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Integrity of liposomes in presence of cyclodextrins: Effect of liposome type and lipid composition

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Abstract

Liposome stability during incubation in presence of cyclodextrins (CDs) is studied. Dried-rehydrated vesicle (DRV), multilamellar vesicle (MLV) and small unilamellar vesicle (SUV) calcein-encapsulating liposomes, composed of different lipids are formulated, and retention of calcein is followed during vesicle incubation in hydroxypropyl-β-CD (HPβ-CD), HPγ-CD or methyl-β-CD (Meβ-CD), for 24 h. Results demonstrate that liposome integrity in cyclodextrins is affected by lipid composition and type. For the same lipid composition calcein release from vesicles is faster in the order: MLV > DRV > SUV. Meβ-CD influences liposome stability most, compared to the other CD's studied. Vesicles composed of saturated phospholipids were found more stable compared to phosphatidyl-choline (PC) liposomes, suggesting that phospholipid saturation and membrane rigidity influences the interaction between liposomal-lipids and CD molecules. Chol (cholesterol) addition in lipid membrane improves PC-liposome integrity, but has opposite or no effect on liposomes consisting of saturated lipids.

Decrease of vesicle dispersion turbidity and size distribution in presence of CD, implies that Meβ-CD induces vesicle disruption and solubilization (to micelles). Turbidity measurements confirm that DRV liposomes are affected more than SUV.

Keywords: Liposome; Cyclodextrin; Membrane integrity; Stability; Lipid; Membrane; Composition

1. Introduction

Cyclodextrins (CDs) are hydrophilic water-soluble oligosaccharides that form hydrophobic cavities in which they can accommodate water-insoluble drugs ('guests'; Frank, 1975). They received considerable attention in the pharmaceutical field because of the improved characteristics (as aqueous solubility, chemical stability and bioavailability) observed for several drug molecules by inclusion-complex formation (Loftsson and Brewster, 1996).

Previous indications that lipophilic drugs are rapidly released from liposomes after *in vivo* administration (Kirby and Gregoriadis, 1983; Takino et al., 1994) prompted the consideration of an alternative approach for stable encapsulation of lipophilic drugs in the aqueous interior of liposomes, utilizing CDs (McCormack and Gregoriadis, 1994). This approach established a novel system in drug delivery, combining liposomes and cyclodextrin complexes of lipophilic drugs by forming drug-in-cyclodextrin-in-liposome (DCL) preparations. However, it was recently observed (McCormack and Gregoriadis, 1996; Fatouros et al., 2001) that (lipophilic) drug release is still rapid from such DCL systems. This result has been connected with the known ability of CDs to remove lipid components from cell membranes by forming inclusion complexes with them (Fauvelle et al., 1997; Debouzy et al., 1998; Nishijo and Mizuno, 1998). In other words, membrane lipids may enter in the CD cavity and displace the drug from the complex, which in turn is released from the vesicles at the same rate as when it is incorporated into liposomes as plain drug.

For further investigation of the validity of the proposed theory, we previously evaluated the release of prednisolone (PR) from CD-PR-complex-containing liposomes. It was indeed demonstrated that the rate of prednisolone release from liposomes when the drug was encapsulated as a CD–drug complex, was the same as when it was encapsulated as plain drug, especially when the β-CD complex was used (Fatouros et al., 2001).

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In the same study, investigation of the effect of empty CD molecules (encapsulated in liposomes) on vesicle integrity was attempted, however, it was not possible to perform such studies, due to the difficulty to prepare empty-CD–molecule-entrapping liposomes (especially (HPβ-CD) composed of plain egg lecithin or phosphatidyl-choline [PC]).

It was demonstrated (by differential scanning calorimetry), that dimethyl-β-CD (DOMβ-CD) triggered a reduction in the enthalpy of transition of lipid vesicles composed of dipalmitoyl-glycero-PC (DPPC), and this was attributed to the ability of the CD molecule to extract DPPC molecules from the vesicles (Puglisi et al., 1996). However, HPβ-CD did not cause alterations in vesicle thermotropic parameters. In another, study (Nishijo and Mizuno, 1998), the effect of CD molecules on the enthalpy of transition of DPPC dispersions was found to be higher in the order DOMβ-CD > *x*-CD > TOMβ-CD (trimethyl-β-CD). Furthermore, it was found that interactions between CDs and phospholipids depend on the length of the lipid fatty acid chain, as well as the hydrophobicity and cavity size of the CD (Nishijo et al., 2000).

In a recent study it was suggested that methylβ-CD molecules solubilize liposomes (transform liposome dispersions to micelles), after forming complexes with the lipids (Boulmedarat et al., 2005).

Herein, we study the effect of various types and concentrations of cyclodextrins on the integrity of liposomal formulations, by incubating pre-formed liposomes with CDs. Liposomal membrane integrity is measured by the leakage of a highly soluble hydrophilic fluorescent dye (calcein), which is initially encapsulated in the liposomes. Additionally, liposome dispersion turbidity and liposome sizing experiments are performed, in some cases.

The effect of liposome type and liposomal lipid composition is evaluated by studying different types of liposomes (SUV (small unilamellar vesicles), MLV (multilamellar vesicles) and DRV (dried-rehydrated vesicles)) and also different lipid compositions. Three different lipids were used for liposomes preparation; phosphatidyl-choline (PC) or egg lecithin, hydrogenated-PC (H-PC) and distearoyl-glycero-PC (DSPC). These lipids were chosen because they form different types of membranes; PC is a natural phospholipid (egg lecithin) that forms liquid type membranes (considerably more leaky compared to the other lipids used), H-PC is a natural – but hydrogenated – phospholipid (hydrogenated egg-lecithin) that forms gel-type (more rigid and less leaky compared to PC) and finally DSPC is a synthetic phospholipid that forms very rigid membranes (compared to the other two lipids). Cholesterol (Chol) is known to increase the rigidity of membranes, so liposomes containing Chol were also used.

