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Molecular organization and recognition properties of amphiphilic cyclodextrins*

Rita H. de Rossi[1,‡], O. Fernando Silva[1], Raquel V. Vico[2], and Carlos J. Gonzalez[1]

[1]Instituto de Investigaciones en Físico Química de Córdoba (INFIQC), Departamento de Química Orgánica; [2]Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, X5000HUA Córdoba, Argentina

Abstract: The continuing challenge of using cyclodextrins (CDs) for solubilization and drug targeting has led to the preparation of a wide variety of chemically modified derivatives in order to improve the properties of these host molecules. A possible approach for pharmaceutical applications would be to combine the recognition specificity of CDs with the transport properties of organized structures such as vesicles, liposomes, or micelles.

Amphiphilic CDs can be admixed to phospholipid monolayers and to liposomes, and they can be dispersed into nanospheres showing promising properties for drug encapsulation.

Monoacylated derivatives of β-CD, Mod-CD (C*n*), were synthesized in our laboratory from the reaction of alkenyl succinic anhydride with β-CD. We found that the compound with 10 carbon atoms in the alkenyl chain, Mod-CD (C10), can be incorporated into inverted micelles. We studied their properties in solution and at the air–water interface. In solution they have very low critical micellar concentration, and in the aggregates there are two recognition sites: one is the cavity of the CD and the other is formed by the hydrophobic tails. The alkenyl chain interacts with the cavity, but this is not an obstacle for the association with external guests such as 1-amino adamantane, phenolphthalein, or Prodan.

Mod-CD (C*n*) with *n* equal to 10, 14, and 16 (*n* indicates the number of carbons in the alkenyl chain), form stable monolayers at the air–water interface and they adopt an organization very different from those found for persubstituted CDs. The differences are attributed to the higher conformational flexibility of these compounds, which allows the organization of the CD units with the cavity perpendicular to the interface.

Keywords: cyclodextrins; amphiphilic cyclodextrins; molecular recognition; self-organization.

INTRODUCTION

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six (α-CD), seven (β-CD), or eight (γ-CD) glucose units linked by α-1,4-glucosidic bonds [1]. The characteristic toroidal structure with a hydrophilic outer surface and hydrophobic central cavity is responsible for the unique properties of

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‡Corresponding author: E-mail: ritah@fcq.unc.edu.ar

these macrocycles. It is well known that CDs can act as hosts for a wide range of organic guest molecules. The complexation of a guest molecule occurs either by partial or total filling of the hydrophobic cavity. This phenomenon has been extensively applied in pharmacy (drug formulation), environmental protection (reduction of water and soil pollution), food processing, cosmetics, and so forth [2–4]. Besides, CDs can also protect guest molecules from chemical degradation [5,6]. Naturally occurring (native) CDs possess two parallel rims at the opposite ends, which differ in diameter. Primary and secondary hydroxyl groups are placed at the narrow and wide ends of the molecule, respectively. The structure of these compounds is very well known, and the size of the cavity changes as the number of glucose units increases but the height is the same. It is also important to note that the water solubility of these compounds is quite different, the one with seven glucose units (β-CD) being the less soluble. There are several explanations for this phenomenon but one important factor is the seventh-fold symmetry of β-CD and the intramolecular association of the OH groups in carbon 2 and carbon 3 [2].

Many CD studies have been described in the literature. Some of them are made in the area of supramolecular chemistry using CD as host for organic compounds and as model for enzyme-catalyzed reactions [7]. Important applications are derived from the increase in the solubility of hydrophobic drugs in water [8].

The secondary and primary OH located at the rim of the CD cavities can be modified either giving compounds with several substituents or just with one. A large variety of chemically modified CDs have been synthesized to enhance their properties and more specifically their pharmacological potency [9–11]. Among these, amphiphilic derivatives were designed to build organized molecular structures through self-assembling systems such as micelles, vesicles, and monolayers or by incorporation in lipid membranes as well as liposomes [12]. They are expected to improve drug transport and delivery in the organism. They can also be dispersed into nanospheres showing promising properties for drug encapsulation [13].

