β-Cyclodextrin in DNA decompaction: An imaging approach

Alfredo González-Pérez

MEMPHYS - Center for Biomembrane Physics, Department of Physics and Chemistry, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

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1. ABSTRACT

In the present work we investigate the CTAB:DNA complexes decompaction process using β-CD as a decompacting agent. The transition from globules (compacted DNA) to coils (decompacted DNA) was achieved without a coexistence region between coils and globules. This non first-order transition was investigated combining fluorescence microscopy (FM), cryo-transmission electron microscopy (cryo-TEM) and TEM of negatively stained samples. Additionally the presence of multi-globular aggregates in the proximity of the critical transition was elucidated. A possible mechanism for the decompaction process was suggested.

2. INTRODUCTION

It is well known that precise control of DNA morphology is of great biological and technological importance (1, 2). The degree of compaction of DNA mediates the extent of accessibility to the DNA sequences and is therefore indirectly responsible for the control of different processes such as gene expression, recombination and DNA repair (3). DNA compaction has been successfully achieved *in vitro* using many different chemical agents, (4) such as surfactants (5). The morphology of the compacted DNA can either be toroidal, rod-like or spherical, all dependent on the nature and concentration of the compacting agent (6). DNA

decompaction is an equally important step in order to control the behavior of DNA. Different strategies have been used in order to decompact DNA complexes, with the concomitant release of DNA in a coil conformation. Such strategies are obviously strongly dependent on the type of chemical used for the initial compaction of DNA (4). In all cases, DNA compaction and decompaction are generally considered to be first-order transition processes were the system passes through a region of coexistence between coils and globules (7-9).

Cationic surfactants have been successfully used in order to compact DNA (5). The compaction is achieved at lower concentrations than the critical micelle concentration (cmc), through the formation of surface micelles on the DNA strands. Decompaction of such complexes then involves the removal of these micelles; a process that has been achieved using both sodium bromide (NaBr) (9), and anionic surfactants (10, 11). However the decompaction achieved using these agents results in a environment with a high ionic strength or in the case of surfactants the presence of additional self-assembled structures like catanionic micelles or vesicles. Different strategies that result in a clean environment after DNA decompaction are liked.

It has been recently shown that compacted DNA using CTAB can be decompacted using β-cyclodextrin (β-CD) (12). The decompaction was achieved without a coexistence region between coils and globules as was reported by González-Pérez *et al.* (12). Hence DNA decompaction using β-CD was found to be a non-first order transition. Additionally the authors found formation of big globular clusters in a narrow β-CD concentration region below the critical transition. This intriguing phenomenon has never been found before, in either DNA compaction or decompaction, irrespective of the nature of the chemical agents used to compact or decompact the DNA. The ability to decompact DNA, using β-CD, when this was compacted by using gemini amphiphiles have been recently reported by Cao *et al.* (13).

Cyclodextrins are cyclic oligosaccharides composed of five or more α-D-glugopyranoside units. The most commonly used cyclodextrins are the α-, β-, and γ-CDs having 6, 7, and 8 glucose units respectively. These cone-shaped molecules have a hydrophobic cavity while the exterior is hydrophilic. They can encapsulate different hydrophobic molecules like amphiphiles forming a so-called inclusion complex. Cyclodextrins are used in many applications in order to encapsulate different kinds of molecules and have been widely used in the pharmaceutical industry (14). Their ability to form inclusion complexes with many cationic amphiphiles make them very promising decompacting agents.

In order to further investigate the internal structure of globular clusters as well as the non-first order transition, we combined fluorescence microscopy (FM), TEM of negatively stained samples and cryo-transmission electron microscopy (cryo-TEM). The FM, TEM and cryo-TEM are commonly used techniques for studying DNA compaction and decompaction (6, 15). The experimental

work presented here has been performed under identical conditions as were used in the previous work (12). The results seem to confirm the non first-order transition, and the multi-globular cluster formation before decompaction was investigated. A mechanism for the decompaction process was finally suggested.

3. MATERIALS AND METODS

3.1. Materials

Coliphage T4DNA 166kbp was supplied by Wako Nippon Gene. Hexadecyltrimethylammonium bromide (CTAB) was obtained from Sigma and re-crystallized twice in acetone. Fluorescent dyes 4',6-diamidinio-2-phenyl-indole (DAPI) and GelStar® nucleic acid gel stain were obtained from Sigma and Cambrex respectively. The latter is supplied as a 10,000× concentrated stock solution in DMSO. The antioxidant Ascorbic acid and β-Cyclodextrin (β-CD) were purchased from Sigma. Uranyl acetate with a purity of 98.0 % was purchased from Fluka.

3.2. Sample preparation

All stock solutions were prepared in 10mM Tris-Cl buffer (pH 7.6). DNA molecules were diluted in the 10 mM Tris-Cl buffer containing 4% of ascorbic acid and fluorescent dye. The final concentration of DNA was 0.5 μM in nucleotide units. DNA was compacted by using a CTAB concentration of 2.42×10^{-4} M. At this concentration all of the DNA molecules were compacted and no coexistence between coils and globules was found. The concentration of β-CD was varied in the decompaction studies and all other parameters were kept constant. The experiments were conducted with GelStar and DAPI. (The former gave a higher contrast). The experiments on negative staining TEM and cryo-TEM were performed without fluorescent dye.

