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Study of the relationship between lipid binding properties of cyclodextrins and their effect on the integrity of liposomes

G. Piel *, M. Piette, V. Barillaro, D. Castagne, B. Evrard, L. Delattre

Laboratory of Pharmaceutical Technology, Department of Pharmacy, University of Liège, CHU, Tour 4, Bat. B36, 1 av. de l'Hôpital, B-4000 Liège, Belgium

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Abstract

It is well known that cyclodextrins are able to extract lipids constituting membranes, increasing their fluidity and permeability. This behaviour towards biological membranes is directly linked to the toxicological effects of methylated cyclodextrins. However, confusion is currently made in the literature between the different methylated cyclodextrin derivatives. Moreover, a new methylated cyclodextrin derivative recently occurred in the market, the Crysmeb®. We wanted to compare and understand the effect of the most currently used cyclodextrins on a model membrane. We studied the influence of natural cyclodextrins (βCD and γCD), methylated derivatives (2,6-dimethyl-βCD (Dimeb), 2,3,6-trimethyl-βCD (Trimeb) and randomly methylated-βCD (Rameb), as well as the new derivative Crysmeb), hydroxypropylated derivatives (HPβCD of different substitution degrees and HPγCD) and the sulfobutylated derivative (SBEβCD) on the release of a fluorescent marker encapsulated in the inner cavity of liposomes. It was shown that the observed effect on calcein release can be directly related to the affinity of cyclodextrins for both lipid components of liposomes, cholesterol and phosphatidylcholine. From this relationship, we were able to determine, for each cyclodextrin, a theoretical concentration giving rise to 50% or 100% calcein release. This theoretical concentration was confirmed experimentally. We have also showed that cyclodextrins which provoke calcein release also induce large structure modifications of liposomes.

Keywords: Liposome; Cyclodextrin; Cholesterol; Calcein; Membrane permeability

1. Introduction

Cyclodextrins are widely used in the pharmaceutical field because of their ability to include a large variety of organic molecules in their hydrophobic cavity. In recent years, cyclodextrins have been used as drug penetration enhancers (Merkus et al., 1996; Irie and Uekama, 1999; Redenti et al., 2001; Chavanpatil and Vavia, 2004). The mechanism of absorption enhancement has been explained by the fact that cyclodextrins extract lipids constituting cell membranes, increasing their fluidity and permeability. This behaviour towards biological membranes, directly linked to the haemolytic and cytotoxic effect of methylated β-cyclodextrins (Frijlink et al., 1991; Leroy-Lechat et al., 1994), has been widely accepted and related to a mechanism of lipid depletion through their complexation by cyclodextrins. More recently, cyclodextrins have been used as tools to manipulate the lipid composition of biological and model membranes, particularly the membrane cholesterol content. It has been reported that cholesterol can be extracted by cyclodextrins from both cell and monolayer membranes (Kilsdonk et al., 1995; Yancey et al., 1996; Ohvo and Slotte, 1996; Ohvo et al., 2000; Nishijo et al., 2004). Methylated cyclodextrins have also been used to clarify whether membrane proteins exist at an association with specialized microdomains, called lipid rafts, by depleting cholesterol contained in them. It is clear that cholesterol forms complexes with cyclodextrins in aqueous solution. Yancey et al. have shown that cholesterol molecules can diffuse directly from the plasma membrane into the hydrophobic core of a cyclodextrin molecule packed near the membrane surface (Yancey et al., 1996). Recently,

Abbreviations: CD, cyclodextrin; Rameb, randomly methylated β-cyclodextrin; HPβCD, hydroxypropyl-β-cyclodextrin; Dimeb, 2,6-dimethyl-β-cyclodextrin; Trimeb, 2,3,6-trimethyl-β-cyclodextrin; SBEβCD, sulfobutylether-β-cyclodextrin; D.S., substitution degree; SPC, soybean phosphatidylcholine; CHOL, cholesterol; SA, stearylamine; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; PCS, photon correlation spectroscopy; $K_{1:1}$, stability constant; S_0, intrinsic solubility of the drug; CE, complexation efficiency

* Corresponding author. Tel.: +32 4 366 43 07; fax: +32 4 366 43 02.
E-mail address: Geraldine.piel@ulg.ac.be (G. Piel).

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Giocondi showed that the effect of methylated cyclodextrins on cell membranes only in terms of cholesterol movements have to be treated with caution (Giocondi et al., 2004). In fact, this author showed that methylated cyclodextrins can extract sphingomyelin from liposomes, resulting in the formation of holes. Interaction of cyclodextrins with phospholipidic chains has also been demonstrated (Nishijo and Mizuno, 1998; Nishijo et al., 2000; Anderson et al., 2004).

Methylated cyclodextrins are known to have a great affinity towards lipidic components. However, confusion is currently made in the literature as "methylated cyclodextrin" is often used indifferently for both 2,6-dimethyl-βCD (Dimeb) and randomly methylated-βCD (Rameb). Moreover, a new methylated cyclodextrin derivative recently occurred in the market which following the manufacturer, seems to be less toxic than other methylated cyclodextrins. It seems important to us to make the difference between these three different methylated cyclodextrins and avoid dangerous confusions if toxicological differences are observed.

