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A novel and versatile design of DNA-lipid conjugates is presented. The assembly of the DNA headgroups into G-quadruplex structures is essential for the formation of micelles and their stability. By hybridization with a complementary oligonucleotide the micelles were destabilized, resulting in cargo release. In combination with a hairpin DNA aptamer as complementary strand, the release is obtained selectively by the presence of ATP.

The molecular-recognition properties and polymorphisms of nucleic acids make them ideal scaffolds for the design of complex supramolecular structures that allow for control over composition, structure and function.[1–5] In addition to the well-known double helix conformation, DNA is also able to fold into non-canonical structures such as G-quadruplexes, i-motifs and A-motifs.[6–8] Among these, G-quadruplexes (G-4s) are of particular interest because of their well-defined conformation, high stability and versatility.[6,9] G-4s are composed of planar guanine tetrads that are able to accommodate small molecules via π-π stacking interactions[10–12] and recent studies have linked the formation of G-4s *in vivo* to be relevant for biological processes, such as gene transcription and telomerase inhibition.[13]

Here, we present a novel design of DNA G-4 based micelles, in which the assembly of the headgroup in a G-4 proved to be crucial for the self-aggregation of these amphipiles into micelles. Modulation of micelle stability can be achieved by introduction of a complementary oligonucleotide that hybridizes with the lipid headgroup, unfolding the G-4 and leading to the release of a dye (fig. 1). Furthermore, we show that this concept is highly versatile and applicable in more complex systems. Through this approach we engineered DNA G-4 micelles that release their cargo in response to a target molecule, such as adenosine 5′-triphosphate (ATP).

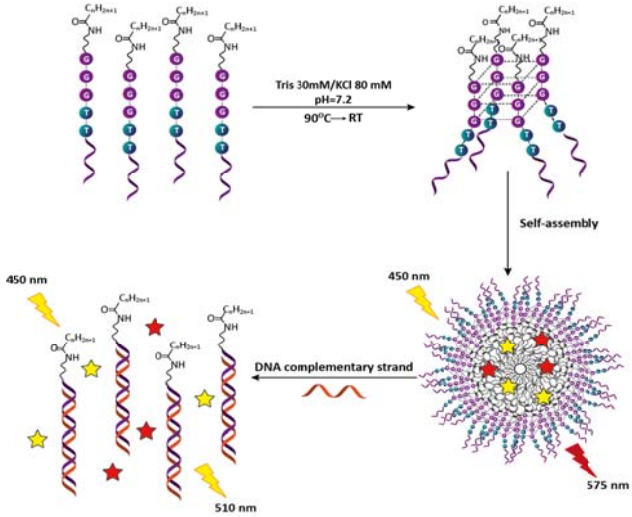

Fig. 1 Schematic representation of G-4 micelle assembly and destabilization. At high K^+ concentrations, the G-rich sequence adopts a tetramolecular parallel G-4 conformation. The resulting amphiphile is able to self-assemble in solution, forming stable micelles. Upon addition of a complementary strand the micelles are disrupted, leading to release of the encapsulated dyes.

Inspired by the hierarchical self-assembly of the G-4s many research groups have exploited these non-canonical structures as scaffolds for the development of new biomolecular systems with different applications, such as sensing,[14,15] catalysis,[16,17] energy transfer[18,19] or as potential nanostructures for drug delivery.[20]

In a recent report Wilner et al. engineered lipid micelles with a 2′OMe RNA sequences able to self-assemble in G-4 structures.[20] These micelles displayed high stability and allowed for the controlled release of a cargo upon destabilization of the quadruplex with an antisense RNA oligonucleotide.

Our design relies on a commercially available 5′-amino-modified DNA strand, that was subsequently conjugated on the 5′-terminus to lipophilic tails of different length (C_{12}-$C_{18:1}$). In the presence of K^+, the short G-rich DNA oligonucleotide

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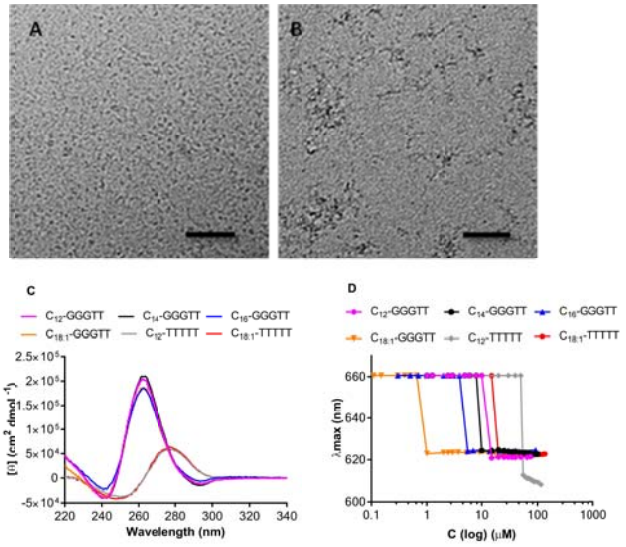

