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Bridging β -Cyclodextrin prevents self-inclusion and allows formation of supramolecular polymers: self-assembly and cooperative interaction with nucleic acids

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In memory of Prof. Ronald Breslow a continued source of scientific inspiration

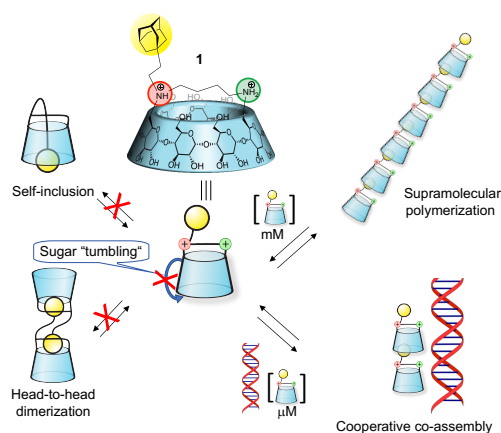
Abstract: To imitate Nature's ability to form cooperative self-assemblies using entirely artificial molecular tools and to confer them with properties is still a true challenge. Here we show that cyclodextrins bridged with an ammonium linker which bears a hydrophobic substituent efficiently form supramolecular polymers at millimolar concentrations, avoiding the competing self-inclusion and head-to-head processes. Notably, such assembly can occur at micromolar concentrations in the presence of genomic DNA, resulting in efficient DNA compaction that is reversible upon addition of a competitive guest, and demonstrating their highly cooperative interaction. We finally show that the supramolecular polymer also cooperatively interacts with siRNA and allows its transfection.

Cooperative self-assembly^[1,2,3] is a powerful tool to construct elaborate architectures in a bottom-up approach. For instance, Nature heavily relies on cooperativity to build the highly complex components of organisms from smaller units.^[4] A simple archetypical example of such constructs is the Tobacco Mosaic Virus (TMV) constituted of only two cooperatively interacting partners: a coat protein and a nucleic acid (NA) template. The outline of the complex assembly process is that the coat protein assembles only in small entities unless the NA is present and induces its full coverage in a highly cooperative manner. In reverse, release of the NA upon chemical or biological triggers^[5] results in the efficient disassembly of the supramolecular architecture into small entities. Thus, mixing NAs with self-assembling systems to form complex architectures, sometimes with the goal of biomimetic functions such as transfection, is a subject of wide interest.^[6,7,8] Different types of such assemblies can be distinguished. The simplest ones are those where a reformed supramolecular assembly, or more specifically a supramolecular polymer (SMP), interacts with NAs (DNA or RNA, single stranded or double stranded).^[9,10,11] In these cases, the assembly is not cooperative as the size of the object is determined by the primary assembly. DNA cooperative templating was

performed in the classical system developed by Meijer and Schenning,^[12] where ss-DNA oligothymine templates the co-assembly of chromophores, but the unicity of the base used precludes this assembly from biological applications. More sophisticated designs utilize the same basic construction as helical viral capsids and therefore logically uses peptides. There are two beautiful such systems constructed with the same three distinct components: a peptide-based self-assembling core, a cationic head to interact with the NA, and a hydrophilic tail to impart water-solubility to the system. These systems are rather large (>5kDa).^[9,13]

We report here on the careful design of a completely artificial, non peptide-like, small-molecule (<1.5 kDa) that self-assembles non-cooperatively through host-guest interactions at mM concentrations but that cooperatively co-assembles with a NA even at μ M concentrations. This ultimately enabled us to perform transfection of cells with siRNA.