Two classes of substituted CDs can be considered: amphiphilic CDs bearing multiple hydrophobic chains, extensively investigated, and amphiphilic CDs obtained by grafting a single hydrophobic anchor, which have been less frequently studied.

POLYSUBSTITUTED CYCLODEXTRINS

Polysubstituted amphiphilic CDs are obtained by persubstitution of the CD primary and/or secondary hydroxyl groups. There are three types of persubstituted compound, and these are called bouquet-, skirt-, and medusa-like (Fig. 1) [14].

Compounds with persubstitution of the primary side with amino-, amido-, sulfo- or thio-alkyl chains are called medusa-like. They are able to form stable Langmuir–Blodgett (LB) layers [15–18]. CD inclusion of naphthalene derivatives was found to occur with LB films of medusa-like derivatives [16].

More recently, novel medusa-like amphiphilic CDs self-assembling into nanospheres were obtained by persubstitution on the primary side with ester of perfluoroalkyl chains [19,20].

CD derivatives obtained by attaching alkyl chains via an ester link to the secondary alcohol groups lead to ''skirt-shaped'' derivatives. They were also found to self-organize into CD nanospheres [21], allowing the encapsulation of drugs such as tamoxifen, used in breast cancer treatment [22]. More recently, nanoparticles has been generated from an enzymatically prepared mixture of poly-esterified skirt-shaped β-CDs [23]. A β-CD heptakis (hexanoyl derivative) was incorporated into lipid membranes to give stable mono- and multilayers with dimyristoyl phosphatidylcholine (DMPC) [24].

Bouquet-like derivatives contain hydrocarbon chains on both sides of the CD cavity, increasing their hydrophobicity. The derivatives obtained by grafting one poly(oxyethylene) or poly(methylene) as well as *O*-alkyl chain, on each side of the CD cavity, were designed to mimic transmembrane ion channels after insertion into lipid membranes [25]. A new very promising CD family, bearing hydrophilic chains on one side and hydrophobic chains on the other side, designed to enhance their amphiphilicity,

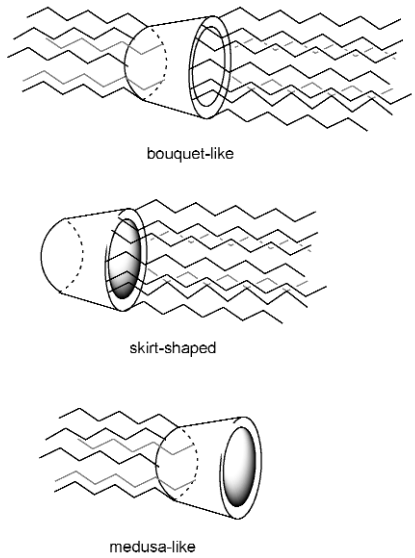


Fig. 1 Schematic representation of polysubstituted CDs.

led to self-assembling derivatives able to form bilayer vesicles [26]. These vesicles consist of bilayers of CDs, in which the hydrophobic ''tails'' are directed inward and the hydrophilic macrocycle ''head groups'' are facing water, thereby enclosing an aqueous interior. The presence of hydrophilic oligomers at the surface of the vesicles increases their colloidal stability while potentially decreasing their adverse immune response in drug delivery systems [27]. Neutral vesicles obtained with CD substituted on one side by poly(ethylene glycol) and with thio-alkyl chains on the other [28] were shown to bind small guest molecules such as adamantane carboxylate [29] and even polymers [30,31] by their inclusion in the CD cavities. Insertion of a labile disulfide bond between the hydrophobic chain and the CD secondary side, lead to vesicles or nanoparticles which could be disrupted upon disulfide cleavage following cellular uptake [32]. Charged vesicles were also described by grafting a cationic ω-amino poly(ethylene glycol) to the CD primary hydroxyl group [33]. Positively charged nanoparticles obtained from the same derivative were shown to encapsulate an anionic porphyrin which could be used in photodynamic cancer therapy [34].