Three different cyclodextrin molecules are used: hydropropyl-β-cyclodextrin (HPβ-CD), hydropropyl-γ-cyclodextrin (HPγ-CD) and methyl-β-cyclodextrin (Meβ-CD). These three types were selected in order to evaluate the effect of cyclodextrin cavity size (HPγ-CD cavity is larger than HPβ-CD) and cyclodextrin cavity hydrophobicity (Meβ-CD has more hydrophobic cavity compared to HPγ-CD and HPβ-CD), on the interaction of CDs with liposomes.

2. Materials and methods

Egg phosphatidylocholine (PC), hydrogenated egg phosphatidylocholine (H-PC) and distearoyloglycero-phosphatidylcholine (DSPC) were purchased from Lipoid, GmbH (Ludwigshafen, Germany) and were demonstrated to give single spots on TLC (New and Liposomes, 1990). HPβ-CD (MS = 0.8), HPγ-CD (MS = 0.6) and Meβ-CD were from Aldrich (Sigma-Aldrich OM, Athens, Greece). Cholesterol (Chol), calcein, Triton X-100, and all other reagents used were of analytical grade and purchased from Sigma–Aldrich OM, Athens, Greece.

2.1. Liposome preparation

Multilamellar vesicles (MLV) were prepared by the thin film hydration method. In brief, the lipid (PC, H-PC or DSPC) mixed (or not) with cholesterol (Chol) [at lipid/Chol, 1:1 mol/mol ratio] was dissolved in chloroform/methanol (2/1, v/v) and the lipid solution was evaporated to form a thin film. For complete removal of organic solvent the film was flashed-dried with nitrogen for 3–5 min. The lipid film was hydrated with 1 mL of Tris buffer pH 7.40 (5 mM tris) containing 20 mM NaCl and 100 mM calcein, or with plain buffer. The resulting liposome dispersions were placed in a bath-type sonicator for 15 min. Small unilamellar vesicles (SUV) were prepared by probe sonication of the MLV dispersions using a vibra cell sonicator (Sonics and Materials, UK), equipped with a tapered micro-tip, for at least two 10 min cycles. In all cases the initially turbid liposomal suspension was well clarified after sonication. Following sonication, the liposome suspensions (MLV and SUV) were left to stand for 2 h at a temperature higher than the transition temperature of the lipid used in each case, in order to anneal any structural defects. The Ti-fragments and any multilamellar vesicles or liposomal aggregates were removed by centrifugation at 10,000 × *g* for 15 min.

For DRV preparation, the dehydration–rehydration procedure (Kirby and Gregoriadis, 1984) was used. In brief, empty SUV liposomes were initially prepared, and 1 mL of the SUV suspension was subsequently mixed with 1 ml of calcein solution (100 mM), and the mixture was freeze-dried overnight. With controlled rehydration of the dried materials as described previously (Kirby and Gregoriadis, 1984), multilamellar dehydrated–rehydrated vesicles (DRV) were generated.

Liposomes (MLV and DRV) were separated from non-entrapped calcein by centrifugation (Heraeus Biofuge, Germany); two 40 min spins at 15,000 rpm were applied for this. For SUV liposomes gel exclusion chromatography was performed, using a Sephadex G-50 column (1 cm × 35 cm) eluted with Tris buffer pH 7.40. The column was pre-saturated with lipid. A phospholipid colorimetric assay (Stewart, 1980) was used to measure the final lipid concentration of all liposomal dispersions prepared.

2.2. Evaluation of liposome membrane integrity

The membrane integrity of liposomes after incubation in buffer or in presence of CD solutions (40 mg/mL, final CD

concentration) at 37 °C was evaluated by calculating the per cent retention of liposome encapsulated calcein, as previously described (Kokkona et al., 2000). In brief, liposome dispersions were mixed with the appropriate amount of CD solution (lipid concentration was adjusted to 1.3 mM, for MLV and DRV liposomes and 0.65 mM for SUV) and each sample was incubated at 37 °C. At specific time intervals (1 min, 2 h, 4 h and 24 h) the latency and retention of calcein was estimated after mixing 20 μl samples with 4 ml buffer, pH 7.4, and measuring calcein fluorescence (EM-490 nm EX-520 nm) before and after disrupting the liposomes with Triton X-100 (1% final concentration). The possibility of interaction between calcein and CD molecules (that would result in modulation of calcein fluorescence intensity) was ruled out after measuring appropriate control samples.

The percent of calcein latency was determined from the equation:

$$\%\,\text{latency} = \frac{F_T - F_i}{F_T} \times 100$$

where F_i and F_T are the calcein fluorescence intensities of each sample in the absence and presence of 1% Triton X-100, respectively. The values obtained after the Triton X-100 additions were corrected for dilution. Percent of calcein retained in liposomes (% retention) was calculated, by considering the initial latency of calcein in the liposomes at time 0 (i.e. at each time point retention is the percent of initial latency remaining latent).

For each experiment a relevant control experiment was carried out in which the membrane integrity of the same liposome dispersions (that were tested in presence of CDs) were estimated in plain buffer (adjusted – by NaCl addition – to be isoosmolar with the CD solutions used), for comparison.

2.3. Measurement of liposome size and surface charge

Fifty microliters of the liposome dispersions were diluted with 20 ml of filtered buffer (0.22 um pore size, polycarbonate filters, Millipore, UK) and sized immediately by photon correlation spectroscopy (Malvern Instruments, Model 4700C), which enabled the mass distribution of particle size to be obtained. Measurements were made at 25 °C with a fixed angle of 90° and sizes quoted are the *z*-average mean (d*z*) for the liposomal hydrodynamic diameter (nm). In some cases, the particle size distribution of liposome dispersions was measured after in presence of CDs.

