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NOVEL AMPHIPHILIC CYCLODEXTRINS: SYNTHESIS, CHARACTERIZATION AND PROPERTIES

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A new class of amphiphilic cyclodextrins have been obtained by grafting one single cholesterol moiety on the primary face of cyclodextrins. The properties of these new derivatives strongly depend on the structure of the adduct with a very specific effect of the methylation of the cyclodextrin core. The properties of these compounds have been investigated in details in the absence and in the presence of a synthetic phospholipidic matrix by Nuclear Magnetic Resonance (NMR) and scattering techniques (light, X-rays, neutrons). It has been shown that a high diversity of structures (liposomes, vesicles, micelles) can be obtained depending on the structures of the molecule.

Получен новый класс амфифилических циклодекстринов с помощью пересаживания одной единственной сердцевины холестерола на первичной лицевой стороне (полости) циклодекстринов. Особенности этих новых производных строго зависят от структуры аддукта и очень специфического эффекта метилирования ядра циклодекстрина. Детально изучены особенности этих соединений в отсутствии и в присутствии синтетического фосфолипидного матрикса с помощью ядерного магнитного резонанса (ЯМР) и светорассеивания (свет, рентгеновские лучи, нейтроны). Показано, что большое разнообразие структур (липосомы, полости, мицеллы) может быть получено в зависимости от структур молекул.

Ստացվել է ամֆիֆիլային ցիկլոդեքստրինների նոր դաս խոլեստերոլի միայն մեկ միջուկի ցիկլոդեքստրինների առաջնային երեսի վրա պատվաստման միջոցով: Այս նոր ածանցյալների առանձնահատկությունները խիստ կախված են հաղորդակցող տարրի (ադդուկտի) կառուցվածքից ցիկլոդեքստրինի միջուկի մեթիլացման շատ յուրահատուկ ազդեցությունից: Այս միացությունների առանձնահատկությունները մանրամասն ուսումնասիրվել են սինթետիկ ֆոսֆոլիպիդային մատրիքսի առկայությամբ և բացակայությամբ միջուկային մագնիսական ռեզոնանսի (ՄՄՌ) և լուսացրման միջոցով (լույս, ռենտգենյային ճառագայթներ, նեյտրոններ): Ցույց է տրվել, որ կառուցվածքների մեծ բազմազանություն (լիպոսոմներ, խոռոչներ, միցելներ) կարող է ստացվել կախված մոլեկուլների կառուցվածքներից:

Introduction

Since a number of years, a special attention has been given to the preparation of amphiphilic cyclodextrins in order to use them as new compounds for the preparation of novel materials (nanoparticules for example) or to insert them in a preformed lipidic matrix such as liposomes. In both cases, the main objective of this approach was to combine the size specificity of cyclodextrins for hydrophobic guests and the transport properties of a structure of liposomes or analogs. Before making any attempt to prepare new amphiphilic

cyclodextrins two main questions must be addressed i.e. first, how many lipophilic groups will be grafted and second, on which side (primary or secondary or both) the grafting will be achieved. All situations have been encountered in the literature (see refs. 1-7 in [1]). We decided to focus in all cases depicted here on a mono-substitution on a primary hydroxyl site of the cyclodextrin. Although the chemistry of single site modification is sometimes more tedious to afford highly pure compounds, it can be achieved rather easily in reasonable yields. The selection of substitution on the primary side (on one of the CH_2OH group) offers two advantages : First, the wider secondary face is not modified and it is well documented that most guest molecules enter through this face. Second, since we have developed the production of antibodies against natural and modified cyclodextrins [2] which allow a very sensitive and selective method for the quantitative determination of these hosts and since the epitope in the recognition process is the secondary face without any effect the modification of primary hydroxyls side, the latter is the most appropriate for mono-substitution. Our first attempt to investigate the properties of amphiphilic cyclodextrins was made by grafting an aliphatic chain of various length through an amide bond on one single primary position [3]. This led to the family of "lollipops". Unfortunately, although these compounds exhibit interesting properties, it was shown by NMR that the aliphatic chain shows a strong tendency to include in the cavity of its own cyclodextrin carrier, therefore limiting its potential application. (see figure 1a). This problem of self-inclusion of the aliphatic chain could be avoided by using an aliphatic chain carrying a very bulky end-group in place of the methyl end. This was achieved using a *t*-butyloxycarbonyl moiety which cannot include in the cavity of the CD. This new class of amphiphilic compounds has been investigated in details [4] in terms of insertion in model liposomes and of inclusion of guests in the cavity of the CD. These derivatives were called "Cup and Ball cyclodextrins". It was indeed shown that in the absence of a guest the bulky end-group attempts to enter the cavity through the primary face (Fig. 1b). However, when a guest compound is included in the cavity through the secondary cavity, the chain is expelled as displayed in figure 1c.

Figure 1. Schematic structures of "Lollipops" (a) and "cup and ball molecules" without (b) and with an included guest (c).

The liberated chain is then able to be inserted in a phospholipid bilayer matrix. In the present case as well as all other cases considered later in the present paper, the liposome model selected was made of di-myristoyl-phosphatidylcholine (DMPC) the molecular structure of which is displayed in Figure 2.

