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ORIGINAL ARTICLE

Action of α-cyclodextrin on phospholipid assemblies

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Abstract Interactions of α-cyclodextrin (α-CD) with dimyristoylphosphatidylcholine (DMPC) and Egg phosphatidylcholine (Egg-PC) were studied (i) by analyzing surface pressure-area isotherms and surface tension of phospholipid monolayers formed at the interface between air and α-CD aqueous solutions and (ii) by X-ray diffraction performed on fully hydrated α-CD/phospholipid binary mixtures. The cyclodextrin molecules strongly interact with the two-dimension phospholipid assembly. Their addition into the aqueous sub-phase leads to the removal of part of the phospholipids from the air-water interface: the higher the α-CD concentration, the higher the phospholipid depletion. This should preferentially involve interactions between cyclodextrin and the phosphatidylcholine head group as α-CD is water-soluble and not surface-active. At the three-dimension level, the bilayer packing of the phospholipid lamellar phase appears not affected by the presence of cyclodextrin as shown by X-ray scattering at small angles whereas wide-angle diffraction patterns reveal the formation of a crystalline phase organized in a pseudo-hexagonal lattice usually characteristic of α-CD dimers. These results point out that α-CD should interact with bilayer-forming phospholipid molecules but likely according to a process that would preserve intact at least a part of the multilamellar assembly.

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Introduction

Although cyclodextrins (CDs) are widely used in pharmaceutics as water-solubilizing systems [1–2], stabilizers or carriers for biologically active molecules, unfortunately they present secondary systemic effects such as haemolysis that severely limits their use as delivery systems via intravenous administration [3]. Haemolytic effect on blood cells of *β*-cyclodextrin, containing seven glucose units, has been deeply investigated. The main mechanism responsible for haemolysis was attributed to complex formation between *β*-CD and cholesterol which is among the lipid constituents of the erythrocyte membrane [4]. Comparatively fewer works have focused on the smallest cyclodextrin, α-cyclodextrin (α-CD), composed of six glucose units. Its action on biological membranes is more puzzling as its cavity appears too small to include cholesterol. The alternative explanation that has been proposed was the possible existence of interactions between **α-CD** and the other lipids constituting the erythrocyte membrane such as nonspecific extraction of phospholipids [3]. Although it is not thermodynamically favorable, partial complexation of the phospholipid molecules by the cyclodextrin via a single-chain has been suggested [5–9] while a two-chain inclusion has been ruled out owing to the small size of the α-CD cavity [10]. NMR investigations have pointed out the possible existence of interactions between phosphatidylinositol polar head group and α-CD

[5–11]. Nevertheless, no direct evidence of such a mechanism has been provided to date regarding phospholipid molecules bearing choline head groups [12]. In this work, the aim was to investigate the ability of α-CD to interact with simple phospholipid assemblies composed of either dimyristoylphosphocholine (DMPC) chosen as a model synthetic lipid and in reason of its property to form a stable monolayer at the air-water interface [13–14] or egg-yolk extracted phosphatidylcholine (Egg-PC) as a model of natural lipid mixture with C_{14}–C_{18} length distribution of hydrocarbon chains partially unsaturated and able to self-organize in a fluid lamellar phase L_α [15]. First, the interactions between the cyclodextrin and phospholipids were examined at the two-dimension level by recording surface pressure-area isotherms and measuring surface tension of DMPC monolayers at the air-water interface when the sub-phase was an aqueous cyclodextrin solution. Secondly, the action of **α-CD** on the three-dimension bilayer structure formed by Egg-PC in excess water was investigated by X-ray diffraction either at small angles to detect any supramolecular change in the lamellar packing or at wide angles to explore at the molecular scale the eventual occurrence of a cyclodextrin-governed solid phase.

Experimental

Materials

1,2-tetradodecanoyl-*sn*-glycero-3-phosphocholine (dimyristoylphosphatidylcholine, DMPC, purity > 99%) and egg-yolk-extracted phosphatidylcholine (Egg-PC, purity >99%) were obtained from Avanti Polar-Lipids (USA). α-cyclodextrin (α-CD, purity ≥98%) was purchased from Sigma (St Louis, USA). Pure water was obtained through reverse osmosis using the Milli-Ro Plus 6 Millipore system (Bedfor, USA) followed by a two-step distillation (first from acidic $KMnO_4$) with a Jencon Autostill apparatus. Its surface tension was equal to 72 ± 0.5 mN/m at 25 °C.

