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# ESR technique for noninvasive way to quantify cyclodextrins effect on cell membranes

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ABSTRACT

A new way to study the action of cyclodextrin was developed to quantify the damage caused on cell membrane and lipid bilayer. The Electron Spin Resonance (ESR) spectroscopy was used to study the action of Randomly methylated-beta-cyclodextrin (Rameb) on living cells (HCT-116). The relative anisotropy observed in ESR spectrum of nitroxide spin probe (5-DSA and cholestane) is directly related to the rotational mobility of the probe, which can be further correlated with the microviscosity. The use of ESR probes clearly shows a close correlation between cholesterol contained in cells and cellular membrane microviscosity. This study also demonstrates the Rameb ability to extract cholesterol and phospholipids in time- and dose-dependent ways. In addition, ESR spectra enabled to establish that cholesterol is extracted from lipid rafts to form stable aggregates. The present work supports that ESR is an easy, reproducible and non-invasive technique to study the effect of cyclodextrins on cell membranes.



## 1. Introduction

Cyclodextrins (CD) are widely used in the pharmaceutical field and known for their ability to increase the aqueous solubility and the chemical stability of different drugs [1,2]. There are three types of natural CDs which are formed by six, seven or eight D-glucopyranose units denominated, respectively as α-, β- and γ-CD. These molecules look like truncated cones constituted by an inner internal hydrophobic cavity and a hydrophilic surface. Among them, β-CD derivatives (β-CDs) are known to possess the efficacy to make complexes with several drugs [1]. Within this family, the Randomly methylated β-CD derivative (Rameb) has received much attention and has shown a great pharmaceutical interest as it is efficient for its ability to penetrate through nasal mucosa [3] and skin [4].

Therefore, Rameb can readily enter in the composition of different nasal spray (for instance estradiol nasal spray sold as Aerodiol® in Europe). Even if this kind of CDs is one of the most studied and commercialized for its ability to enhance drug delivery, Rameb might still be of a great interest when investigating various membranes.

Numerous works have demonstrated that the plasma membrane contains different domains [5–9], indicating that membranes cannot be defined by only the fluid mosaic model as described by Singer and Nicholson [10]. Recently, it was highlighted that cholesterol distribution in the membrane is heterogeneous and concentrated in cholesterol- and sphingomyelin-rich domains (lipid rafts). These lipid rafts are involved in significant cell function and can be isolated in the form of patches resistant to cold non ionic detergent [5,11].

However, the existence of these domains is still the subject of an intense debate in the scientific community and their morphology, size, density and molecular composition are also under consideration. Known for their cholesterol affinity, the β-CDs like Rameb [3,12] may interact with lipid rafts and could become an interesting tool to understand the function of lipid rafts.

The most common way to quantify the cholesterol depletion due to β-CDs is to make a lipid extraction and use a cholesterol assay kit [13]. In order to obtain the percentage of cholesterol into the membrane rafts which are identified as low density membrane fraction, a separation of these domains is generally realized by a sucrose gradient (with or without detergent extraction) [14]. One of the main disadvantages of the cholesterol extraction is that different amounts of cholesterol are obtained depending on the experimental protocol used to isolate the membrane [14]. Electron Spin Resonance (ESR) is a unique technique able to provide information about the structure and the dynamics of biological systems in a noninvasive way [15]. However, despite this great advantage, no study has been undertaken by ESR to accurately investigate the interaction between β-CDs and membranes. The present work demonstrates that ESR is an easy and reproducible technique to

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quantify the damage caused by the β-CDs and could be a new advantageous tool for a better understanding of these actions on cell membrane.

## 2. Materials and methods

### 2.1. Cell cultures

The human colon carcinoma (HCT-116) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Belgium) supplemented with 10% fetal calf serum (Invitrogen, Belgium), 2 mM of L-glutamine, penicillin (100 U/ml), streptomycin (100 U/ml) (Invitrogen, Belgium) and was maintained in a humidified 37 °C, 5% $CO_2$ incubator. Cells were not used at passages higher than 20.