Water-soluble SMPs have been studied for some time,^[14] and many systems are now available, including some based on cyclodextrins (CDs).^[15] CDs appeared as an ideal platform for our purpose because they contain a built-in cavity for a host-guest interaction in water, and a large number of hydroxyl groups positioned ideally for external functionalization. Moreover, regioselective multi-hetero-functionalization of CDs is now possible.^[16] Furthermore, CDs have been used in conjunction with NAs in many transfection systems,^[17,18,19] but where the cooperativity of the assembly has not previously been demonstrated.^[20,21,22]



Scheme 1. Bridging of functionalized CDs to avoid self-inclusion by tumbling and dimerization, and to allow supramolecular polymerization and cooperative assembly-induced by nucleic acid (NA)

The straightforward design of a functional CD which will form a SMP is the appendage of a hydrophobic moiety onto the CD

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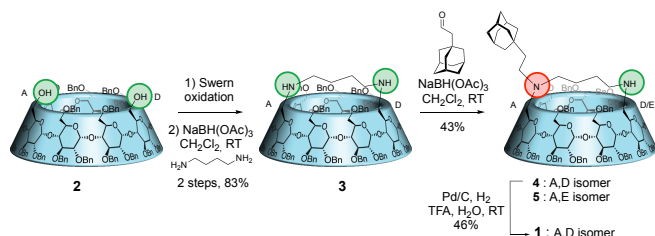
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ring.^[23] However, this simple design has inherent drawbacks for the formation of large CD-based SMPs, as various factors interfere with their formation. Firstly, relatively low solubility and their tendency to aggregate is characteristic of CDs and often interfere with their analysis, for example by light scattering. Furthermore, competitive self-inclusion of the appended hydrophobic group and/or the possibility of forming head-to-head dimers are detrimental to the formation of SMPs. Even when these competitive complexes are not formed the degree of polymerization (DP) of CD-based SMPs is rather low. This is linked to the low affinity of guests typically used in the studies and to the relatively low water-solubility of the CD monomers.^[24] Interestingly, the classical adamantane/CD host guest system, which displays among the highest affinity constant has only been seldomly used in this kind of assemblies. This is probably due to lack of solubility of the resulting conjugate.^[25] We have recently synthesized such a molecule, but, although the adamantane is too large to penetrate the CD cavity from the upper rim, the CD-adamantane conjugate was shown to exist in solution exclusively as a self-included species.^[26] This was attributed to a so-called "tumbling" phenomenon, consisting in the turnaround of the sugar unit attached to the hydrophobic conjugate, a motion which can then place the attached group inside the cavity of the CD. This underestimated phenomenon is becoming more widely accepted. It can occur with a flexible chain attached to the CD rim,^[27] but also with a more rigid one such as a triazol,^[28] or a phenyl ring.^[29] To address all these problems simultaneously, we propose here an original design in which the hydrophobic moiety is placed on a bridge linking diametrically opposed sugars. Hence self-inclusion, tumbling and head-to-head dimerization should be avoided. Furthermore, the bridge is linked to the CD by ammonium groups to increase its solubility and to interact with NAs. (Scheme 1)

The synthesis of the designed monomer **1** started from native β -CD using the now classical benzylation/regioselective bis-debenzylation^[30] to afford diol **2**. A Swern oxidation of diol **2** afforded the corresponding dialdehyde that was bridged by a double reductive amination using putrescin giving **3** in 83% yield over two steps. Another reductive amination using 1-adamantaneacetaldehyde yielded 43% of the two regioisomeric singly functionalized CDs **4** and **5** together with unreacted **3** (21%) and bifunctionalized CD (13%), which were successfully separated by a silica gel flash chromatography (see SI). The isolated major regioisomer **4** was deprotected through catalytic hydrogenation under acidic conditions to furnish the corresponding CD **1** in 46% yield after purification using reverse phase chromatography (Scheme 2).



Scheme 2. Synthesis of monomer **1**.

