

# Ionic Channel Behavior of Modified Cyclodextrins Inserted in Lipid Membranes

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We study the insertion and behavior of modified amphiphilic cyclodextrins in suspended bilayer lipid membranes by electrophysiological methods. We observe that our molecules build single well-defined ionic channels. The pore conductance is measured in two lipid membranes differing by their composition. These measurements reveal two distinct behaviors. In the case of thin membranes, we observe single channels, whereas in the case of thick membranes, we only detect a large number of aggregated channels. In a few experiments, we have been able to monitor the transition between the two behaviors by modifying slightly the swelling of the lipid bilayers by decane. The precise structure of the channels is yet unknown; however, we deduce from our measurements an estimation of the channel diameter.

The transport of ions through lipid membranes mediated by molecular channels is the subject of intense research in biology, biophysics, and chemistry for more than 40 years.<sup>1</sup> 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.<sup>2</sup> These models are also interesting for their applications as drugs or sensors.<sup>3,4</sup> A striking example is  $\alpha$ -hemolysin,<sup>4,5</sup> which is also used to study the translocation of macromolecules, in particular, DNA.<sup>6,7</sup> For a long time, the only model molecules available were small polypeptide chains such as gramicidin<sup>8</sup> or alamethicin.<sup>9,10</sup> Other nonpeptidic types of molecules have also been proposed on the basis of, in particular, macrocyclic architecture, such as cyclodextrins.<sup>11,12</sup> It has been shown that modified cyclodextrins could be inserted in lipid vesicles membrane,<sup>13</sup> but their action as ionic channels has never been demonstrated.

In this article, we are interested in the study of a new type of amphiphilic modified cyclodextrins. These molecules have been synthesized in our laboratory and will be described in detail elsewhere.<sup>14</sup> Their precise chemical name is heptakis-6-thiol-6-deoxyheptakis (2,3-trimethylsilyl)-B-cyclodextrin (that we will abbreviate in the following as CD). Each of the seven sugars of the

cyclodextrin bears two trimethylsilyl groups on one side and a thiol group on the other side (cf. Figure 1). In preliminary studies, we observed that these molecules were interacting with lipid membranes and we studied the structure of mixed vesicles containing CD and a lipid with static and dynamic light scattering.<sup>15</sup> In this work, we focus on the behavior of these modified cyclodextrins in a planar lipid bilayer.

We use classical electric measurements to determine the interaction between the cyclodextrin molecules and the lipid membrane, detect their eventual insertion into it and observe their final organization. In our experiments, the membrane thickness can be slightly varied as we change the nature of the lipid and as the membranes are slightly swollen by the solvent of the lipid used to make the membrane, in our case, decane. We use two types of membranes (made respectively of asolectin and diphytanoyl-phosphatidylcholinelecithin), and we find similar results in both cases. 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, we evaluate the membrane conductance and observe the formation of conducting channels through the membrane. In the case of thick membranes, we observe that cyclodextrin molecules are self-assembled in the lipid membrane under the form of large aggregates with a large fluctuating electrical conductance; whereas, in the case of thin membranes, they are arranged in the bilayer under the form of single isolated channels with quantified conductances. Assuming that the channels in the aggregates have the same unitary conductance as the isolated channels we estimate indirectly the size of the aggregates. We argue that the observed behavior of aggregate formation is related to the higher energy cost of insertion of the amphiphilic cyclodextrins in thick membrane, which leads to elastically induced attraction between them.

## Material and Methods

**Formation of Cyclodextrin Pores in Vertical Bilayers.** We form the bilayer according to standard techniques<sup>16</sup> in the

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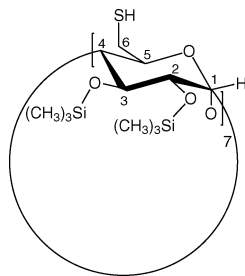
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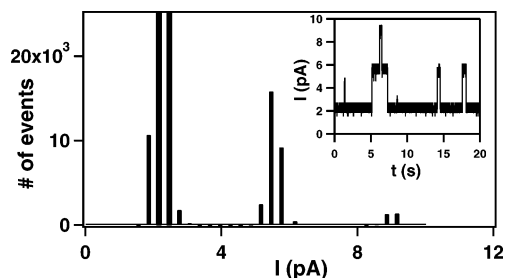
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**Figure 1.** Structure of the modified  $\beta$ -cyclodextrin molecule used in this study.



