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Quantification of Randomly-methylated-β-cyclodextrin effect on liposome: An ESR study

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ABSTRACT

In the present work, the effect of Randomly-methylated-β-cyclodextrin (Rameb) on the microviscosity of dimyristoyl-L-α phosphatidylcholine (DMPC) bilayer was investigated using the electron spin resonance (ESR) technique. The ability of Rameb to extract membrane cholesterol was demonstrated. For the first time, the percentage of cholesterol extracted by Rameb from cholesterol doped DMPC bilayer was monitored and quantified throughout a wide Rameb concentration range. The effect of cholesterol on the inner part of the membrane was also investigated using 16-doxyl stearic acid spin label (16-DSA). 16-DSA seems to explore two different membrane domains and report their respective microviscosities. ESR experiments also establish that the presence of 30% of cholesterol in DMPC liposomes suppresses the jump in membrane fluidity at lipids phase-transition temperature (23.9 °C).

Introduction

Cyclodextrins (CDs) have a large field of applications including in cosmetic, food and pharmaceutical industry [1]. CDs are formed by six, seven or eight D-glucopyranose units named, respectively, as α-, β- and γ-cyclodextrin. These molecules are a truncated cone constituted by an internal hydrophobic cavity and an external hydrophilic surface. This particular structure confers on the CDs the ability to include a large number of organic molecules [2]. Methylated β-CDs are also well known for cholesterol and lipids extraction from both cell and monolayer membranes [2,3]. Moreover, Randomly-methylated-β-cyclodextrins (Rameb) are also known for their ability to penetrate through skin [4] and nasal mucosa [3].

Lipid vesicles such as liposomes are now routinely used not only as a good cell membrane model [5], but also as a medical drug vector [6,7]. Recently, CDs have been described as a drug release modulator within liposomes [3]. The understanding of the mechanism of interaction between CDs and classical liposomes or liposomes doped with cholesterol would improve the potentiality and the efficiency of CDs in this new field of pharmaceutical applications.

Previous and recent researches have focused on the mechanism of action of the CD on liposomes and on the influence of the CD on physical stability as well as on the integrity of the vesicle bilayer [2,8–11].

Some teams have also studied the mechanism of cholesterol removal by CD by means of a liposomial stability kinetic analysis [11,12]. However, despite a wide range of methods explored, none, to the best of our knowledge, has been able to quantify precisely and in a noninvasive way the percentage of cholesterol extracted by the CD. Electron spin resonance (ESR) is not commonly used in the study of cyclodextrin. However this is a highly efficient technique able to give information concerning the structure and the dynamics of biological systems in a non-destructive way. Spin labelling fits well in with the study of membranes and particularly with the changes in lipid bilayer organisation induced by drug [13]. Nitroxide labelled fatty acid probes (n-DSA) can be useful for determining the order degree of the probe surrounding. Absolute values of microviscosity have been obtained after prior calibration of the ESR spectra of nitroxide probes in solvent mixture of known microviscosity [14]. Microviscosity is then defined as the homogenous solution viscosity, which results in the same spectrum as that in the microenvironment [14]. In this work, we will show that ESR is a noninvasive, easy, rapid and reproducible method to quantify accurately the damage caused by cyclodextrin on liposomes doped in cholesterol.

In the present work, we have investigated the CD action in well-defined liposomes systems composed of only one sort of phospholipid and doped with a well-known percentage of cholesterol.

Materials and methods

Preparation of liposomes labelled with n-DSA. Stearic acid derivatives (Aldrich, USA) labelled by stable doxyl radical ring at the C-5

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(5-DSA) or at the C-16 (16-DSA) position (counted from the carboxylic group of the stearic acid derivative) were used to study the membrane properties. The location of the spin labels (n-DSA) in liposomes is well known and it is established that 5-DSA explores the polar part of liposome while 16-DSA explores the hydrophobic core [15].