Hybrid Langmuir and LB films consisting of amphiphilic CDs and hydrophobic azobenzene were fabricated [18]. Due to the larger cross-sectional area of the CD core compared with the sum of the cross-sectional areas of the attached alkyl chains, the films have free space in the hydrophobic regions and the azobenzenes can isomerize upon irradiation. Since the isomerization of azobenzenes is accompanied by changes in the physicochemical properties of the molecules such as dipole moment and geometrical shape, molecular films incorporating azobenzenes have been applied to photofunctionalized systems having the ability of switching wettability and electric conductivity [35,36]. Since *trans*-to-*cis* photoisomerization of azobenzene is accompanied by an increase in cross-sectional area of the molecule [37], densely packed molecular films are not suitable for photoswitching. In the films formed by the medusa-like compounds there is enough free space in the hydrophobic regions and the azobenzenes can isomerize upon irradiation, which then can be used as a photoswitch [38].

Receptor clustering is a powerful tool that cells and bacteria use to tune affinity and select competing ligands on their membrane surface [39]. In chemical terms, this phenomenon is an example of dynamic molecular recognition through multivalent interaction [40]. It has been shown that some amphiphilic CDs can form an artificial membrane with embedded receptor molecules that recognizes and binds a suitable ligand via multivalent interaction with a small cluster of receptors [41].

The transport of ions through lipid membranes mediated by molecular channels is a subject of intense research in biology, biophysics, and chemistry [42]. The structure of the biological channels and their mechanisms of transport are complex and difficult to elucidate; this has prompted the search for relatively simple synthetic artificial models. It has been shown that the substituted CD **1** shown in Fig. 2, where each of the seven sugars of the CD bears two trimethylsilyl groups on one side and a thiol group on the other side, interact with lipid in a planar lipid bilayer [43].

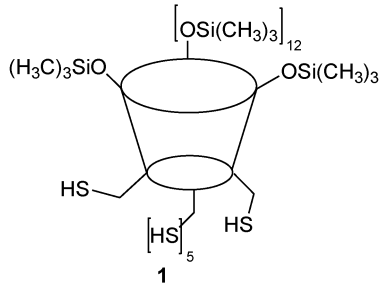


Fig. 2 Polysubstituted CD used to model ion channels in membranes.

Electric measurements were used to determine the interaction between the CD molecules and the lipid membrane, to detect their eventual insertion into it and to observe their final organization. From the values and variations of the ionic current through a bilayer lipid membrane submitted to an electrical difference of potential on the order of 100 mV, the authors evaluate the membrane conductance and observe the formation of conducting channels. The main qualitative result obtained from those studies is that it was proven without ambiguity that modified amphiphilic CD **1** inserted into a lipid membrane form ion-conducting channels. The results indicate that the insertion of the CD into the membrane depends strongly on the thickness of the membranes and two distinct types of CD organization were observed: single channels and aggregates. The type of organization depends on the apparent membrane thickness and not on the membrane nature (asolectin or diphytanoyl membrane). These observations may be useful to design other transmembrane molecules on the basis of CDs and to control their insertion and interactions in lipid bilayers [43].

MONOSUBSTITUTED CYCLODEXTRINS

Two monosubstituted CDs were synthesized by grafting the cholesterol moiety on the primary hydroxyl face of the CD molecule through spacers of various lengths (Fig. 3) [12]. The incorporation into lipid model membranes such as DMPC was investigated.

Several complementary techniques were used, i.e., small-angle X-ray scattering (SAXS), differential scanning calorimetry (DSC), and ^{31}P NMR in order to characterize the organized systems. The compound with the longer connecting chain (**2a**) is incorporated into the bilayer phospholipidic system at high molar ratio and gives rise to separated bilayer systems. On the other hand, the other compound (**2b**) is also incorporated into the bilayer, although it does not produce a segregated phase but rather it induces a higher disorder in the phospholipid bilayer. These results emphasize the influence of the length of the spacer on the overall behavior of the mixed phase.