**Figure 2.** The membrane is prepared by mixing 15  $\mu\text{L}$  of a 12.5  $\mu\text{g}/\text{mL}$  solution of cyclodextrin in decane with 250  $\mu\text{L}$  of a 1% solution of asolectin in decane. The applied voltage is  $\Delta V = 150$  mV. The inset depicts a current trace with three jumps corresponding to three openings of channels. The current histogram is plotted in the main window.

hole of a perforated vessel (Warner Instruments, Hamden, USA). The pinhole size is about 150  $\mu\text{m}$ . It is coated by carefully putting a droplet of cyclodextrin-asolectin or cyclodextrin-diphytanoyl phosphatidylcholine (-PC) solution in decane on each side of the pinhole. The decane is evaporated after 20–30 min drying at room temperature.

The vessel is put in a two-chambered apparatus. The two chambers are filled with 250 mL of a 1 M KCl solution. A pipet is quickly dipped into the same lipid-CD solution. It is used to produce an air bubble, which brushes the coated aperture.

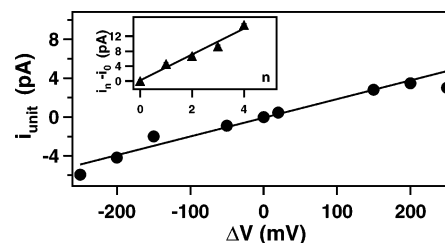
We follow the bilayer formation by measuring the current between two Ag-AgCl electrodes. Typically, the bilayer formation is indicated by a strong current variation from the saturation value of the amplifier to about 0 pA.

**Preparation of the Cyclodextrin Solution.** We prepare a cyclodextrin solution (12.5  $\mu\text{g}/\text{mL}$ ) in decane. We mix it in various amounts with 250  $\mu\text{L}$  of 1% asolectin (Fluka, Buchs, Switzerland) solution in decane or of 1% diphytanoyl-PC (Avanti Polar Lipid, Alabaster, U.S.A.) solution in decane. The volume of cyclodextrin solution  $V$  can vary between 5 and 60  $\mu\text{L}$ . In this way, we can monitor indirectly the amount of CD in the membrane, which we cannot impose directly because we cannot control the final state of the lipid bilayer.

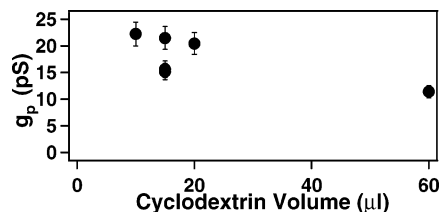
**Data Acquisition and Analysis.** The current flow through the bilayer membrane is measured with a BLM 120 amplifier (Bio-Logic, Claix, France). Data are filtered at 10 kHz and acquired at 100  $\mu\text{s}$  intervals with the DigiData 1322A digitizer coupled with Clampex software (Axon Instruments, Union City, U.S.A.). We use also a PCI-6014 acquisition card (National Instrument, Austin, U.S.A.) (at 1 ms intervals) coupled with a free software Win-CP (University of Strathclyde, Glasgow, UK). The duration of one data acquisition sequence for one applied voltage is most often thirty minutes long but can exceed 1 h. The data are analyzed with Igor Pro software (WaveMetrics, Lake Oswego, U.S.A.) and Win-CP.

## Results

**Case of Asolectin Membranes.** One example of our initial observations is shown in the inset of Figure 2: well-defined current jumps appear as we apply a voltage difference (here  $\Delta V = 150$  mV) across an asolectin membrane containing modified cyclodextrins. There are several well-defined quantified jumps. One can see on



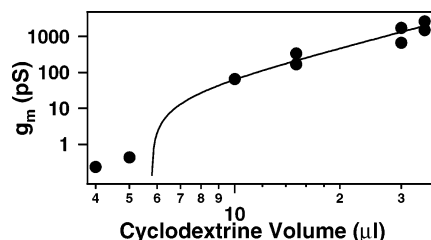
**Figure 3.** The membrane is prepared by mixing 15  $\mu\text{L}$  of the solution of cyclodextrin and 250  $\mu\text{L}$  of the solution of asolectin. Inset: we measure the unit current  $i_{\text{unit}} = 3.5$  pA  $\pm$  0.35 pA when the applied voltage is  $\Delta V = 200$  mV. In the main window, we plot the unit current vs the applied voltage. The slope of the line corresponds to the channel conductance 19.2 nS.



**Figure 4.** Pore conductance  $g_p$  of membranes prepared by mixing different volumes  $V$  of a 12.5  $\mu\text{g}/\text{mL}$  cyclodextrin solution in decane with 250  $\mu\text{L}$  of a 1% asolectin solution in decane.