Fig. 2 Characterization of the DNA G-4 conjugates. A) Cryo-TEM images of C12-GGGTT and B) C16-GGGTT. Scale bar represents 50 nm. B) CD spectra of the G-4 conjugates dissolved in Tris-HCl 30 mM KCl 80 mM pH=7.2 at 25 ˚C. C= 30 μM D) CMC determination of the oligonucleotide-lipids in Tris-HCl 30 mM KCl 80 mM pH=7.2 incubated with 2.5 μM Nile Red.

assembled into a parallel G-4, bringing the four hydrophobic tails in proximity and forming the surfactants, that once in solution, self-organize into stable micelles. The DNA-lipids were synthesized by reaction of the activated *N*-hydroxysuccinimide (NHS) esters of carboxylic acid of different lengths with the 5'-amino-modified oligonucleotide (Scheme S1). As a negative control we also synthesized oligonucleotide-lipids that are unable to assemble into a G-4 structure (5'-TTTTT-3'). After purification by reversed-phase HPLC, the conjugates were characterized by UPLC-MS (Fig. S2, ESI).

The purified oligonucleotide-lipids were assembled into a G-4 by annealing in buffer (30 mM Tris-HCl, 80 mM KCl, pH=7.2) and subsequently analysed by CD spectroscopy to confirm the formation of G-4 (fig. 2c). The CD spectra of the oligonucleotide-lipids showed a positive band at 260 nm and a negative band at 240 nm, characteristic of a tetramolecular parallel G-4.[21,22] For the control conjugates, a negative band near 250 nm and a positive band near 280 nm were observed, confirming that in this case no G-4 structure was formed.

Next, we set out to study the aggregation behaviour of our oligonucleotide-lipid conjugates in solution. Cryo-TEM studies showed that the G-4 surfactants are self-assembling in structures that because of their size can be attributed to micelles. DLS measurements of the C16-GGGTT and C18:1-GGGTTT surfactans confirmed the presence of aggregates with 1-3 nm average radius (Fig. S10). In contrast, no micellar structures were detected in the Cryo-TEM studies for the conjugates with the 5'-TTTTT-3' sequence (fig. 2 and fig. S4, ESI), which suggests that either under these conditions micelles are not formed, or they are too small to be observed. This result highlights that the assembly of the oligonucleotide headgroup into a G-4 is important for the self-assembly of the surfactants and hence in favouring micelles formation.

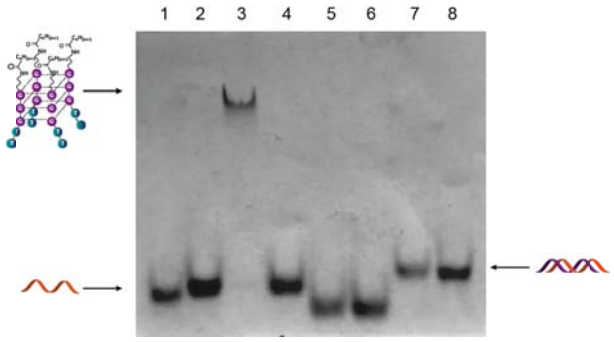

Fig. 3 Monitoring the destabilization of the G-4 using native 10% polyacrylamide gel electrophoresis. Lane 1: OL1; Lane 2: OL2; Lane 3: $C_{18:1}$-OL1; Lane 4: $C_{18:1}$-OL2; Lane 5: c-OL1; Lane 6: c-OL2; Lane 7: $C_{18:1}$-OL1 + c-OL1; Lane 8: $C_{18:1}$-OL2 + c-OL2.

OL1: 5'-GGGTTTAAGTGTAGTT-3'; **OL2:** 5'-TTTTTTAAGTGTAGTT-3'.