A large variety of cyclodextrins are now available and their inclusion behaviour differs. The β-cyclodextrin (βCD), when compared with other "natural" cyclodextrins, α and γ-cyclodextrin (γCD), has the highest affinity for encapsulating sterols, in particular cholesterol (Yancey et al., 1996). Chemical modifications of the hydroxyl groups of cyclodextrins often enhance both their solubility in water and their ability to dissolve hydrophobic compounds. α, β and γCD are the less hydrophobic cyclodextrins and hydroxypropyl-βCD (HPβCD), with the same cavity diameter as βCD, is even less hydrophobic. On the other hand, Dimeb and 2,3,6-trimethyl-βCD (Trimeb) have a deeper cavity and are more hydrophobic than βCD. These results suggest that Dimeb and Trimeb have a strong ability to form soluble complexes with cholesterol in aqueous solution, but that HPβCD has only a weak ability (Nishijo et al., 2000, 2003; Anderson et al., 2004). However, complexes of cholesterol with HPβCD have been characterized (Williams et al., 1998). The interaction of cholesterol with several cyclodextrins has been investigated by Nishijo et al., using the solubility method (Nishijo et al., 2003, 2004). It was found that Dimeb and Trimeb form two types of soluble complex, with molar ratios of 1:1 and 1:2. Neither a soluble nor insoluble complex was formed between cholesterol and α, β and γCD, but a minor soluble complex formation was observed between cholesterol and HPβCD. More recently, a new methylated βCD derivative, Crysmeb, has been developed, with a lower molar substitution degree. Inclusion behaviour of this derivative needs to be investigated and compared to other methylated derivatives.

Based on these results, it has been postulated that, when cyclodextrins interact with lipidic components of liposome membranes, they may produce damage. This could destabilize the bilayers to some extent, enabling partial or complete leakage of drug content from vesicles. In monitoring the leakage of a fluorescent marker as done recently by Hatzi et al. (2007), it is hoped to obtain informations on the interaction between cyclodextrins and liposome membranes.

In this paper, liposomes constituted of soya phosphatidylcholine, with or without cholesterol, entrapping calcein in the inner aqueous phase, were prepared. The effect of the cyclodextrin concentration on the interaction with liposomes was studied from the amount of leaked calcein. A screening of the different cyclodextrins available and currently used in Pharmaceutical Technology (βCD, γCD, HPβCD, HPγCD, Dimeb, Rameb, Crysmeb, Trimeb and SBEβCD) was studied. The influence of the substitution degree of HPβCD (D.S. 0.4, 0.6, 0.8) on the calcein leakage was also tested.

In order to explain the results obtained with calcein and, since soluble complex formation between cholesterol and cyclodextrin is strongly suggested, we therefore investigated quantitatively the interaction of cholesterol with these cyclodextrins in aqueous solution and related these results to those of calcein leakage. The effect of cyclodextrins on the structure of liposomes was also investigated.

A detailed knowledge of the extraction of cholesterol and other lipid components by cyclodextrins can help us to better understand and predict what will happen when cell membranes are exposed to cyclodextrins.

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (SPC, purity: 99%), cholesterol (CHOL, purity: 99%), stearylamine (SA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and calcein were purchased from Sigma–Aldrich (Bornem, Belgium).

β-Cyclodextrin (βCD, Eur. Pharm. 5th ed., 7.58% H_2O), hydroxypropylated β-cyclodextrin (HPβCD, Eur. Pharm. 5th ed., D.S. 0.63, 3.22% H_2O) and Kleptose® Crysmeb (Crysmeb, D.S. 0.5, 4.29% H_2O) were kindly donated by Roquette Frères (Lestrem, France). γ-Cyclodextrin (γCD, 4.25% H_2O), randomly methylated β-cyclodextrin (Rameb) BETA W7 M1.8 (D.S. 1.8, 3.81% H_2O), HPβCD (Eur. Pharm. 5th ed., D.S. 0.8, 4.55% H_2O) and hydroxypropylated γ-cyclodextrin (HPγCD, D.S. 0.7, 2.85% H_2O) were a gift from Wacker-Chemie GmbH (Munich, Germany). 2,6-Dimethyl-β-cyclodextrin (Dimeb), 2,3,6-trimethyl-β-cyclodextrin (Trimeb) and HPβCD (Eur. Pharm. 5th ed., D.S. 0.4, 4.98% H_2O) were obtained from Cyclolab (Budapest, Hungary). Sulfobutylether-β-cyclodextrin (SBEβCD, D.S. 0.98, 3.2% H_2O) came from Cydex (Kansas, USA).

Betamethasone was purchased from Medeva (Braine L'Alleud, Belgium).

All other reagents and solvents were of analytical grade.