Self-assembly was first studied by NMR. The ^1H NMR of CD **1** was monitored during dilution (SI, Fig. S11) and showed some chemical shift variations for adamantyl proton signals indicating intermolecular associations. Moreover, ^1H signals of the CD become broader when its concentration increases, suggesting the

formation of larger species at high concentration. The inclusion of the adamantyl group in the CD cavity was confirmed by a T-ROESY experiment (Fig. 1 and SI, Fig. S12). Indeed, strong correlations were observed between adamantyl protons (Hc and Hb) and the H-3 protons located inside the CD cavity. Such correlations are in accordance with intermolecular inclusion of the adamantyl group across the secondary rim of the CD of another monomer. These observations demonstrate the importance of the bridge that prevents the self-inclusion process which otherwise operates as in the case of compound **6** (Fig. 2) devoid of the bridge.^[Erreur ! Signet non défini.] The bridging of the CD therefore forces intermolecular inclusion of the adamantyl substituent. A DOSY experiment was also performed to evaluate the size of the self-assembly of CD **1** at different concentrations (Fig. 2). Increasing the concentration of **1** led to a non-linear decrease in its diffusion coefficient. This dependency is indicative of polymerization of repeat units.^[31] In contrast, the diffusion coefficients of **6** and **7** (structures in SI) remained constant and similar to the one of native β -CD over the same concentration range. These observations indicate that competing aggregation processes did not interfere,^[32] and that the intermolecular inclusion of the adamantyl group is necessary for the formation of the oligomers. Finally, a competition experiment was performed using adamantane carboxylate as a stopper. We showed by DOSY NMR that the SMP could be disassembled using this competitive guest (SI, Fig. S14).

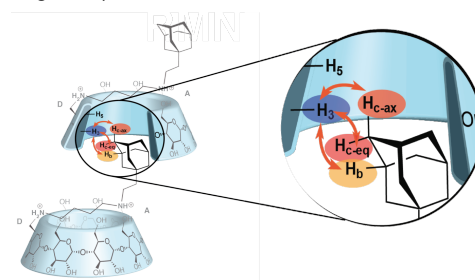


Figure 1. TROESY cross-correlations (red arrows) for CD **1** at 6.3 mM (600 MHz, D_2O , 300K).

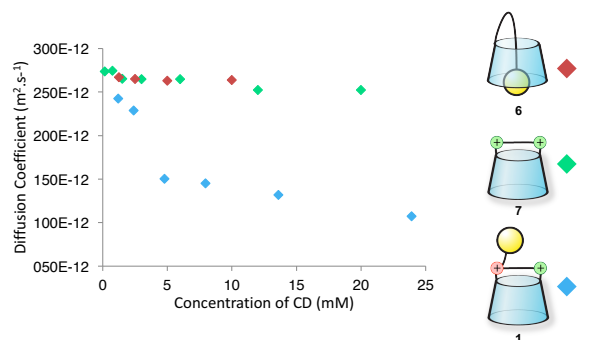


Figure 2. Concentration dependence of diffusion coefficient D for **1**, **6** and **7** (from ^1H NMR spectroscopy, 600 MHz, D_2O , 300K).

The self-assembly of CD **1** into a SMP was confirmed by viscometry. The non-linear and strong dependence of the relative viscosity of CD **1** with concentration indicates associative behavior of this compound (SI, Fig. S15). We also performed Small-Angle Neutron Scattering (SANS). At 6.9 mM CD **1** was found to form cylinders with a diameter of about 0.97 nm and a length of 6.6 nm, which corresponds to an assembly of 7 monomers (Fig. 3). We therefore proved by NMR, viscometry and SANS that CD **1** self-assembles through intermolecular inclusion

of its adamantyl substituent. To quantify this interaction, we performed Isothermal Titration Calorimetry (ITC). Dilution of CD **1** solutions at initial concentrations ranging from 0.5 to 13 mM produced a strongly concentration dependent signal (SI, Fig. S16) that was assigned to the endothermic dissociation of CD/adamantyl complexes. After integration of the heat flow curves, the data for CD **1** was fitted to an isodesmic model,^[33] and gave a K_a of 1.1×10^4 L/mol and ΔH_{assoc} of -2.9 kcal/mol. With the association constant of CD **1** in hand, we can compare the DPs obtained both by DOSY NMR, using Tirado-Garcia de la Torre equation,^[34] and by SANS experiments to the predicted values for an isodesmic model (see SI). The various approaches show DPs in the same range.

