Supramolecular Polymers

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Bridging β-Cyclodextrin Prevents Self-Inclusion, Promotes Supramolecular Polymerization, and Promotes Cooperative Interaction with Nucleic Acids

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Dedicated to Professor Pierre Sinaÿ on the occasion of his 80th birthday

It is still a great challenge to imitate nature's ability to form cooperative self-assembled structures using entirely artificial molecular tools and to confer them with properties. Here we show that cyclodextrins bridged by an ammonium linker with a hydrophobic substituent efficiently form supramolecular polymers at millimolar concentrations and avoid the competing self-inclusion and head-to-head processes. Notably, such assembly can occur at micromolecular concentrations in the presence of genomic DNA, thereby resulting in an efficient compaction of DNA 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[1–3] is a powerful tool to construct elaborate architectures in a bottom-up approach. For example, 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), which is formed from only two cooperatively interacting partners: a coat protein and a nucleic acid (NA) template. The principle feature 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.[6–8] Different types of such structures can be distinguished. The simplest ones are those where a preformed supramolecular assembly, or more specifically a supramolecular polymer (SMP), interacts with NAs (DNA or RNA, single-stranded or double-stranded).[9–11] In these cases, the assembly is not cooperative as the size of the object is determined by the primary assembly. Cooperative templating with DNA was performed in the classical system developed by Meijer, Schenning, and co-workers,[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 from 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, but these systems are rather large (>5 kDa).[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 millimollar concentrations but cooperatively coassembles with an NA even at micromolar concentrations. This ultimately enabled us to transfect 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 to be an ideal platform for our purpose because they contain a built-in cavity for a host–guest interaction in water as well as a large number of hydroxy 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,[17–21] but the cooperativity of the assembly has not previously been demonstrated.[21–23]

The straightforward design of a functional CD which will form a SMP is through appendage of a hydrophobic moiety onto the CD ring.[15] However, this simple design has inherent

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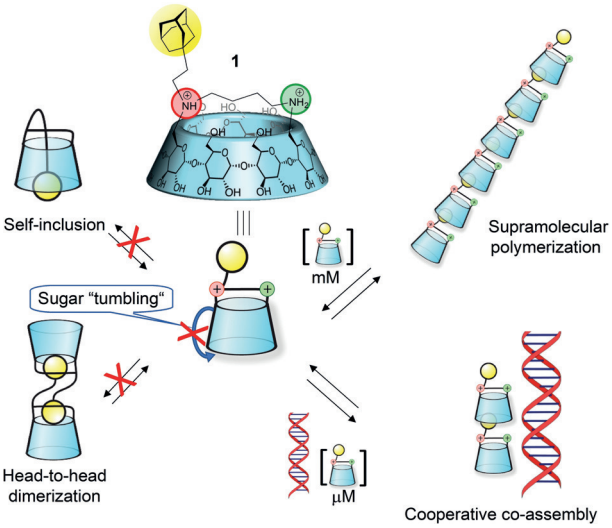
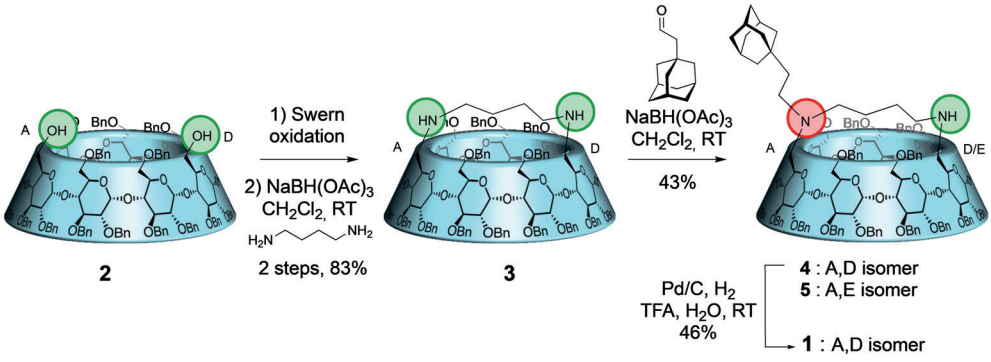
drawbacks for the formation of large CD-based SMPs, as various factors interfere with their formation. Firstly, relatively low solubility and their tendency to aggregate are characteristic of CDs and often interfere with their analysis, for example, by light scattering. Furthermore, competitive self-inclusion 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,[25] which has one of the highest affinity constants, has only seldomly been used in this kind of structures. This is probably due to the lack of solubility of the resulting conjugate.[26] 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.[27] This was attributed to a so-called "tumbling" phenomenon, which consists of 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,[28] but also with a more rigid one such as a triazol[29] or a phenyl ring.[30] 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 sugar moieties. Hence self-inclusion, tumbling, and head-to-head dimerization should be avoided. Furthermore, the bridge is linked to the CD through ammonium groups to increase its solubility and to interact with NAs (Scheme 1).