2.2. Liposome preparation

Unilamellar vesicles made from SPC:CHOL:SA (60:30:10 mol%) or SPC:SA (90:10 mol%) were prepared by hydration of lipid films. In practice, the required amounts of lipids were dissolved in chloroform in a round-bottomed flask and were dried under vacuum using a rotary evaporator. The resulting lipid film was hydrated using 3 mL of calcein dissolved in 0.22 μm-filtered 10 mM HEPES solution, containing 67 mM NaCl and adjusted to pH 7.4 with 0.1N NaOH solution.

Suspensions were thoroughly mixed using a vortex mixer and then extruded through Nucleopore polycarbonate membranes of successive 0.4 and 0.2 μm pore diameters. In order to detect a leakage-induced effect, the self-quenching fluorescent dye calcein was entrapped in these vesicles at a concentration of 50 mM. At this concentration, calcein shows minimal fluorescence, owing to the formation of ground state dimers. Any fluorescence measured will be due to calcein leakage and dilution in the exterior aqueous media. After extrusion, external calcein was removed from liposome-encapsulated calcein by four repeated ultracentrifugations at 35,000 rpm for 2 h at 4 °C. The supernatant was removed and the pellet was resuspended in 10 mM HEPES solution, containing 145 mM NaCl in order to obtain isoosmotic media inside and outside liposomes, and adjusted to pH 7.4 with 0.1N NaOH solution.

2.3. Evaluation of liposome integrity by calcein leakage

The membrane integrity of liposomes at 37 °C was evaluated by calculating the percent retention of liposome encapsulated calcein. The leakage of liposomal contents into the medium is indicative of changes in the membrane permeability. Liposomes were placed in contact with a cyclodextrin buffered solution of a final concentration of between 0 and 100 mM (except for βCD and Crysmeb, for which maximal concentrations tested were 7.5 and 50 mM, respectively, because of their solubility limit and the dilution factor inherent in the protocol). In practice, all runs were initiated by adding 100 μL of liposome suspension (adjusted to a 0.15 mM SPC concentration) to 100 μL of a buffered solution of cyclodextrin or a HEPES buffered solution (blank test) or a 2% Triton X-100 solution, which had been previously placed in a 96-well plate and heated at 37 °C. The release of calcein from the liposomes was measured fluorometrically (SpectraMax Gemini XS), excitation and emission wavelengths were 490 and 520 nm, respectively, and widths of excitation and emission slits were 5 nm) immediately after the addition of the liposome suspension and after 2.5, 5.0, 7.5, 10.0, 15.0, 20.0 and 30.0 min. This assay method was successfully validated. The amount of calcein released was calculated by the following equation:

$$\%\,\text{calcein released} = \frac{I_t - I_0}{I_T - I_0}$$

where I_t is the fluorescence intensity at 520 nm and at time t after the addition of the liposome suspension, I_0 the fluorescence intensity of calcein loaded liposome suspension at 520 nm in a buffered solution without cyclodextrin, and I_T is the fluorescence intensity at 520 nm and after complete destruction of the liposomes by Triton X-100.

Data were determined from the average of at least three determinations.

2.4. Evaluation of liposome size and integrity by PCS analysis

Liposome dispersions were sized by photon correlation spectroscopy (PCS) (HPPS, Malvern Instruments). Measurements were made at 25 °C with a fixed angle of 90° and sizes quoted were the average mean for the liposomal hydrodynamic diameter (nm). Size was measured before, immediately after the addition of the cyclodextrin solution, and after 3 h. Measures were realized at least in triplicates.

2.5. Phase solubility diagram of cholesterol and SPC

Affinity of cholesterol and SPC for the different cyclodextrins was measured using the phase solubility method (Higuchi and Connors, 1965). Excess amounts of cholesterol or SPC were added to various concentrations of cyclodextrins in 3 mL of HEPES buffer (10 mM HEPES, 145 mM NaCl, pH 7.4). Each concentration of cyclodextrin was tested in triplicate. The suspensions placed under inert atmosphere (nitrogen) were shaken in a water bath at 25 °C for 48 h. After filtration through a 0.45 μm filter, cholesterol was assayed by the following validated HPLC method: HPLC was performed using a system consisting of a LaChrom Merck Hitachi system L-7100 pump, an L-7400 UV detector, an L-7200 autosampler and a D-2500 chromato integrator. Fifty microlitres samples were injected into a Lichrocart column (250 mm × 4 mm i.d.) prepared with an octylsilane (C8) phase LiChrospher (Merck) and were maintained at 35 °C. The mobile phase consisted of a 20:31:32:17 (v:v) mixture of methanol, acetonitril, tetrahydrofuran and water HPLC grade. The flow rate was 1.7 mL/min. All the samples were analysed in duplicate. Each sample was diluted with mobile phase before injection into the HPLC system. Cholesterol was detected at 214 nm.