The n-DSA were added to a chloroform solution (Merck, Germany) of 5 mg/ml dimyristoyl-L-α phosphatidylcholine (DMPC) (Sigma, USA) which corresponded to a total lipid concentration of 7.4 mM. The probes were also added to a chloroform solution of a DMPC:Cholesterol (Merck, Germany) mixture (i.e. 70:30 mol%). The mixture consisted of a total lipid cholesterol concentration of 7.4 mM. The mixture was stirred for 5 min and the solvent was evaporated under vacuum. The formed lipid film was suspended in a phosphate buffer solution pH 7.0 (1/15 M; Na_2HPO_4–KH_2PO_4, from Riedel-de Haën, Belgium) at a temperature above the DMPC phase-transition temperature (23.9 °C), and stirred by vortex mixer in order to obtain large multilamellar vesicles (MLV) [13]. After this hydratation, five freeze–thaw cycles using liquid nitrogen were carried out to allow a better incorporation of the labelled stearic acid or cholesterol into the liposomes phospholipidic bilayers. The MLV suspensions were transferred into an extruder (Lipex Biomembrane, Canada), in which the unilamellar liposomes were formed by passing the suspensions through polycarbonate filters (0.1 μm pore size, Nucleopore, CA), under a pressure up to 6895 Pa of nitrogen. The procedure was repeated ten times at a temperature above the phase transition temperature of the phospholipids and it resulted in unilamellar liposomes with a mean size of about 90 nm and a very low polydispersity [16]. Randomly-methylated-β-cyclodextrins (Rameb) kindly donated by Dr. G. Piel (Laboratory of pharmaceutical technology, Belgium) were dissolved in PBS solution and sterilized using an Acrodisc syringe filter of 22 mm (VWR, Belgium) in order to obtain a 35 mM stock solution. Liposomes were then incubated with Rameb at the desired concentration during 5 minutes.

Liposome preparation for microviscosity measurements. As the addition of beta-cyclodextrins to membranes is able to expulse phospholipids [3,8,9], and also n-DSA out of the membrane (see Results), a special protocol was used to measure liposomes microviscosity: liposomes without n-DSA were prepared as described above and then incubated with the desired Rameb concentration for one hour at 37 °C. In order to remove the residual Rameb, liposomes solutions were ultra-centrifuged three times for two hours at 120000*g*. The resulting pellet was suspended in the buffer solution and incubated with 10^{-4} M of DSA during 5 minutes.

Preparation of liposomes labelled with cholestane. 3β-Doxyl-5α-cholestane (cholestane) (Sigma, USA) solubilized in chloroform was used as a probe to investigate liposomes membranes. Liposomes doped with 30% of cholesterol and not labeled with n-DSA were prepared as described above. Once created, the vesicles were incubated for 20 minutes with 0.1 mM of cholestane. The suspension was then incubated with Rameb in the desired concentration during 5 minutes.

ESR experimental conditions. All ESR experiments were performed at 9.5 GHz using a Bruker ESR 300E spectrometer (Bruker, Germany) equipped with a variable temperature controller accessory and operating at a centre field strength of 3360 G with 120 G as scan range, a modulation amplitude of 2.0 G and 20 mW microwave power. These parameters were used for all the liposomes containing different concentrations of cholesterol.

ESR spectra simulation. The modelization of ESR n-DSA spectra was carried out according to the method presented by Arzov et al. [17,18] while experimentally acquired spectra were simulated using the software EPRSIM (version 4.99 2005) based on experimental data. The spectrum-simulation model assumes that each spectrum can be a superimposition of the spectral components that identify the membrane heterogeneity. Each component reflects the properties of a particular type domain which is highlighted by the values of the chosen parameter set. The set corresponds with order parameter *S*, effective rotational correlation time τ_c, hyperfine and Zeeman tensor's polarity correction factors and weighting factors *w*. EPRSIM allowed to decompose a spectrum into at most five different spectral components.