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 acids (NAs).

The synthesis of the designed monomer **1** started with the now classical benzylation/regioselective bis-debenzylation[31] of native β-CD to afford diol **2**. A Swern oxidation of diol **2** afforded the corresponding dialdehyde that was bridged through a double reductive amination with putrescin to give **3** in 83% yield over two steps. Another reductive amination using 1-adamantaneacetaldehyde afforded the two regioisomeric singly functionalized CDs **4** and **5** in 43% yield together with unreacted **3** (21%) and bifunctionalized CD (13%), which were successfully separated by flash chromatography on silica gel (see the Supporting Information). The isolated major regioisomer **4** was deprotected by catalytic hydrogenation under acidic conditions to furnish the corresponding CD **1** in 46% yield after purification by reverse-phase chromatography (Scheme 2).

The self-assembly of **1** was first studied by NMR spectroscopy. The ^{1}H NMR spectrum of CD **1** was monitored upon dilution (see Figure S21 in the Supporting Information) and showed some variations in the chemical shifts of the adamantyl proton signals, thus indicating intermolecular associations. Moreover, the ^{1}H signals of the CD became broader when its concentration increased, thus 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 (Figure 1 and see Figure S22 in the Supporting Information). Indeed, strong correlations were observed between the 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 a CD of another monomer. These observations demonstrate the importance of the bridge in preventing the self-inclusion process which otherwise operates, as in the case of compound **6** (Figure 2) which is devoid of the bridge.[27] 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-assembled structure of CD **1** at different concentrations (Figure 2). Increasing the concentration of **1** led to a nonlinear decrease in its diffusion



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

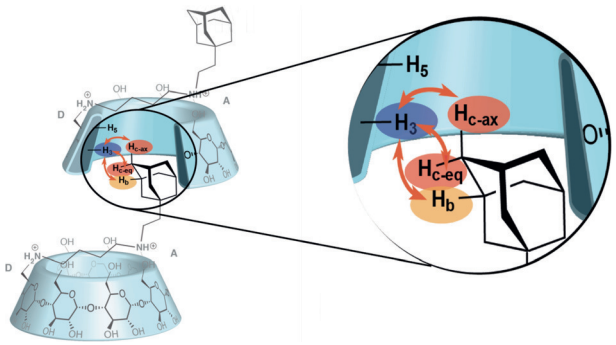


Figure 1. T-ROESY cross-correlations (red arrows) for CD **1** at 6.3 mM (600 MHz, D_2O, 300 K).