### 2.2. Exposure of HCT-116 cells to Randomly methylated β-cyclodextrin (Rameb)

Cells from two T175 flasks (90% confluent) were collected by trypsinization, centrifuged for 7 min at 240*g* and suspended in 10 ml of medium (DMEM) containing Randomly methylated β-cyclodextrin (Rameb) which was sterilized by filtration in aseptic conditions at desired concentration. The cells ($15 \times 10^6$) were then incubated at 37 °C for 1 h in a humidified atmosphere with 5% of $CO_2$. Rameb was obtained from Wockes Chemie GmbH (Munich, Germany).

### 2.3. Cell viability

After 1 and 2 h of Rameb incubation (10 mM), as described above, cells were centrifuged for 5 min at 200*g* and the pellet was resuspended in 10 mM of fresh DMEM. The cell viability was then determined using both a Cell Proliferation Reagent WST-1 (Roche, Germany) and the Trypan blue dye exclusion assay.

In order to perform the WST-1 cell viability assay according to the manufacturer instructions [16], the cells ($10^4$ cells by well by 100 μl) were placed in microtiter plates (10 min after 1 h period of incubation). Absorbance of the colored formazan was determined using an automated microplate reader at 450 nm wavelength. The mean absorbance of control wells (cells not incubated with Rameb) represented 100% cell viability. Viability of cells treated with Rameb was measured in triplicate and compared to the absorbance of control cells.

### 2.4. Cell sample preparation for ESR measurements

It is known that addition of β-CDs to membrane models expels phospholipids [3,17,18]. Consequently stearic acid derivative labeled by stable doxyl radical ring (*n*-DSA) is also expelled out of the cell membrane (see results, Fig. 1). This property helped us to define an appropriate protocol in order to measure the microviscosity. The 1 h incubation of cells with Rameb (as described above) was ended by centrifugation for 5 min at 200*g*. After dilution of the cell pellet in 1 ml of sterile phosphate buffer saline solution (PBS), an amount of stearic acid derivative labeled by stable doxyl radical ring at the C-5 level (5-DSA) (Aldrich, USA) was added to achieve $10^{-4}$ M concentration and incubated less than 5 min. A 5-DSA stock solution was first obtained in ethanol ($10^{-2}$ M) and the added 5-DSA solution was less than 1% of total volume. Final solutions were then transferred into a quartz cell (500 μl) for ESR measurements.

3β-Doxyl-5α-cholestane (cholestane) (Sigma, USA) dissolved in chloroform was also used as a probe on HCT-116 membranes. In these experiments, collected and centrifuged cells ($15 \times 10^6$) were suspended in 1 ml of PBS containing $10^{-4}$ M cholestane and incubated for 10 min. The incubation was ended by the addition of sterilized stock Rameb solution ($10^{-1}$ M in PBS) to achieve the final concentration of $2.5 \times 10^{-3}$ M. HCT-116 suspensions were then transferred into a quartz flat ESR cell.

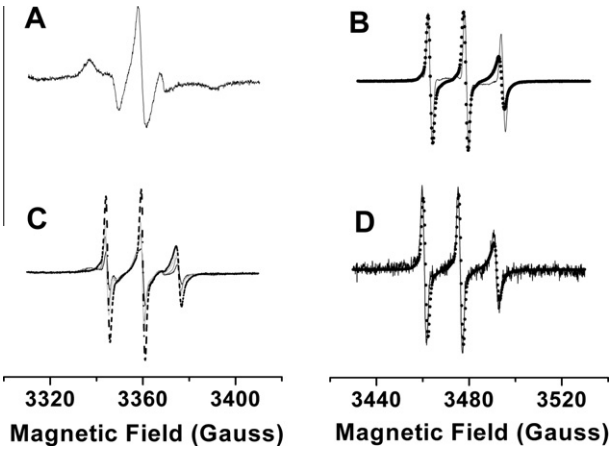


**Fig. 1.** Rameb influence on 5-DSA ESR spectra: (A) 0.001 M 5-DSA in cells membrane. (B) 0.001 M 5-DSA dissolved in PBS (full line) or in PBS containing 10 mM of CD (dotted line). (C) Spectral ESR evolution of 5-DSA initially embedded in cell membranes and incubated with 1.5 mM (black line), 2 mM (gray line), 6 mM (light gray line) and 10 mM of Rameb (dotted line). (D) ESR spectrum of 5-DSA in the supernatant of cells incubated with 10 mM of CD (full line) and 5-DSA solubilized in PBS containing 10 mM of CD.