2.4. Liposome dispersion turbidity experiments

CD-induced vesicle size alterations were evaluated by measuring liposome dispersion turbidity in absence (initial value) and presence of increasing CD concentrations (ranging between 35 and 210 mg/ml). Turbidity was measured with a Shimatzu RF-1501, spectrofluorophotometer equipped with a thermostated sample holder and a magnetic stirrer (emission and excitation both set at 500 nm, slit 10–10), before, and 1 min, 1 h and 2 h after mixing the liposome dispersions with the appropriate amount of CD. Turbidity decrease due to sample dilution was corrected after performing control experiments (by mixing the samples with plain buffer).

2.5. Statistical treatment of experimental results

For the statistical evaluation of differences between results, a number of statistical tests were utilized. The paired *t*-test, to check the significance between calcein retention or turbidity differences – under the same conditions (time and CD) – of different liposome types. Single sample *t*-tests, for checking the significance of turbidity reduction (relative turbidity (%) values compared with 100). Finally two-way analysis of variance, to check the significance of the effect of liposome type and lipid composition on CD-induced modifications of calcein retention or relative turbidity (SPSS v14.0).

In all cases, a probability value of less than 0.05 was considered to be significant.

3. Results

3.1. Liposome properties

In all cases, the DRV and MLV liposomes were not homogenous in size. For the DRV about 75% of the vesicles have mean diameters between 310 and 480 nm, and for MLV between 3.6 and 5.6 μm. No significant differences were found between the sizes of liposomes with different lipid compositions.

For SUV liposomes, mean diameters and corresponding polydispersity indexes are presented in Table 1. In all cases, the differences between samples with different lipid compositions are not statistically significant ($p > 0.05$), however Chol addition in vesicle membranes always results in slight increase of their mean diameter.

3.2. Membrane integrity of liposomes in absence and presence of different cyclodextrins

The retention of vesicle-encapsulated calcein was monitored after incubation of the vesicles in presence of plain buffer (control) or CD solutions, at 37 °C for 24 h. The results of these studies are presented in Figs. 1–3.

In Fig. 1 the integrity of DRV liposomes, composed of PC, H-PC or DSPC (graph-sets A–C, respectively) and containing Chol (right-side graphs) or not (left-side graphs), is presented.

Table 1
Mean diameter of SUV liposomes with different lipid compositions

Lipid composition	Mean diameter
PC	89 (±14) [*P* 0.48]
PC/Chol	118 (±21) [*P* 0.56]
H-PC	107 (±2.0) [*P* 0.38]
H-PC/Chol	119.6 (±4.2) [*P* 0.36]
DSPC	116 (±2.3) [*P* 0.47]
DSPC/Chol	122 (±2.9) [*P* 0.41]

Four different samples were measured five times and the mean (as well as the standard deviation of the mean) is presented. Polydispersity index values are presented in square brackets.