SPC was assayed by an enzymatic method (Phospholipids B, Wako, Neuss, Germany). The principle of this enzymatic assay is the following: this assay consists of the cleavage by phospholipase D of SPC in choline, which is oxidized in betaine with the simultaneous production of hydrogen peroxide. The hydrogen peroxide, which is produced quantitatively, oxidatively couples 4-aminoantipyrine and phenol. Peroxydation results in the generation of a coloured compound, quinoneimine, quantified by spectrophotometry at 505 nm (spectrophotometer Perkin-Elmer Lambda 11).

3. Results and discussion

Unilamellar liposomes constituted of soya phosphatidylcholine, with or without cholesterol, entrapping calcein in the inner aqueous phase, were prepared. Size was around 200 nm and the polydispersity index was always lower than 0.2, proof that samples are monodisperse. Once separated from the non-entrapped calcein fraction, there is typically some residual dye outside the vesicles that gives rise to a small fluorescence signal even in impermeable intact vesicle samples. To correct for this, control samples were prepared in parallel to the samples submitted to cyclodextrins, and were treated identically. The residual amount of fluorescence present before the addition of cyclodextrin was equal to $10.8 \pm 0.8\%$ ($n = 5$). This basal fluorescence level did not increase significantly even after 1 month's storage at 4 °C under nitrogen, calcein remaining entrapped within the vesicles.

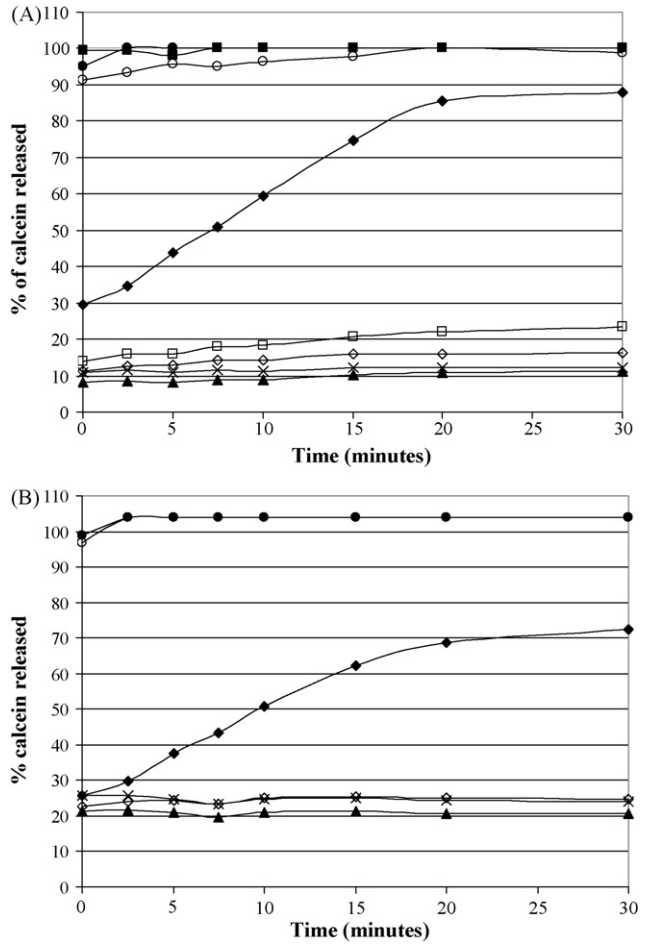

Fig. 1. Percentage of calcein released from SPC:CHOL:SA (60:30:10) liposomes (A) or from SPC:SA (70:30) liposomes (B) as a function of time in the absence of CD (×), in the presence of Trimeb (◊), Rameb (□), γCD (△) and Dimeb (◯). Open symbol = 20 mM and black symbol = 50 mM ($n > 3$).

When calcein was able to leak out from the vesicles into the surrounding buffer, the fluorescence increased dramatically, due to the relief of self-quenching that occurs. Control wells contained the detergent Triton X-100 to rupture the vesicles and caused complete calcein release. This allowed us to determine maximum fluorescence for proper normalization of results. The leakage of calcein from the interior of liposomes was monitored at 37 °C as a function of time and cyclodextrin concentration. Eleven kinds of cyclodextrin, the most commonly used in pharmaceutics, were studied: βCD, γCD, HPβCD at three different substitution degrees (D.S. 0.4, 0.6, 0.8), as well as HPγCD, Dimeb, Rameb, Crysmeb, Trimeb and SBEβCD. The results of the addition of cyclodextrins are shown in Fig. 1A for SPC:CHOL:SA liposomes and in Fig. 1B for the same liposomes without cholesterol (SPC:SA liposomes). All cyclodextrins were tested at the same concentration but for more clarity, these figures only show cyclodextrins that have a significant influence on calcein leakage. Error bars are not indicated for the same reason but each point represents the mean of at least three measurements.

