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Features of the Interaction between Cyclodextrins and Colloidal Liposomes

I. PUSKÁS[1], L. BARCZA[2], L. SZENTE[3], and F. CSEMPESZ[1,*]
[1]*Department of Colloid Chemistry Eötvös University, P.O. Box 32 H-1518, Budapest 112, Hungary;* [2]*Department of Inorganic and Analytical Chemistry Eötvös University, P.O. Box 32H-1518, Budapest 112, Hungary;* [3]*Cyclolab Cyclodextrin Research & Development Laboratory Ltd., H-1097, Budapest, Illatos u. 7., Hungary*

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Abstract

Interactions in aqueous dispersion between dipalmitoyl phosphatidyl choline (DPPC) liposomes and dissolved cyclodextrins (CD) of different chemical compositions, respectively, were studied. Liposomal dispersions with small unilamellar vesicles (SUV) of monomodal size distribution were prepared and the physical stability of the vesicles both in the presence and absence of cyclodextrin was investigated. For the characterization of the kinetic stability of the dispersions under various conditions, size-distribution functions determined by photon-correlation spectroscopy (PCS) were used. The affinity of cyclodextrins to the liposomes was characterized by 'binding isotherms' determined under equilibrium conditions at 25 °C. Based on the quantity of the cyclodextrins bound to the DPPC bilayers, stability constants for the associates were estimated. The physical stability of the liposomes and the possible control of stability were also investigated.

Introduction

Considerable efforts in pharmaceutical research have been made recently to develop new drug delivery systems, which enhance the efficacy and safety of existing drugs [1]. Colloidal carriers (nanoparticles, microspheres, liposomes, etc.) and also, 'molecular- or nanocapsules' (such as cyclodextrins) provide numerous opportunities for the formulation of controlled release and site-specific drug delivery systems [2]. Biologically active materials can be adsorbed on the surface of the particles or incorporated into the carrier. The encapsulation (or adsorption) efficiency, the organ distribution of the carrier and accordingly, the therapeutic activity of the drug can be controlled by modifying the composition and/or the surface properties of the carrier [3].

Liposomes find perhaps the most extensive use as drug carriers [4]. Due to the bipolar character of phospholipid bilayers or multilayers, both hydrophilic and lipophlilic compounds, i.e. in principle almost any type of drug can be encapsulated into vesicula [5].

It is well known that cyclodextrins (CDs) are capable of forming inclusion complexes with many drugs and other compounds by taking up a molecule (or its hydrophobic part) into the cavity [6]. The complexation will affect many of the physicochemical properties, e.g. the aqueous solubility of sparingly soluble drugs. A hard obstacle in the use of cyclodextrins is that certain CD derivatives can interact with the membrane components of living cells resulting in irreversible changes in the structure of the membrane [7]. Such structural damages may lead to considerable reduction in the physical stability and/or alterations in the permeability for a drug of the cell membrane [8, 9].

The purpose of the present work is 2-fold. First is to test the effect of natural CDs on the kinetic stability of colloidal dipalmitoyl phosphatidyl choline (DPPC) liposomes, and second, to characterize the stability of possible DPPC–CD complexes. To this end, the mean size, size distribution and polydispersity of the liposomes and the change in time of these characteristics have been measured in the presence and absence of dissolved CDs. In addition, the sorbed amount of CD derivatives onto DPPC bilayers and the stability constants of the possibly formed complexes have been calculated.

Experimental

Materials

Dipalmitoyl phosphatidyl choline (DPPC) from Sigma Chemical Co. (St. Louis MO) was used for the preparation of liposomes.

The *α-, β-, γ-cyclodextrin* and the chemically modified *heptakis(2,6-di-O-methyl)-β-cyclodextrin* (*DIMEB*) used in these investigations were the products of Cyclolab Cyclodextrin Research & Development Laboratory Ltd., Budapest.

* Author for correspondence. E-mail: csf@ludens.elte.hu

The phospholipid and the CD derivatives were of pharmaceutical or analytical grade.