### 2.5. Liposome preparation

Lipid vesicles were prepared from Dimyristoylphosphatidylcholine (DMPC) (Sigma, USA) and cholesterol (CHOL) (Merck, Germany). Made from DMPC:CHOL (70:30 mol%), the liposomes were prepared by hydratation of lipid films. Briefly, the required amount of lipids (3.51 mg/mL of DMPC and 0.86 mg/mL of CHOL) was dissolved in chloroform. The total lipid cholesterol concentration was 7.4 mM. The mixture was then stirred for 5 min and the solvent was evaporated under vacuum. The resulting lipid film was suspended in PBS pH 7.0 (1/15 M; $Na_2HPO_4$–$KH_2PO_4$, from Riedel-de Haën, Belgium) at 35 °C, and stirred by vortex mixer in order to obtain large multilamellar vesicles (MLV) [19]. 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 (Air Liquid, Belgium). The procedure was repeated 10 times at 35 °C and resulted in unilamellar liposomes [20]. Once the liposomes were made, cholestane (stock solution in chloroform ($10^{-1}$ M)) was added and the final solution ($10^{-4}$ M) was then transferred for ESR experiments.

### 2.6. ESR experimental conditions

All ESR experiments were performed at 9.56 GHz using a Bruker ESR 300E spectrometer (Bruker, Karlshrure-Germany) equipped with a variable temperature controller accessory and operating at center field strength of 3480 G with 100 G as sweep width, modulation amplitude of 2.55 G and 20.2 mW microwave power. The time constant and the conversion time were respectively 2.56 and 163.84 ms. Three scans were acquired for each measurement.

### 2.7. ESR spectra simulation

ESR *n*-DSA modeling spectra were carried out according to the method reported by Arsov et al. [21,22]. The experimentally ac-

quired spectra were simulated using the software EPRSIM (version 4.99 2005). 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 domain type which is highlighted by the values of the chosen parameter set. This one corresponds to order parameter $S$, effective rotational correlation time $\tau_c$, hyperfine and Zeeman tensor's polarity correction factors and weighing factors $w$.

### 2.8. Microviscosity determination

The mobility of $n$-DSA in the explored medium (liposomes and HCT-116 cells) was quantified by order parameter ($S$) as described in point *2.4*. In the case of weakly to moderately immobilized probes ($\tau_c < 3 \times 10^{-9}$ s) $\tau_c$ is used, whereas, for strongly immobilized labels ($\tau_c > 3 \times 10^{-9}$ s), $S$ is better suited [23,24]. Previously established standard curves of microviscosity versus correlation time or order parameter [25] were used to convert measured parameters into membrane microviscosity. Each measure was repeated at least five times and microviscosity standard deviation was calculated to be 3%.

### 2.9. Membrane cholesterol measurement

Cells from two T175 flasks (90% confluent) were collected by trypsinization, centrifuged for 7 min at 240$g$ and suspended in 10 ml DMEM containing Rameb sterilized by filtration in aseptic conditions at desired concentration. The cells ($15 \times 10^6$) were then incubated at 37 °C for 1 h in a humidified atmosphere with 5% of $CO_2$. The 1 h incubation was ended by centrifugation during 5 min at 200$g$ in order to remove the Rameb. The cell pellet was then suspended in DMEM to be able to count the required amount of cells to perform a lipidic extraction. Briefly, cells (750,000) were put in contact with 300 µl of 1% Triton distilled in water. After 5 min of incubation, 1.8 ml of a chloroform/methanol (2:1) mixture was added. The mixture was stirred for 10 min. To obtain a good phase separation, 300 µl of Tris (0.2 M) at pH 6.5 was added, and the mixture was centrifuged for 10 min (240$g$) at 37 °C. To isolate the lipidic phase, the chloroform phase was recovered and evaporated under a flow of nitrogen. The membrane cholesterol levels were then measured using an Amplex Red Cholesterol Assay Kit according to the manufacturer's instructions (Molecular Probes). Briefly, the membrane fractions were dissolved within 800 µl of buffer (PBS, pH 7.4) then incubated with Amplex Red reaction mixture for 30 min at 37 °C and analyzed using a fluorimeter with an excitation wavelength of 570 nm and emission wavelength of 590 nm.