At 37 °C, SPC:CHOL:SA liposomes naturally released calcein (1.2% in 30 min) in contrast with the stability results observed at 4 °C. Dimeb at a concentration higher than 20 mM, induced a total calcein leakage immediately after its addition. No significant increase of the calcein leakage was observed after the addition of βCD, Crysmeb, HPβCD (whatever the substitution degree), HPγCD and SBEβCD. In the presence of γCD, Rameb and Trimeb, calcein was released at a level dependent on the cyclodextrin concentration. At 20 mM, Rameb induced a calcein leakage of 23% after 30 min of contact. At 30 mM, the leakage reached 90% (not shown in the figure) and reached 100% at 50 mM. The effect of Trimeb was also dependent on the concentration but seemed to be lower than that of Rameb. The 90% calcein release level was reached with 50 mM Trimeb instead of 30 mM Rameb. The effect of γCD was weaker than with methylated cyclodextrins. A 50 mM solution did not induce a significant calcein leakage but a 100 mM solution induced a leakage of around 30% after 30 min. The amount of calcein released was clearly dependent on the cyclodextrin concentration. After the same period of time and the same cyclodextrin concentration, the amount of calcein released increase in the order Dimeb > Rameb > Trimeb > γCD.

In order to understand whether the leakage observed is due to membrane cholesterol extraction, we performed the same study with liposomes lacking in cholesterol. SPC:SA (90:10) liposomes were produced with the same preparation method. Results are shown in Fig. 1B.

The basal fluorescence level (24.7 ± 0.9%) present before the addition of cyclodextrin was greater than that observed with SPC:CHOL:SA liposomes, confirming the most important permeability of this kind of liposome. Cholesterol is known to stabilize liposome membranes. Only Dimeb, Rameb and Trimeb induced a significant calcein leakage. Dimeb induced a total calcein leakage immediately after its addition at a concentration of 20 mM. Rameb and Trimeb, as for liposomes containing cholesterol, induced a calcein release, which was clearly dependent on the cyclodextrin concentration. The efficiency of these cyclodextrins for calcein leakage was greater for Rameb than for Trimeb. The other cyclodextrins did not significantly increase the calcein leakage. This may indicate that methylated cyclodextrins, apart from Crysmeb, interact with lipid molecules and, as a consequence, the small calcein molecules escape from the liposome vesicles through pores (or else disruptions of the membrane continuity), which are formed on the membranes during lipid removal (Kokkona et al., 2000; Fatouros et al., 2001).

From Fig. 2, it can be observed that, when membranes contain cholesterol, liposomes are more sensitive to methylated cyclodextrins. The same amount of cyclodextrin induces a greater calcein release from liposomes containing cholesterol than from liposomes which do not contain cholesterol. After 30 min of contact with a 50 mM Crysmeb solution, the calcein release observed is not significantly different from that observed without cyclodextrin. For cyclodextrins, the presence of cholesterol has the opposite effect than that observed for conventional detergents (Schnitzer et al., 2005). Cholesterol does not stabilize membranes from the effect of cyclodextrins. This may be explained by the high affinity of these cyclodextrins for cholesterol.

In order to explain the results observed for calcein, phase solubility studies of cholesterol and SPC in the presence of

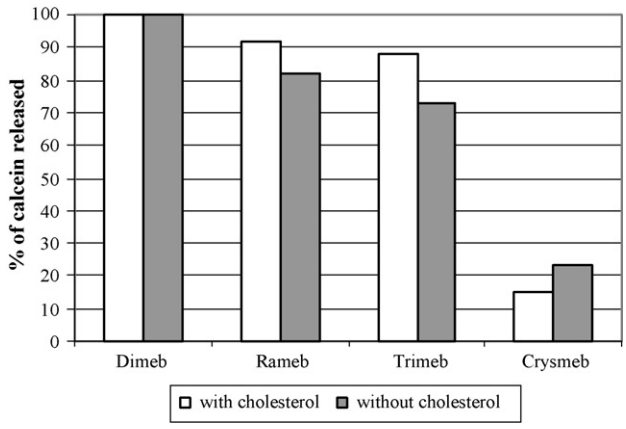

Fig. 2. Comparison of the effect of methylated cyclodextrins (50 mM) on the calcein release from liposomes, after 30 min of contact at 37 °C ($n > 3$).

different cyclodextrins were performed (Fig. 3). Phase solubility diagrams of cholesterol with Rameb, Dimeb, Crysmeb, Trimeb and HPβCD (whatever the substitution degree) are of the Ap type (following the Higuchi and Connors classification (Higuchi and Connors, 1965)), confirming the formation of a soluble complex. Therefore, it is presumed that, in aqueous solution, cholesterol forms two types of complex with these cyclodextrins, having molar ratios of 1:1 and 1:2. βCD, γCD, HPγCD and SBEβCD do not form soluble inclusion complexes with cholesterol. Due to the very low undetectable cholesterol concentration in the absence of cyclodextrin, complexation efficiency (CE) was calculated from the slope of phase-solubility diagrams. CE is independent of the intrinsic solubility of the drug (S_0), thus showing less variation than the $K_{1:1}$ values (Loftsson et al., 2005):

$$\mathrm{CE} = S_0 K_{1:1} = \frac{[\mathrm{D/CD}]}{[\mathrm{CD}]} = \frac{\mathrm{slope}}{1 - \mathrm{slope}}$$