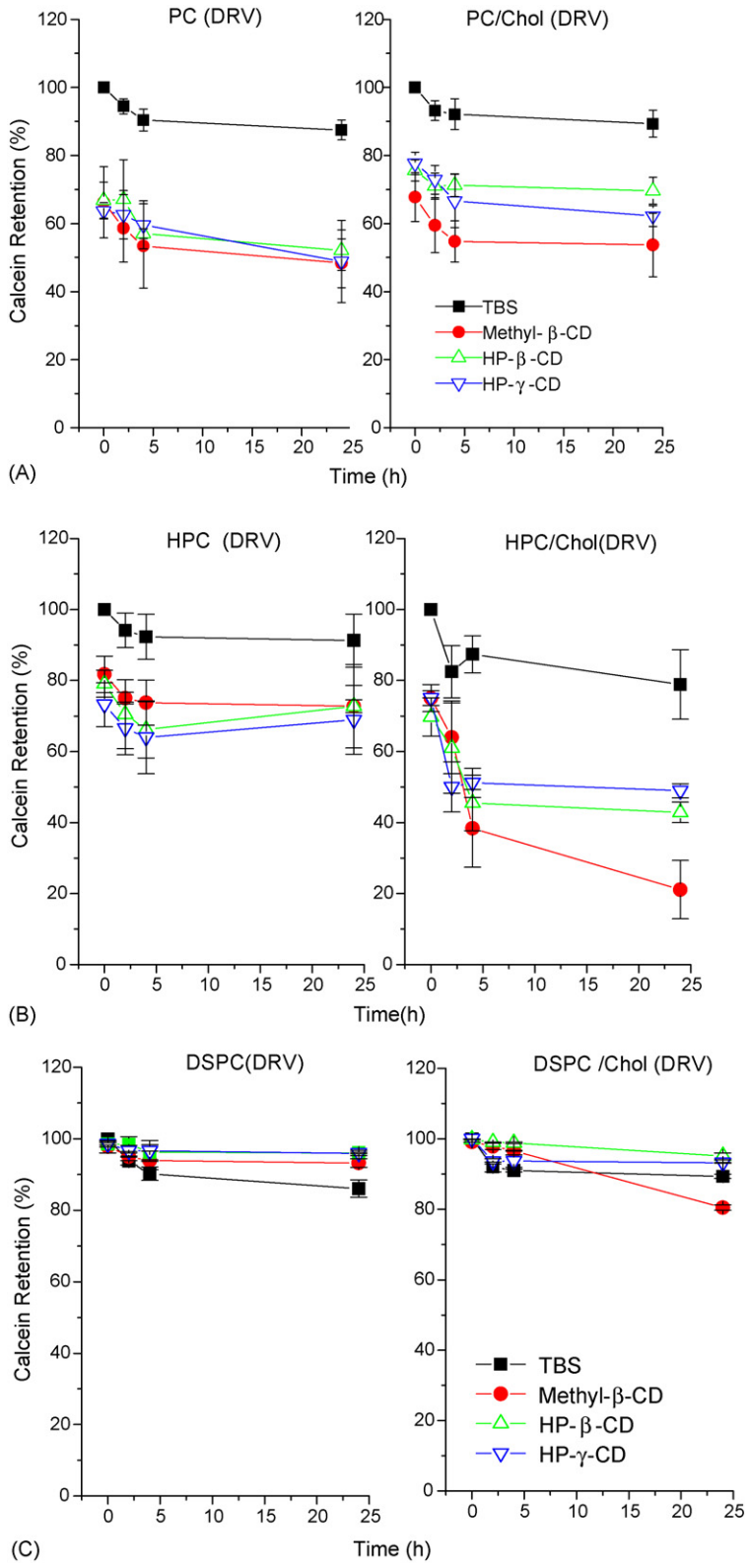

Fig. 1. Retention of vesicle-encapsulated calcein (%) in DRV liposomes (lipid concentration is 1.3 mM) during incubation in presence of various CD molecules (40 mg/ml). (A) PC and PC/Chol liposomes, (B) H-PC and H-PC/Chol liposomes and (C) DSPC and DSPC/Chol liposomes. Each point is the mean of at least four experiments (bars represent S.D. values). In each case a control experiment (incubation in buffer [TBS]) is also performed for comparison. The symbol key is presented on the graph.

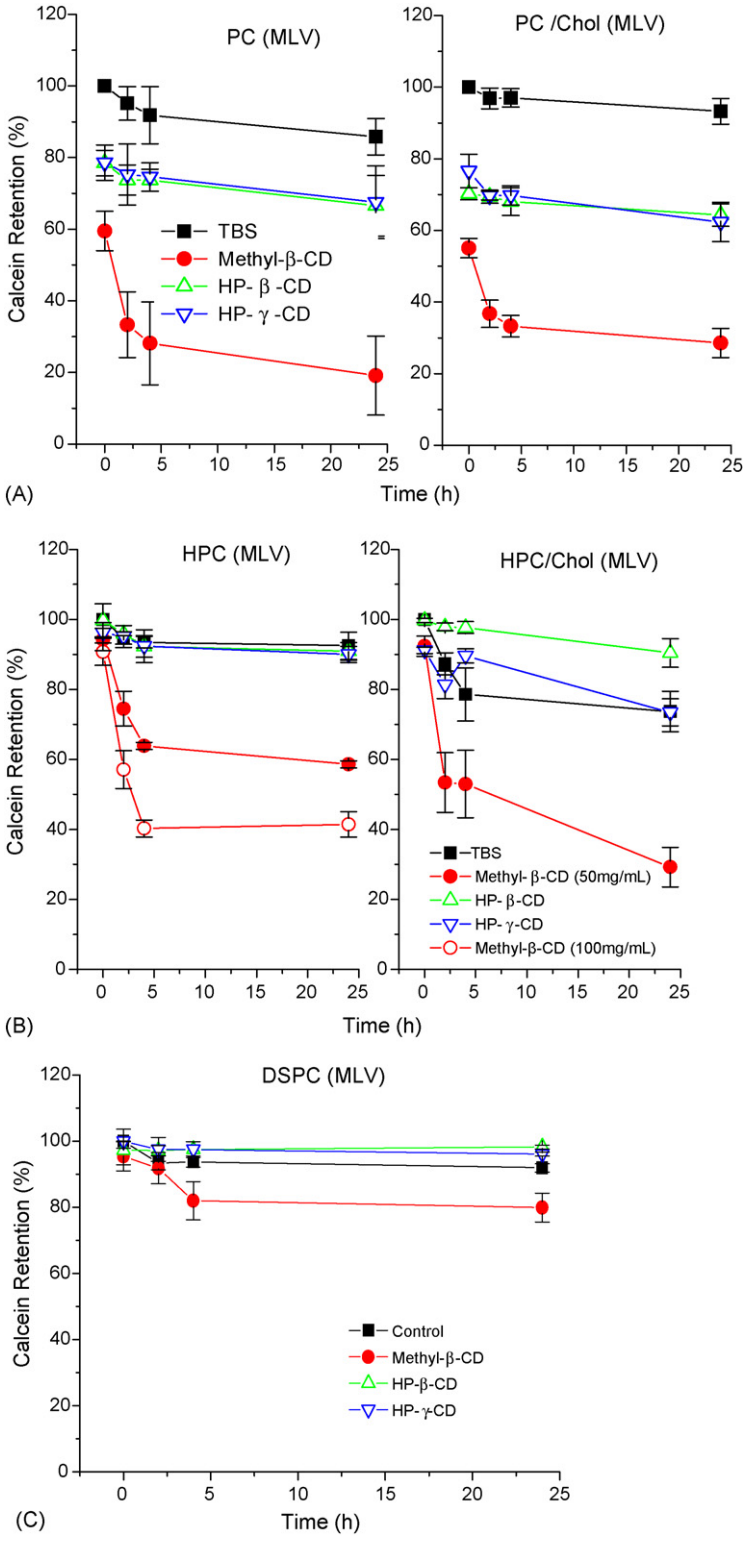

Fig. 2. Retention of vesicle-encapsulated calcein (%) in MLV liposomes (lipid concentration is 1.3 mM) during incubation in presence of various CD molecules (40 mg/ml). (A) PC and PC/Chol liposomes, (B) H-PC and H-PC/Chol liposomes and (C) DSPC liposomes. Each point is the mean of at least four experiments (bars represent S.D. values). In each case a control experiment (incubation in buffer [TBS]) is also performed for comparison. The symbol key is presented on the graph.