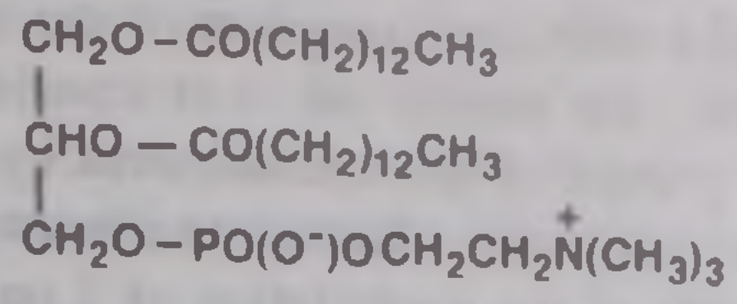
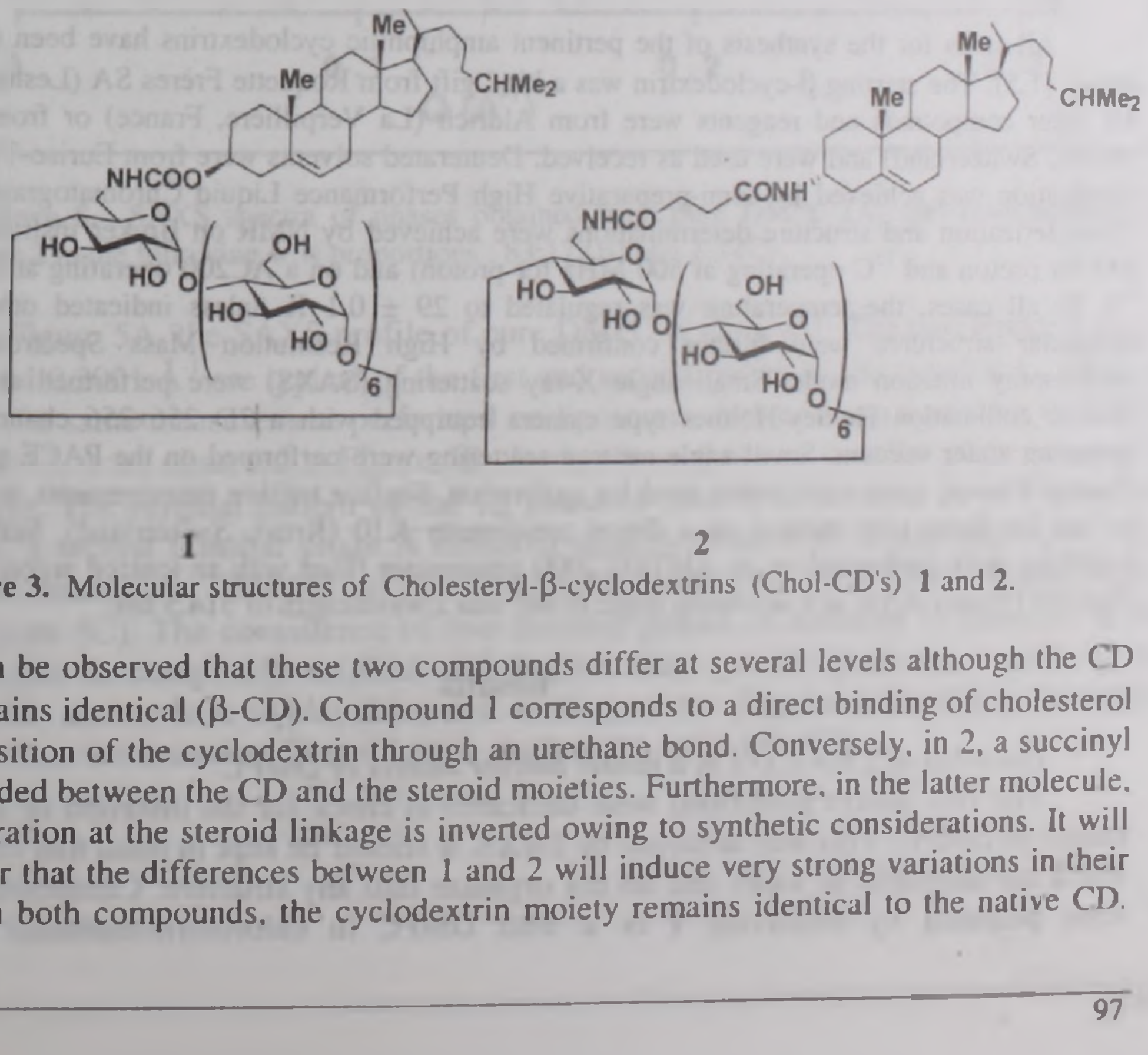


Figure 2. Structure of Dimyristoyl-phosphatidyl-Choline (DMPC).

This matrix was selected for several reasons :

- Hydration leads to the formation of large liposomes formed by well characterized bilayers.
- Sonication leads to small (single-layered) vesicles (average diameter 30 nm)
- The transition temperature between the gel (frozen chains) and the fluid states (molten chains) is ca. 23°C. This allows to make all experiments in a reasonable temperature range to retain the more biologically relevant fluid state.

The use of various techniques indeed allowed to show that the "Cup and Ball" molecules, when loaded by a guest, include in DMPC liposomes or vesicles [4]. The insertion is characterized by a partition coefficient indicating the proportion of amphiphilic complex inserted in the DMPC matrix. However, the insertion level did not reach very high values whatever the length of the chain. We then attempted to overcome these limitations by using a different nature for the hydrophobic moiety of the amphiphilic cyclodextrin. The basic principle of a single grafting at the primary position was retained. The choice was made to use cholesterol or derivatives as hydrophobic part since these compounds are well known to insert easily into phospholipid bilayers and play a key role in the properties of living cell membranes. In a first step two types of molecules were prepared as shown in Figure 3.



1 2

Figure 3. Molecular structures of Cholesteryl-β-cyclodextrins (Chol-CD's) **1** and **2**.

It can be observed that these two compounds differ at several levels although the CD moiety remains identical (β-CD). Compound 1 corresponds to a direct binding of cholesterol on the 6 position of the cyclodextrin through an urethane bond. Conversely, in 2, a succinyl spacer is added between the CD and the steroid moieties. Furthermore, in the latter molecule, the configuration at the steroid linkage is inverted owing to synthetic considerations. It will be seen later that the differences between 1 and 2 will induce very strong variations in their behavior. In both compounds, the cyclodextrin moiety remains identical to the native CD.

All OH groups are unmodified with the exception of the single primary one used for the grafting. In a second step of the results we will consider the effects of the selective replacement of the hydroxyl groups of the cyclodextrin by methoxy groups and it will be shown that very different and surprising effects are observed. We therefore found of very important to examine the effects of the methylation of 2 on its properties. The synthesis of the derivative to be considered further has been described in details in [5]. It corresponds to the structure of 2 but all OH groups in positions C2 and C6 have been methylated. The molecular structure of this compound further labeled as 3 is displayed on Figure 4.