In our experiments, despite the Ap type diagram obtained, r^2 values obtained from linear regression were always higher than 0.96 for Rameb, HPβCD, Crysmeb, Dimeb and Trimeb. CE were determined for cholesterol and for SPC. Results are

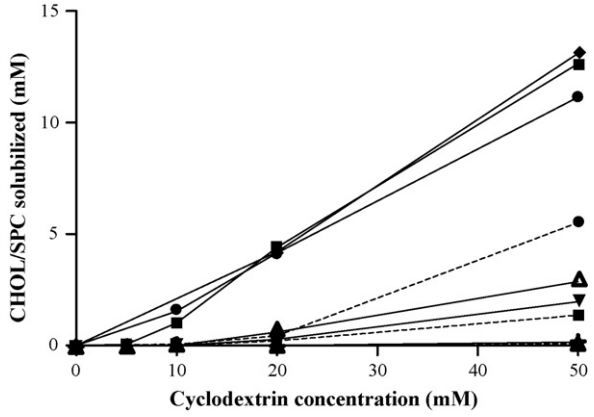

Fig. 3. Phase solubility diagram of cholesterol (continuous line) and SPC (dotted line) in the presence of different cyclodextrins: Trimeb (♦), Rameb (■), Dimeb (●), Crysmeb (△) and HPβCD (▼) ($n = 3$).

Table 1
Complexation efficiencies[a] of cholesterol and phosphatidylcholine with different cyclodextrins

Cyclodextrin	Cholesterol	Phosphatidylcholine
Rameb	0.54	0.10
HPβCD	0.07	0.00
Crysmeb	0.16	0.01
Dimeb	0.41	0.16
Trimeb	0.55	0.05

[a] Determined in HEPES buffer, pH 7.4, 25 °C.

shown in Table 1. Results show that the affinity of cyclodextrins for cholesterol increases in the order βCD = γCD = HPγCD = SBEβCD < HPβCD < Crysmeb < Dimeb < Rameb = Trimeb. It must be noted that no differences were found for the different substitution degrees of HPβCD. Dimeb, Trimeb and Rameb have a chemical structure in which hydroxyl groups of βCD are partially or completely methylated. Therefore, they have a deeper cavity and are more hydrophobic than other cyclodextrins. This might enable these methylated derivatives to form inclusion complexes with the much more hydrophobic compound cholesterol by hydrophobic interaction. Our results are in good accordance with those of Yancey et al. (1996) and Nishijo et al. (2003). Concerning SPC, the order of affinity was βCD = γCD = HPγCD = SBEβCD < HPβCD < Crysmeb < Trimeb < Rameb < Dimeb but complexation efficiencies were systematically lower than for cholesterol.

The case of the β-cyclodextrin methylated derivative Crysmeb seems to be particular. Compared to Rameb, Dimeb and Trimeb, the low substitution degree of this derivative (0.5) seems to decrease its affinity for cholesterol and phosphatidylcholine. However, Crysmeb seems to have good solubilizing properties for other substances or active drugs (Piel et al., 2006). The selective solubilizing properties of this derivative for active substances instead of lipid components may be an interesting property.

We tried to determine the relationship between cholesterol solubilizing properties of cyclodextrins and their effect on

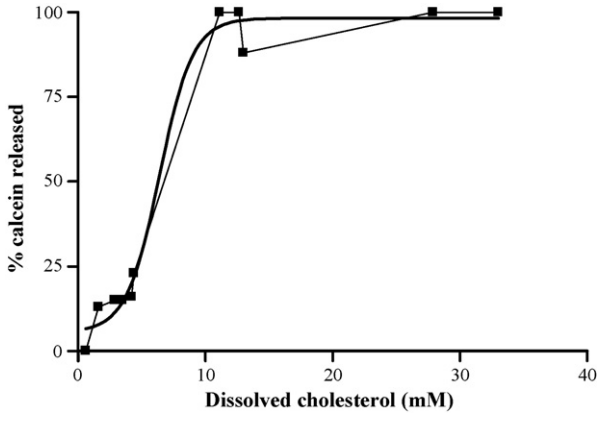

Fig. 4. Relationship between the percentage of calcein released after 30 min from SPC:CHOL:SA liposomes in the presence of a determined concentration of cyclodextrin and the quantity of cholesterol dissolved (mM) by the same quantity of the same cyclodextrin.