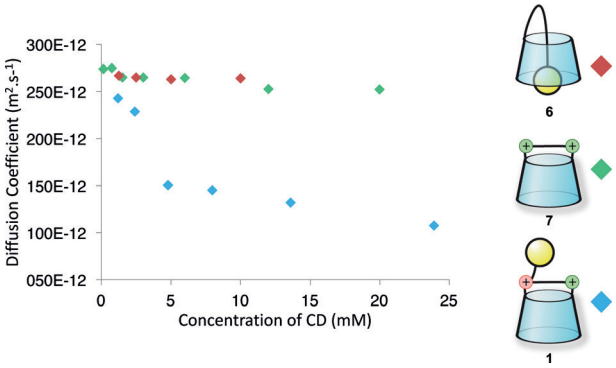


Figure 2. Concentration dependence of the diffusion coefficient *D* for **1**, **6**, and **7** (from ^{1}H NMR spectroscopy, 600 MHz, D_2O, 300 K).

coefficient. This dependency is indicative of the polymerization of repeat units.[32] In contrast, the diffusion coefficients of **6** and **7** (structures in the Supporting Information) remained constant and similar to that of native β-CD over the same concentration range. These observations indicate that competing aggregation processes did not interfere,[33] 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 spectroscopy that the SMP could be disassembled by using this competitive guest (see Figure S24 in the Supporting Information).

The self-assembly of CD **1** into a SMP was confirmed by viscometry experiments. The nonlinear and strong dependence of the relative viscosity of CD **1** with concentration indicates the associative behavior of this compound (see Figure S25 in the Supporting Information). We also performed small-angle neutron scattering (SANS) measurements. 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 seven monomers (Figure 3). We therefore proved by NMR, viscometry, and SANS measurements that CD **1** self-assembles through intermolecular inclusion of its adamantyl substituent. To quantify this interaction, we performed isothermal titration calorimetry (ITC). Dilution of solutions of CD **1** at initial concentrations ranging from 0.5 to 13 mM produced a strongly concentration-

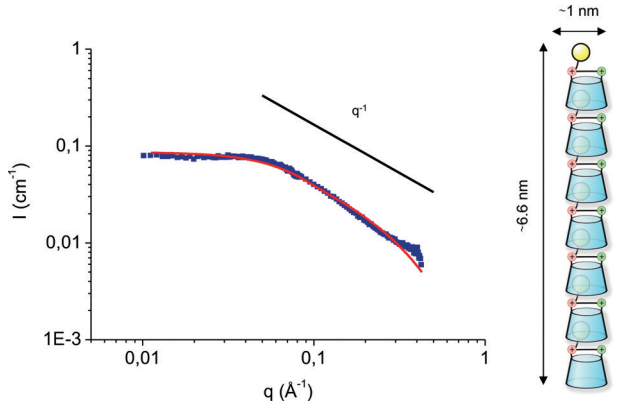


Figure 3. SANS intensity (*I*) versus scattering vector (*q*) for a 6.9 mM solution of CD **1** in D_2O. The plain curve is a fit according to a model for a cylindrical rod of length 6.6 nm and diameter 0.97 nm (see the Supporting Information).

dependent signal (see Figure S26 in the Supporting Information), which was assigned to the endothermic dissociation of CD/adamantyl complexes. After integration of the heat-flow curves, the data for CD **1** were fitted to an isodesmic model,[34] which gave a K_a value of 1.1×10^4 L mol^{-1} and a ΔH_{assoc} value of -2.9 kcal mol^{-1}. With the association constant of CD **1** in hand, we can compare the degrees of polymerization (DPs) obtained both by DOSY NMR spectroscopy, using the Tirado–Garcia de la Torre equation,[35] and by SANS experiments with the predicted values for an isodesmic model (see the Supporting Information). The various approaches show DP values in the same range.

We have, therefore, proven that CD **1** assembles into a SMP and follows the isodesmic model. Furthermore, we overcame the long-standing limitations of CD-based SMP formation: self-inclusion through bridging, low degree of polymerization (DP) by using the adamantane-β-CD interaction, and the low solubility by 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 in, interactions with nucleic acids, namely DNA compaction and siRNA transfection.