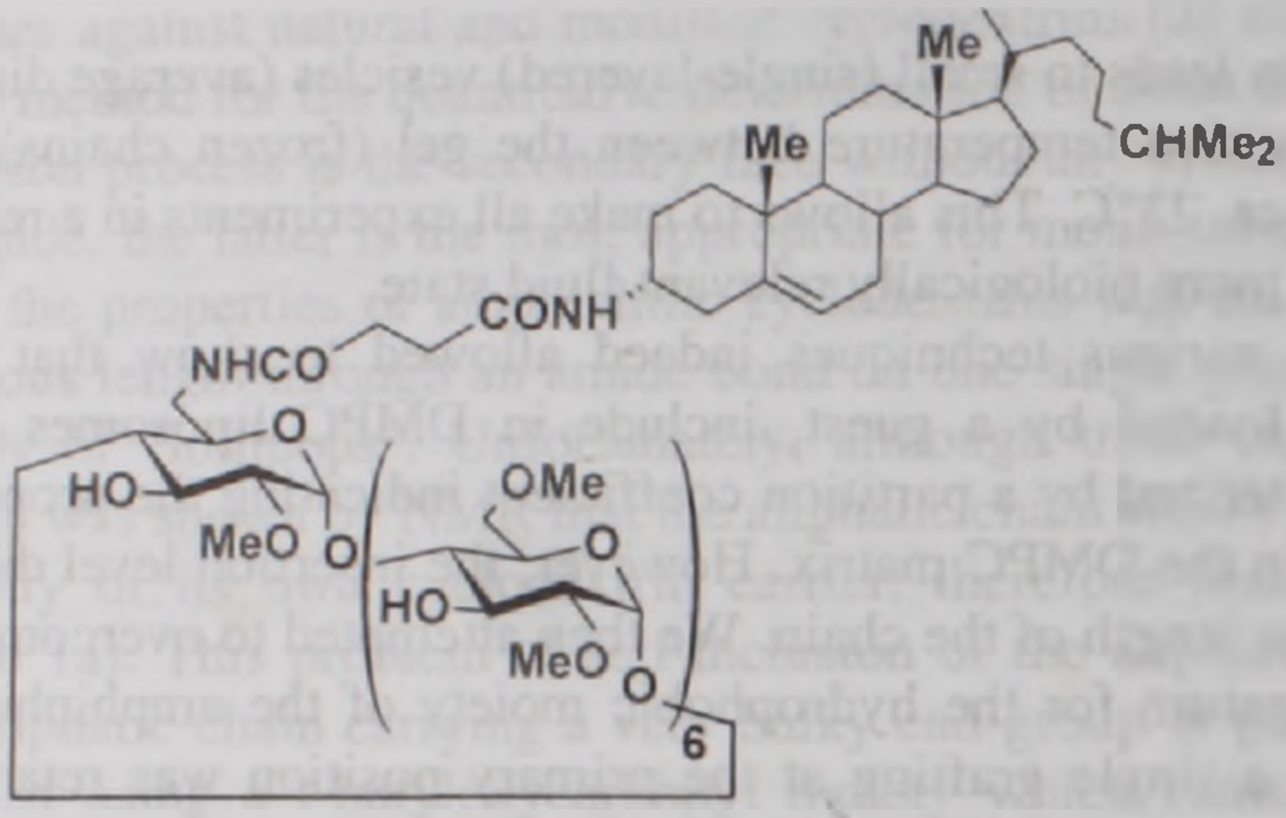


Figure 4. Molecular structure of **3** (Chol-dimeb).

Materials and methods

All steps for the synthesis of the pertinent amphiphilic cyclodextrins have been described in details [1,5]. The starting β-cyclodextrin was a kind gift from Roquette Frères SA (Lestrem, France). All other compounds and reagents were from Aldrich (La Verpillière, France) or from Fluka AG (Buchs, Switzerland) and were used as received. Deuterated solvents were from Euriso-Top (France). Purification was achieved by semi-preparative High Performance Liquid Chromatography (HPLC). Characterization and structure determinations were achieved by NMR on Bruker instruments (DRX 500 for proton and ^{13}C operating at 500 MHz for proton) and on a AC200 operating at 81 MHz for ^{31}P. In all cases, the temperature was regulated to 29 ± 0.1 K unless indicated otherwise. The molecular structures were further confirmed by High Resolution Mass Spectrometry using electrospray infusion mode. Small angle X-ray scattering (SAXS) were performed at 30°C on a pinhole collimation Huxley-Holmes type camera equipped with a 2D 256x256 channels detector operating under vacuum. Small angle neutron scattering were performed on the PACE setup at LLB (Saclay, France) pure water being used for calibration. Surface tension measurements were achieved by the Du Nouy ring method on a digital tensiometer K10 (Krüss, Switzerland). Static and light scattering were performed on an AMTEC 2000 goniometer fitted with an ionized argon laser source (Spectra Physics 2016) at a scattering angle of 90° and a wavelength of 514.5 nm.

Results

Insertion of Chol-CD's in a model bilayer matrix of DMPC

The first assays performed were dedicated to check for the insertion of **1** and **2** in a matrix of DMPC. This was achieved by SAXS. It should be kept in mind that compounds **1** and **2** are insoluble in water and do not organize into any structure. Composite liposomes were prepared by dissolving **1** or **2** with DMPC in chloroform-methanol (2:1, v:v).

evaporating to dryness under vacuum to a glassy film, hydration in excess water and vortexing to obtain a milky suspension of the liposomes. This was done using various ratios of **1** or **2** vs. DMPC in order to follow the evolution of the structure(s). In all cases presented in this work, the total lipid:water ratio was set to 1:4