Methods

SUV (small unilamellar vesicles) liposomes were prepared by a modified method described by Kremer *et al.* [10]. 0.050 g DPPC was dissolved in 2.50 cm^3 abs. ethanol and 240 μl of this solution was added to 4.50 cm^3 aqueous solution of CD by a micropipette. The sample was then sonicated for 5 min by an ultrasound equipment type Realsonic 40SF (max. electric power: 300 W, frequency of ultrasound: 37 kHz). The bath of the sonicator was incubated at constant temperature in the range of 25.0 – 45.0 °C. The homogenous dispersion of the freshly prepared DPPC liposomes exhibited a slight opalescence.

The change in time of the particle size, size distribution and polydispersity are sensible markers of the kinetic stability of dispersions. All these parameters in the dispersions of DPPC liposomes at several molar ratios of DPPC and CD have been determined by photon correlation spectroscopy (PCS) at 25 °C, using a Zetasizer 4 (Malvern Inst. UK) instrument.

Results and discussion

Size distribution of pure DPPC-liposomes

The dispersions used both in the stability measurements and the investigations of sorption were formulated at 36.5 °C. The size distribution functions, number (or volume) average of the vesicles and their polydispersity index were determined by PCS analysis. Since the loading capacity of vesicles closely correlates with the entrapped volume, in this study the volume average diameter and/or the volume distribution function (Figure 1) were usually given as characteristics for a liposome dispersion.

These results demonstrate that SUV liposomes with narrow size distribution were prepared by the method improved as described above. The adequacy of the results of PCS analysis has been confirmed by electron microscopy, as well. In Figure 2, a freeze-fracture (electron) micrograph of the liposomes is shown. The diameter of the spherical particles is in the range of 40–50 nm.

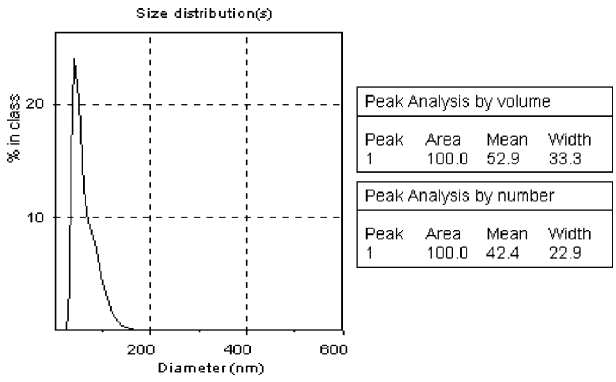

Figure 1. Size distribution function of dipalmitoyl phosphatidyl choline liposomes formulated at 36.5 °C.

Binding of cyclodextrin molecules on the membrane of liposomes

Possible interactions between dissolved cyclodextrins and the liposomes were studied by 'sorption' investigations. In these measurements, the liposomes were prepared in cyclodextrin solution of known concentration and stored for 2 days at 25 °C. After the 'sorption period', the samples were ultracentrifuged (30,000 rpm) for 30 min. in order to separate the vesicles together with the bound CD molecules from the medium. The concentration of the free cyclodextrin in the equilibrium solution ([CD]) was determined by a spectrophotometric assay described by Buvári and Barcza [11], and the amount of CD molecules bound onto the (separated) DPPC bilayers (c_{CD}-[CD]) has been calculated.

The affinity of cyclodextrins for the DPPC membrane was characterized by 'binding isotherms', shown in Figure 3.

In the Figure, the molar ratio of the bound cyclodextrin (n_{CD}) to the phospholipid (n_{DPPC}) is plotted against the CD concentration in the equilibrium solu-

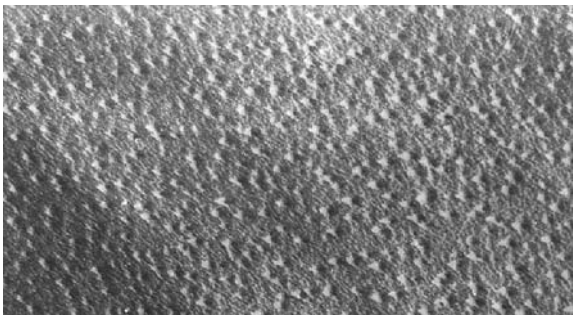

Figure 2. Electron micrograph of the dipalmitoyl phosphatidyl choline liposomes [particle concentration: 0.1%(w/w)].