Surface pressure and surface tension measurements

Phospholipid monolayers were formed by spreading aliquots of a DMPC solution (1.10^{17} molecules/mL in chloroform) at the air-water interface or at the interface between air and **α-CD** aqueous solutions.

Surface pressure isotherms were recorded on a Langmuir-type film balance (MCN Lauda, Germany) according to a procedure already described [16, 17] by spreading DMPC chloroform solution by means of a precision micropipette (Microman Gilson 25 μL) over the maximum available area (780 cm^2) at 25 °C. After complete evaporation of the solvent, the measurements were performed at the compression rate of 51 cm^2/min.

Surface tension measurements were carried out by the Wilhelmy plate method, at 23 ± 1 °C using a thermostated automatic digital tensiometer (model Krüss K10T, Germany) and a 10 cm^3 circular glass cell of 11.7 cm^2 surface area. All the measurements were taken without detaching the plate from the interface and the data were continuously plotted on a calibrated chart recorder until a constant value had been reached. DMPC monolayers were formed by spreading a chloroformic solution deposited with a precision micropipette (Microman Gilson 10 μL) on water surface: successive aliquots (2 μL) were added every one hour to reach equilibrium before further addition. The surface pressure as a function of surface density of DMPC (δ) at the air-water interface was deduced from the surface tension measurements according to equation $\pi_{DMPC} = \gamma_{H_2O} - \gamma_{DMPC}$. For the experiments of cyclodextrin injection into the aqueous sub-phase, the cell was equipped with a lateral tube allowing liquid addition and mounted onto a magnetic stirrer [16]. The DMPC monolayer was left to set equilibrium for 1 h before aliquots of a concentrated solution of α-CD (80 mM) were injected into the aqueous sub-phase to obtain the desired cyclodextrin concentration in the 10^{-4}–10^{-2} M range. The sub-phase was then slowly stirred for 2 min with a teflon-coated stir bar before measurement.

X-ray diffraction

Small- and wide-angle X-ray scattering (SAXS and WAXS, respectively) measurements were carried out using a monochromatic focused X-ray beam (0.8×0.8 mm^2, $\lambda = 1.54$ Å) on the Austrian SAXS beamline at ELETTRA, Trieste, Italy (H. Amenitsch, http://www.ibr.oeaw.ac.at/beamline/index.html). For all the experiments a specially designed sample holder (MICROCALIX) which allows X-ray diffraction patterns to be recorded at a controlled temperature (±0.5 °C), developed by Keller et al. [18], was used. SAXS and WAXS intensities of the diffraction lines were simultaneously recorded from the same sample using two one-dimension position-sensitive linear detectors (1024 channels, filled with argon-ethane mixture). The calibration of the detectors was performed with crystalline β form of highly purified tristearin (repeat distances of 44.95 ± 0.05 Å at small angles and 4.59, 3.85, 3.70 ± 0.01 Å at wide angles)

[19]. Data were collected at 25 °C with a time frame of 30 s at 8 keV (λ = 1.54 Å) by a National Instrument LabVIEW supported data acquisition system (H. Amenitsch, HCI, Hecus M. Braun-Graz GmbH).

Data analysis was performed using IGOR Pro 4.07 and Bragg reflections plotted as a function of the scattering vector q ($q = 4\pi\sin\theta/\lambda$, 2θ scattering angle). The q values at the maximum of the reflection peaks (q_{max}) were determined from Gaussian fit of the peaks and the repeat distances d characteristic of the structures were calculated as $d = 2\pi/q_{max}$.

Aliquots of Egg-PC supplied as chloroform solutions were placed into vials to form pure lipid films by removing chloroform under a nitrogen stream followed by drying under vacuum for 12 h. Lipid contents were determined by weight (precision, 5×10^{-5} g). Pure water or α-cyclodextrin solution (80 mM) was then added to obtain a final hydration level of 80% by weight and mixtures homogenized by vortex at 20 °C for 20 min. Samples (20 μL) were introduced into thin glass capillaries (GLAS, Muller, Berlin, Germany) for X-ray diffraction recordings.