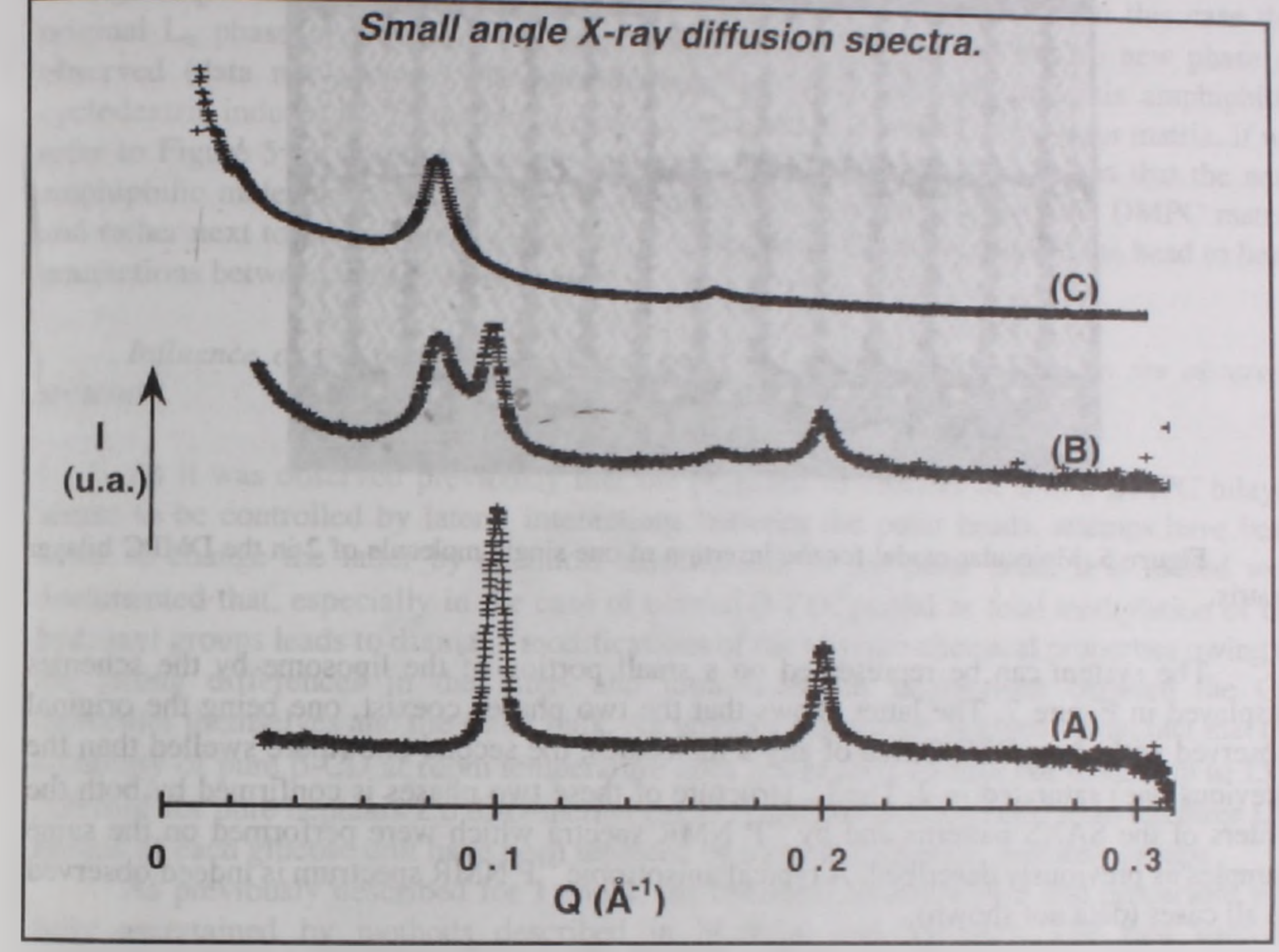


Figure 5. SAXS spectra of phases obtained from pure DMPC (A) and from mixtures of DMPC and **2** in the following w:w proportions : 83:17 (B) and 2:3 (C).

In Figure 5A, the SAXS profile of pure DMPC is observed The two Bragg peaks at 0.1005 and 0.2001 $Å^{-1}$ are typical of the first and second orders of the lamellar L_α phase of DMPC. The same measurement performed on liposomes derived from a mixture of DMPC and **2** (83:17, w:w) observed in Figure 5B clearly shows that two lamellar phases coexist in the sample. The original pattern of the L_α phase of DMPC is retained as in Figure 5A. However, a second lamellar phase is observed with two orders at 0.0845 and 0.1672 $Å^{-1}$. Further increasing the DMPC:**2** to 2:3 (w:w) leads to the pure "new phase" further called $L_{\alpha CD}$ (Figure 5C). The coexistence of two lamellar phases of different thicknesses in the same sample is completely original and implies that specific processes involving the cyclodextrin moiety have to play a key role in this process. The insertion of **2** in the matrix of DMPC is schematized on Figure 6 for a single guest molecule of **2**.