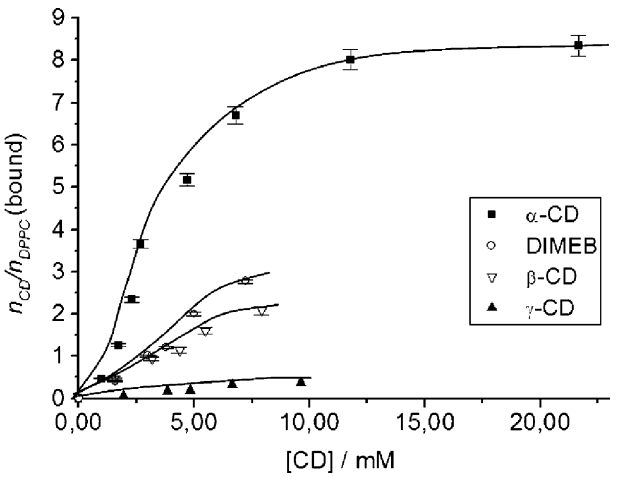

Figure 3. Binding isotherms for cyclodextrins on dipalmitoyl phosphatidyl choline membranes.

tion ([CD]). The results well demonstrate the considerable differences in the affinity of the different cyclodextrins for the lipid membranes. The amount of cyclodextrin bound by 1 mol DPPC increases in the order: γ-CD < β-CD, (DIMEB) < α-CD. This order is presumably due to the differences in the cavity size of the cyclodextrin molecules. In addition, the methylated β-CD (DIMEB) exhibits a slightly higher affinity for the vesicles than the natural β-CD, indicating that the surface groups of the β-CD derivative might also contribute to the CD–phospholipid interaction.

These results provide experimental evidences for the idea that molecular complexes with different molar ratio may form when the cyclodextrins bind to the DPPC membrane.

Theoretical approach to the CD–DPPC association

The mean value (Q) of the number of cyclodextrin molecules in the associate bound to the DPPC, (represented by 'binding isotherms' in Figure 3 can be given as

$$Q = \frac{c_{CD} - [CD]}{c_{DPPC}} \left(= \frac{n_{CD}}{n_{DPPC}} \right), \qquad (1)$$

where c_{CD} is the total (known) concentration of cyclodextrin added, [CD] is its (measured) equilibrium concentration and c_{DPPC} is the molar concentration of DPPC.

Assuming that different types of associates are formed:

$$c_{CD} = [CD] + \sum q\left[(DPPC)_p(CD)_q\right], \qquad (2)$$

where $[(DPPC)_p(CD)_q]$ represents the equilibrium concentration of the associate formed in the following interaction:

$$p\text{DPPC} + q\text{CD} \Leftrightarrow (\text{DPPC})_p(\text{CD})_q, \qquad (3)$$

Similarly, the total DPPC concentration can be given as

$$c_{DPPC} = [DPPC] + \sum p[(DPPC)_p(CD)_q]. \qquad (4)$$

The general formation constant for any associate (Equation (3)) can be defined as:

$$\beta_{pq} = \frac{\left[(DPPC)_p(CD)_q\right]}{[DPPC]^p[CD]^q}, \qquad (5)$$

and the generalized forms of the total concentrations are:

$$c_{CD} = [CD] + \sum q\beta_{pq}[DPPC]^p[CD]^q, \qquad (6)$$

$$c_{DPPC} = [DPPC] + \sum p\beta_{pq}[DPPC]^p[CD]^q. \qquad (7)$$

Before starting the evaluation, some assumptions have to be made:

- (*a*) two equilibria exist in the system: one of them is valid for the equilibrium between the CD and DPPC molecules in the aqueous phase (characterized by equations (5)–(7)) and the second one for the same species in the colloidal phase;
- (*b*) the total and constant concentrations in the aqueous phase are guaranteed by the presence of the colloidal phase since fast and continuous exchange exists among them;
- (*c*) the supernatant after ultracentrifugation is the aqueous phase alone, it contains practically no colloidal DPPC (i.e. the CD content measured is really identical with [CD]);
- (*d*) by analogies of many inclusion complexes [6], the p value (the number of monomeric DPPC molecules in the associate) can be assumed as constant 1;
- (*e*) in spite of this – referring to Schlenk and Sand [12] – the q value (the number of CD molecules in the complex) can be assumed higher than 1 (see for comparison the 'necklace' structures, e.g. [13])
- (*f*) being DPPC a dipalmitoyl derivative, the q values can be changed by pairs. In the first step, the Q values were calculated from the experimental data listed in Table 1.