Figure 2 that the value of the second jump is twice the value of the first jump. The measured histogram of the current value is also plotted on the same figure. One observes three peaks. The most important one is the base current  $i_0$ , the other peaks correspond to the quantified current jumps. By fitting each peak with a Gaussian function, we deduced the unitary current jumps that we note as  $i_{\text{unit}}$ . The current value of the jump number  $n$  is then  $i_n = i_0 + ni_{\text{unit}}$ . By plotting  $(i_n - i_0)$  as a function of  $n$ , we obtain the line drawn in the inset of Figure 3. The slope of the line is  $i_{\text{unit}} = 3.5$  pA  $\pm$  0.35 pA when  $\Delta V = 200$  mV. Note that the applied voltage cannot exceed 250 mV (in absolute value) without leading to the destruction of the membrane. The unit current  $i_{\text{unit}}$  increases linearly within experimental uncertainty as we increase the applied voltage  $\Delta V$  (Figure 3) (within the range  $\pm 250$  mV). This shows that the structure associated with the unitary current follows Ohm's law. The slope of the curve defines its conductance as  $g_p \approx 19.2 \pm 4.7$  nS, that one may call "unitary conductance". Finally we plot on Figure 4 the unitary conductance  $g_p$  as a function of the volume of the 12.5  $\mu\text{g}/\text{mL}$  cyclodextrin solution in decane mixed with the initial 250  $\mu\text{L}$  of asolectin solution in decane; this conductance value remains approximately constant and equal to about 17 nS, except for one measurement that may be aberrant. In this last case (where  $g_p \approx 330$  nS), the membrane capacitance is found to be surprisingly large ( $C = 230$  pF) compared to the usual capacitance (30 pF). This would mean that the membrane is abnormally thin and has a high conductance.

**Case of Diphytanoyl Phosphatidylcholine Membranes.** To study the influence of the nature of the lipid on the insertion on the CD molecules in the membrane,<sup>17</sup> we also used diphytanoyl-PC, a well-known synthetic lipid, leading to a very stable and impermeable bilayer. Surprisingly, the observed experimental results were strikingly different than those obtained with asolectin. When a voltage was applied to a diphytanoyl-PC bilayer where our CD molecules had been premixed as before, we did not observe at first any well-defined unitary structure



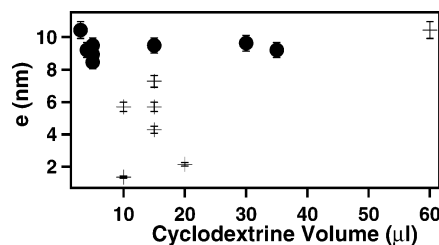
**Figure 5.** Conductance  $g_m$  of membranes prepared by mixing different volumes  $V$  of a 12.5  $\mu\text{g/mL}$  solution of cyclodextrin in decane with 250  $\mu\text{L}$  of a 1% solution of diphytanoyl-PC in decane.

associated with quantified conductivity jumps, although the membrane was conducting and dependent on the CD content. We first studied the base current through the membrane  $i_0$  as a function of the applied voltage  $\Delta V$  and checked that the membrane had a well-defined Ohmic behavior associated with a well-defined membrane conductance  $g_m$ . We plot on Figure 5 the membrane conductance  $g_m$  as a function of the volume of cyclodextrin solution mixed with the initial 250  $\mu\text{L}$  of diphytanoyl-PC solution in decane. If the cyclodextrin volume is low ( $V < 5 \mu\text{L}$ ), the membrane conductance has the usual value of 5 nS associated with pure lipid membranes. This is a check that the lipid membrane had a normal behavior and the lipid molecules were not degraded. If  $V > 5 \mu\text{L}$ , the  $g_m$  values are rather high, mainly in the range between 10 and 1  $\mu\text{S}$ .

### Discussion

We thus observe two radically different behaviors according to the nature of the lipid (asolectin or diphytanoyl-PC) we use. In each case, however, we observe that the cyclodextrin molecules, which are amphiphilic, interact strongly with the membrane (as expected). The behavior observed with asolectin seems simple to interpret. The cyclodextrin molecules inserted in the lipid membrane build well-defined isolated transient channels, which are responsible for the observed quantified conductance jumps. In the first case, the opening of a single channel is observed. A first question is then: what is the structure of the cyclodextrin channels? In the second case of diphytanoyl-PC membranes, we do not observe in the preceding results any singular individual behavior, but as the membrane conductance is 100–1000 times higher than a pure lipid membrane, one must conclude that there is also a permeability to ions of the diphytanoyl-PC bilayers induced by the cyclodextrin molecules. The interpretation of this new behavior raises a second question. We will first try to answer this second one before coming back (incompletely) to the first.