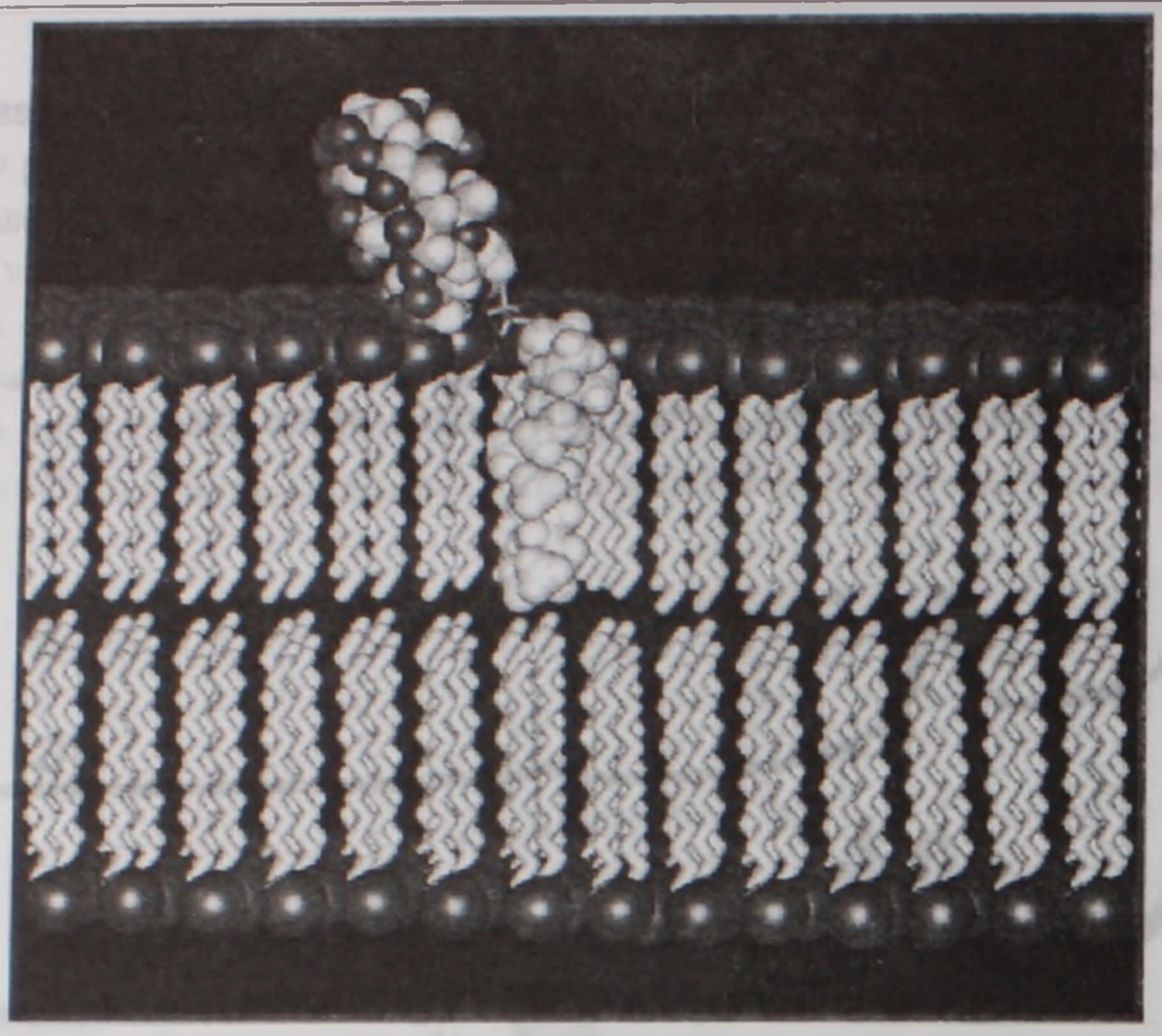


Figure 6. Molecular model for the insertion of one single molecule of **2** in the DMPC bilayer matrix.

The system can be represented on a small portion of the liposome by the schemes displayed in Figure 7. The latter shows that the two phases coexist, one being the original observed with pure DMPC free of any **2** molecules, the second one (more swelled than the previous one) saturated in **2**. The L_α structure of these two phases is confirmed by both the orders of the SAXS patterns and by ^{31}P NMR spectra which were performed on the same samples as previously described. A typical anisotropic ^{31}P NMR spectrum is indeed observed in all cases (data not shown).