## 3. Results and discussion

### 3.1. Cell viability study

Based on WST-1 assay, fluorometric measurements revealed that the cell viability was more than 94% after an incubation of the highest concentration of Rameb (10 mM) for 1 and 2 h (data not shown). Moreover, at the concentration used to obtain an optimized ESR signal ($10^{-4}$ M 5-DSA), no cytotoxic effect of the spin probe was observed (98% of cell viability). These results were also confirmed by Trypan blue exclusion assay. It is important to notice that another type of cell could reveal much higher mortality with similar concentration of Rameb [13].

### 3.2. Effects of Rameb on 5-DSA probe

Stearic spin labels (5-DSA) were used as probes to analyze the interaction between Rameb and the cell membrane fluidity. Spectrum on Fig. 1A is characteristic of a probe which invested the hydrophilic cell membrane compartment at 25 °C [26].

Fig. 1B highlights the affinity of the 5-DSA with the Rameb. Indeed, a significant difference has been observed between the probe spectrum in PBS (Fig. 1B, full line) and the one obtained for 5-DSA in PBS containing Rameb (Fig. 1B, dotted line) which is asymmetric and distorted; such a spectrum is characteristic of probe aggregates in micelle structures as previously reported [17,27,28].

The addition of increasing concentrations of Rameb (1.5, 2, 6 and 10 mM, respectively) to HCT-116 cells containing 5-DSA induces a progressive transformation of the ESR spectrum (Fig. 1C) which tends to be similar to the spectrum shown in Fig. 1B (dotted line). This result reveals that the Rameb affinity with 5-DSA is strong enough to lead to the extraction of all the probes initially embedded in the membrane bilayer and that the extracted probe is not free in solution but aggregates in micellar structure. This was confirmed by the analysis of the ESR spectrum of the cell supernatant that has been in contact with Rameb (Fig. 1D, full line) which is similar to the 5-DSA spectrum in PBS and containing Rameb (Fig. 1D, dotted line).

Fig. 1C also puts in evidence that 5-DSA are extracted in a dose-dependent way. Indeed, the highest the Rameb concentration is, the most intense the three peaks of the ESR spectrum are.

A spectral simulation has been done to confirm this behavior (Fig. 2A-a and B-a, gray line). The simulated spectrum of 5-DSA incorporated into cell membranes, which were in contact with 1.5 mM of Rameb, corresponds to the addition of two components characteristic of two different domains. The first one (Fig. 2A-1b) corresponds to probes embedded into the cell membrane and the second domain (Fig. 2A-2b) to probes extracted from the cell membrane and aggregated within micelles (20% for this Rameb concentration). The spectral simulation obtained with cell membranes in contact with 10 mM of Rameb has only one component indicating that all the probes are extracted from the membrane (Fig. 2B-b). This result is consistent with the fact that Rameb acts in a dose-dependent manner.

### 3.3. Effects of Rameb on cholestane probes

The ESR spin labeling method relies on the presence of probes inside the bilayer. The $n$-DSA spin label (which has almost the