Results and discussion

Interaction of α-CD with DMPC monolayers

To investigate whether α-CD is able to act on DMPC organized in monolayers, a preliminary experiment was performed in dynamic conditions by measuring the surface pressure of DMPC layers, deposited either on pure water or on a 10^{-3} M α-cyclodextrin solution, when the monomolecular film was compressed at a constant rate. Resulting surface pressure versus molecular area curves (π-A isotherms) are shown in Fig. 1.

On pure water, the π-A isotherm mainly corresponds to the expanded liquid state of the film. The collapse pressure was recorded at 44 ± 0.5 mN/m and extrapolation of the rising linear part to zero pressure yields a molecular area value of 83 Å^2, both in agreement with literature data [13]. In the presence of α-CD in the sub-phase, the π-A isotherm was still consistent with the formation of a fluid mononuclear film but different in overall shape. The variation of the surface pressure as a function of available area followed a two-step process. Until the surface pressure reached 26 mN/m, the compressibility curve showed a lower rising slope than that recorded on pure water. Extrapolation to zero pressure leads to a molecular area close to 100 Å^2 that is clearly larger than expected for DMPC molecules at the air-water interface. Under surface pressure higher than 26 mN/m the π-A curve

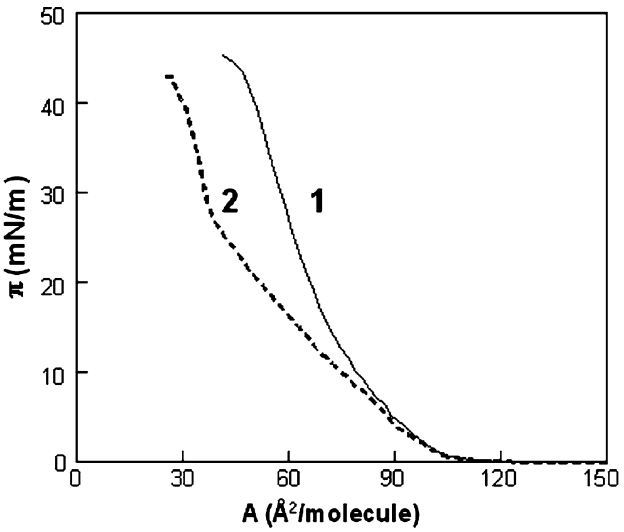

Fig. 1 Surface pressure (π)-area (**A**) isotherms of DMPC monolayers spread on pure water (curve 1) and on a 10^{-3} M α-CD aqueous solution (curve 2) at 23 °C; compression rate 51 cm^2/min

looked in shape like that of the DMPC isotherm recorded on pure water but it was markedly shifted to the left. These results demonstrate that the interfacial behaviour of the phospholipid molecules is modified by the cyclodextrin. In no way, the observed changes in the isotherms could arise from additional α-CD molecules at the air-water interface since they are not surface-active. Indeed, surface tension of α-CD aqueous solutions ranging from 10^{-4} to 10^{-2} M were found rigorously equal to that of pure water (data not shown). Then, the first step of the film compression under moderate surface pressure that corresponds to a deviation of the π-A curve, undoubtedly reveals the existence of interactions between α-CD and DMPC. These interactions transitorily induce an increase of the apparent molecular area of the phospholipid in the expanded liquid state of the film. The second step of the film compression, from the critical surface pressure of 26 mN/m, may be explained by the depletion from the interface of part of the DMPC molecules. Indeed, not only the π-A curve becomes parallel to that for pure DMPC on water (curve 1, Fig. 1) but also the collapse pressure of the film occurs at 44 mN/m that identifies pure DMPC monolayers. The shift of the isotherm towards lower molecular areas would be apparent and would correspond to loss of phospholipid molecules. The interactions between α-CD and DMPC would be then strong enough to drive a part of the phospholipid molecules into the sub-phase.

To verify this last hypothesis, surface tension measurements were undertaken. Indeed, the surface pressure π or the surface tension γ ($\pi = \gamma_{sub} - \gamma$, where

γ_{sub} is the surface tension of the aqueous sub-phase) of a monomolecular DMPC film formed at the air-water interface directly inform on the physical state and surface concentration of the phospholipid molecules. According to a typical behaviour [20] and in agreement with the Langmuir isotherm in Fig. 1, the expanded liquid state corresponds to DMPC molecular areas ranging from 107 down to 45 $Å^2$/molecules, before monolayer collapse under a surface pressure of 44.9 ± 0.5 mN/m (Fig. 2). Moreover, the surface pressure π and consequently the surface tension γ linearly depend on the surface density of the phospholipid molecules. In these conditions, depletion of DMPC molecules from the interface should lead to a proportional π decrease (increase in γ, respectively).