As seen, for all CDs studied, when liposomes are prepared without Chol (left-side graphs), their membrane integrity is affected more (by CDs) in the order PC > H-PC > DSPC. In fact DSPC liposomes are very stable in presence of almost all the CDs studied, with the exception of Meβ-CD in the case of DSPC/Chol liposomes (24 h incubation period). On the other hand, the integrity of both, PC and H-PC DRV liposomes is decreased by CDs in all cases. Indeed, immediately after liposomes are mixed

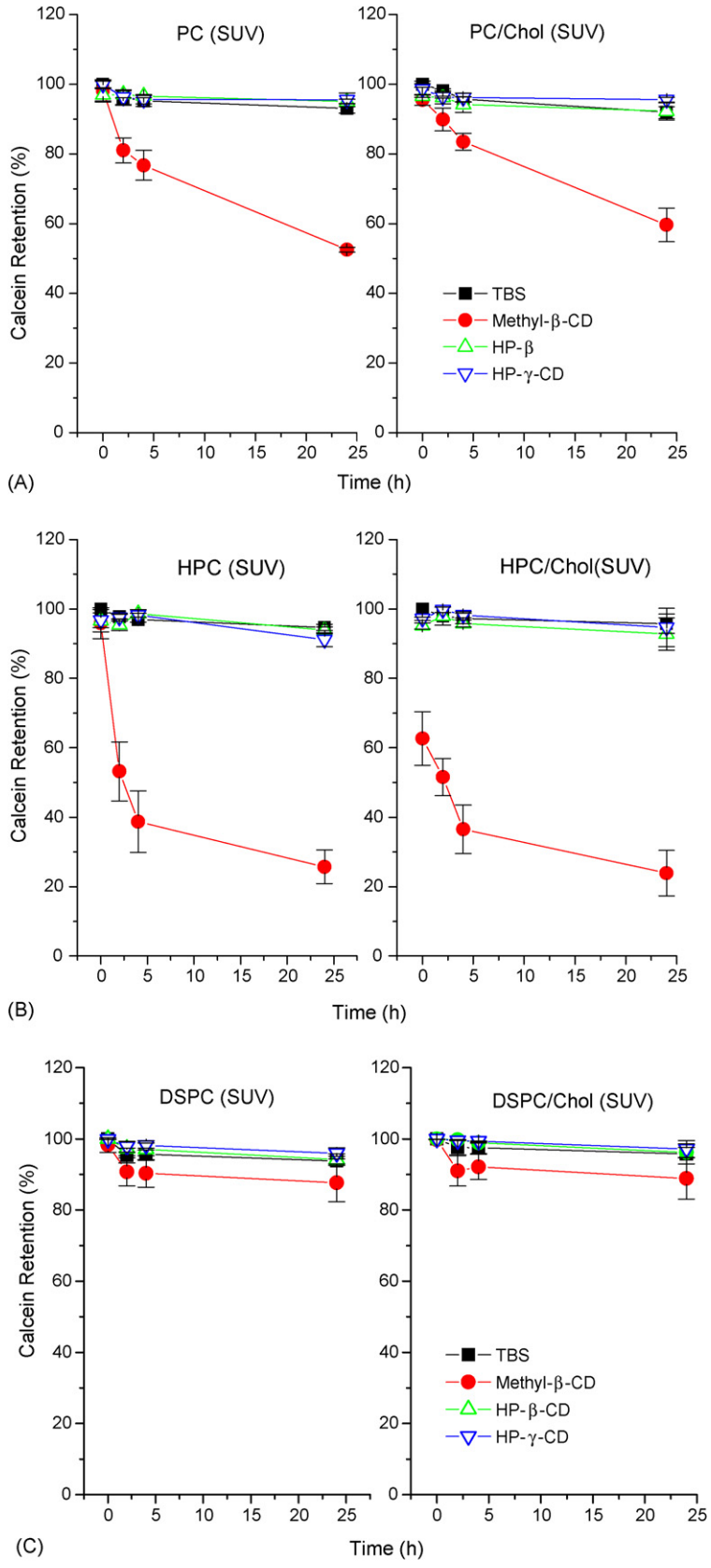

Fig. 3. Retention (percent) of vesicle-encapsulated calcein in SUV liposomes (lipid concentration is 0.65 mM) during incubation in presence of various CD molecules (40 mg/ml). (A) PC and PC/Chol liposomes, (B) H-PC and H-PC/Chol liposomes and (C) DSPC and DSPC/Chol liposomes. Each point is the mean of at least four experiments (bars represent S.D. values). In each case a control experiment (incubation in buffer [TBS]) is also performed for comparison. The symbol key is presented on the graph.

with CDs they lose a significant amount of their calcein content (between 33 and 36% for PC-liposomes and 19–27% for H-PC liposomes). However, after this initial (significant [$p < 0.01$]) decrease of calcein retention, no further influence on vesicle integrity is demonstrated for the full 24 h incubation period. This observation implies that when DRV liposomes are mixed with CDs (at this specific CD/lipid analogy [mol/mol ratio = 22]), rapid leakage of vesicle encapsulated dye is induced possibly due to interaction between CD molecules and liposome membrane constituents that results in some kind of "de-stabilization" of the lipid vesicle membrane (or extraction of lipid molecules from the liposome membrane). Following this initial intense effect, CD molecules do not influence membrane stability further (for the 24 h incubation period studied), implying that perhaps the lipid membrane is re-organized (in its initial state).

When cholesterol is included in the DRV liposome membranes (Fig. 1, right-side graphs) although the PC-based liposomes are stabilized by Chol (release less calcein in presence of CDs, as concluded by comparing the left and right-side graphs), the H-PC based liposomes are considerably destabilized, especially in presence of Meβ-CD (Fig. 1B, right- and left-side graphs). Similarly, a significant de-stabilization in presence of Meβ-CD was demonstrated for the DSPC-based DRVs, when Chol was added in their membrane (Fig. 1C, 24 h time point). Thereby, we may conclude that addition of Chol in liposomes composed of saturated phospholipids results in decreased vesicle integrity in presence of CDs. This can be explained if we accept that interaction between CD and cholesterol is faster and of higher degree, compared to that between CD and saturated lipids (H-PC and DSPC). Extraction of Chol-molecules from the vesicle membrane (Kilsdonk et al., 1995) and concurrent leakage of the vesicle-encapsulated calcein, can thus explain the destabilization of the vesicles, when Chol is added in their membrane.