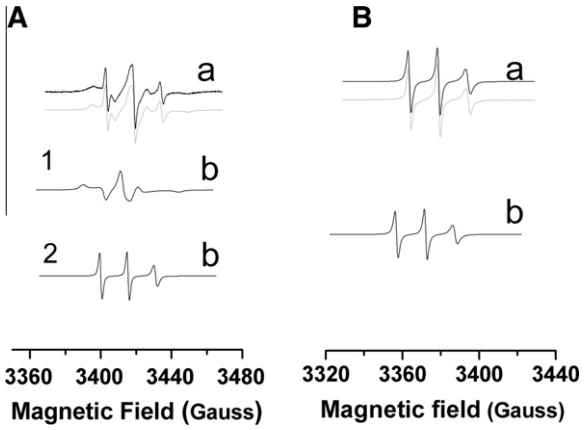


**Fig. 2.** ESR spectra of 5-DSA in the supernatant of cells being in contact with 1.5 mM (A) and 10 mM of Rameb (B). (a) Black line: experimental spectrum. Gray line: the best fit to the experimental spectrum, taking into account the superimposition of possible coexisting domains shown in (b). (b) A: computer simulation of the specific spectra of the two coexisting domains explored by the 5-DSA: 1; first domain ($S = 0.906$, $\tau = 0.419$ ns, $W = 0.702$), 2; second domain ($S = 0.631$, $\tau = 0.557$ ns, $W = 1.714$). (b) B: computer simulation of the specific spectrum of the only one domain ($S = 0.028$, $\tau = 0.557$ ns, $W = 0.728$) explored by the probe.



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same behavior as the phospholipids) has been first used, confirming that the Rameb is able to interact with them. To better apprehend the CDs action on membranes another kind of probe has been used: the cholestane, a membrane spin label with a chemical structure similar to cholesterol.

Fig. 3A shows the ESR spectrum of cholestane embedded in cell membrane (light gray line). The large distorted peak observed results in an important spin–spin nitroxide interaction favoured by a high local concentration of cholestane. Available knowledge indicates that cellular membranes manage their vast chemical diversity by sorting into specialized microdomains [29] which are referred to under the general heading of lipid raft. Cholesterol plays a fundamental role in maintaining their structure and function [30]. The cholestane spin probe probably lies close to each other in these lipid rafts, leading to this kind of self-broadening.

To support this hypothesis, DMPC liposomes doped with 30% of cholesterol and containing cholestane have been prepared (Fig. 3B). These structures have been made to incorporate the cholesterol uniformaly in the membrane (no raft). The corresponding ESR spectrum is reported in Fig. 3B; no broad peak as in Fig. 3A is observed indicating that probes are not close to each other like in cell membrane. This result tends to confirms that cholesterol might be organized in rafts within cell membrane and that cholestane like cholesterol has an affinity for these microdomains. As probe and cholesterol have almost similar chemical structure, the cholestane probably reflects the cholesterol behavior.

The evolution of probe ESR spectrum in cells incubated with Rameb (Fig. 3A) clearly demonstrates that cholestane is no more embedded in membrane. The resulting spectrum is close to the spectrum of cholestane solubilized in PBS containing Rameb (Fig. 3C dotted line). The difference between cholestane ESR spectrum in PBS and the one obtained in PBS containing Rameb (Fig. 3C) reflects once again the ability of Rameb to form micellar aggregates with the probes. To sum up, ESR results show that Rameb not only extracts different components from the plasma membrane to form stable aggregates but it is also able to interact with lipid rafts.

### 3.4. Effects of Rameb on membrane microviscosity

As cholesterol and phospholipids are essential constituents of the membrane, their extraction ought to necessarily affect the degree of order of the bilayer, and so the microviscosity.

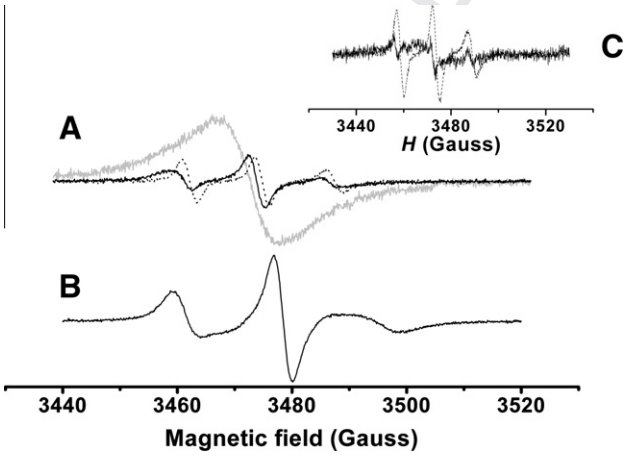


**Fig. 3.** (A) ESR spectra of cholestane (0.1 mM) in cell membrane (gray line), in cell membrane being in contact with 2.5 mM of CD (black line) and solubilized in PBS containing 2.5 mM of CD. (B) Spectrum of cholestane (0.1 mM) embedded in liposomial bilayer (70:30 mol%). (C) Spectra of cholestane (0.1 mM) solubilized in PBS (black line) and in PBS containing 2.5 mM of Rameb (dotted line).