Microviscosity determination. The simulated spectra were used to obtain τ_c or *S*. These two values quantify the mobility of n-DSA in the explored medium (liposomes) [19]. They are expressed in function of the anisotropy of the microenvironment. In the case of weakly to moderately immobilized probes ($\tau_c < 3 \times 10^{-9}$ s), τ_c is used, whereas, for strongly immobilized ones ($\tau_c > 3 \times 10^{-9}$ s) *S* is better suited [19]. Recently we have established standard curves of microviscosity versus correlation time or order parameter that enable to convert measured parameters into membrane microviscosity [14]. Each measure was repeated at least four times and standard deviation of the microviscosity was calculated to be 4%.

Results and discussion

The n-doxyl stearic acid spin labels (n-DSA) have been used as probes to investigate the membrane fluidity of liposomes at different depths at 25 °C. ESR spectra were collected for both 5-DSA and 16-DSA probes. The first step was the evaluation of microviscosity changes induced by the incorporation of cholesterol in DMPC unilamellar liposomes.

Cholesterol effects on the hydrophilic part of the membrane

Local microviscosities of liposome membranes measured near the polar head group (5-DSA) were calculated using the *S* parameter. Fig. 1A shows the increase of the DMPC bilayer microviscosity induced by the addition of cholesterol at varying percentages. These results highlight the idea that cholesterol can act as a bulky group and that it reduces the mobility of phospholipids and DSA head groups, which consequently reduces the local fluidity. This finding is in agreement with previous works showing that cholesterol condenses the membrane above the transition temperature [13].

The highest condensing effect of cholesterol on the membrane is observed for liposomes dopped with 30% of cholesterol. Therefore, we used that value to assess the role of cholesterol on the

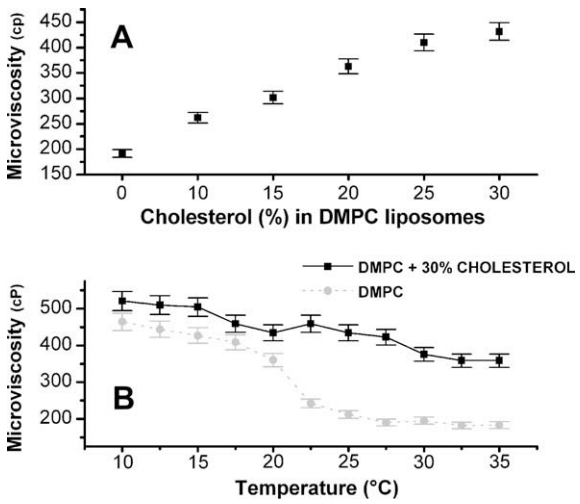

Fig. 1. (A) Modification of microviscosity of liposomes bilayer in function of their cholesterol content. (B) Dotted line: influence of temperature on the microviscosity of DMPC liposomes. Full line: Influence of temperature on the microviscosity of (70:30 mol%) liposomes.

microviscosity variation of liposomes related to the temperature. Fig. 1B shows both microviscosities explored by 5-DSA of DMPC bilayer doped or not with cholesterol in function of temperature. The microviscosity of (70:30 mol%) bilayer revealed a tidy linear decrease (Fig. 2. solid line) in function of temperature. However, no real microviscosity change was observed. On the contrary, for the DMPC liposomes, an abrupt decrease of microviscosity was observed near the gel-to-fluid transition temperature (23.9 °C) (Fig. 1B, doted line). When the temperature ($T > 25$ °C) raised over the transition temperature, the DMPC bilayer reached a lower and constant microviscosity value that is about 200 cP (Fig. 1B, doted line). These ESR experiments confirm that the presence of 30% of cholesterol in DMPC liposomes abolishes the endothermic transition of DMPC. These findings are in accordance with the fluorescent study of V.G. Bieri et al. [20].