When comparing the effects of the different types of CDs on the integrity of DRV liposomes, it is observed that, plain lipid DRV-liposomes are affected the same way by all three CD-types used, while Chol-containing DRV liposomes are affected more by Meβ-CD, compared to the other CDs (HP-CDs). This implies a higher affinity of Meβ-CD towards Chol.

For MLV and SUV liposomes calcein retention values are presented in the same type of graphs as those of Fig. 1, in Figs. 2 and 3, respectively. For both of these liposome types it is obvious that the membrane destabilizing effect of Meβ-CD is always higher, compared to the other CD's studied. In fact, in all cases (lipid compositions) of MLV and SUV liposomes, the liposome integrity is drastically affected by the presence of Meβ-CD and the leakage of vesicle-encapsulated calcein caused by this specific CD is always substantially higher compared to that caused by the HP-CDs.

Comparing the calcein retention results obtained for MLV and DRV liposomes (Figs. 1 and 2) we conclude that their behavior in presence of CDs is similar, with some slight exceptions: (i) H-PC MLV liposomes are not destabilized by HP-CDs, as was demonstrated for H-PC DRV liposomes. (ii) Addition of Chol in plain PC MLVs does not result in increased stability in presence of HP-CDs (as observed for DRV liposomes). However, SUV liposomes demonstrate a different behavior in respect to their integrity in presence of CDs. Indeed, SUV liposomes are very stable (Fig. 3) in presence of both HP-cyclodextrin's used, although the CD/lipid ratio used in the experiments with SUVs was two times higher (44) from that used for other liposome types (DRV and MLV). Only the CD with the most lipophilic cavity, Meβ-CD, causes significant decrease of encapsulated calcein retention in SUV liposomes composed of H-PC and PC (that are slightly more stable when Chol is included in their membrane), while DSPC-based SUV liposomes were found very stable in all cases.

This enhanced stability of the SUV vesicle could imply that the curvature of the lipid membrane of substantially smaller SUVs (compared to DRV and MLV), does not allow HP-CD molecules to establish the contact angle needed in order to interact with the lipid membrane (and extract lipid or cholesterol molecules from the lipid bilayer).

3.3. Turbidity studies of liposome dispersions in absence and presence of different cyclodextrins

Liposome dispersion turbidity monitoring can be used as a method to detect alterations of vesicle size (Kokkona et al., 2000). This technique is used here, in order to understand the mechanism of the CD-induced decrease of liposome integrity. The question we want to answer is, if the observed CD-induced release of vesicle encapsulated calcein, is related with the loss of lipid membrane components due to their extraction by CD molecules (that would probably result – if extensive enough – in a decrease of vesicle size) and furthermore, if complete solubilization of the vesicles can take place (by formation of CD–lipid complexes, from each one the liposome membrane components, or micelles), and if yes, at which CD concentration.

From the relative turbidity values presented in Fig. 4, it is obvious that PC SUV liposome turbidity is only slightly decreased during incubation in presence of both HPβ-CD and Meβ-CD, up to a CD concentration of 80 mg/mL. This observation does not correlate well with the fact that at a concentration of 40 mg/mL in the membrane integrity study (Fig. 3A) the same liposomes were stable in presence of HPβ-CD but very unstable in Meβ-CD. This implies that interaction between PC SUV liposomes and Meβ-CD is initiated before any effect on vesicle size is obvious (due to extraction of lipid molecules from membrane). Indeed, it is logical to hypothesize that a considerably large number of lipid molecules should be extracted from the liposomes before any measurable decrease in their size occurs. On the other hand, calcein, which is a small molecule can leak out from vesicles (from the pores formed during lipid extraction) even if only a few lipid molecules are extracted from the vesicle membranes. However, at higher cyclodextrin concentrations turbidity decreases caused by HPβ-CD are substantially lower than those caused by Meβ-CD. Thereby, by combining the results of the two types of studies we may suggest that at lower CD concentrations interaction between Meβ-CD and PC SUV liposomes results in release of some encapsulated molecules from the vesicles, whilst only at higher CD concentrations vesicles

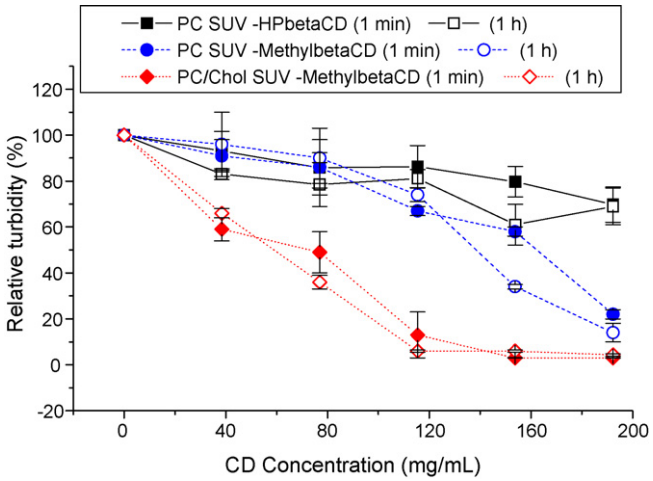

Fig. 4. Relative turbidity (%) measured for SUV liposomes with different lipid compositions (PC or PC/Chol) after incubation for 1 min (solid symbols) or 1 h (open symbols) in increasing concentrations of HPβ-CD or Meβ-CD. Details are presented in Section 2.4. Each point is the mean of at least three experiments (bars represent S.D. values). In each case a control experiment (dilution with equivalent volumes of buffer [TBS]) was also performed, and the corrected values are presented. The symbol key is presented on the graph.