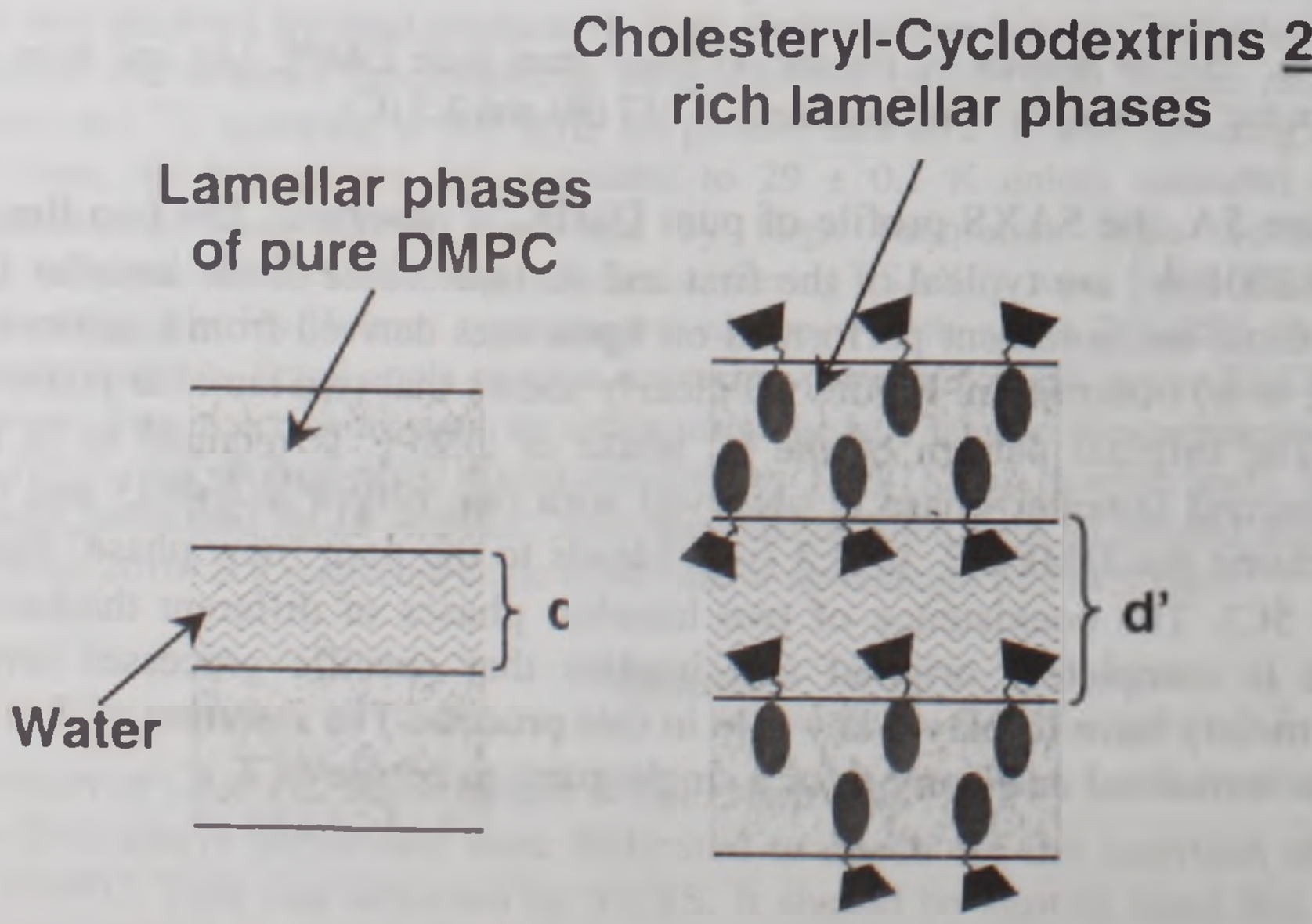


Figure 7. Structures of the two coexisting phases in mixtures of DMPC and **2**

The original behavior of these samples implies that the CD moiety plays a very important role in the structure of the mixed samples. It can be explained by strong interactions between the CD polar heads leading to clusters saturated in **2** in the presence of normal pure DMPC bilayer domains. These "lateral interactions" will be confirmed later in this paper. The presence of a spacing group between the CD and the cholesterol derivative is of high importance since the same phenomenon is not observed with **1**. In this case the original L_α phase of DMPC is strongly perturbed and less ordered but no new phase is observed (data not shown). The specific behavior of **2** indicates that this amphiphilic cyclodextrin induces the formation of clusters saturated in **2** in the DMPC layer matrix. If we refer to Figure 5 in which one single molecule of **2** is represented, it means that the next amphiphilic molecule will also insert in the matrix but not anywhere in the DMPC matrix and rather next to the previously installed **2** molecule in order to optimize the head to head interactions between the CD head-groups.

Influence of the chemical modification of the cyclodextrin moiety on the observed structure.

As it was observed previously that the presence of clusters of **2** in a DMPC bilayer seems to be controlled by lateral interactions between the polar heads, attemps have been made to change the latter by chemical modification of the polar head. It is indeed well documented that, especially in the case of normal β-CD, partial or total methylation of the hydroxyl groups leads to dramatic modifications of the physico-chemical properties owing to the strong differences in the inter- and intra-molecular interactions between the CD molecules themselves and the water bulk. An obvious illustration is given by the fact that the solubility of pure β-CD at room temperature does not exceed 15 mM but climbs up to 150-200 mM for pure heptakis 2,6 di-*O*-methyl β-CD (DIMEB) although two from the three OH groups of each glucose unit have been replaced by more hydrophobic methoxy groups

As previously described for **1** and **2**, the chemical structure of **3** and precursors was fully ascertained by methods described in Material and Methods. The first surprise concerning this compound is that it is highly soluble in water reaching 800g/L in water at 25°C. It gives a single isotropic line in ^{31}P NMR indicating the absence of very large objects. This encouraged us to attempt surface tension measurements to look for the presence of a critical micellar concentration (CMC). It was indeed found [5] that a clear transition is observed vs. concentration of **3** in water indicating a CMC of ca. $5\ 10^{-6}$ M at 25°C; Dynamic and static light scattering confirmed that the observed objects are not small vesicles but compact objects with an average radius of 30 Å. The exact shape of the latter was confirmed by SANS and the experimental data fitted with several models. The SANS profile and fittings are reported on Figure 8.

It clearly appears from this figure that the objects obtained in water with **3** correspond to almost monodisperse spherical micelles with an average number of 24±4 molecules per micelle and an average diameter of 5 nm. A schematic representation of the latter object (containing 24 monomers) is displayed on Figure 9.