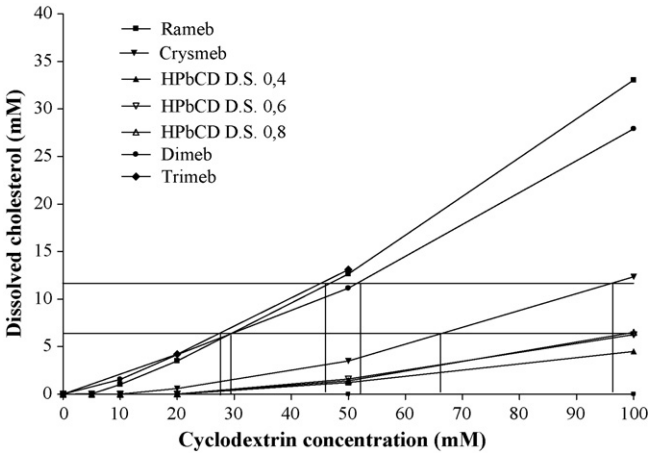

Fig. 5. Phase solubility diagram of cholesterol in the presence of different cyclodextrins with theoretical concentration of cholesterol solubilization reported.

calcein release. Fig. 4 shows the relationship between the percentage of calcein released after 30 min from SPC:CHOL:SA liposomes in the presence of a determined concentration of cyclodextrin and the quantity of cholesterol solubilized by the same quantity of the same cyclodextrin. Observation of this graph allowed us to extrapolate the limit of cholesterol solubility that provokes 50% or complete disruption of these liposomes. The theoretical concentration of solubilized cholesterol that provokes 50% calcein release is around 6 and 12 mM for total calcein release. If we report these limit concentrations on the phase solubility diagrams (Fig. 5), we can determine for each cyclodextrin a theoretical concentration above which they will partially or completely disrupt liposomes. These theoretical limit concentrations are around 65 mM for Crysmeb and 30 mM for Rameb, Trimeb and Dimeb for the 50% calcein leakage and around 100 mM for Crysmeb and 50 mM for the other methylated derivatives to obtain a complete calcein leakage. In order to have unambiguous proof of the latter hypothesis, we tested the effect of Rameb, Trimeb and Dimeb at concentrations lower than 20 mM. Fig. 6A shows that Rameb and Trimeb confirm our hypothesis, since these cyclodextrins do not provoke calcein leakage at a concentration lower than 20 mM. These results are in accordance with those of Anderson et al. (2004) who showed that disruption of phospholipid membranes may be minimized if the concentration of Rameb is kept below about 15 mM. On the other hand, Dimeb provoked complete calcein release even at 10 mM as well as a partial release at 5 mM. This may be explained by the fact that Dimeb has also a strong affinity for SPC. This is confirmed by Fig. 6B, which shows that, at 10 mM, Dimeb provokes a complete leakage of calcein from SPC:SA liposomes. In a previous paper (Boulmedarat et al., 2005), we showed by turbidity experiments that, whatever the lipid composition, continuous addition of Rameb to vesicles led to a progressive decrease in sample turbidity, evidencing the ability of Rameb to damage gradually liposome structure by a solubilization process. The solubilization experiments allowed, for given Rameb to lipid ratios, the determination of domains of vesicle-to-mixed aggregate transition. Referring to this previ-

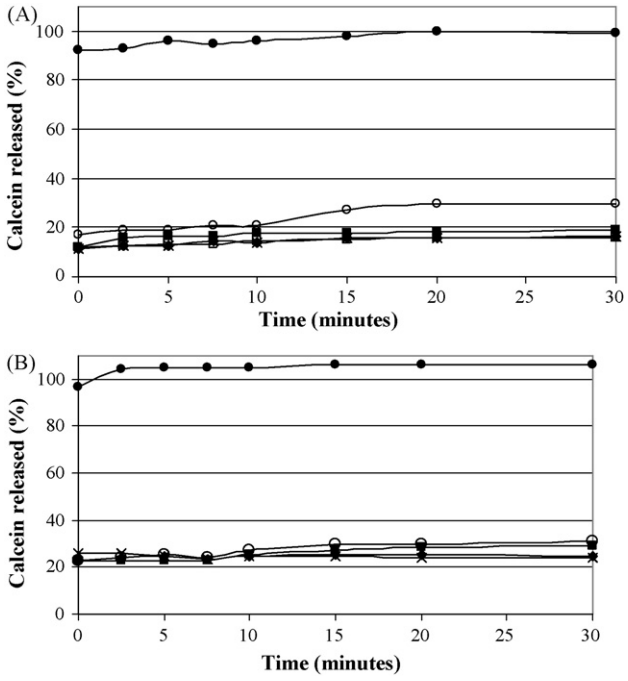

Fig. 6. Percentage of calcein released from SPC:CHOL:SA (60:30:10) liposomes (A) or from SPC:SA (70:30) liposomes (B) as a function of time in the absence of CD (×), in the presence of Rameb 10 mM (■) or Dimeb 5 mM (◯) and 10 mM (●) or Trimeb 20 mM (♦) ($n > 3$).

ous paper, when calcein liposomes were incubated with 30 mM Rameb, some lipid vesicles were then still intact coexisting with lipid–Rameb mixed micelle like aggregates. This means that Rameb had already affected partially liposome structure and could induce high levels of released calcein. It is therefore assumed that permeabilization of lipid vesicle membrane by Rameb was due to the formation of transient pores, probably due to lipid and cholesterol solubilization as shown in the previous study (Boulmedarat et al., 2005). In the present experiment, when Rameb concentrations over 50 mM were placed in contact with liposomes a complete leakage of calcein occurred, confirming the theoretical concentration obtained and the results obtained by turbidity measurements in the previous paper.