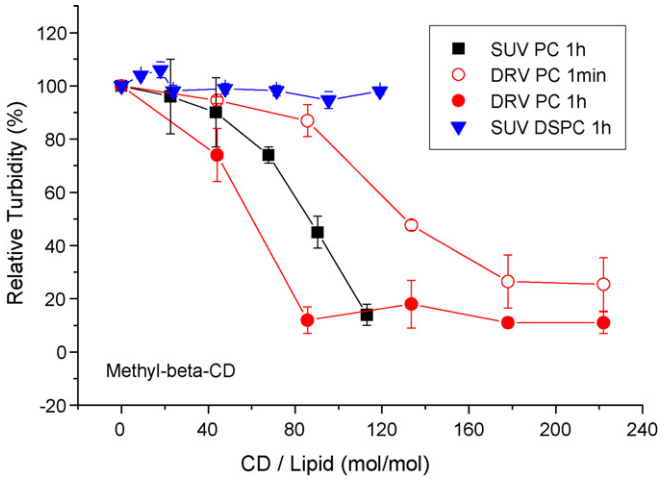

Fig. 5. Relative turbidity (%) measured for SUV or DRV liposomes with different lipid compositions (PC or DSPC) after incubation for 1 min (solid symbols) or 1 h (open symbols) in increasing concentrations of Meβ-CD. Details are presented in Section 2.4. Each point is the mean of at least three experiments (bars represent S.D. values). In each case a control experiment (dilution with equivalent volumes of buffer [TBS]) was also performed, and the corrected values are presented. The symbol key is presented on the graph.

are completely solubilized. It should be mentioned at this point that in all cases in which the relative turbidity of the samples measured was below 10%, visual observation of the dispersion revealed that the dispersions were completely clear (no floating aggregates were present on their surface), favoring the conclusion that vesicles are completely solubilized.

The turbidity measurement results for the PC/Chol SUV liposomes (Fig. 4) show a drastic decrease in vesicle turbidity, even at lower CD concentrations (compared to the PC [without Chol] liposomes). In fact these liposomes are completely solubilized when dispersed in a medium containing ∼100 mg/mL Meβ-CD. Thereby, when Chol is included in the vesicle membrane perhaps due to a faster extraction of Chol molecules from the liposome bilayers, the vesicles are completely lysed (or solubilized) at lower Meβ-CD concentration, compared to liposomes without Chol.

Another fact that should be mentioned is that in all cases of SUV liposomes the relative turbidity measured was not affected by incubation time (values measured 1 min after mixing liposome dispersions with CDs were unchanged during the full 24-h period studied).

A turbidity experiment was also carried out for PC DRV liposomes, in presence of Meβ-CD. These later results are presented in Fig. 5 in combination with the results of PC SUV liposomes, for direct comparison. Since lower lipid concentration had to be used in the case of the DRV liposomes due to their higher turbidity (as expected by their larger size), the values are shown as relative turbidity versus CD/lipid mol/mol ratio. As seen, relative turbidity values measured for DRV liposomes are incubation-period dependent (between the 1 min and 1 h period). However, no further decrease in relative turbidity was noticed after 1 h (not shown here). Since this was not the case for the SUV liposomes it is logical to hypothesize that it is connected with the increased lamellarity of DRV liposomes (compared to unilamellar SUVs).

The turbidity values measured for DRV and SUV liposomes under the same conditions show that DRV liposomes are less stable (compared to SUV) in presence of Meβ-CD, correlating well with the membrane integrity results (Figs. 1A and 3A). Additionally, the very high stability of DSPC SUV liposomes in presence of Meβ-CD, is confirmed by this study (Fig. 5, triangles).

3.4. Size distribution studies of liposome dispersions in absence and presence of different cyclodextrins

In order to have extra proof that the decreases in relative turbidity measured when CDs were mixed in the liposomes, is attributed to vesicle size reduction we performed a small scale study with MLV and SUV liposomes composed of PC and PC/Chol. The vesicle mean diameters were measured immediately after preparation and also after incubation in presence of high concentrations (ranging between 80 and 125 mg/mL) of HPβ-CD Meβ-CD. The results of this study are presented in Table 2. As seen, for the PC-MLV liposomes, vesicle size is not affected by HPβ-CD but is substantially decreased by Meβ-CD. At very high CD/lipid ratio (379) the liposomes are completely dissolved (or solubilized) as seen by visual observation of the samples. This is also the case for the PC/Chol MLV liposomes, that are also solubilized when incubated at high CD/lipid ratio (379) of both HP- and Meβ-CD.

The results of the size measurements of the SUV's studied also provide proof that when liposomes are incubated in presence of CD molecules, especially when high CD/lipid ratios apply, their size is significantly decreased, Although, most or the measurements in presence of Meβ-CD could not be performed due to the high foaming of the samples (air bubble interference), or if performed their polydispersity was too high (0.90–1.0, this is why they are not presented in Table 2), upon visual observation,

Table 2
Mean diameters of MLV and SUV liposomes composed of PC or PC/Chol, before and after incubation in presence of different concentrations of CDs

Lipid composition	CD	CD/lipid	Mean diameter
MLV liposomes (mean diameter in μm)			
PC	No CD	0	3.57
	HPβ-CD	76	3.48
		379	3.62
	Meβ-CD	76	2.91
		379	Clear[a]
PC/Chol	No CD	0	4.19
	HPβ-CD	76	3.84
		379	Clear[a]
	Meβ-CD	76	3.76
		379	Clear[a]
SUV liposomes (mean diameter in nm)			
PC	No CD	0	118.5 ± 9.8 [0.523]
	HPβ-CD	76	118 ± 12 [0.484]
		379	16.0 ± 3.1 [0.373]
	Meβ-CD	76	Not measurable[b,c]
		379	36.8 ± 7.1 [0.370]
PC/Chol	No CD	0	93 ± 29 [0.592]
	HPβ-CD	76	108.1 ± 5.6 [0.643]
		379	47 ± 17 [0.563]
	Meβ-CD	76	Not measurable[b,c]
		379	Not measurable[b,d]

For MLV liposomes the uniformity value of all measurements ranged between 0.31 and 0.55. For the SUV liposomes, polydispersity index values are presented in square brackets.
[a] The dispersion is completely clear by visual observation. No obscuration can be achieved, too low signal.
[b] The samples could not be measured due to very high foaming.
[c] The samples could not be measured due to very high polydispersity.
[d] The samples could not be measured due to very low signal.

the samples of PC/Chol SUVs incubated with the highest Meβ-CD concentration used (Meβ-CD/lipid mol/mol = 379), were completely clear, indicating that the vesicles are completely dissolved. This observation provides further proof of the good correlation between relative turbidity and mean size measurements.