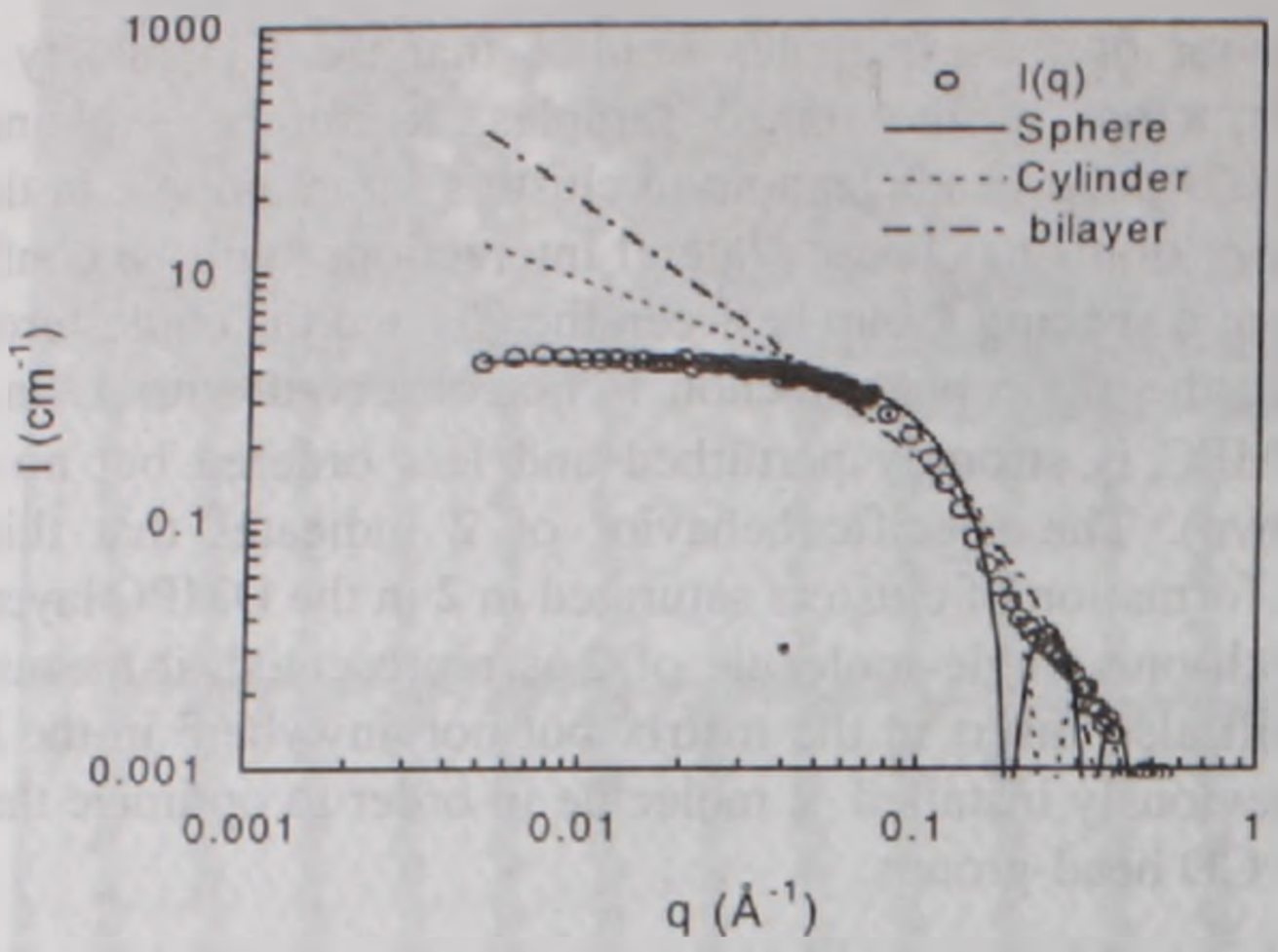


Figure 8. SANS of micelles obtained from **3** in water and demonstration of the spherical shape by attempts to fit the results with other models (cylinders and bilayers)

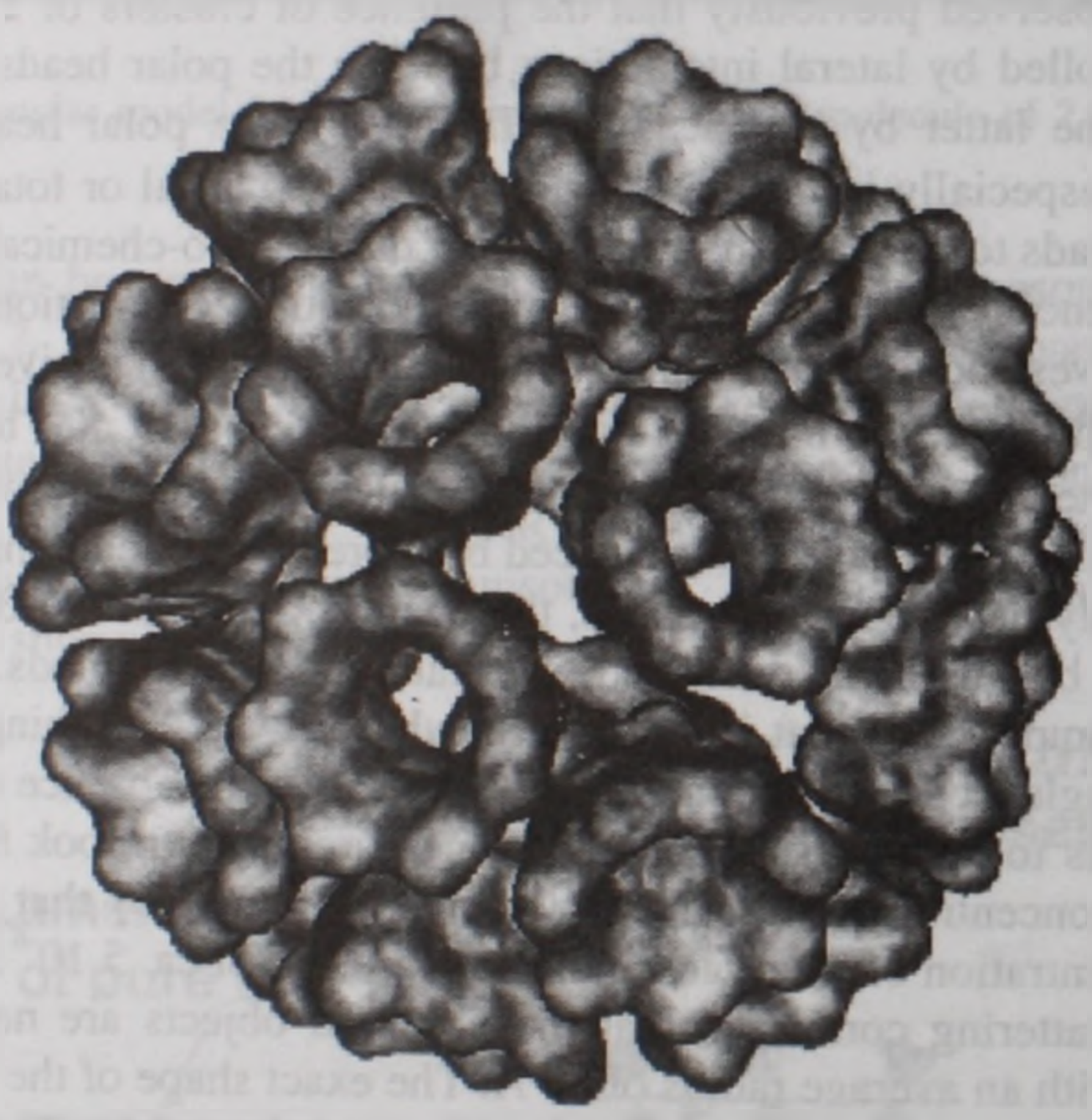


Figure 9. Molecular modeling of a micelle formed by **3** in water, the inner core formed by cholesterol being not visible on this picture.

The density profiles afforded by both X-Ray and Neutron scattering fully support that the CD cavities are exposed to the water interface as shown in Figure 9. Since these structures are clearly defined, two further questions must be addressed :

- Does the cavity of the cyclodextrin retains its ability to include guests ?
- How do these micelles behave in the presence of a liposome matrix as observed for **1** and **2** with bilayers of DMPC ?

To provide answers to the question, a selection of guests well identified to include in the cavity of DIMEB was assayed on **3** in the micellar form using dedicated NMR techniques combining diffusion methods and dipolar interactions (nOe pumping). These procedures are described in details in [6]. Furthermore, the SAXS and SANS methods used previously for the micelles alone were repeated in the presence of potential guests for the cavity of the cyclodextrin. These techniques were assayed for both charged and uncharged guests. It was shown that the inclusion of charged guests (inducing the presence of electric charges on the surface did not modify in a significant way the size and shape of the micelles and that even strong lateral interactions could not change the area per molecule of **3** from its original value (340 $Å^2$). This implies that the packing of **3** molecules into homogeneous micelles is exceptionally robust.