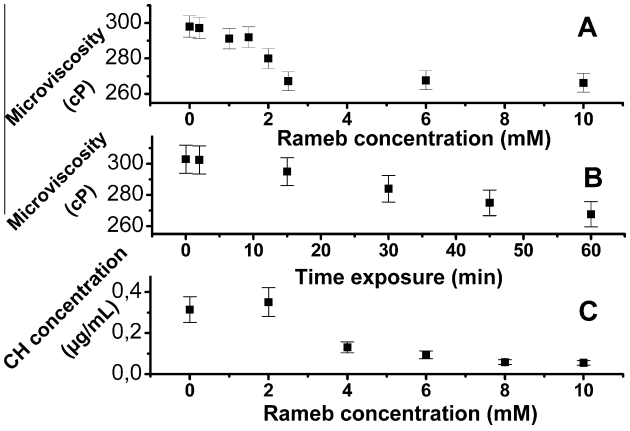


**Fig. 4.** (A) Influence of various concentrations of Rameb (0, 0.25, 1, 1.5, 2, 2.5, 6 and 10 mM) on the microviscosity of HCT-116 membranes. Cells have been in contact with the CD during 1 h at 37 °C. (B) Influence of 6 mM Rameb time exposure on microviscosity of cell membranes. (C) Residual cholesterol content of HCT-116 after treatment with various concentrations of Rameb (0, 2, 4, 6, 8 and 10 mM) for 1 h at 37 °C.

Fig. 4A shows the microviscosity sensed by 5-DSA probes incorporated in HCT-116 cell membrane in function of the Rameb concentration. In the absence of Rameb, the microviscosity measured on HCT-116 membrane, near the polar head group, is 298 cP. This value decreases as the Rameb concentration increases and stabilizes when reaching 265 cP at $2.5 \times 10^{-3}$ M of cyclodextrin. Up to $10^{-2}$ M of Rameb, the microviscosity remains nearly constant.

Fig. 4B puts in evidence the microviscosity sensed by 5-DSA probes incorporated in HCT-116 cell membrane as a function of time exposure for unique Rameb concentration of 6 mM. It appears that the more the cyclodextrin is in contact with the cells the more the microviscosity is low. A linear decrease of 35 cP is measured during the first hour.

In order to correlate this microviscosity decrease with the membrane cholesterol extraction, the membrane cholesterol concentration was measured using a cholesterol assay kit.

Fig. 4C shows that the cholesterol concentration of cells without Rameb is 0.31 ± 0.06 µg/mL. This concentration decreases when cells were incubated in presence of Rameb. Beyond an exposure of 6 mM of CD, the cholesterol level remains nearly constant (0.054 ± 0.011 µg/mL). The comparison between Fig. 4A and C clearly shows that the microviscosity and the cellular concentration of cholesterol decrease with a similar Rameb dose dependence, supporting the hypothesis that the membrane microviscosity is directly linked to the cholesterol cell concentration. ESR microviscosity results corroborate our previous work using liposomes as a membrane model and confirm that the cholesterol extraction due to the Rameb is the major cause of the membrane microviscosity decrease [17].

## 4. Conclusions

This study allows to confirm the ability of Rameb to extract cholesterol and 5-DSA in cells in a dose-dependent way. ESR results demonstrate that the extracted cholesterol and 5-DSA formed stable aggregates and that the lipid rafts micodomain are damaged by the CD. Moreover, this work shows that the membrane microviscosity is directly linked to the cholesterol content of the cell. Interestingly, based on our ESR results, Rameb appears as efficient agent to extract cholesterol depending on time and concentration. In summary, this study has demonstrated that ESR is an easy and reproducible tool able to give a lot of information on the ability of a drug to interact with cell membranes.

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