As the chemical and structural difference between the asolectin and diphytanoyl-PC bilayers are at first sight rather small, we find it initially very difficult to explain our observations. We first suspected possible artifacts such as chemical degradation of the CDs or lipids. This was not the case. We finally propose that the large differences of behavior between the two experimental situations that we have investigated can find their origin in the difference of membrane thickness and mechanical stresses that exist between the different bilayers that we have used and may control, according to recent theories,<sup>18–21</sup> the cyclodextrin insertion and the interactions between the inserted molecules. To estimate the membrane thickness, we



**Figure 6.** Apparent thickness of membranes prepared with different solutions of cyclodextrin and diphytanoyl-PC (●) or asolectin (+).

measure the membrane capacitance  $C$  at the beginning of each experiment and use the classic electrical model of a lipid bilayer, a capacitance in parallel with a low conductance. The membrane capacitance  $C$  is a function of its thickness  $e$

$$C = \epsilon_0 \epsilon \frac{S}{e}$$

where  $\epsilon_0$  is the electric constant,  $\epsilon = 2$ , the dielectric constant of a biological membrane, and  $S$  is the membrane surface. Because we cannot determine the final shape of the membrane in the pinhole, we will assume in the following that  $S$  is the nominal pinhole surface of the vessel. As a consequence, the values that we estimate from the capacitance measurements are only apparent, but not absolute, values of the thickness of the membrane.

In the case of the asolectin bilayers, we observe a high variation of the membrane capacitance from a sample to another (the extreme values are 30 and 230 pF), whereas the diphytanoyl-PC membrane capacitance remains constant (about 30 pF). We plot in Figure 6 the apparent membrane thickness as a function of the volume of cyclodextrin solution in the initial decane–lipid solution. The apparent thickness varies between 4 and 8 nm for the asolectin bilayers, whereas we measure 9 nm for the diphytanoyl-PC one. We conclude from these experiments that the insertion of the cyclodextrin molecules is probably easier in the thinnest membranes (i.e., in the asolectin membranes) than in thickest one. This may also be used to interpret our observation that the CD molecules do not insert in the diphytanoyl-PC bilayers below a certain concentration.

Before analyzing the interactions between cyclodextrins inserted in the membrane, let us now consider the structure of the channels in the simplest case of the isolated channels of the asolectin bilayers. Assuming the validity of Ohm's law at the level of a channel, we can use the measurements of electrical conductivity to estimate the size of a channel. Assuming a cylindrical geometry, the pore conductance  $g_p$  is a function of pore diameter  $D$ :

$$g_p = \frac{\kappa \pi D^2}{4e}$$

where  $\kappa = 11.2 \text{ Sm}^{-1}$  is the conductivity of 1 M KCl solution, and  $e$  is the pore length. Assuming that  $e$  is given by the measured apparent membrane thickness, deduced from the capacitance, we can estimate an apparent pore diameter  $D$  from the measurements of  $g_p$  and  $C$ :

$$D = \sqrt{\frac{4\epsilon_0 \epsilon g_p S}{\pi \kappa C}}$$

We plot on Figure 7 the measured values of the apparent channel diameter  $D$  as we vary the cyclodextrin proportion

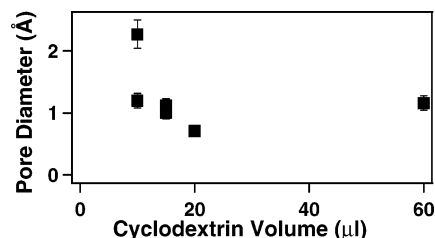
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**Figure 7.** Measured diameter of the cyclodextrin channels performed on different samples as we change the cyclodextrin volume in the membrane solution.