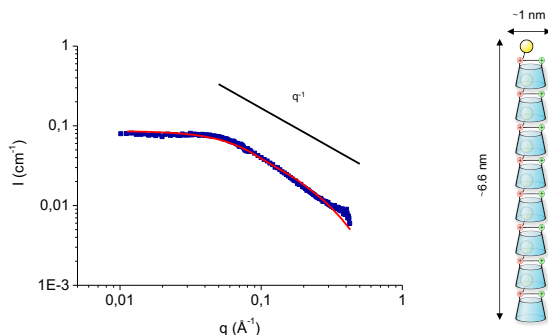


Figure 3. SANS intensity (I) versus scattering vector (q) for a 6.9 mM solution of CD **1** in D_2O . The plain curve is a fit according to a model for a cylindrical rod of length 6.6 nm and diameter 0.97 nm (see SI).

We have therefore proven that CD **1** assembles into a SMP and follows the isodesmic model. Furthermore, we solved some of the long-standing challenges of CD-based SMP formation: self-inclusion through bridging, low DP using the Adamantane- β -CD interaction, and the low solubility adding ammonium groups on the CD. We next analyzed how the unique self-assembly character of these cationic CDs was affected by or could be applied for interactions with nucleic acids, namely DNA compaction and siRNA transfection.

We first explored the behaviour of CDs in the presence of a giant genomic duplex DNA, T4DNA (166 000 base pairs), in highly diluted solutions ($0.1 \mu\text{M}$ in DNA phosphate in 10 mM Tris-HCl buffer). Under these conditions, the conformational state of a large number of individual DNA molecules (> 100 per condition) in solution could be precisely assessed by means of fluorescence microscopy. In the absence of CD, all molecules appeared as large fluctuating coils (Fig. 4, yellow bar) due to electrostatic repulsions between phosphate groups along the DNA backbone. Addition of unmodified β -CD up to millimolar concentrations did not affect this conformational state of DNA. In contrast, submillimolar addition of modified CDs induced a dramatic conformational change of individual DNA molecules in solution. At very low CD concentrations, all molecules remained in the coil state but, above a critical concentration, all DNA molecules appeared as bright fast-diffusing globules, a typical signature of DNA in a compact state (Fig. 4, blue bar). Between these two regimes, we identified an intermediate zone where molecules either in coil or compact states co-existed (Fig. 4 green bar). This allowed us to establish compaction curves, i.e., the fraction of DNA molecules in the compact state as a function of [CD] (SI, Fig. S17A), and determine the [CD] at which this fraction was 50%, referred to as C_{50} . Interestingly the compaction ability of the

different CDs was strongly dependent on both their charge and their assembly propensity (Fig. 4).

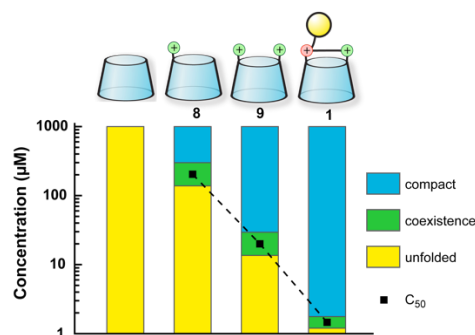


Figure 4. DNA conformational state diagram in the presence of various CDs as determined by FM. The yellow, green and blue bars depict the range of CD concentration corresponding to $\geq 80\%$ of unfolded state, coexistence of unfolded and compact states, and to $\geq 80\%$ of compact state, respectively. The black square represents the CD concentration at which 50% of DNA molecules are in the compact state (C_{50}). Conditions: [T4 DNA] = $0.1 \mu\text{M}$ in 10 mM Tris-HCl (pH = 7.4); [YOYO-1 iodide] = $0.01 \mu\text{M}$.