We first explored the behavior of CDs in the presence of a giant genomic duplex DNA, T4DNA (166 000 base pairs), in highly diluted solutions (0.1 μM 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 DNA molecules appeared as large fluctuating coils (Figure 4, yellow bar) as a result of electrostatic repulsions between phosphate groups along the DNA backbone. The addition of unmodified β-CD up to millimolar concentrations did not affect this conformational state of the DNA. In contrast, the addition of submillimolar concentrations of modified CDs induced a dramatic conformational change in the individual DNA molecules in the solution. At very low CD concentrations, all the molecules remained in the coil state but, above a critical concentration, all the DNA molecules appeared as bright fast-diffusing globules, a typical signature

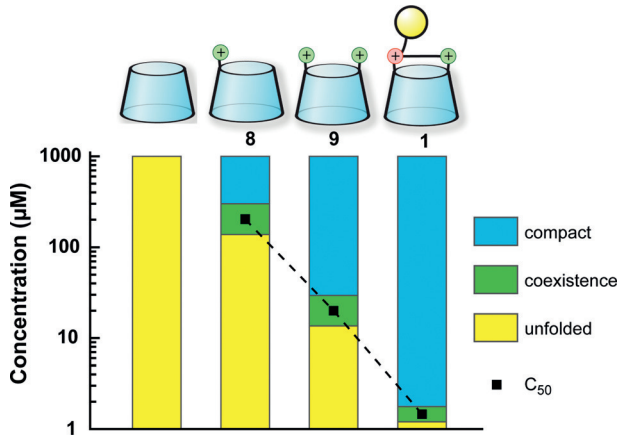


Figure 4. Conformational state diagram of DNA in the presence of various CDs as determined by fluorescence microscopy. The yellow, green, and blue bars depict the range of CD concentrations corresponding to ≥80% of the molecules in the unfolded state, coexistence of unfolded and compact states, and to ≥80% of the molecules in the compact state, respectively. The black square represents the CD concentration at which 50% of the DNA molecules are in the compact state (C_{50}). Conditions: [T4DNA] = 0.1 μM in 10 mM Tris-HCl (pH 7.4); [YOYO-1 iodide] = 0.01 μM.

of DNA in a compact state (Figure 4, blue bar). Between these two regimes, we identified an intermediate zone where molecules in either the coil or compact states co-existed (Figure 4 green bar). This allowed us to establish compaction curves, namely, the fraction of DNA molecules in the compact state as a function of the CD concentration (see Figure S27A in the Supporting Information) 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 propensity to undergo assembly (Figure 4).

Increasing the number of ammonium groups from one to two was accompanied by a strong decrease in the C_{50} value (200 and 20 μM for CD **8** and CD **9**, respectively), a known behavior explained by a more entropically favorable ion exchange when the charge on the compaction agent increased.[36] Moreover, the addition of the adamantane group at a constant electrostatic charge was accompanied by a decrease in the C_{50} value by one further order of magnitude (1.3 μM 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 far below their critical micellar concentration.[37] 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 compacted DNA in the presence of a fixed concentration of CD **1** (3 μM). Notably, the addition of 10 and 50 equivalents of adamantane carboxylate resulted in the unfolding of 52% and 96% of DNA molecules, respectively (see Figure S27B in the Supporting Information). This clearly indicates that cooperative binding occurred with DNA through the formation of adamantane-β-CD by inclusion. We thus found that, in the presence of DNA, the adamantane-β-CD host–guest interaction could occur at micromolar concentrations of CD **1**, which is three orders of magnitude smaller than that of the same CD **1** alone in solution, an effect that has not been 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 μM, a concentration comparable and even smaller than that of the naturally occurring tetravalent compaction agent spermine (around 5 μM under the same conditions).[39] The general applicability of the concept and the importance of the bridge were confirmed using another set of CDs: a bridged self-assembling CD and a non-bridged self-included one (see Figures S34 and S35 in the Supporting Information). Finally, compared to highly charged compaction agents such as poly-L-lysine, which can compact DNA close to charge neutralization,[38] 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 acids for application in siRNA transfection for gene silencing. We chose GL3 siRNA, an NA directed against the expression of luciferase. A 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)[39] 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 adamantane carboxylate as a competitive guest and stopper. The addition of two equivalents of adamantane carboxylate resulted in most of the siRNA being released, while eight equivalents fully released the siRNA (Figure 5a). Thus, retardation of the 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 efficiently protect siRNA (see Figure S36 in the Supporting Information). Finally, GL3 siRNA complexes formulated with **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 the CD 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, thus proving that the SMP can, indeed, induce transfection of siRNA (Figure 5b).