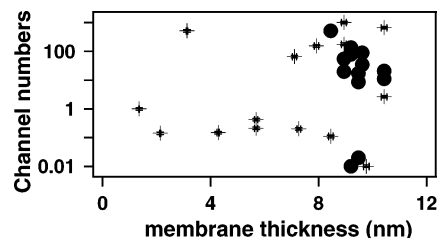
in the asolectin bilayers. We find a constant value approximately equal to 1 Å, except in one experiment where a value of  $D \approx 2$  Å is observed. In this last case, the apparent membrane thickness  $e$  is found to be abnormally small ( $e \approx 1.4$  nm), and this might correspond to a case where the real membrane area is much smaller than the pinhole size. The estimated value of the diameter (1 Å) is rather small and undoubtedly shows that the observed channels have a molecular origin. However, there is a rather large difference from the value of the diameter of a  $\beta$ -CD cavity, which is 7.8 Å.<sup>22</sup> One possible explanation is that the direct application of Ohm's law neglects the effects of dielectric image charges, which create a potential barrier and diminish the mobility of ions in the channel.<sup>23</sup> One thus underestimates the channel size. There is also a considerable uncertainty regarding to the channel length  $e$ . The lipid bilayer thickness, typically about 5 nm, is much larger than the thickness of one CD molecule, and there must be a stack of several CDs to form one channel. It is, however, difficult to imagine the structure of the stacking at this stage of our knowledge. The simplest hypothesis would be to assume that the CDs are arranged as dimers, with their hydrophobic parts facing each other, and that there is only one dimer in the bilayer. If one assumes that the thickness of our modified CD is on the order of 1 nm, this lead to a dimer thickness on the order of 2 nm, which is much smaller than the thickness of the lipid bilayer unless the latter is strongly pinched. One could assume more dimers, but the stability of the channel would be more difficult to explain. Clearly more experiments or numerical simulations are needed to elucidate the CD's channel structure.

We now come back to our measurements on diphytanoyl-PC membrane. The simplest hypothesis is to assume that the measured conductivity is due to the collective behavior of channels of the same type as observed in asolectin membranes. The conductance of each channel is in parallel with the others. The total membrane conductance is

$$g_m = \sum_{k=1}^n g_p + g_0 = ng_p + g_0$$

where  $g_0$  is the membrane conductance without channels and  $n$  the number of channels inside the membrane. As the membrane conductance ( $g_0$  on the order of 5 nS) is much smaller than the pore conductance  $g_p \approx 19.2$  nS, we can use a simplified relation to calculate the number of channels as  $n \approx g_m/g_p$ .

We observe (cf. Figure 5) that the estimated number of channels is, as expected, an increasing function of the initial cyclodextrin volume in the lipid solution; the more



**Figure 8.** Number of cyclodextrin channels as a function of apparent membrane thickness  $e$  in the case of asolectin (+) or diphytanoyl (●) membranes. The cyclodextrin volume is either 10 or 15  $\mu$ L.

we add cyclodextrin to the membrane solution, the more we count channels in the membrane.

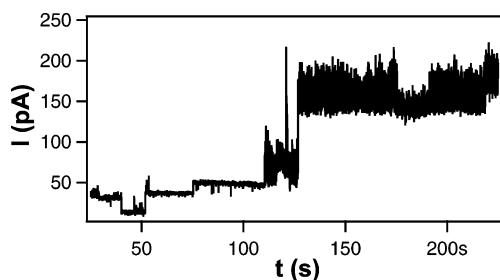
To study the correlation between the insertion of the CD molecules and the elastic properties of the membranes, we plot on Figure 8 the number of channels as a function of the measured apparent membrane thickness  $e$ , the cyclodextrin volume being either 10 or 15  $\mu$ L. There is an unavoidable scatter of the data, but one can clearly distinguish two sets of points, a set corresponding to zero or one channel at small thicknesses, lower than about 7 nm, and a set corresponding to a proliferation of channels at a large thickness  $e > 7$  nm. The transition as we vary either the thickness or the cyclodextrin proportion (cf. Figure 5) is abrupt. This leads us to suggest by analogy with micelle formation in a bulk three-dimensional solution that the modified CD molecules might form some kinds of aggregates or micelles in the membrane. In bulk water, some modified cyclodextrin molecules form micelles because of their amphiphilic properties, and an aggregation number of 24 molecules have been observed.<sup>24</sup> Here also, further structural studies are needed.