3.3. Fluorescent microscopy

The fluorescence microscopy study was conducted by placing a drop of the sample on a thoroughly cleaned microscope slide and then placing a cover slip on top of it. The samples were illuminated with a UV-mercury lamp and the fluorescence images of single DNA molecules were observed using a Zeiss Axioplan microscope, equipped with a 100U oil-immersed objective lens, and digitized on a personal computer through a highly sensitive SIT C-video camera and an image processor, Argus-20 (Hamamatsu Photonics, Japan). The apparent long-axis length of the DNA molecules, L, was deemed as the longest distance in the outline of the fluorescence image of single DNA. Images of the dynamic motion of single DNA-lipid complexes in solution were recorded by using the C-image software obtained from Hamamatsu. The observations were carried out at room temperature.

3.4. Negative staining TEM

A copper grid _200 mesh was used, and the final samples were negative stained with 2% uranyl acetate, and observed with Philips CM120 BioTwin Bio Cryo electron microscope operated at 120 kV. For each sample, 7 μl of sample was placed on a carbon grid and was dried after 2

minutes. This process was repeated three times in order to increase the amount of globules on the carbon grid. Afterwards, 5 µl of an aqueous solution of 2% uranyl acetate (UA) (pH=7) was placed on the carbon grid, and after 30 s the grid was blotted dry using a filter paper. Finally, the carbon grid was washed twice to remove any excess of the staining agent and left to dry. Some samples were stained by using a 2% phosphotungstic acid (PTA). The samples were observed with Philips CM120 BioTwin Cryo electron microscope operated at 120 kV.

3.5. Cryo Transmission Electron Microscopy Cryo-TEM

The samples were prepared according to a conventional cryo-TEM protocol, using a controlled environment vitrification system (CEVS). Samples were prepared on hydrophilised (glow discharged) lacey carbon grids on which 5 µl of the sample solution was placed. The excess fluid was gently blotted away using filter paper and plunged into liquid ethane (freezing point -180 °C). All grids were stored in liquid nitrogen (-196 °C) until being transferred to the TEM. The vitrified sample was transferred to a liquid-nitrogen-cooled TEM cryo-holder (Oxford Instruments CT-3500) using a cryotransfer station designed for minimal air exposure and heat loss. Samples were imaged using a Philips CM120 BioTwin Cryo electron microscope operated at 120 kV. Images were recorded with a GIF 100 (Gatan Imaging Filter), using a CCD camera (MSC 791) in low-dose mode, giving a low electron beam intensity of around 10 e-/Å^2(16).

4. RESULTS AND DISCUSSION

4.1. From globule to coil: A non-first order transition

In previous work we have shown that using β-CD induces the transition of globules to coils. This transition did not show a coexistence region between coils and globules. However, in a narrow concentration range close to the critical transition the presence of big aggregates was found. In order to elucidate the intrinsic nature of such aggregates we investigate again the conformational state of T4DNA by fluorescence microscopy (FM) following the protocol described in the Experimental Section. T4DNA was compacted using CTAB and by increasing the concentration of β-CD, the T4DNA was decompacted. We investigate the narrow region above and below critical β-CD concentration (critical transition concentration). Figure 1 shows fluorescence images and the intensity profiles of CTAB:T4DNA aggregates at different β-CD concentrations. In figure1a at low β-CD concentration the results indicate a negligible effect on the decompaction of CTAB:T4DNA complexes since all the DNA is in a globular state (Figure 1a). However, when the β-CD concentration is close to the critical transition concentration, larger clusters appear in coexistence with individual globules (Figure 1b). At this β-CD concentration, using CTAB:β-CD equilibrium constants reported by Cabaleiro-Lago et al. (17) and assuming that all the CTAB is free in solution, it has previously been estimated that the CTAB:β-CD complex lead out values round 85 % (17). The cluster formation in the proximity of the critical cyclodextrins concentration remains unexplained.

In order to gain additional information about these properties of this aggregates we studied in a first step the CTAB interaction with T4DNA and with β-CD in a narrow concentration range around the critical transition concentration. Subsequently we investigate the interaction of β-CD with the CTAB:T4DNA globules in the same concentration range around the critical transition concentration.

4.2. T4DNA interaction with CTAB and β-CD

The low solubility of β-CD in aqueous solution compared with the α- and γ-CD has been a matter of discussion. Coleman et al. (18) showed that at a concentration around 4.4 mM, β-CD forms aggregates with sizes of 200 nm. Results published by Gonzalez-Gaitano et al. (19) suggest that at concentrations below 3 mM, β-CD in water can form sheet-disks with sizes ranging from 142 nm to 172 nm. Such sheet-disks formation was further confirmed by the results reported by Bonini et al. (20) using dynamic light scattering and cryo-TEM. In their work, they showed that above 3 mM, the structures have sizes of 100 nm and growth-forming layers.