The first series of experiments was performed by spreading DMPC molecules on aqueous α-CD solutions of various concentrations. The amount of DMPC deposited onto the water surface was chosen constant and such as the phospholipid molecules develop a surface pressure corresponding to a tightly close packed monolayer on pure water (δ = 2.5 10^{14} molecules/cm^2; π = 38 mN/m; γ = 34 mN/m). The surface tension versus time curves are shown in Fig. 3. On pure water, the DMPC monolayer well stabilized around the expected γ value of 34 mN/m. In the presence of 10^{-3} M α-CD, the surface tension was immediately decreased by DMPC spreading but stopped at a transitory value higher than 34 mN/m. This was followed by a progressive increase in surface tension before final stabilization at a γ value of 49.1 ± 0.8 mN/m significantly higher than that expected if all the DMPC molecules had been at the interface. The most marked effect was obtained at 10^{-2} M α-CD as the surface tension nearly reached the value found for pure water in the absence of phospholipid. This result demonstrates that α-CD hinders the formation of the DMPC monolayer by likely inducing phospholipid desorption from the interface to the aqueous sub-phase. This supports the interpretation proposed for the two-step process of DMPC film compression observed in dynamic conditions and confirms the existence of interactions between α-CD and phospholipid molecules. However, if these interactions are strong enough to remove DMPC from the air-water interface, the amount of cyclodextrin molecules necessary to desorb one phospholipid molecule is very high. This can be estimated from the ratio of the surface pressures π deduced from each surface tension measurement since π is proportional to the surface density of DMPC at the interface. Then a 10^{-3} M cyclodextrin concentration, that is nearly 6.10^{18} molecules contained in the measure 10-cm^3 cell, leads to about 100.[1–(72–49.1)/(72–34)] = 40% of DMPC molecule depletion, that is nearly 10^{15} molecules (2.5 10^{14} × 11.7 molecules). In the same way, total depletion requires about 10^4 cyclodextrin molecules per phospholipid. These proportions do not correspond at all to usual stoechiometries of cyclodextrin inclusion complexes which appears then very likely unprobable.

Taking into account that α-CD is not surface-active, its interaction with the DMPC molecules spread at the interface should mainly involve the phospholipid head

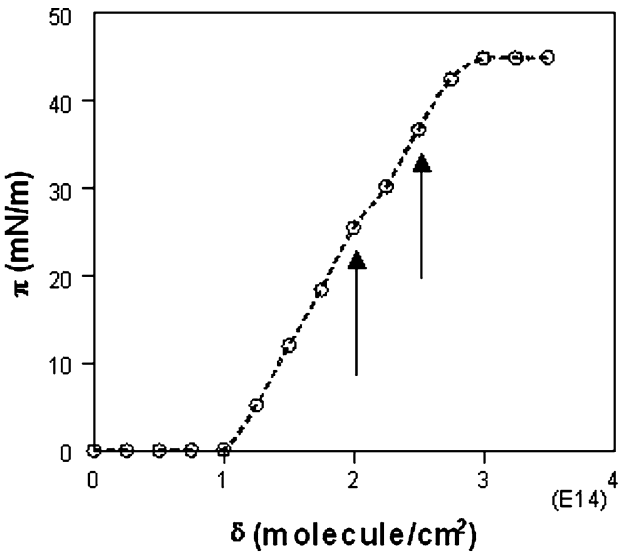

Fig. 2 Surface pressure (π) – surface density (δ) of DMPC molecules spread on water at 23 °C. Arrows delimit the DMPC surface densities used for the surface tension measurements in the presence of α-CD solutions (Fig. 3, 4)