The formation of aggregates composed of elementary well-defined channels is supported by supplementary experiments done on thick asolectin bilayers and thin diphytanoyl membranes that we succeeded in making after numerous attempts using, as already stated, the slightly variable swelling of our lipid bilayers by decane. The apparent thickness of the asolectin membranes varies between 7.1 and 10.4 nm. In this case, we do not observe any jumps of conductance, but the membrane conductance is, however, very high ( $20S > g_m > 1.3S$ ). This is the same behavior as for the thick diphytanoyl-PC membrane discussed above. With the same hypothesis as above, we deduce a number of channels between 65 and 1000 in the membrane. Then, if the apparent membrane thickness is larger, we observe the increase of the membrane conductance, a signature of the insertion of stable aggregates. In the case of the new experiments with diphytanoyl bilayers, we succeeded in decreasing the apparent thickness of the membrane. After a succession of destruction and formation of the membrane using the same lipid reservoir, we obtain a high capacitance membrane (larger than 40–50 pF instead of 30 pF). The final apparent membrane thickness is about 7 nm. In this case, the most striking result is that we indeed succeeded in observing sequentially during the same experiment a single channel behavior after a multichannel behavior. The corresponding electrical current is shown in Figure 9 as a function of time. We measure a unitary pore conductance of  $g_p = 67$  nS and an apparent pore size of 6 Å, larger than the one measured on asolectin membranes and in good agreement with the diameter of the inner cavity of a  $\beta$ -cyclodextrin.<sup>22</sup> We even succeeded in observing directly the insertion of

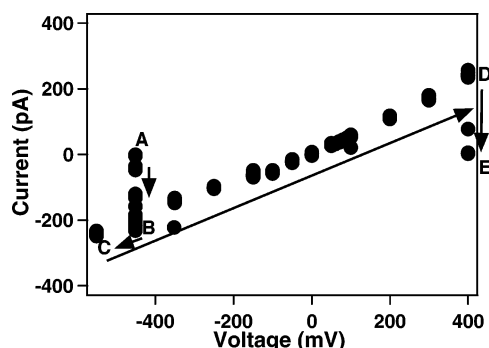
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**Figure 9.** Measured intensity vs time. Membrane prepared with a solution of cyclodextrin (10  $\mu$ L) and diphytanoyl(-PC) (250  $\mu$ L).  $C = 49$  pF ( $e = 6.38$  nm). The applied voltage is  $\Delta V = 150$  mV.



**Figure 10.** Measured current vs applied voltage. We started from the point (A). We observed the inclusion of an aggregate (strong increase of the current (B)). We changed the applied voltage from (C) until (D). After this point, we observed the separation of the aggregate from the membrane (strong decrease of the current (E)).

one cyclodextrin aggregate. The sequence of events is shown on Figure 10 as we vary the electrical potential. We start from an insulating membrane without channels (conductance  $g_m = 4.6$  nS), which we submit suddenly to a difference of potential  $\Delta V = -450$  mV (point A). The electrical current, initially vanishing, suddenly reaches the value  $-223$  pA (from A to B on Figure 10). One could object that the large voltage value that we have imposed could possibly create spontaneous electroporation holes in the membrane.<sup>25</sup> However, the observed structure was stable when we decreased the absolute value of the

potential  $|\Delta V|$ . The electrical current was increasing linearly with the potential  $\Delta V$  as we were reversing the membrane polarity (from point B to point D). We thus could measure the aggregate conductance as  $g_a \approx g_m = 476$  nS. We could change the voltage until the positive value of  $\Delta V = +400$  mV. At this point (D), we observe a strange decrease of the current until 4 pA (E), incompatible with electroporation; the only interpretation is that the aggregate leaves the membrane. After this, the membrane conductance is about 10 nS (point E). From the conductance measurements, we deduce that the aggregate is a small micelle made of  $n = g_a/g_p = 7$  cyclodextrin channels.

Several recent theories describe the insertion of inclusions in a lipid membrane<sup>18,19</sup> and the elastically induced interaction (in most cases attractions) between membranes.<sup>20,21</sup> We still lack precise experimental information to support or infirm these theories. But we note that there are qualitative agreements with our observations. It is easier to insert our CD inclusions in thin membranes than in thick ones, and the elastically induced attractions between CD molecules in distorted thick membranes explain our observation.

## Conclusion

Our main qualitative result is that we prove without ambiguity that modified amphiphilic cyclodextrins inserted in a lipid membrane form ion-conducting channels. We have investigated the condition of insertion of the cyclodextrins in the membrane and have shown that it depends strongly on the thickness of the membranes.

We have observed two distinct types of cyclodextrin organization: single channels and aggregates. We have shown that the type of organization depends on the apparent membrane thickness and not on the membrane nature (asolectin or diphytanoyl membrane). These observations will be useful to design other transmembrane molecules on the basis of cyclodextrins (which offer the advantage that they can be easily modified chemically) and to control their insertion and interactions in lipid bilayers.

Another goal will be to obtain more detailed information on the structure of the channels.

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