] D. Gabel, S. Foster, R. Fairchild, The Monte Carlo simulation of the biological effect of the 10B(n, α)7Li reaction in cells and tissue and its implication for boron neutron capture therapy, Radiat. Res. 111 (1987) 14–25.
[7] T. Kobayashi, K. Kanda, Analytical calculation of boron-10 dosage in cell nucleus for neutron capture therapy, Radiat. Res. 91 (1982) 77–94.
[8] V.I. Bregadze, Dicarba-closo-dodecaboranes $C_2B_{10}H_{12}$ and their derivatives, Chem. Rev. 92 (1992) 209–223.
[9] J.F. Valliant, K.J. Guenther, A.S. King, P. Morel, P. Schaffer, O.O. Sogbein, K.A. Stephenson, The medicinal chemistry of carboranes, Coord. Chem. Rev. 232 (2002) 173–230.
[10] A.H. Soloway, W. Tjarks, B.A. Barnum, F.-G. Rong, R.F. Barth, I.M. Codogni, J.G. Winson, The chemistry of neutron capture therapy, Chem. Rev. 98 (1998) 1515–1562.
[11] Z.J. Lesnikowsky, J. Shi, R.F. Schinazi, Nucleic acids and nucleosides containing carboranes, J. Organomet. Chem. 581 (1999) 156–169.
[12] A.H. Soloway, J.-C. Zhuo, F.-G. Rong, A.J. Lunato, D.H. Ives, R.F. Barth, A.K.M. Anisuzzaman, C.D. Barth, B.A. Barnum, Identification, development, synthesis and evaluation of boron-containing nucleosides for neutron capture therapy, J. Organomet. Chem. 581 (1999) 150–155.
[13] W. Tjarks, The use of boron clusters in the rational design of boronated nucleosides for neutron capture therapy of cancer, J. Organomet. Chem. 614 (2000) 37–47.
[14] R.F. Schinazi, N.M. Goudgaon, G. Fulcrand, Y. El Kattan, Z. Lesnikowsky, G. Ullas, J. Moravek, D.C. Liotta, Cellular pharmacology and biological activity of 5-carboranyl-2′-deoxyuridine, Int. J. Radiat. Oncol. Biol. Phys. 28 (1994) 1113–1120.
[15] N.S. Mourier, A. Eleuteri, S.J. Hurwitz, P.M. Tharnish, R.F. Schinazi, Enantioselective synthesis and biological evaluation of 5-o-carboranyl pyrimidine nucleosides, Bioorg. Med. Chem. 7 (1999) 2759–2766.
[16] R.F. Schinazi, S.J. Hurwitz, I. Liberman, A.S. Juodawlkis, P. Tharnish, J. Shi, D.C. Liotta, J.A. Coderre, J. Olson, Treatment of isografted 9L rat brain tumors with β-5-o-carboranyl-2′-deoxyuridine neutron capture therapy, Clin. Cancer Res. 6 (2000) 725–730.
[17] I. Gay, D.R. Lorey, R.F. Schinazi, G.H. Morrison, S. Chandra, Dynamic SIMS ion microscopy imaging of intracellular boron accumulation from carboranyl nucleosides in glioma cells, Anticancer Res. 21 (2001) 2369–2375.
[18] K. Shelly, D.A. Feakes, M.F. Hawthorne, P.G. Schmidt, T.A. Krish, W.F. Bauer, Model studies directed toward the Boron Neutron Capture Therapy of cancer-boron delivery to murine tumors with liposomes, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 9039–9043.
[19] D.A. Feakes, K. Shelly, C.B. Knobler, M.F. Hawthorne, $NA_3[B_{20}H_{17}NH_3]$—Synthesis and liposomal delivery to murine tumors, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 3029–3033.
[20] D.A. Feakes, K. Shelly, M.F. Hawthorne, Selective boron delivery to murine tumors by lipophilic species incorporated in the membranes of unilamellar liposomes, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1367–1370.