In order to get information on the nature of the CTAB:β-CD interaction in buffer solution and in absence of T4DNA we performed TEM experiments using the negative staining technique and imaging the samples in a TEM as well as free stained samples using cryo-TEM. The results of the TEM experiments on negatively stained samples and cryo-TEM are shown in Figure 2. We investigated the same narrow β-CD concentration range above and below the critical transition concentration as in the previous fluorescence microscopy experiments shown in Figure 1.

Figure 2a and 2b show the presence of sheed-like aggregates and layers respectively. At low β-CD concentration the sheed-like aggregates are polydisperse and we can observe different sizes that range from 500 nm to several microns. High β-CD concentration favors the formation of long layers with a well defined geometry. In Figure 2c we show a cryo-TEM image of an analogous layer to the one shown in Figure 2b. We can confirm that at high β-CD concentration the sheet-like structures evolve into layered structures, similar to those described by Bonini et al. (20). However in our work, the β-CD concentration at which CTAB:β-CD aggregates are formed was found to be below that reported by Bonini et al. (20). A possible explanation for this is that the buffer enhances the effect of the aggregation. The CTAB should not have a strong influence on the sheet-disks or layer formation. Note that these structures were not observed in the fluorescent experiments because the fluorescence dye only binds the T4DNA.

We conclude that the presence of different kinds of aggregated structures, namely disk-like and layers, can be formed in buffer solution at the CTAB: β-CD concentrations under investigation. The structures are analogous to those presented by Bonini et al. (20) and we expect that this aggregation phenomenon will also appear when T4DNA is included in the solution.
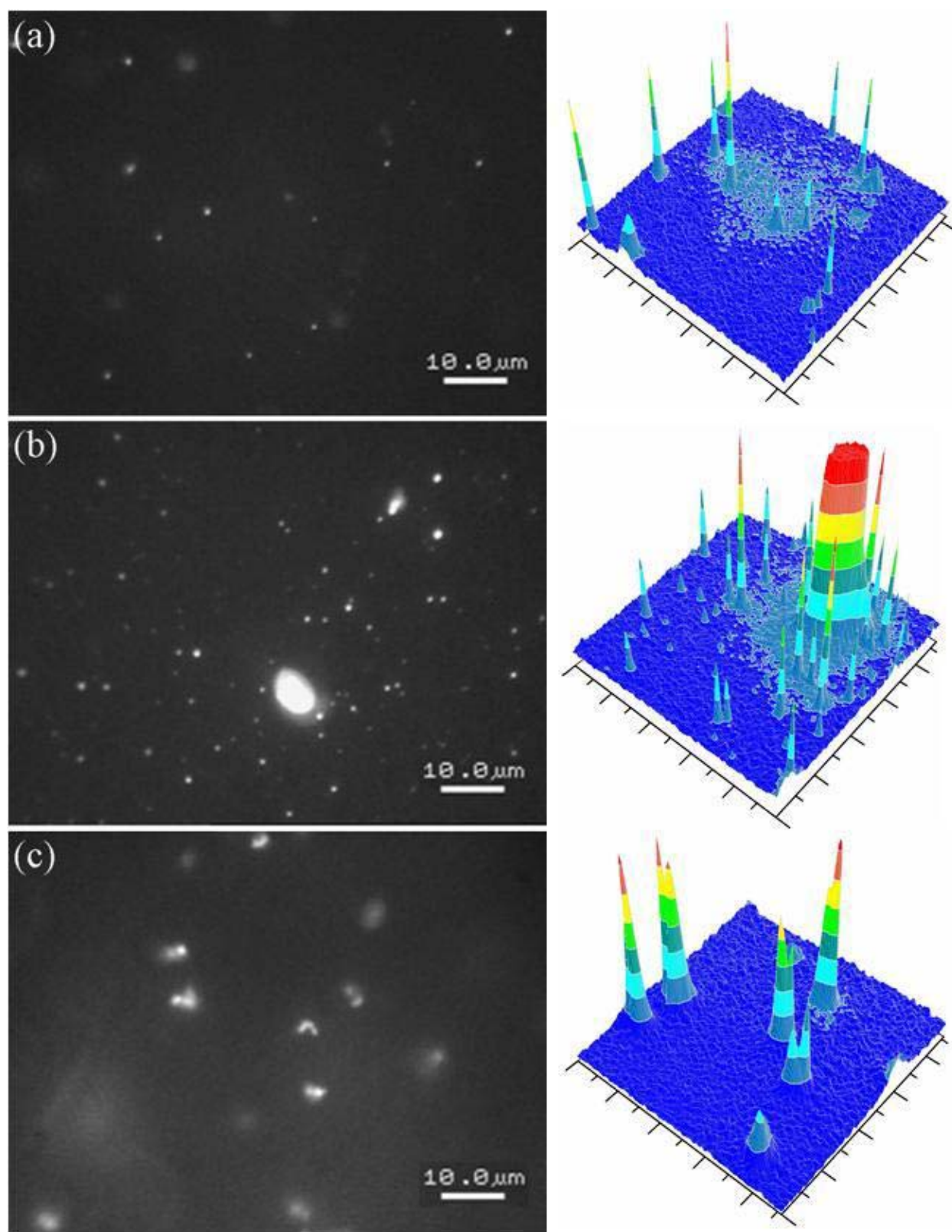
Figure 1. To the left are shown the fluorescence images of T4DNA:CTAB at different β-CD concentrations: (a) globules at 3.16 x 10^{-4} mol kg^{-1} β-CD, (b) individual globules and some multi-globular aggregates at 8.91 x 10^{-4} mol kg^{-1} and (c) coils at 3.16 x 10^{-3} mol kg^{-1}. To the right are shown the intensity profiles for a selected region from the left hand images.
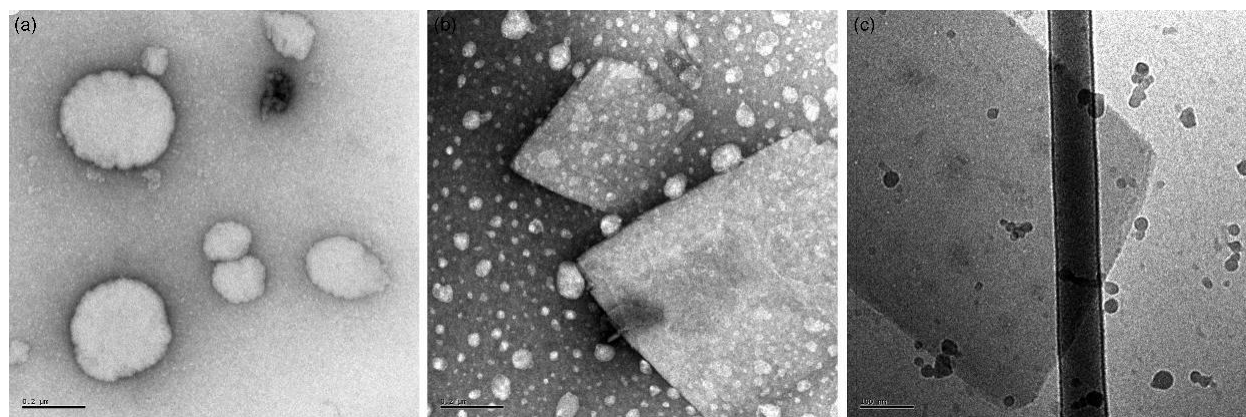
Figure 2. (a) and (b) show the TEM negative stained samples of CTAB: β-CD at pH 7.6 for a β-CD concentration of 6.31 x 10^{-4} and 1 x 10^{-3} mol Kg^{-1} respectively. (c) Shows similar results for 1 x 10^{-3} mol kg^{-1} β-CD concentration obtained from cryo-TEM.