Results obtained on model membranes can be related to in vivo results. Asai showed that, when the nasal mucosa is exposed to HPβCD at a concentration of 20%, no tissue damage is observed, whereas severe damage to the integrity of nasal mucosa was observed when using 20% Rameb (∼150 mM), (Asai et al., 2002).

Here, in order to confirm that Rameb, Trimeb and Dimeb induce a complete destruction of liposomes at concentrations higher than 50 mM, the size of liposome dispersions was measured in the presence of cyclodextrins. PCS experiments of SPC:CHOL:SA liposomes were performed before, directly after the addition of the cyclodextrin solution (T_0) and 3 h later ($T_{3\,h}$). A control was performed by measuring the size of liposomes without cyclodextrin after 3 h in the same conditions. Fig. 7 shows results obtained for Rameb and Crysmeb. Trimeb and Dimeb gave similar results to Rameb. These analyses confirmed that Rameb, Trimeb and Dimeb at 50 mM completely modify the

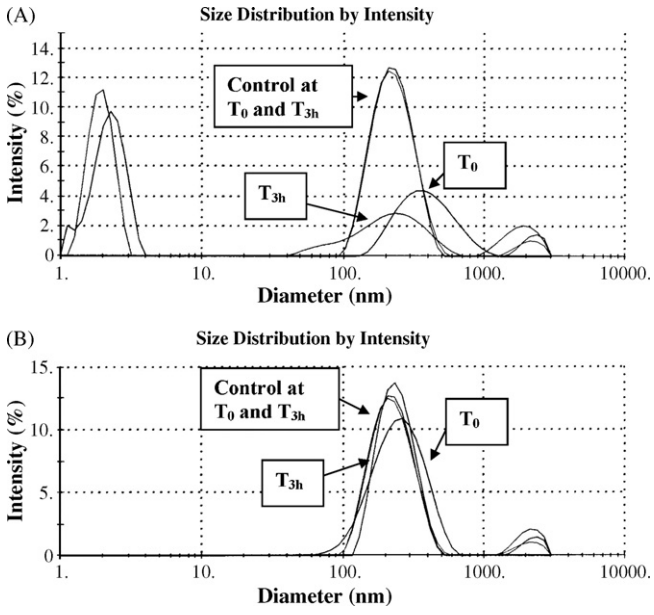

Fig. 7. PCS analysis at 25 °C of SPC:CHOL:SA liposomes before (control), directly after the addition of the cyclodextrin solution (T_0) and 3 h later ($T_{3\,h}$). The cyclodextrin concentration is 50 mM. (A) Rameb and (B) Crysmeb.

size and probably the structure of liposomes, while Crysmeb at the same concentration does not.

4. Conclusions

This study has shown that the effect of cyclodextrins on calcein release can be directly related to the affinity of these cyclodextrins to the lipid components of liposomes. The effect of cyclodextrins on calcein release from liposomes can be classified as follows: Dimeb > Rameb > Trimeb > γCD > βCD = HPβCD (whatever its substitution degree) = HPγCD = SBEβCD = Crysmeb. From the relationship between the percentage of calcein released after 30 min in the presence of a determined concentration of cyclodextrin and the quantity of cholesterol dissolved by the same quantity of the same cyclodextrin, we were able to determine for each cyclodextrin a theoretical concentration giving rise to 50% or 100% calcein release. The theoretical limit concentration to obtain 50% calcein leakage is around 30 mM for the three cyclodextrins, Rameb, Trimeb and Dimeb, and around 50 mM to obtain a complete calcein leakage. These theoretical concentrations were confirmed experimentally for Rameb and Trimeb, since these cyclodextrins do not provoke calcein leakage at a concentration lower than 20 mM. On the other hand, Dimeb provokes complete calcein release, even at 10 mM, as well as a partial release at 5 mM, due to its high affinity for SPC. When membranes are enriched in cholesterol, liposomes are more sensitive to methylated cyclodextrins. For cyclodextrins, the presence of cholesterol has the opposite effect than that observed for conventional detergents, due to the high affinity of cyclodextrins for cholesterol. We also showed that calcein release is due to modifications of liposome structure.

The case of Crysmeb seems to be particular. Compared to other methylated derivatives, the low substitution degree of this derivative seems to decrease its affinity for cholesterol and phosphatidylcholine. However, Crysmeb has good solubilizing properties for other substances. The selective solubilizing properties of this derivative for active substances instead of lipid components may be an interesting property, since cyclodextrin behaviour towards biological membranes, and especially their haemolytic and cytotoxic effects, are widely accepted to be related to lipid complexation and depletion.

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