[21] S.C. Mehta, J.C.K. Lai, D.R. Lu, Liposomal formulations containing sodium mercaptoundecahydrododecaborate (BSH) for boron neutron capture therapy, J. Microencapsul. 13 (1996) 269–279.
[22] M.F. Hawthorne, K. Shelly, Liposomes as drug delivery vehicles for boron agents, J. Neuro-Oncol. 33 (1997) 53–58.
[23] A.M. Moraes, M.H.A. Santana, R.G. Carbonell, Preparation and characterization of liposomal systems entrapping the boronated compound o-carboranylpropylamine, J. Microencapsul. 16 (1999) 647–664.

[24] F. Pavanetto, P. Perugini, I. Genta, C. Minoia, A. Ronchi, U. Prati, L. Roveda, R. Nano, Boron-loaded liposomes in the treatment of hepatic metastases: preliminary investigation by autoradiography analysis, Drug Deliv. 7 (2000) 97–103.
[25] M. Johansson, N. Bergstrand, K. Edwards, Optimization of drug loading procedures and characterization of liposomal formulations of two novel agents intended for boron neutron capture therapy (BNCT), J. Liposome Res. 9 (1999) 53–79.
[26] A. Donati, S. Ristori, C. Bonechi, L. Panza, G. Martini, C. Rossi, Evidences of strong C—H···O bond in an o-carboranyl-lactoside in solution, J. Am. Chem. Soc. 124 (2002) 8778–8779.
[27] S. Morandi, S. Ristori, D. Berti, L. Panza, A. Becciolini, G. Martini, Association of sugar-based carboranes with cationic liposomes: an electron spin resonance and light scattering study, Biochim. Biophys. Acta 1664 (2004) 53–63.
[28] G. Martini, S. Morandi, S. Rossi, S. Ristori, Characterization of carriers for drug delivery in boron neutron capture therapy. Electron spin resonance study of nitroxide-containing liposomes, Prog. Colloids Surf. Sci. 126 (2004) 146–150.
[29] S. Martini, S. Ristori, A. Pucci, C. Bonechi, A. Donati, A. Becciolini, G. Martini, C. Rossi, Boronphenylalanine insertion in cationic liposomes for boron neutron capture therapy, Biophys. Chem. 111 (2004) 27–34.
[30] K. Edwards, M. Johnsson, G. Karlsson, M. Silvander, Effect of polyethyleneglycol-phospholipids on aggregate structure in preparations of small unilamellar liposomes, Biophys. J. 73 (1997) 258–266.
[31] N. Bergstrand, K. Edwards, Aggregate structure in dilute aqueous dispersions of phospholipids, fatty acids, and lysophospholipids, Langmuir 17 (2001) 3245–3253.
[32] S. Rossi, G. Karlsson, G. Martini, K. Edwards, Combined cryogenic transmission electron microscopy and electron spin resonance studies of egg phosphatidylcholine liposomes loaded with a carboranyl compound intended for boron neutron capture therapy, Langmuir 19 (2003) 5608–5617.
[33] D. Marsh, D. Kurad, V.A. Livshits, High-field electron spin resonance of spin labels in membranes, Chem. Phys. Lipids 116 (2002) 93–114.
[34] L.J. Korstanje, E.E. van Faassen, Y.K. Levine, Reorientational dynamics in lipid vesicles and liposomes studied with ESR: effects of hydration, curvature and unsaturation, Biochim. Biophys. Acta 982 (1989) 196–204.
[35] Z.-C. Liang, P.-O. Westlund, G. Wikander, G. Lindbloem, A quantitative electron-spin-resonance line-shape study of the order–disorder transition in the lamellar phase of the palmitoyllysophosphatidylcholine-water system, Mol. Phys. 85 (1995) 757–767.
[36] J. Strancar, M. Sentjurc, M. Schara, Fast and accurate characterization of biological membranes by EPR spectral simulations of nitroxides, J. Magn. Reson. 142 (2000) 254–265.