It is well known that T4DNA can be compacted by using CTAB as was reported by many authors (21). In our work we studied a very precise CTAB:T4DNA ratio that ensures the full compaction of T4DNA, been far from the region of coexistence between coils and globules. In order to confirm the presence of globules we investigated the CTAB:T4DNA samples by using TEM of negative stained samples and the results are shown in Figure 3.

In Figure 3 we show a region with a high number of globules. The average sizes of the globules are in good agreement with those reported by other authors using dynamic light scattering (DLS). TEM images of negatively stained samples show that the size of the globules at low β-CD concentration is around 70 nm (see Figure 3), which is in good agreement with previous studies by Dias *et al.* (11). We should stress that such high number of globules was reached by the addition and drying of successive sample drops from the stock solution. This method avoid the use of a high T4DNA:CTAB concentration. In Cryo-TEM experiments the globules are difficult to find because of the low concentration of the stock solution. We avoid an increase of the DNA:CTAB concentration in order to neglect the globule-globule interaction. The increase in the concentration of T4DNA can affect the DNA conformation as well as the interaction between globules or coils. The interaction of double-stranded structures of increasing DNA concentration, the so called Holliday junctions, was confirmed by Yakbovskaya *et al.* (22) using electron microscopy experiments.

4.3 β-CD as a decompacting agent

After having investigated the CTAB: β-CD interaction as well as the different kinds of aggregated structures we will study the influence of the β-CD concentration on the decompation of T4DNA:CTAB complexes. At low β-CD concentration below the critical transition concentration we performed TEM experiments of negative stained samples using the same protocol described for the imaging of globules. The results are show in Figure 4.