Knowing the total concentrations (c_{CD} and c_{DPPC}) as starting data and measuring the equilibrium concentration of cyclodextrin [11], the values of stability constants can be calculated using Equations (1) and (6)–(7) by an iterative computer program (with different sets of equilibrium constants), which searches for the best fit between the experimental and computed values.

Assumptions *d-e-f* can be checked by supposing different species in the computer calculation and see the fit with the experimental data. Assumption *d* (i.e. the p value is always 1) is highly verified, while the Q values (primary data in Table 1) themselves prove the reality of assumption *e*. Assumption *f* is rather sophisticated and will be discussed later.

Assumptions *b* and *c* are of first importance and the possibility of calculations itself gives positive answer. To ensure point *b*, different concentrations of DPPC were also used (see Table 1) and samples stored more than 2 days (then ultracentrifuged) were also investigated, but the results (i.e. the measured Q values) fit well to the others proving this way the correctness of assumption *b* (and to some extent that of assumption *c*). As a direct proof, the phosphorus content of the supernatant was

Table 1. Initial concentration of dipalmitoyl phosphatidyl choline and cyclodextrins and the corresponding (relative) bound quantity (Q) of cyclodextrins

CD	c_{DPPC}/M	c_{CD}/M	Q
γ-CD	1.40×10^{-3}	2.02×10^{-3}–1.02×10^{-2}	0.06–0.38
DIMEB	1.40×10^{-3}	2.17×10^{-3}–1.11×10^{-2}	0.41–2.77
β-CD	1.40×10^{-3}	2.17×10^{-3}–1.08×10^{-2}	0.46–2.08
α-CD	7.00×10^{-4}	1.57×10^{-2}	7.30
	1.05×10^{-3}	1.24×10^{-2}–2.71×10^{-2}	6.10–8.16
	1.40×10^{-3}	1.66×10^{-3}–3.33×10^{-2}	0.47–8.25
	1.75×10^{-3}	2.11×10^{-2}–2.18×10^{-2}	6.87–7.09

determined by ICP-AES (LABTAM 8440 – Melbourne, Australia) at 213.618 nm wavelength. The concentration was below 0.60 mg/dm^3 indicating that more than 98.6 % of the species containing DPPC were removed by ultracentrifugation.

Complex stability constants

The relative bound quantity of cyclodextrins onto DPPC membranes was found to be independent of the phospholipid content of the dispersions in the concentration range of $7.00 \times 10^{-4} - 1.75 \times 10^{-3}$ M.

Since in the case of γ-cyclodextrin Q is less than 1 at any CD-concentration, formation of 1:1 ($p = q = 1$) complex could only be supposed, but the calculations gave better fit when both 1:1 and 1:2 associates were included. (The fit is worse assuming 1:2 complex alone.)

In spite of the fact that Q is less than 3 both in the β-CD and DIMEB complexes of DPPC, the best fit between the measured and calculated values can be obtained with the whole series of $q = 1$–4 ($q = 1$–3 is less effective). The computations give nearly similar (a little bit worse) fits when 1:2 and 1:4 species are assumed. The numerical values of association constants are higher than that of γ-CD but they are – as expected – very similar.

DPPC forms the most stable and most complicated complexes with α-CD and the whole series up to 1:10 must be considered for finding a good fit. Since the last value seems too high for *one* palmitoyl (i.e. hexadecanoic) group [12], the collateral participation of both substituents is highly indicated by the stoichiometric ratios of α-CD associates. Really, the best fit was not obtained by taking the whole series but by the combination of 1:2–1:4–1:6–1:8–1:10 mol/mol associates.

The results are collected in Table 2. The standard deviations of the constants are within ±10–15%.

These results provide theoretical basis for the sorption measurements. They confirm possible formation of molecular complexes in cyclodextrin-containing liposomal dispersions. Considering the relative amount of the CD and the DPPC molecules in an associate, it is reasonable to assume that the cyclodextrin molecules are 'strung out' (like beads) on the apolar chains of phospholipid.