Concerning the second point, the investigation of the interaction of the previously described micelles with DMPC liposomes showed that mixed micelles between **3** and DMPC can be obtained and that isotropic solutions can be attained indicating a completed destruction of the bilayer phase. This implies that these compounds have strong detergent properties and could be used to destroy phospholipidic bilayers in order to isolate membrane proteins. This point is clearly evidenced by ^{31}P NMR on vesicles of DMPC as displayed on Figure 10.

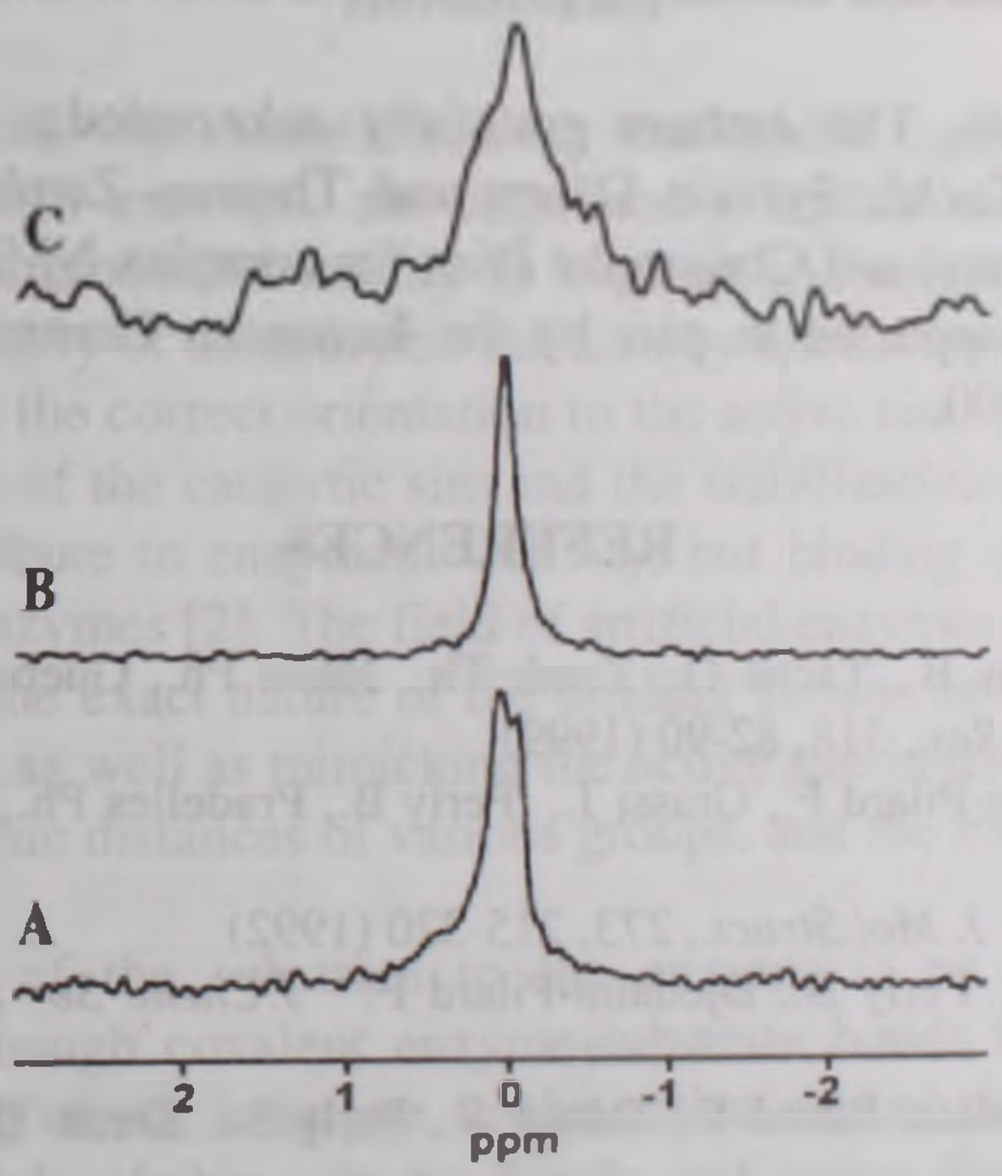


Figure 10. ^{31}P NMR Spectra of small unilamellar vesicles of pure DMPC 15 mM (A) and in the presence of DIMEB (C) or Chol-dimeb **3** (B) For B and C the ratio between DMPC and the CD derivatives is 15:3 (mM:mM).

In the case of Figure 10A, a sharp doublet is observed. The two lines correspond to the ^{31}P signals from the outer and inner lipids in the vesicle. This pattern is typical of small unilamellar vesicles. The corresponding solution is translucent. In Figure 10B, a clear solution is observed and a single sharp line is observed on the spectrum as expected for micelles. Complementary light scattering experiments showed that the mixed micelles have an average diameter of 13 nm. In 10C, the addition of the CD moiety alone (DIMEB) leads to a milky biphasic solution and to a very broad spectrum indicating that the original

vesicles are destroyed leading to large objects. The solubilization properties of these novel amphiphilic cyclodextins are under further investigation and will be described in the future.

Conclusions

A novel class of amphiphilic cyclodextrins has been presented here. They have all been fully characterized in terms of chemical structures and purity. Their interactions with preformed model phospholipid bilayers (DMPC liposomes) have been investigated using a very large variety of techniques which all converge to identical results. A number of different structures has been encountered (liposomes with or without lateral phase separation, spontaneous and mixed micelles). It was also shown that the cavity of the CD moiety retains a full capacity to include external guests which is of considerable importance for potential applications. The key-point in this study is that going from one type of structure to another one is achieved by very small modifications of the cyclodextrin moiety (presence or absence of a spacing arm, methylation or not of the OH groups ...) Further investigations involving the same strategies and techniques but replacing the cholesterol by another highly hydrophobic structure are on the way and will be presented in a near future. The main conclusion is that this quite novel class of amphiphilic molecules open a very large field for basic research as well as for applications in a wide variety of domains ranging from pharmacy, cosmetics, food and household processes to basic chemistry and physics.

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