Increasing the number of ammonium groups from 1 to 2 was accompanied by a strong decrease of C_{50} (200 and $20 \mu\text{M}$ for CD **8** and CD **9**, respectively), a known behavior explained by a more entropically favorable ion-exchange when the compaction agent charge increased.^[35] Moreover, the addition of the adamantane group at constant electrostatic charge was accompanied by a decrease by one further order of magnitude of C_{50} ($1.3 \mu\text{M}$ for CD **1**). This was reminiscent of the enhanced DNA compaction ability of amphiphilic molecules due to their highly cooperative binding to DNA where they could locally assemble much below their critical micellar concentration.^[36] To determine whether this enhancement effect was due to hydrophobic interactions between adamantyl substituents or specific Adamantane- β -CD inclusion, we performed a competition assay by adding adamantane carboxylate to a solution of fully compact DNA in the presence of a fixed concentration of CD **1** ($3 \mu\text{M}$). Notably, the addition of 10 and 50 equivalents of adamantane carboxylate resulted in the unfolding of 52% and 96% of DNA molecules, respectively (SI Fig. S17B). This clearly indicates that cooperative binding occurred on DNA through the formation of adamantane- β -CD inclusion. We thus found that, in the presence of DNA, adamantane- β -CD host-guest interaction could occur at micromolar concentrations of CD **1**, i.e., 3 orders of magnitude smaller than the same CD **1** alone in solution, an effect that was not reported before. This resulted in a remarkable DNA compaction ability of CD **1**, which was able to fully compact DNA with only two cationic charges at $3 \mu\text{M}$, a concentration comparable and even smaller than the one for the naturally occurring tetravalent compaction agent spermine (around $5 \mu\text{M}$ under same conditions).^[37] The general applicability of the concept and the importance of the bridge are confirmed by the similar results obtained with a distinct cyclodextrin (see SI).