Effect of cyclodextrins on the stability of the liposomes

The effect of dissolved CDs on the physical stability of DPPC liposomes was investigated by measuring in time the average size of the vesicles, formulated in CD-solutions. Figure 4a shows the relative increase in the mean size of the liposomes (D/D_0) as compared to size of the vesicles prepared in absence of cyclodextrin (D_0). The temperature of the storage was 25 °C and the molar ratio of the DPPC and the cyclodextrin (n_{DPPC}/n_{CD}) was 1:7.

Monomodal size distribution for the pure DPPC dispersion was detected after a 7-day storage, as well.

Table 2. Formation constants of dipalmitoyl phosphatidyl choline-cyclodextrin complexes

	1:1	1:2	1:3	1:4	1:6	1:8	1:10
γ-CD	3.6×10^1	1.2×10^3					
DIMEB							
(a)	1.7×10^2	3.6×10^4	7.6×10^6	1.2×10^9			
(b)		7.8×10^4		1.3×10^9			
β-CD							
(a)	2.9×10^2	4.6×10^4	4.3×10^6	3.8×10^8			
(b)		7.9×10^4		3.3×10^8			
α-CD		1.5×10^5		3.0×10^{10}	2.0×10^{15}	3.0×10^{19}	3.0×10^{22}

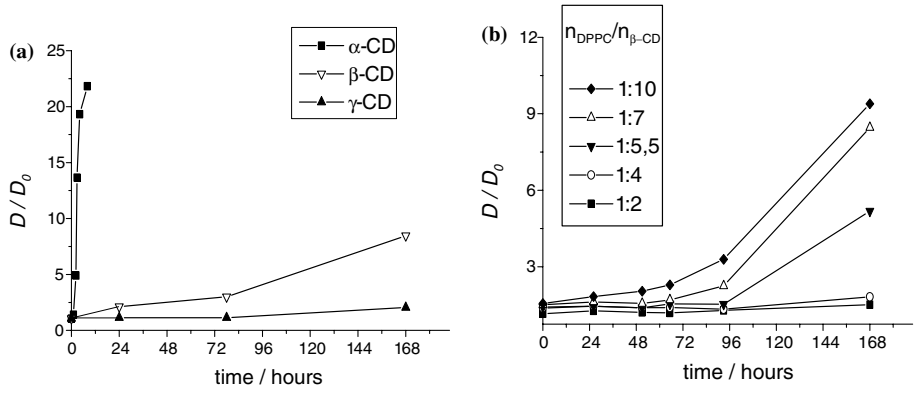

Figure 4. Relative increase in size of dipalmitoyl phosphatidyl choline liposomes in dispersions (a) in the presence of natural cyclodextrins, (b): in the presence of β-cyclodextrin of various concentration.

However, in presence of cyclodextrin the average size of the vesicles increased significantly even after a few days, and the samples became highly polydisperse. These changes clearly show that due to the dissolved CD molecules, the physical stability of the liposomal dispersion definitely decreased and aggregation and/or fusion of the vesicles took place.

The order of reducing the physical stability of the DPPC membranes (above a critical CD/DPPC ratio) was found to be the same as that of the affinity for the phospholipid membranes of the CDs, (i.e.: γ-CD < β-CD < α-CD) obtained from the sorption measurements. In addition, it is well demonstrated that the effect of the different cyclodextrins on the DPPC bilayers also depended on the phospholipid/CD ratio. The relative increase of the vesicle size in presence of β-CD (Figure 4b) indicates that β-cyclodextrin brings about notably diminished membrane stability only above a DPPC/CD ratio of 1:5. Similar effects with the α- and the γ-CD were also found indicating that dissolved cyclodextrins, under certain conditions, may cause considerable damages in the structure of the phospholipid membranes.

Conclusions

In this work interactions in binary systems between (SUV) DPPC-liposomes and cyclodextrins were studied. It was found that α, β, γ-CDs form complexes of different stoichiometry with dipalmitoyl phosphatidyl choline. The stability constants for relevant complexes at various compositions were calculated. The sorption of cyclodextrin molecules onto DPPC liposomes increases in the order: γ-CD < β-CD, (DIMEB) < α-CD. The order of reducing the physical stability of the DPPC due to the dissolved cyclodextrins was the same, i.e.: γ-CD < β-CD < α-CD.

Acknowledgement

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