In Figure 4a we show the presence of different globular aggregates in coexistence with individual globules. The sizes range from a few nanometers, corresponding to individual globules, to microns. In figure4b and 4c we show a big globular aggregate and the arrows indicate small globular aggregates resulting from the hierarchical aggregation of individual globules. In the bottom of Figure 4b we show a sheed-like structure formed by CTAB: β-CD analogous to those shown in Figure 2a. The samples under investigation below the critical transition concentration didn't show the presence of T4DNA in a coil conformation. It is well known that the formation of multi-globular aggregates from individual globules, appear at relatively high concentration of CTAB as was found by. Melnikov *et al.* (23) who reported the formation of aggregates from two or more globules at high concentration of cationic surfactants. In our system the formation of aggregates was not observed in the stock T4DNA:CTAB solution and this low concentration was kept constant for all the different β-CD concentrations under investigation. This ensures that the aggregation of globules is not due to changes in the CTAB concentration. Below the critical transition concentration DNA, can only be in a globular state.

At high β-CD concentration, above the critical transition concentration, different samples have been investigated by using the negative staining technique and imaged in a TEM. In this case we used PTA as a stained agent because of the better contrast in the images compared with the UA staining agent. The results are shown in Figure 5.

In Figure 5 we show the coexistence of CTAB: β-CD layers and densely packet T4DNA in a coil conformation. The sizes of the layers as well as the geometry are in good agreement with those shown in Figure 2b and c. In Figure 5d we show the presence of a dense region with T4DNA in a coil conformation. The different samples studied above the critical transition concentration did not indicate the presence of any individual globule or multi-globular aggregate.

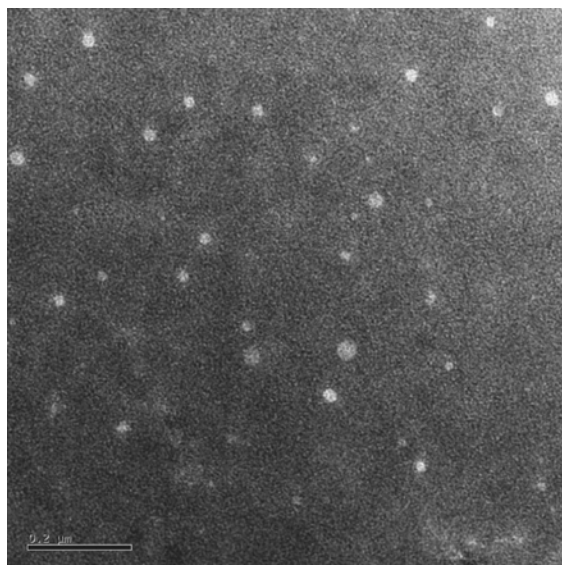In order to investigate the internal structure of the aggregates and elucidate the mechanism of multi-

Figure 3. TEM image of a negative stained samples of T4DNA:CTAB (globules) using 2% Uranil acetate.
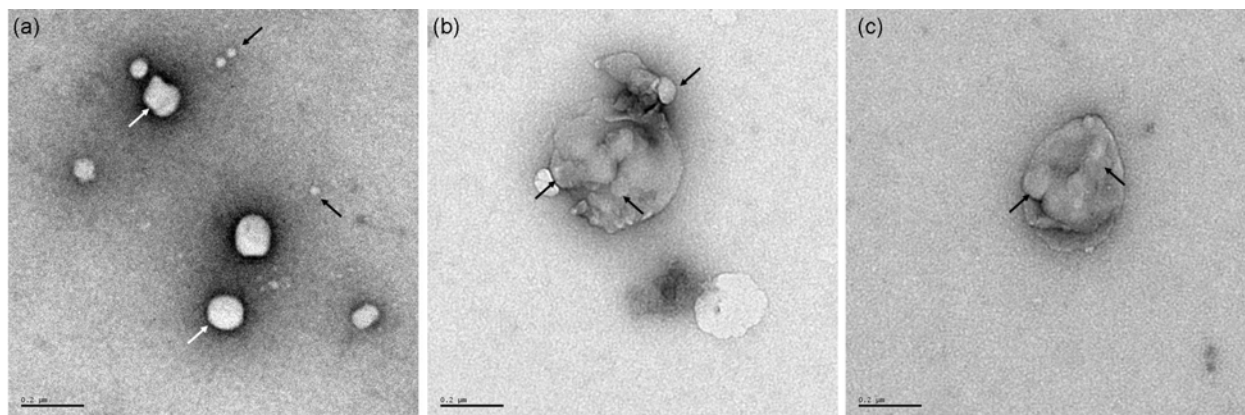

Figure 4. Negative stained TEM images of T4DNA:CTAB complexes at different β-CD concentrations. The samples in (a), (b) and (c) were prepared at 8.91×10^{-4} mol kg^{-1} β-CD concentration. The white arrows show the presence of individual globules as well as multi-globular aggregates. In (b) and (c) we show big globular clusters. The black arrows show small clusters with globular conformation. In (b) at the bottom we can see a seed-like aggregate formed by CTAB: β-CD inclusion complexes.

globular aggregate disruption we performed cryo-TEM experiments in the narrow β-CD concentration range close to the critical transition concentration. The results are shown in Figure 6

In Figure 6a we imaged a single multi-globular aggregate and from the image we can observe the internal structure formed by the individual globules (white arrow). In Figure 6b we show the presence of rod-like fibers. It is assumed that these fibers are formed by CTAB:β-CD inclusion complexes. Similar fibers have been described by Bonini *et al.* (20) in sonicated solutions of β-CD at concentrations up to 6 mM. In our system, this could indicate that the dissolution of the globular clusters is an early stage of the decompaction process. These fibers or "rod-like structures" can then be interpreted as being formed by aggregation of free CTAB:β-CD that remains in solution. We can conclude that clusters formed by

individual globules dissolve to give rise to fibers after a slight increase in β-CD concentration. Again at a higher β-CD concentration, no globules are present, and the T4DNA attains a coil conformation. The image has been obtained by slowly under focus. The coils were found in dense packet regions which is in good agreement with the result shown in Figure 5d for negative stained samples.