Cholesterol effects on the hydrophobic part of the membrane

The effect of cholesterol on the inner part of the liposome membrane was also investigated using 16-DSA at 24 °C. Thanks to its nitroxide radical brought on by the doxyl stearic acid chain at the sixteenth carbon position, 16-DSA explores the hydrophobic part of the bilayer [13]. ESR spectra of 16-DSA incorporated inside liposomes were simulated.

Fig. 2A (a) (black line) shows the 16-DSA experimental spectrum inside DMPC liposomes doped with 10% of cholesterol. Superimposed in gray on Fig. 2A (a), we set the best fit to the experimental spectrum. To obtain the best simulation, we were obliged to consider that 16-DSA was in two different areas. Fig. 2A (b1 and b2) show the spectra of the two components of the simulation. Each component corresponds to domains which have two distinct microviscosities. The existence of these two microdomains explored by the 16-DSA remains valid whatever the percentage of cholesterol inside the lipid bilayer is (even 0%) (Fig. 2B). ESR results confirm that 16-DSA explores mainly the hydrophobic part of the bilayer but has a non-zero probability of being located in the bilayer polar part. Indeed it has been shown that the 16-DSA probe is oriented and linked like the lipids in the membrane and is flexible in DMPC liposomial membrane. This flexibility leads to a change in the radical position: the fatty acid chain tilts and carries the radical nitroxide closer to the polar head group [21,22].

Fig. 2B shows the evolution of the microviscosities of the two microdomains varies in function of the increase of the percentage of cholesterol (from 0% to 30%) in DMPC liposomes. At 25 °C, the microviscosities sensed by 16-DSA in these two microdomains varies differently in function of the cholesterol incorporation. In the first microdomain, microviscosity increases from 27.6 to 198 cP. Inversely, for the second microdomain microviscosity decreases from 73.8 to 16.7 cP. A possible explanation could be that the more cholesterol is embedded in the membrane the closer the probe (which is in the "tilt" mode) is of the bulky cholesterol steroid and the more it perceives a stiffening of the membrane. That leads to an increase of microviscosity as for the 5-DSA. We can assume that cholesterol condenses the top of the membrane where steroid rings take place and lets more freedom to the end of the phospholipidic carbon chain which is very flexible [23]. This could be an explanation of the second microviscosity of the nitroxide which is positioned deeper into the membrane and so could sense its microviscosity lessening due to the increase of carbon chains disorder.

From Fig. 2B we can also notice that the microviscosity of each domain explored by 16-DSA is always lower than the microviscosity near the polar head group region explored by 5-DSA. This observation is in accordance with the fact that even if 16-DSA is able to tilt, the nitroxide always remains positioned deeper in the membrane. It concords with the fact that 16-DSA gives information about the hydrophobic part of the membrane which is more fluid than the hydrophilic part [24]. To summarize, 16-DSA is a flexible probe able to give information on a microdomain positioned deep in the membrane (extended probe) but also on a microdomain close to the polar head group (bended probe). This last microdomain is still deeper than the microdomain explored by the 5-DSA. Both microdomains are sensitive to the cholesterol presence in the membrane. The complex analysis of the microviscosities explored by the 16-DSA probe will not allow an interpretation of the Rameb action on liposomes as easily as for the 5-DSA probe.