4. Discussion

Herein the effect of three different cyclodextrin molecules on the membrane integrity and relative size of liposomes was studied. Liposomes of different type (size and lamellarity) and lipid composition were investigated. The results obtained can be useful when preparation of stable liposomal formulations of lipophilic drugs – by encapsulating drug–cyclodextrin-complexes in aqueous compartments of lipid vesicles – is applied (McCormack and Gregoriadis, 1996; Fatouros et al., 2001; Maestrelli et al., 2006; Piel et al., 2006; Hagiwara et al., 2006).

The experimental results prove that liposomes are destabilized (their integrity decreases) by some cyclodextrins, as previously documented (Nishijo et al., 2000; Boulmedarat et al., 2005). However, the novelty of this study is that it is proven herein that CD-induced liposome destabilization depends on both; liposome type and lipid composition. Additionally, the type of cyclodextrin is also a major determining factor affecting not only the degree of vesicle destabilization but also the mechanism by which it occurs.

In respect to cyclodextrin type, for all cases studied Meβ-CD was found to cause higher destabilization of liposomes, compared to HPβ-CD and HPγ-CD. In fact, both HPβ- and HPγ-CD, had more or less the same result on the vesicle membrane integrity, for all the vesicles studied. This suggests that for destabilization of liposomes by cyclodextrins the lipophilicity – and not the size – of the cyclodextrin cavity is most important, implying that cyclodextrin ability to permeate in the vesicles is crucial for substantial decrease of liposome membrane integrity and final vesicle "solubilization" to occur. It has been previously reported that Meβ-CD destabilizes liposomes by a surfactant-like mechanism (CD molecules form mixed micelles with lipids, as is the case with surfactants). The decrease in turbidity noticed is most possibly due to extraction of lipid components from the vesicle bilayers and re-organization of the remaining lipids into smaller vesicles, or finally micelles. When relative turbidity of the vesicle dispersions approaches zero, a large number (or perhaps all) of the lipid molecules of the vesicles are probably in the form of CD-complexes or micelles. This was confirmed by vesicle size measurements and visual observation of the samples. The fact that Meβ-CD (the most lipophilic) is the only CD type demonstrated to dissolve liposomes totally (under the conditions applying in the membrane integrity and turbidity studies), suggests that perhaps internalization of CD molecules in lipid membranes is a prerequisite for total vesicle disruption, when CD concentrations are lower than 100 mg/mL.

Interestingly, liposome type seems to be a determining factor in the extent of membrane integrity decrease caused by the various cyclodextrin molecules studied. Indeed: (i) SUV liposomes, the only liposome type of the three studied that are unilamellar, were found to be the most stable (Figs. 1–3). A plausible explanation for the high stability demonstrated by SUV liposomes, which were only affected by Meβ-CD (the most lipophilic of the CD molecules used), may be that curvature of the vesicle surface does not permit initial interaction between membrane components and CD molecules, for the CD molecules that interact with the liposomes only by surface contact. However, Meβ-CD that passes through the bilayer into the liposomes, as also proposed by others (Nishijo et al., 2000), interacts also with the lipids of the inner side of the membrane bilayer. Nevertheless, SUV liposomes are more stable compared to DRV even in presence of Meβ-CD as demonstrated also by turbidity results (Fig. 5) (higher concentrations of Meβ-CD are needed for initiation and completion of vesicle solubilization). This may imply that initial contact of CD with the liposome membrane is important also for Meβ-CD–membrane interaction and vesicle internalization.

DSPC SUV liposomes are stable even in presence of Meβ-CD (Fig. 3), implying that lipid membrane rigidity is another factor that plays a key role in the interaction between CD molecules and liposomes (possibly because it affects the ability of Meβ-CD to enter into the vesicles, or its initial interaction with membrane components).

(ii) DRV vesicles show the same stability in presence of all cyclodextrins studied, with a small exception of H-PC/Chol

and DSPC/Chol liposomes, which, after 24 h of incubation are affected slightly more by Meβ-CD compared to the other CD molecules (Fig. 1). This is a very important finding when the drug-in-CD-in-liposome system is considered, since the DRV liposome is the vesicle type, which offers very high (aqueous phase) entrapment possibilities. Thereby, in applications where high entrapment values and also high retention of the drug in the liposomes is required, perhaps the best formulation will be a cyclodextrin-complex of the drug entrapped in DRV liposomes composed of DSPC, the ones demonstrated to be most stable.

Nevertheless, this last piece of practical advice should be also confirmed in cases of specific drug applications, since the specific drug–CD association constant is also important when drug-in-CD-in-liposome systems are used. When a drug is tightly associated with a specific CD molecule, and cannot be displaced by Chol or other lipid components of liposome membranes, then perhaps even CD molecules that induce membrane solubilization may be used if that specific CD offers other advantages for the specific drug that is being formulated, as high entrapment efficiency. This was demonstrated recently with betamethasone (Piel et al., 2006).

Concluding, although the mechanisms implicated are not fully elucidated, the results presented here, together with data concerning the association of the drug with different cyclodextrin molecules, can provide a useful platform for formulation development when a lipophilic drug is to be formulated into liposomes.

In addition, the present results are also useful when applications of naked drug–CD complexes for iv-administration are considered (for drugs with low solubility). In such cases, interaction of CD molecules with Chol of red blood cells may lead to drug displacement from the complexes and subsequent precipitation.

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