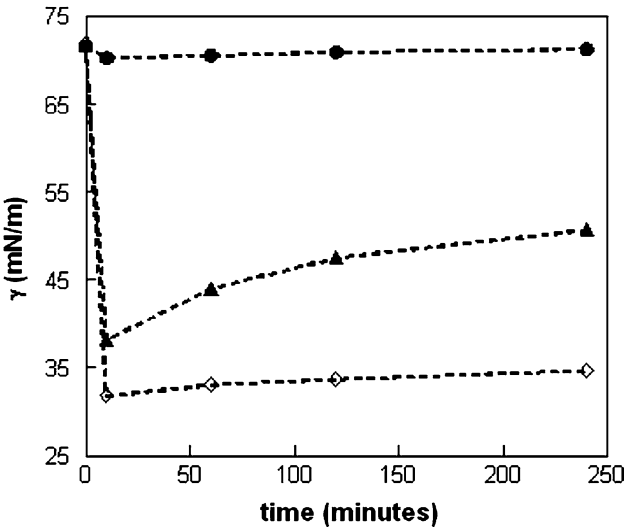

Fig. 3 Variation of the surface tension as a function of time of DMPC monolayers spread on pure water (◇) or on α-CD aqueous solutions of 10^{-3}M (▲)and 10^{-2}M (•)

group. To ensure this conclusion, a second series of surface tension measurements was performed by adding the cyclodextrin molecules into the aqueous subphase once the DMPC monolayer was formed and stabilized at the air-water interface at a surface pressure of 38 mN/m that corresponds to tightly packed phospholipids (Fig. 2). In these conditions, the probability that the cyclodextrin molecules could interact with the phospholipid alkyl chains was even more restricted than when a chloroformic solution of DMPC was deposited onto the α-CD solutions. Results are shown in Fig. 4. While the DMPC surface tension was quite stable on pure water over the time interval studied, it was rapidly increased by addition of α-CD. The variations of γ was very similar to those observed upon DMPC deposit onto cyclodextrin solutions and notably reached the surface tension of water at 10^{-2} M α-CD. This definitively confirms the important role of the phosphatidylcholine group in the cyclodextrin-phospholipid interactions. Indeed, during this last experiment, the DMPC molecules were already arranged so that the hydrocarbon chains were oriented towards air leaving only the polar head groups in direct contact with the water phase containing the cyclodextrin molecules.

Interaction of α-CD with Egg-PC lamellar phase

Knowing that α-CD interactions with phospholipids were strong enough to extract molecules from monolayers at the air-water interface, X-ray diffraction experiments were performed to investigate the ability of such interactions to destabilize three-dimension assemblies composed of phospholipid bilayers. Fig. 5 shows the small-angle X-ray scattering (SAXS) patterns obtained at 25 °C for fully hydrated Egg-PC lamellar phase alone or in mixture with α-CD.

The pure Egg-PC hydrated phase yielded two Bragg reflections characteristic of the long-range packing in the phospholipid lamellar phase L_α , with a bilayer repeat distance of 61.97 ± 0.2 Å, in agreement with literature data [21]. Wide-angle diffraction recordings in the 0.8–1.6 $Å^{-1}$ range showed a broad peak of very low intensity corresponding to an average distance of 4.5 Å between the phospholipid hydrocarbon chains in the liquid state. Cyclodextrin addition did not change both the number and the positions of the small-angle reflections which still coincide with those obtained for the Egg-PC L_α phase. In contrast, supplementary narrow diffraction peaks were noticed at wide angles and revealed the formation of a crystalline phase with repeat distances at 16.0 Å and 13.8 Å, characteristic of α-CD dimers in a pseudo-hexagonal lattice [22]. This implies that the solubility properties of the cyclodextrin were affected since, at the sample concentration studied, α-CD should be entirely solubilized in the water phase. Moreover, α-CD does not usually form dimers in the solid state except in association with exogenous species [23]. Then, wide-angle diffraction data could not be explained otherwise by the existence of cyclodextrin-phospholipid interactions. However, these interactions seem not efficient enough to perturb the Egg-PC lamellar assembly. On the basis of SAXS data, no insertion of α-CD into the bilayers occurs which indeed would have led to the shift or shape-modification of the Bragg reflections. Nevertheless, at