We can now suggest the following possible mechanism in order to explain the decompation process (see Figure 7). The decompaction proceeds as following. Starting with compacting T4DNA with a CTAB, we add β-CD and raise its concentration while keeping the CTAB:T4DNA concentration constant. An increase in the β-CD concentration will result in the formation of inclusion complexes between the CTAB free in solution and β-CD. The number of the CTAB: β-CD inclusion complexes increase when the β-CD concentration increases. In the
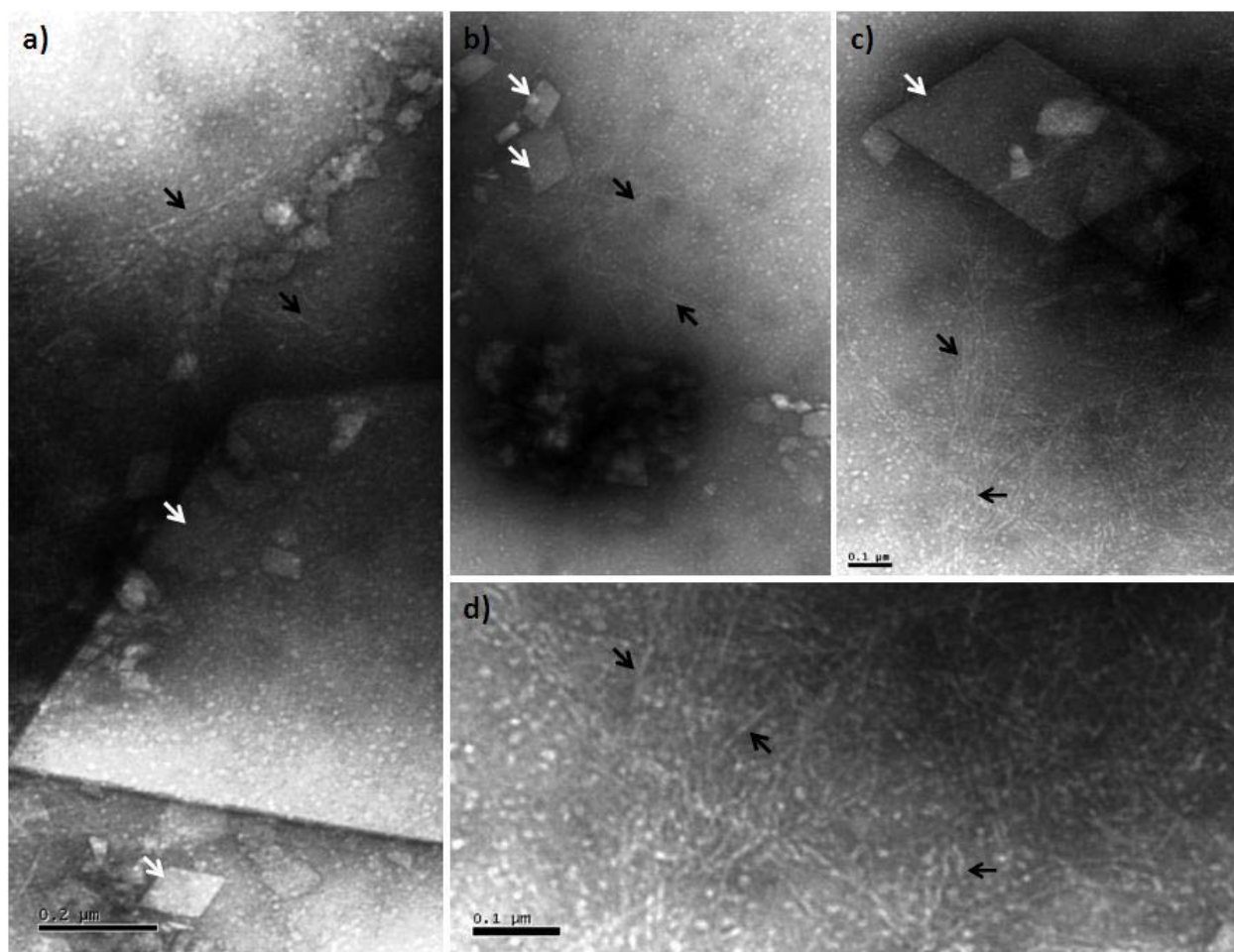
Figure 5. Negative stained samples of T4DNA:CTAB at 1.05 x 10^{-3} β-CD concentration. The white arrows show the layers formed by CTAB: β-CD and the black arrows the T4DNA in a coil conformation.

globules, the surface micelles with a characteristic aggregation number will reorganize to maintain equilibrium with the free monomers in solution (those that are not complexes with β-CD). Close to the critical transition concentration, just before the decompaction, almost all the CTAB is complexed and the negatively charged globules can now fuse through the stacking of CTAB: β-CD inclusion complexes that can also form layers and seed-like structures in coexistence with the DNA.