[37] I. Perissi, S. Ristori, S. Rossi, L. Dei, G. Martini, Electron spin resonance and differential scanning calorimetry as combined tools for the study of liposomes in the presence of long chain nitroxides, J. Phys. Chem., B 106 (2002) 10468–10473.
[38] D. Marsh, Membrane Spectroscopy, Springer, Berlin, 1981.
[39] G. Wikander, P.O. Ericksson, E.E. Burnell, G. Lindbloem, ESR line shapes in lyotropic systems: the micellar and liquid-crystalline phases of the dodecyltrimethylammonium chloride/water system, J. Phys. Chem. 94 (1990) 5964–5972.
[40] E. Meirovitch, D. Igner, G. Moro, J.H. Freed, Electron-spin relaxation and ordering in smectic and supercooled nematic liquid crystals, J. Chem. Phys. 77 (1982) 3915–3938.
[41] D.J. Schneider, J.H. Freed, in: L.J. Berliner (Ed.), Biological Magnetic Resonance. Spin Labeling. Theory and Applications, vol. 8, 1989, (New York).
[42] D.E. Budil, S. Lee, S. Saxena, J.H. Freed, Nonlinear-least-squares analysis of slow-motion EPR spectra in one and two dimensions using a modified Levenberg–Marquardt algorithm, J. Magn. Reson. 120 (1996) 155–189.
[43] V.R. Jarugula, R.F. Schinazi, G. Fulcrand, Y.E. Kattan, D.C. Liotta, F.D. Boudinot, Pharmacokinetics of 5-carboranyl-2′-deoxyuridine in rats, J. Pharm. Sci. 83 (1994) 1697–1699.
[44] L. Ciani, S. Ristori, A. Salvati, L. Calamai, G. Martini, DOTAP/DOPE and DC-Chol/DOPE lipoplexes for gene delivery: zeta potential measurements and electron spin resonance spectra, Biochim. Biophys. Acta 1664 (2004) 70–79.
[45] M. Ge, J.H. Freed, Electron-spin resonance study of aggregation of gramicidin in dipalmitoylphosphatidylcholine bilayers and hydrophobic mismatch, Biophys. J. 76 (1999) 264–280.
[46] M.F. Ottaviani, P. Baglioni, G. Martini, Micellar solutions of sulfate surfactants studied by electron spin resonance of nitroxide radicals: 1. Use of neutral and positively charged spin probes, J. Phys. Chem. 87 (1983) 3146–3153.
[47] H. Caldararu, Structural aspects in self-assembled systems of polyoxyethylene surfactants, as studied by the spin-probe technique source, Spectrochim. Acta, Part A: Mol. Biomol. Spectrosc. 54 (1998) 2309–2336.
[48] M.P. Veiga, J.L.R. Arrondo, F.M. Goñi, A. Alonso, D. Marsh, Interaction of cholesterol with sphingomyelin in mixed membranes containing phosphatidylcholine, studied by spin-label ESR and IR spectroscopies. A possible stabilization of gel-phase sphingolipid domains by cholesterol, Biochemistry 40 (2001) 2614–2622.
[49] K. Tajima, Y. Imai, T. Horiuchi, M. Koshinuma, A. Nakamura, ESR study on DMPC and DMPG bilayers in the ($L\alpha+H_2O$) phase, Langmuir 12 (1996) 6651–6658.
[50] R.A. Demel, B. Dekruyff, The function of sterols in membranes, Biochim. Biophys. Acta 457 (1976) 109–132.
[51] Y. Lou, M. Ge, J.H. Freed, A multifrequency ESR study of the complex dynamics of membranes, J. Phys. Chem., B 105 (2001) 11053–11056.
[52] P. Hoffmann, K. Sandhoff, D. Marsh, Comparative dynamics and location of chain spin-labelled sphingomyelin and phosphatidylcholine in dimyristoyl phosphatidylcholine membranes studied by EPR spectroscopy, Biochim. Biophys. Acta 1468 (2000) 359–366.