Modified CDs **2a** and **2b** with a hydrophobic chain are insoluble in water, therefore, they were methylated in the OH-2 and OH-6 groups of the CD to yield a compound, chol-DIMEB, with good solubility in water [44]. It is known that methyl substitution of some of the OH group of CD gives compounds with higher solubility. For instance, the solubility of β-CD is 16 g/dm^3 while the solubility of dimethylsubstituted β-CD at the OH-2 and OH-6 groups (DIMEB) is 570 g/dm^3 [44]. It is supposed

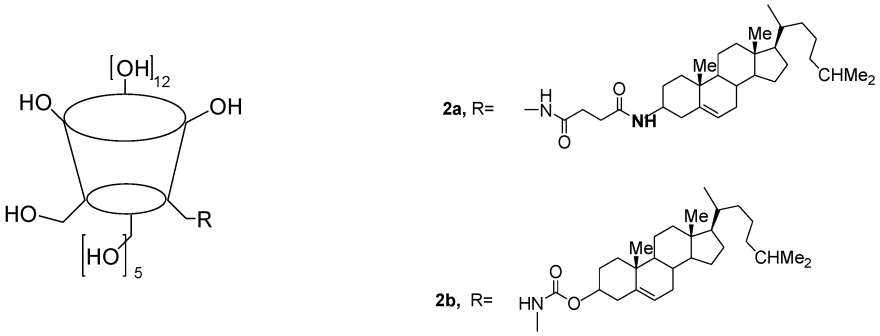


Fig. 3 Schematic representation of two monosubstituted CDs.

that breaking the symmetry of β-CD leads to strong perturbations of its interactions with the network of water, inducing increased solubility even if the grafted substituent is hydrophobic [45]. Monodisperse spherical micelles with a well-defined aggregation number $N = 24$ have been evidenced for aqueous solutions of chol-DIMEB using various scattering techniques. This aggregation number is lower than the value expected from geometrical considerations alone, signaling that the methylation of the polar head and hydration effects play key roles in the final area per polar head in the micelles. A very low free energy of micellization has been found ($cmc = 5 \times 10^{-6}$ M, $\Delta G°_{mic} = -40$ kJ/mol), indicating a highly associative behavior for chol-DIMEB molecule. Moreover, the core-shell structure was retained in these original nonionic spherical aggregates, making the CD moieties available for the inclusion of hydrophobic guests [44].

We have synthesized a series of monoacylated CDs [46,47], and their aggregation properties are discussed here.

Monoacylated CDs were obtained from the reaction of alkenyl succinic anhydride **3** with β-CD (Fig. 4). In this paper, we name them Mod-CD (C*n*), where *n* indicates the number of carbons in the alkenyl chain (Fig. 4, $n = m + 4$). These compounds form aggregates in water, and we have studied and characterized them for the derivative with 10 carbon atoms in the alkenyl chain [48]. The critical aggregation concentration ($c_{agg}c$) determined at pH 10.6 in buffer CO_3^{-2}/HCO_3^- was 1.7×10^{-4} M and the aggregation number was 144, indicating that the aggregates are large and certainly not spherical. Although the exact shape is unknown, they probably have rod-like structure.

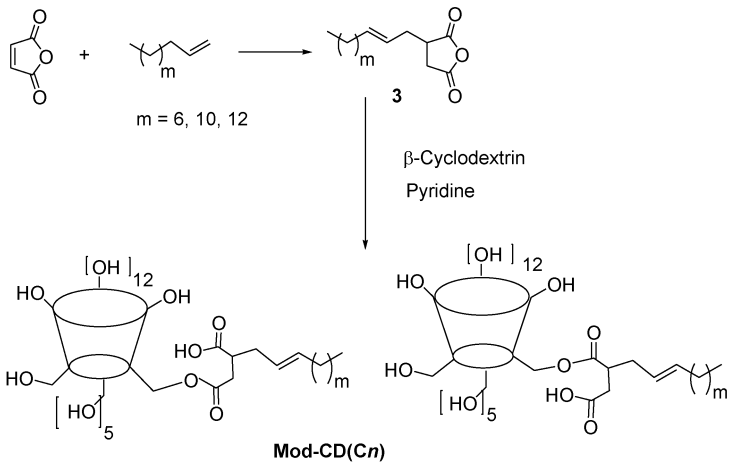


Fig. 4 Synthesis of monoacylated CD.