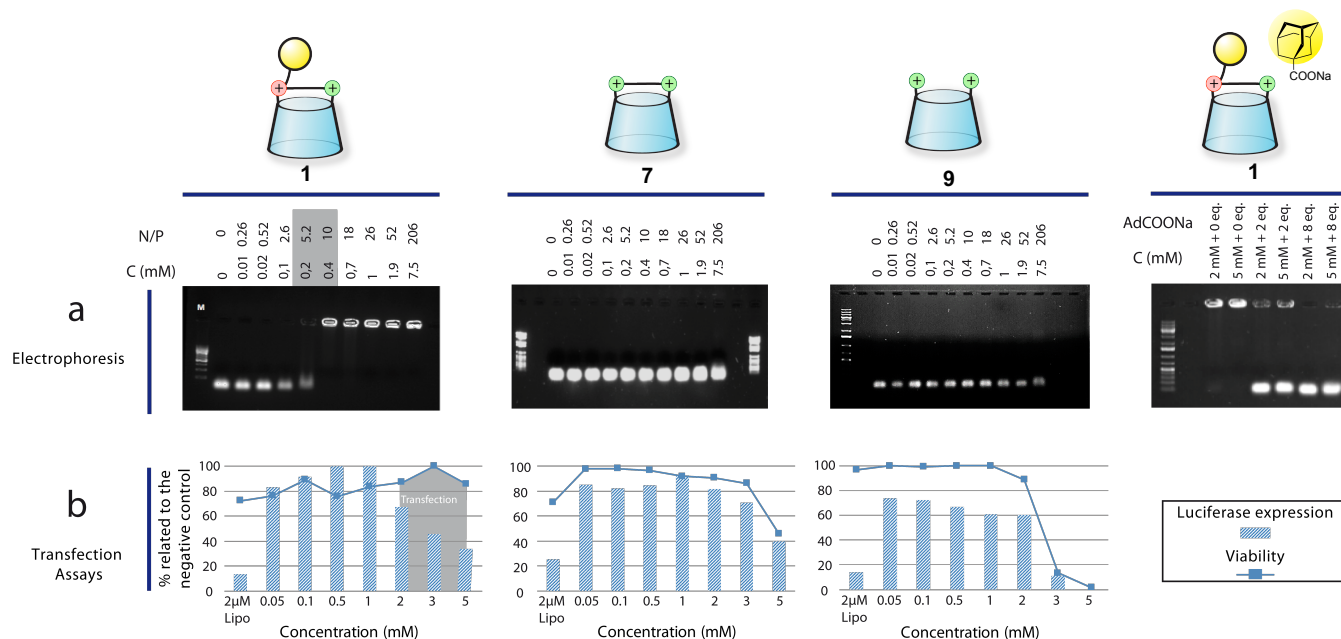


Figure 5. a, Gel retardation assay using an agarose gel electrophoresis for siRNA (1.72 μM) complexes formulated with **1**, **7** and **9** at different concentrations of CD. EtBr was used as the staining reagent. b, Cell viability and in vitro transfection efficiency in HEK-293 cells for siRNA complexes formulated with **1**, **7**, **9** and 5 pmole of siRNA. Lipo = Lipofectamine 2000.

Finally, compared to highly charged compaction agents such as poly-L-lysine, that can compact DNA close to charge neutralization,^[37] CD **1** offers the additional possibility to easily unfold DNA once compacted by simply adding an inclusion competitor.

Finally, we exploited the ability of CD **1** to strongly interact with nucleic acid for application in siRNA transfection for gene silencing. We chose GL3 siRNA, a NA directed against the expression of luciferase. Gel retardation assay using an agarose gel electrophoresis showed that, whatever the concentration, derivatives **7** and **9** (which cannot form SMPs) are not able to complex siRNA to inhibit its migration. In contrast, CD **1**, was able to prevent siRNA from migrating, even at concentrations as low as 0.4 mM (N/P=10)^[38] for which CD **1** alone should only form dimers. Furthermore, the importance of the supramolecular assembly to interact efficiently with siRNA, and therefore shield the negative charges of the siRNA, was studied by competition experiments using AdCOONa as a competitive guest and stopper. Upon the addition of 2 equivalents of AdCOONa, most of the siRNA was released, while 8 equivalents fully released the siRNA (Fig. 5a). Thus, retardation of migration of siRNA by dicationic CDs is only possible when the CD self-assembles, and therefore interacts more efficiently with the polyanion in a cooperative and multivalent manner. Moreover, nuclease protection assays were carried out and showed the ability of our SMPs to protect efficiently siRNA (SI, Fig. S18). Finally, GL3 siRNA complexes formulated with either **1**, **7** or **9** were tested in gene delivery assays towards HEK-293 cells, which express the firefly luciferase GL3. Derivatives **7** and **9** showed no transfection ability, as the decrease in the expression of luciferase at high

concentrations of CD was correlated with cell death. In contrast, the expression of luciferase clearly decreased when cells were treated with complexes of **1** and siRNA, at concentrations of CD **1** above 2 mM, while no toxicity on cells was observed, proving that the SMP can, indeed, induce transfection of siRNA (Fig. 5b).

We have therefore unambiguously shown that the bridging of the CD with an ammonium linker which bears a hydrophobic substituent efficiently forms SMPs, avoiding the competing self-inclusion and head-to-head processes. Furthermore, this self-assembling CD derivative interacts in a highly cooperative manner with DNA as demonstrated by compaction experiments. It also interacts cooperatively with siRNA and allows its transfection. All those properties were clearly attributed to the ability to form SMPs through host-guest interactions by competition experiments. We now have to decipher the hierarchical assembly between CD **1** and NAs.

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- [38] Here, we consider that all amines are charged, i.e. that all CDs are doubly charged.