Note that the surface of individual T4DNA globules can then be covered by CTAB:β-CD inclusion complexes because the globule surface is slightly negatively charged and the hydrophobic tail of CTAB is encapsulated within the β-CD. The CTAB:β-CD inclusion complexes play a role of linkers between individual T4DNA globules. When the critical transition concentration is overcome, the thermodynamic equilibrium between the surface micelles (inside the globules and multi-globular aggregates) and the monomers free in solution cannot be adjusted anymore because there are no monomers left in solution (all are complexed with β-CD). The result is a complete transition from globule to coil.

However after decompaction some CTAB:β-CD complexes can remain attached to the coils as was previously suggested by Cao *et al.* (13). Note that the values for the long axis length in coils reported by González-Pérez *et al.* (12) from fluorescence microscopy experiments are longer than those reported by Yoshikawa *et al.* (23). This effect cannot be due to the intrinsic nature of the dye because the values for the globule size are analogous to those reported by Yoshikawa *et al.* (23) using a different dye.

5. CONCLUSIONS AND PERSPECTIVES

To summarize, T4DNA compacted with CTAB can efficiently be decompacted using β-CD through a non first-order transition. This phenomenon is likely to be related to the disruption of the surface micelles that keep the globular conformation stable. The thermodynamic stability of the surface micelles in the globules can be altered in the presence of β-CD that form inclusion complexes with the free CTAB monomers in solution. Additionally the formation of aggregated structures (seed-like and layers) based on CTAB:β-CD inclusion complexes in buffer, was confirmed and is in good agreement with
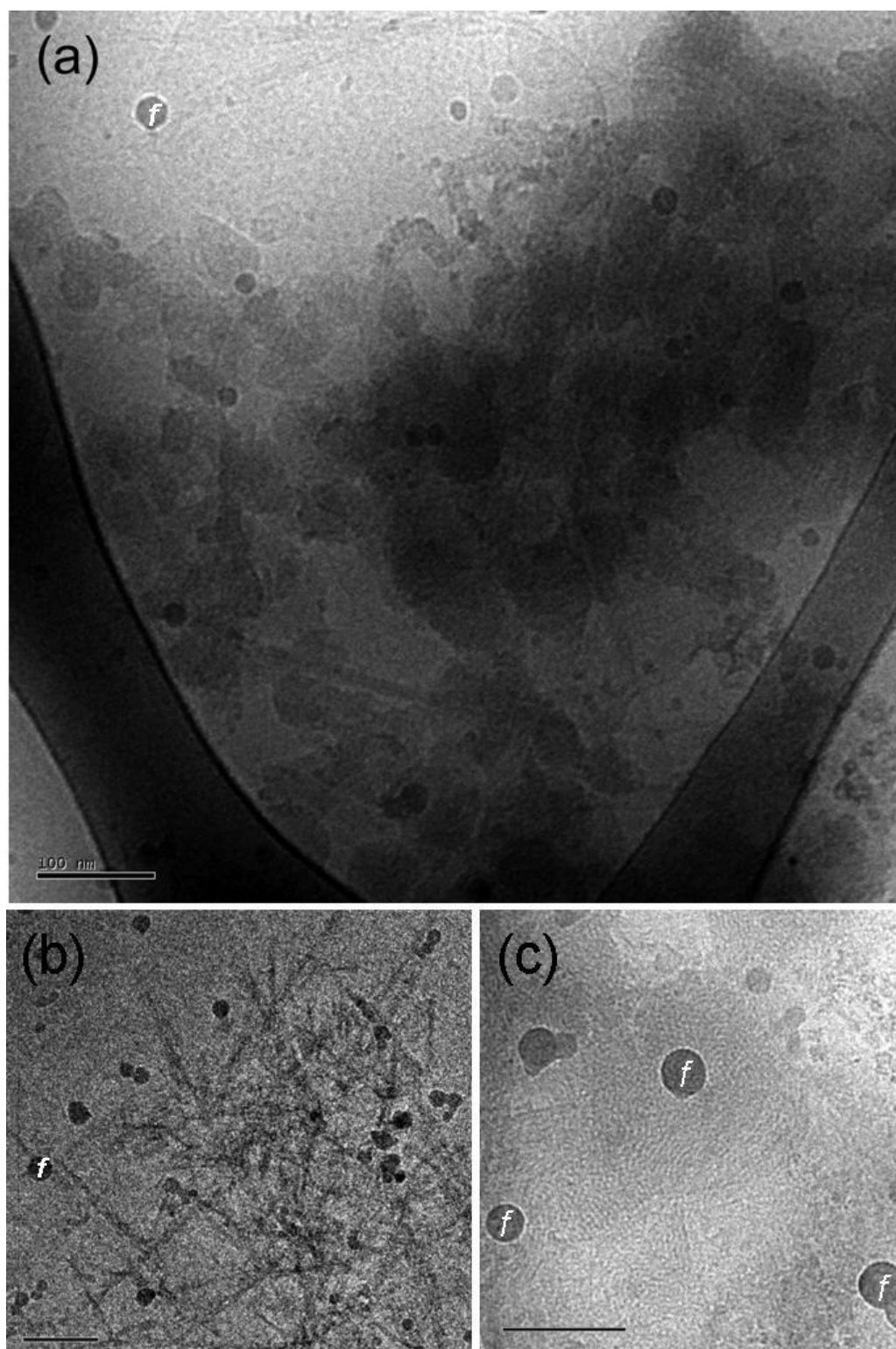
Figure 6. Cryo-TEM images of the globule clusters (a), CTAB: β-CD rod-like fibers (b) and DNA in a coil conformation (c) studied at 8.91 x 10^{-4} mol kg^{-1} (a-b) and 3.16 x 10^{-3} mol kg^{-1} (c) β-CD concentration.