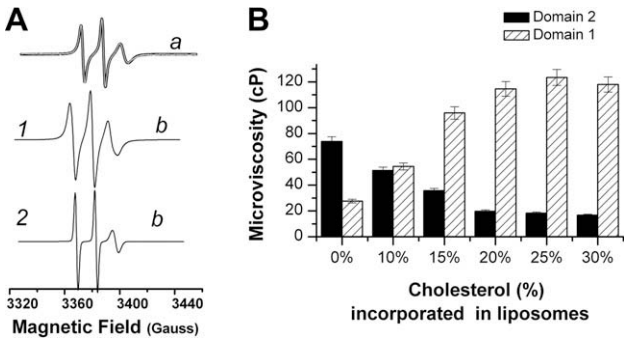

Fig. 2. (A) Gray: Typical ESR spectrum of 16-DSA in the bilayer of (90:10 mol%) liposomes. Black line: experimental spectrum. Gray line: the best fit to the experimental spectrum, taking into account the superimposition of spectra of two coexisting domains shown in (b). *b*: computer simulation of the specific spectra of the two coexisting domains: 1; first domain ($S = 0.19$, $\tau = 0.69$ ns, $W = 1.88$), 2; second domain ($S = 0.02$, $\tau = 1.11$ ns, $W = 0.33$). (B) The columns represent the influence of cholesterol on the microviscosity of the two coexisting domains explored by the 16-DSA.

Rameb effects on n-DSA and cholestane probes

Our method relies on the presence of probes in the bilayer. In this section all liposomes were formed with 30% of cholesterol. We were first interested in studying the possible action of the Rameb on n-DSA and cholestane which is a membrane spin label with a chemical structure similar to cholesterol.

The comparison between the spectrum of probes (cholestane and n-DSA) dissolved in PBS and probes solubilized in PBS containing Rameb shows a significant difference especially for the n-DSA (Fig. 3A and Fig. 3C: spectrum 1 and 2). Indeed, the spectrum of probes in PBS containing Rameb is asymmetric and distorted (Fig. 3A: doted line and Fig. 3C: spectrum 2). Our results show that a higher amount of cyclodextrin (from 10 to 50 mM) in the PBS solution induces a higher distortion in the n-DSA spectrum (data not shown). These kinds of spectra are typical of the n-DSA embedded in micelles structures [25].

Fig. 3B (full line) and Fig. 3C (spectrum 5) respectively show the ESR spectrum of 5-DSA and cholestane in lipid bilayer at 25 °C. Both spectra are characteristic of the spin label strongly immobilized in the membranes. The addition of increasing concentrations of Rameb to DMPC liposomes containing probes induces 3-line ESR spectra (Fig. 3B and Fig. 3C: spectrum 3 and 4). The resulting ESR spectrum is similar to those obtained with probes solubilized in PBS containing Rameb. We can also notice that the extraction of cholestane probes from the bilayer increases with Rameb concentration (Fig. 3C: spectrum 3 and 4). Our results show that Rameb is able to extract cholestane and n-DSA initially embedded in the liposomial membrane in a dose-dependant manner. The extracted probes are not free in solution but aggregate with Rameb in a micellar structure. This behaviour is in perfect agreement with the literature data [26].

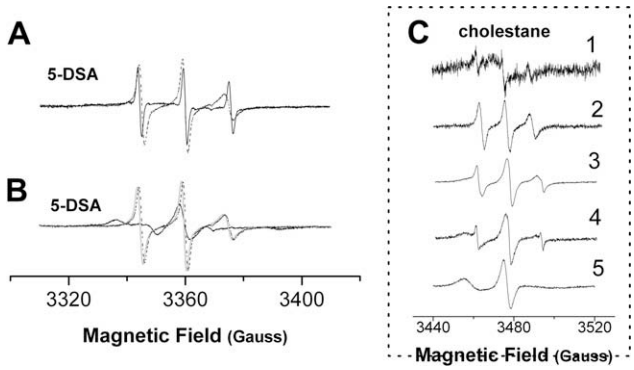

Fig. 3. (A) Full line: typical spectrum of 0.01 M 5-DSA in PBS. Dotted line: spectrum of 0.01 M 5-DSA dissolved in PBS containing 50 mM Rameb. (B) Black line: spectrum of 0.01 M 5-DSA in DMPC liposomes bilayer. Gray line: spectrum of 0.01 M 5-DSA in DMPC liposomes bilayer and in contact with 50 mM Rameb. Black dotted line: spectrum of 0.01 M 5-DSA dissolved in PBS containing 50 mM Rameb. (C) 1; spectrum of 10^{-4} M cholestane in PBS. 2; spectrum of cholestane (10^{-4} M) distilled in PBS containing 2.5 mM of Rameb. 3; ESR spectrum of 10^{-4} M cholestane after preincubation with 30 mM Rameb on liposomes (70:30 mol%). 4; ESR spectrum of 10^{-4} M cholestane after preincubation with 2.5 mM Rameb on liposomes (70:30 mol%). 5; spectrum of 10^{-4} M cholestane incorporated in liposomes (70/30 mol%).