Using NMR spectroscopy, we could determine the type of interactions in the aggregates. In the methyl proton region (Fig. 5), three different types of signals are observed: a quartet-like signal arising from superposition of two (maybe three) methyl triplets at 0.96 ppm, a very broad signal at 0.89 ppm, and a triplet at ~0.86 ppm. The intensity and chemical shift of the triplet at ~0.86 ppm varies depending on the concentration and the pH of the sample. This signal is more intense at pD = 11.9. The chemical shift of the group of signals at 0.96 ppm also varies with concentration but not much with pH. The line broadening of the signal at 0.89 ppm infer a very short T2 and probably suggest that this signal belongs to methyl groups of Mod-CD species forming micelles. Besides, there is also a very broad signal on the left side of the sharp doublet at 5.02 ppm (H-1), which may correspond to the anomeric proton of the species forming micelles (Fig. 5, inset).

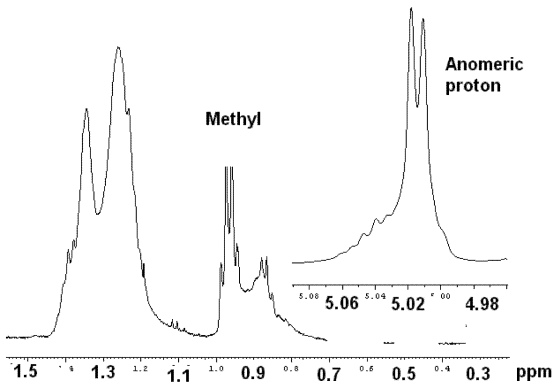
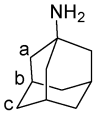


Fig. 5 Methyl region of the ^{1}H NMR of Mod-CD (C10) in D_2O (inset: the anomeric proton of CD).

We have also performed a series of 1D ^{1}H NMR experiments of Mod-CD (C10) at different concentrations (1, 2, and 4 mM) at pD = 10.4. It was observed that the signals of H-3 and H-5, corresponding to the hydrogens that are inside the cavity of CD, shift upfield as the concentration increases. These shifts suggest a concentration-dependent inclusion process going on. Besides, the methyl signals shift downfield as the concentration increases, and the intensity of the methyl triplet signals at 0.86 ppm slightly increases with the concentration.

Since it is known that adamantane has a high binding constant with β-CD [49] and it could displace other guests from the cavity, we prepared a sample of Mod-CD (C10) (4 mM – pD = 11.9) containing 4 mM of 1-amino adamantane **3**. In the ^{1}H NMR spectrum containing **3**, CD protons H-3 and H-5 shift upfield significantly. On the other side, the methyl signal at 0.86 ppm disappears completely.



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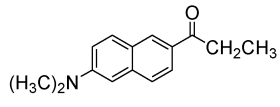

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The T-ROESY (rotating frame Overhauser effect spectroscopy) experiment of this sample shows ROE cross-peak of the 1-amino adamantane protons (a, b, and c) with H-3 and H-5 of the CD glucose units. The methyl signal at 0.86 ppm has disappeared and no longer shows ROE cross-peaks with the sugar protons. However, the methyl protons signals at 0.96 ppm still show ROE with the sugar protons, same as the methylene protons.

The results of the NMR experiments clearly show that there is self-inclusion of the chain and also inclusion of one chain in the cavity of other CDs. Despite that, external guests can interact with the cavity, and this was shown with several compounds. We have determined the association constant with phenolphthalein, and the value obtained is the same as that corresponding to native β-CD, namely, 3.5×10^4 M^{-1}. On the other hand, with Prodan, **4**, which is a molecule frequently used to determine the polarity of interfaces [50], we obtained an association constant that is about twice the value obtained for β-CD, i.e., 4.0×10^3 M^{-1} and 1.6×10^3 M^{-1}, respectively. Besides looking at the fluorescence spectrum we can see that upon addition of Mod-CD (C10) there is an increase in the emission intensity and the maximum moves to lower wavelengths. This behavior is similar to that observed with native β-CD and indicates that the molecule is in a less polar microenvironment as is the cavity of β-CD. In addition, there is an increase in the absorption at about 430 nm, which is attributed to the Prodan molecule located in another less polar microenvironment, probably the micelle core.