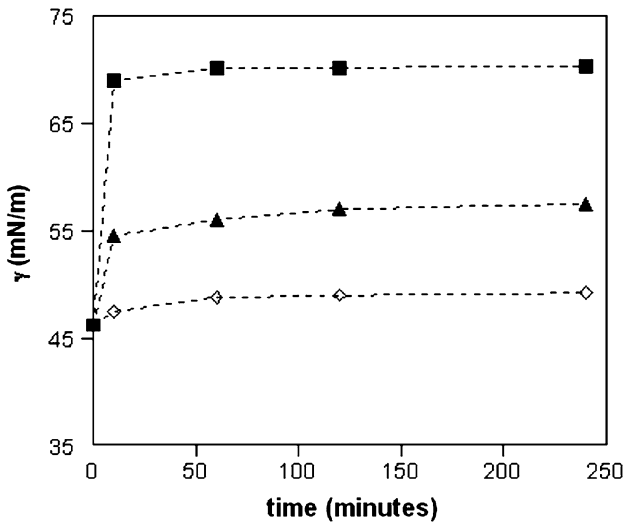

Fig. 4 Surface tension variations as a function of time of stabilized DMPC monolayers at the air-water interface without (◇) or with subsequent addition of α-CD into the aqueous subphase at final concentrations of 10^{-3}M (▲) and 10^{-2}M (■)

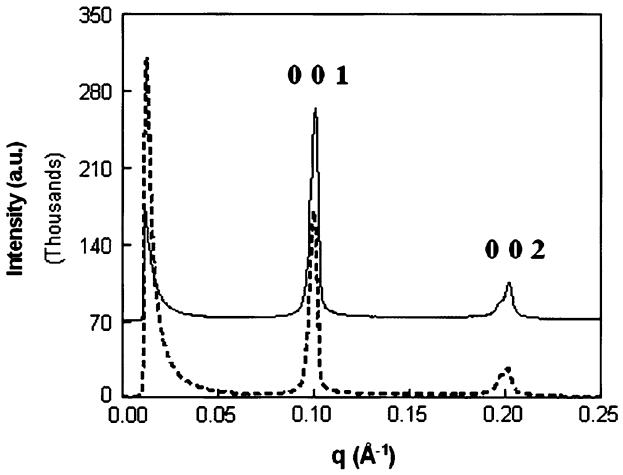

Fig. 5 SAXS patterns at 25 °C of fully hydrated Egg-PC lamellar phase (—) or fully hydrated α-CD:Egg-PC (8.10^{-5}: 2.5×10^{-3}, mol:mol) binary mixture (—). The first- and second-order reflections are indicated on the patterns

this stage of the study the possible extraction of a part of the phospholipid molecules from the bilayers could not be totally excluded although, as previously shown, it is very little probable in the case of phosphatidylcholine derivatives [11–12].

Conclusion

The experiments reported here provided the evidence that α-CD does interact with di-alkyl phosphatidylcholine assemblies, either organized at the two-dimension level in monolayers at the air-water interface or in the three-dimension bilayer packing of the lamellar phase L_α. The cyclodextrin molecules, solubilized into the aqueous sub-phase, are able to remove phospholipids from the air-water interface according to a process that likely involves interactions with the phospholipid polar head group. Such interactions may be also at the origin of the formation of the solid crystalline phase implying α-CD dimers detected in the fully hydrated α-CD:Egg-PC binary mixtures. However, the structure of the phospholipid lamellar phase remains apparently not affected by the cyclodextrin-phospholipid interactions. This could not totally exclude that the cyclodextrin molecules may extract locally only a part of the phospholipid from the bilayers while preserving intact membrane regions. At this stage of the study, stoichiometric association of α-CD and di-alkyl phosphatidylcholines such as inclusion complex formation can reasonably be ruled out. The existence of strong interactions between α-cyclodextrin and polar heads of charged phospholipids organized in vesicles has been previously shown by ^{31}P- and ^{1}H-NMR mainly as the result of electrostatic forces but without involving complex formation [5, 11, 12]. However, in the experimental conditions used, no existence of such interactions for zwitterionic phospholipids could be proved [12]. Regarding both these studies and our findings, it would appear that interactions of α-cyclodextrin with phosphatidylcholines may be highly dependent on their structural organization, either in monolayer or in bilayer, and then to the orientation of their polar heads at the hydrophobic-hydrophilic interface. To understand the role of the choline group and to deeply examine the structural consequences of the action of α-cyclodextrin on bilayer phospholipid assemblies or even possible implication of the phospholipid alkyl chains, further experiments should be undertaken. Beside the use of NMR spectroscopy, investigations by differential scanning calorimetry as well as complementary X-ray diffraction measurements are in progress.

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