In summary, we have unambiguously shown that bridging the CD with an ammonium linker which bears a hydrophobic substituent leads to the efficient formation of SMPs and avoids 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

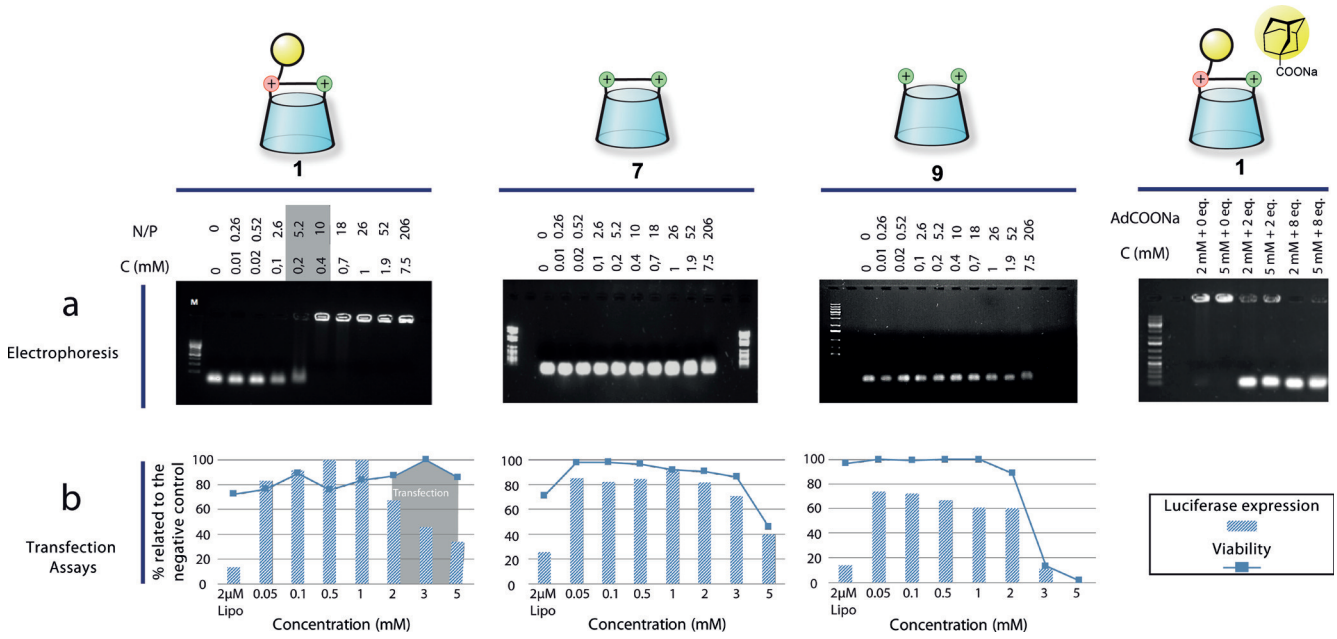


Figure 5. a) Gel retardation assay using an agarose gel electrophoresis for siRNA (1.72 μM) complexes formulated with **1**, **7**, and **9** at different concentrations of CD. EtBr was used as the staining reagent. b) Cell viability and in vitro transfection efficiency in HEK-293 cells for siRNA complexes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

cooperatively with siRNA and allows its transfection. All those properties were clearly attributed to the ability to form SMPs through host–guest interactions, as shown by competition experiments. We now have to decipher the hierarchical assembly formed between CD **1** and NAs.

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Conflict of interest

The authors declare no conflict of interest.

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