It is very well known that CDs accelerate the rate of hydrolysis of esters [51], therefore, we measured the effect of Mod-CD (C10) on the reactions of *p*-nitrophenyl acetate and valerate. In Fig. 6, the data for *p*-nitrophenyl acetate are shown and it can be seen that both, Mod-CD (C10) and β-CD, gave similar behavior.

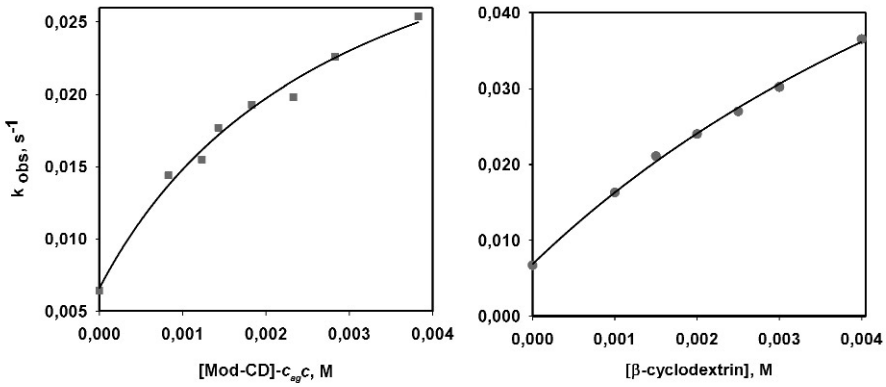

Fig. 6 Effect of Mod-CD (C10) and β-CD on the rate constant of hydrolysis *p*-nitrophenyl acetate at pH = 10.6 and 25 °C.

The accepted mechanism for the CD-mediated hydrolysis of esters [52] involves the association of the guest with the cavity and then nucleophilic reaction of one of the ionized secondary OH groups of CD (Fig. 7). From the kinetic data, the association equilibrium constant and the rate constant for the catalyzed and uncatalyzed reaction were determined and the values are collected in Table 1.

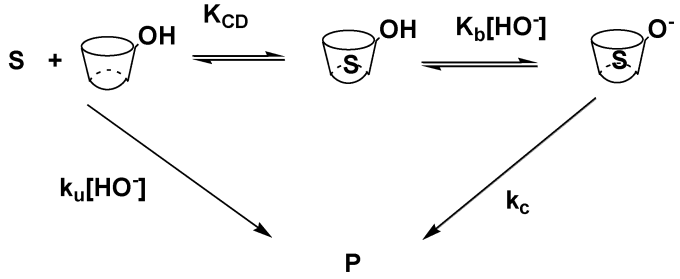

Fig. 7 Mechanism for the CD-mediated hydrolysis of esters.

Table 1 Rate and equilibrium constants for the reactions of *p*-nitrophenyl acetate and valerate at 25 °C and pH 10.6, see Fig. 7 for the meaning of the different terms.

Cyclodextrin	K_{CD}, M^{-1}		k_c, s^{-1}		k_c/k_u	
	Acetate	Valerate	Acetate	Valerate	Acetate	Valerate
β-CD	107	294	104	19	15	5.7
Mod-CD (C10)	335	466	38	13	5.7	4.3

The association of the ester is stronger with Mod-CD (C10) than with native β-CD, but the effect on the rate is quite similar, the small differences probably result from orientation of the substrate in the cavity which is less favorable for the intramolecular reaction.