previous results reported by Bonini *et al.* (20). This phenomenon seems to be a key step in the formation of multi-globular aggregates below the critical transition concentration. The CTAB:β-CD inclusion complexes can
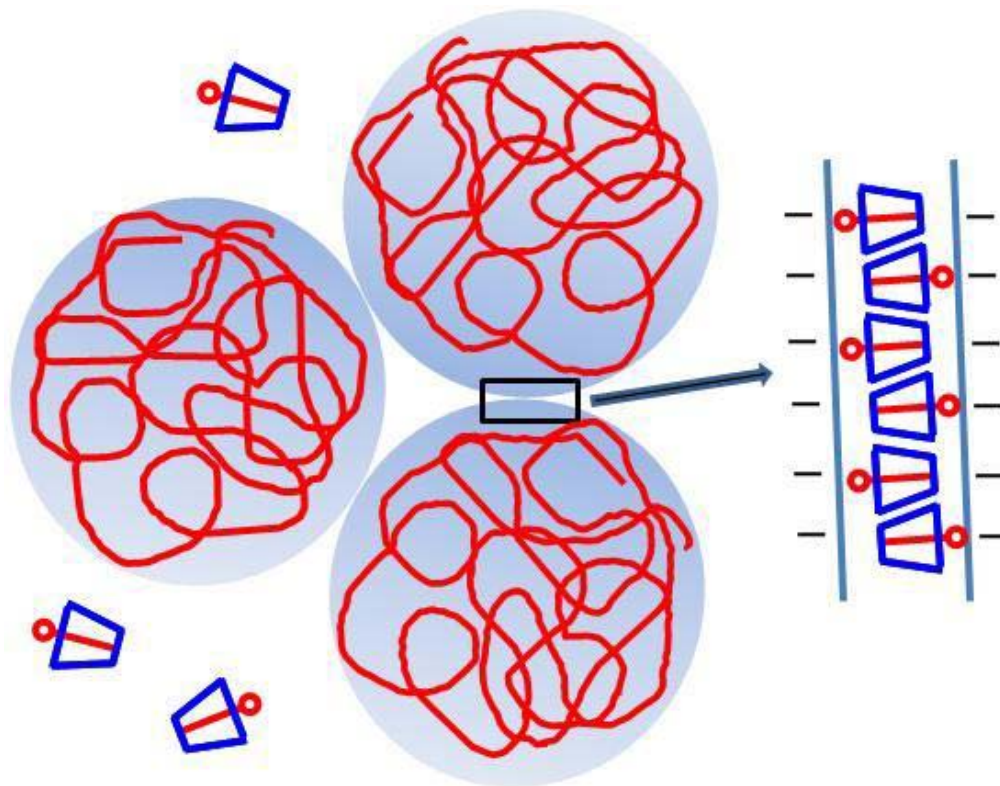
Figure 7. Mechanism of interaction between T4DNA globules (fusion) via CTAB: β-CD inclusion complexes to form multi-globular aggregates.

interact with the individual globules on the surface. They can link two or more globules to form big aggregates. Further studies will be done using other cyclodextrins with higher solubility than β-CD like 2-hydroxiproplyl-β-cyclodextrins (HP-β-CD) in order to confirm these phenomena. A new theoretical approach explaining this phenomenon is still needed and some thermodynamic studies of the decompaction process are under investigation.

6. ACKNOWLEDGEMENTS

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Send correspondence to: Alfredo Gonzalez-Perez, MEMPHYS, Center for Biomembrane Physics, Department of Physics and Chemistry, University of Southern Denmark, Campusvej.55, DK-5230 Odense M, Denmark, Tel: 45-6550 3474, Fax: 45-6550 4048, E-mail: alfredo@memphys.sdu.dk