Rameb effects on the microviscosity of the microdomain explored by 5-DSA

As Rameb is able to extract phospholipids [3,8,9] like n-DSA probes from the vesicle, we have first studied the influence of this phospholipidic extraction on the microviscosity. Fig. 4A shows that the addition of Rameb did not modify significantly the microviscosity of DMPC bilayer. Even if we pay attention to this little microviscosity variation, we observe a restriction of the probes movement (corresponding to a microviscosity increase). As Rameb is not known for penetrating into the membrane bilayer [26], the increase of microviscosity can not result from a congestion of the CD at the polar head group. So, the variation could be due to the phospholipids extraction by cyclodextrin leading to a reduction of the vesicle size. This reduction compacts the phospholipids and consequently decreases the movement of probes.

Fig. 4B shows the variation of (70:30 mol%) bilayer microviscosity as a function of Rameb concentration. The addition of Rameb up to 2×10^{-2} M into liposomes at 25 °C did not modify significantly the microviscosity. However, above this concentration range, a clear decrease of bilayer microviscosity was observed. Our results mentioned above show that the microviscosity modification does not result from lipids extraction by Rameb. So, this microviscosity modification observed in Fig. 4B [5] can only result from a cholesterol extraction. Fig. 1A clearly shows that microviscosity is directly correlated with cholesterol concentration in the bilayers. The combined analysis of Fig. 1A with Fig. 4B allows us to estimate the cholesterol remaining inside the bilayers after Rameb effect. After one hour of 0, 30, 40, and 50 mM Rameb exposure, we obtain liposomes containing respectively 30% (2.22 mM), 22% (1.63 mM), 15% (1.11 mM) and 10% (0.74 mM) cholesterol.

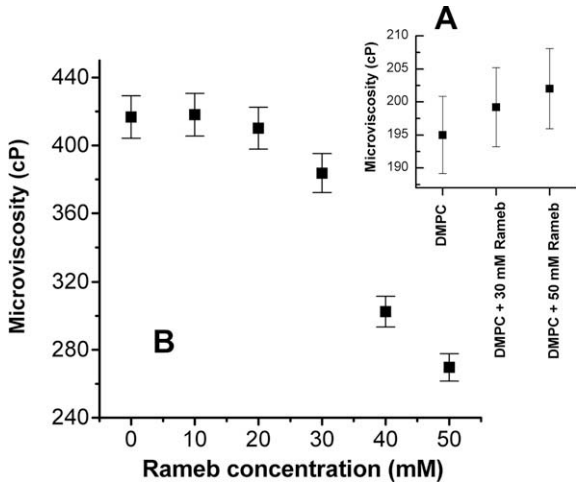

Fig. 4. (A) Influence of Rameb on microviscosity of DMPC liposome membrane. (B) Microviscosity change induced by different Rameb concentration on the membrane of liposomes (70:30 mol%). Vesicles have been in contact with Rameb during 1 h at 37 °C.

Conclusion

In this work, we have confirmed the ability of Rameb to extract cholesterol. Aggregates resulting from this extraction were observed. Moreover, for the first time, our experimental method allows to quantify the amount of cholesterol extracted by Rameb as a function of its added concentration.

These results clearly show that ESR is a reproducible technique which presents the advantage of exploring the membrane bilayer in a non-invasive way. Therefore any drug action able to modify the cholesterol content of a membrane can easily be studied and quantified by this method.

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