Reverse micelles are organized systems formed by a detergent in a nonpolar solvent [53]. We have studied the interaction of reverse micelles of AOT in heptane with several native and modified CDs [54]. We found that none of the native CDs can be incorporated into the reverse micelles whereas hydroxypropil CD (HPCD) and Mod-CD (C10) were incorporated. We have studied the interaction of several guests with the CDs in the reverse micelles, and we have shown that methyl orange interacts with the cavity of HPCD. This was demonstrated by UV–vis spectroscopy and circular dichroism as well. On the other hand Mod-CD (C10), incorporated into the reverse micelles, does not interact with external guests. We concluded that the molecules of HPCD are in the water pool, whereas those of Mod-CD (C10) are in the interface and the cavity is probably occupied by the hydrocarbon solvent.

We have studied the Mod-CD (C*n*) compounds at the air–water interface [55]. We found that compounds with 10, 14, and 16 carbon atoms form stable monolayers which upon compression are arranged in different phase states as is evident from the observation of the surface pressure, surface potential, the compression modulus, and the perpendicular dipolar moment (Fig. 8). The results were also confirmed by Brewster angle microscopy (BAM) and by epifluorescence microscopy (EFM). We concluded that the orientation at the interface for the compressed molecule has the cavity perpendicular to the interface. This contrast with what is usually found for persubstituted CDs where the cavity orients parallel to the interface.

The various independent surface parameters measured concurrently indicate that the monolayer stability, surface electrostatic and molecular packing, is dependent on the type of the acyl chain substituent. However, the reorganization at critical molecular areas of the Mod-CDs (C*n*) at the interface is considerable and rather independent of the chain length. This is entirely different from the behavior of the persubstituted derivatives, and none of the changes exhibited by the monoacylated compounds are consistent with the oligosaccharide ring remaining in a fixed orientation along the interface when the surface pressure is varied, as it was demonstrated repeatedly for the persubstituted CDs. As illustrated in Fig. 8, this offers ample possibilities for varying the surface packing, dipole moment orientation, topography, and/or exposure of the cavity of β-CD to the aqueous milieu through control of the interfacial organization of the monoacyl moiety linked to the oligosaccharide ring and the lateral surface pressure.

From all the measurements at the air–water interface, we conclude that upon compression the CD molecules adopt a conformation where the cavity is perpendicular to the interface, this conformation favors intramolecular hydrogen bonding between two adjacent CD molecules and part of this structure is retained after the first decompression. The intermolecular hydrogen bonding is the cause of the aggregation of β-CD in water solution, which has been recently proved [56]. The organization adopted at the interface by the monosubstituted CD contrasts with that of persubstituted derivatives due to the differences in structural flexibility in the two types of compounds.

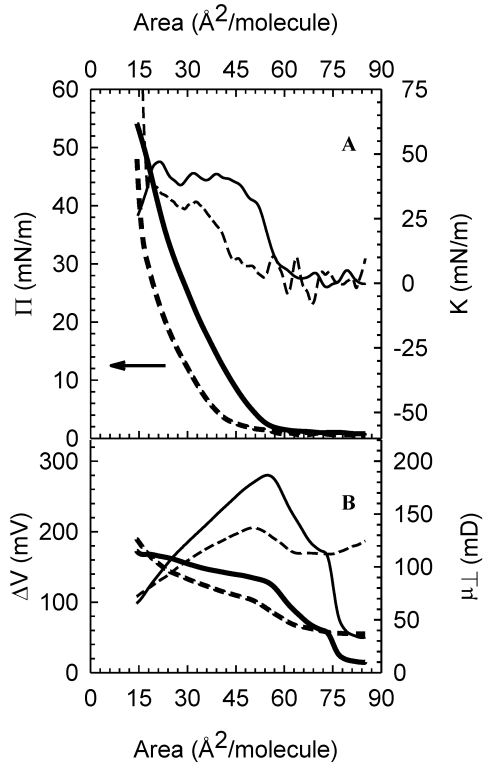

Fig. 8 (A) Variation of surface pressure (thick lines, left axis) and surface compressional modulus (thin lines, right axis) with molecular area for Mod-CD (C16); (B) Variation of surface potential (thick lines, left axis) and perpendicular resultant dipole moment (thin lines, right axis) with molecular area. First compression (solid line), first decompression (dashed line).

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