The critical micelle concentration (CMC) of the DNA-lipid conjugates was determined using Nile Red as fluorescent probe. Nile Red has been extensively utilized to monitor the phase behaviour of amphipiles, due to its sensitivity to the polarity of the microenvironment.[23,24] In particular, Nile Red shows a consistent change in the maximum of its emission (λ_{max}) at surfactant concentrations above the CMC. By plotting the λ_{max} of Nile Red as a function of the conjugates concentration we were able to estimate their CMC, as shown in fig. 2d. It was observed that the CMC is dependent on the length of the hydrophobic tail of the surfactants. In fact, the CMC of $C_{18:1}$-GGGTT (1 μM) is one order of magnitude lower than that of C_{12}-GGGTT (10 μM). Moreover, the results also confirmed that the presence of a G-4 forming sequence in the hydrophilic headgroup of the surfactants leads to a stabilization of the self-assembly: the CMC is significantly higher when the G-4 is not formed (10 μM for C_{12}-GGGTT; 50 μM for C_{12}-TTTT).‡

Both cryo-TEM studies and the measured CMCs demonstrate that the assembly of the headgroup into a G-4 contributes significantly to micelle formation. Moreover, the micelles still maintain the function of G-4, since binding with the cationic porphyrin TMPyP4[11,25] was observed with C_{12}-GGGTT, as evidenced by the red shift (11 nm) and hypochromicity (41 %) of the Soret band (fig S5). In agreement with these results, no change in the UV/Vis absorption was detected in the case of C_{12}-TTTT, which does not form the G-4 structure.

Based on these results we envisioned that destabilization of the G-4 would cause a decrease in the stability of the micelles and potentially to their disruption, allowing the release of a cargo. Toward this end, a new conjugate with a longer oligonucleotide sequence on the 3' terminus (Table S1, ESI) was synthesized ($C_{18:1}$-OL1), such that the addition of a complementary strand would result in the disassembly of the G-4 and formation of duplexes. Also in this case, we synthesized a similar conjugate ($C_{18:1}$-OL2) as a negative control.

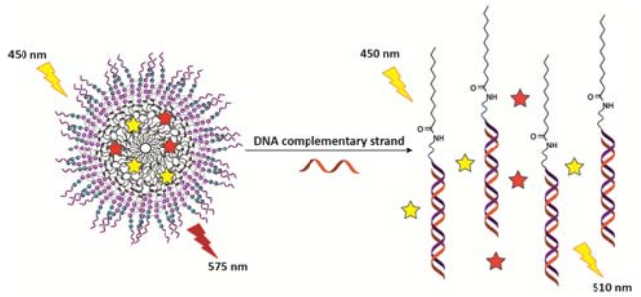

Fig. 4 FRET experiment. When both dyes DiO/DiI are encapsulated energy transfer occurs. Upon addition of the complementary oligonucleotide the dyes are released causing a loss of the FRET efficiency.

Using native polyacrylamide gel electrophoresis it was confirmed that indeed the presence of a complementary sequence destabilizes the G-4. In fact the band of $C_{18:1}$-OL1 (Fig. 3, lane 3) disappeared after incubation with the complementary oligonucleotide c-OL1 (lane 7) while a new band appeared, suggesting disassembly of the G-4 and formation of the duplex. Moreover the gel shows that the presence of the alkyl chain promotes the formation of the G-4 for long oligonucleotides, as can be seen by the different retention of the bands in lane 1 (OL1) and lane 3 ($C_{18:1}$-OL1).

In order to estimate whether micelle stability was affected by disruption of the G-4, we monitored the release of hydrophobic probes from the core of the micelles over time by measuring Förster resonance energy transfer (FRET) efficiency. A well know FRET pair[20,26,27] was chosen, where 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) acts as donor and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) acts as acceptor. When both dyes are encapsulated in one micelle, excitation at 450 nm (excitation of the donor) results in energy transfer due to their close proximity, leading to fluorescence emission at 575 nm (emission of the acceptor). In contrast, when the micelles disassemble, the two dyes are released and in decrease in the FRET efficiency should be observed (fig. 4).

The fluorescence experiments were performed on samples of $C_{18:1}$-OL1 and $C_{18:1}$-OL2 dissolved in a solution of bovine serum albumin (BSA) in PBS (45 mg/mL) at 37 ˚C with λ_{ex} = 450 nm, monitoring the emission in the range of 465-700 nm. Encapsulation of the dyes inside the micelles was confirmed by UV-Vis (fig. S6, ESI). The use of BSA is necessary to avoid precipitation of the dyes after release from the hydrophobic core of the micelles.

The FRET assay indicated a significant difference between $C_{18:1}$-OL1 and $C_{18:1}$-OL2 (fig. S6, ESI). In fact while in the G-4-forming micelles $C_{18:1}$-OL1 energy transfer between the two dyes was observed, no FRET was detected in case of $C_{18:1}$-OL2, suggesting that the micelles formed in this case are not stable enough to keep the two probes in close proximity of each other. For $C_{18:1}$-OL1 a small decrease in the FRET efficiency is initially observed, probably due to the release of some of the dye molecules from the micelles when diluted in BSA solution. After 60 min the FRET ratio no longer changed, suggesting that equilibrium is reached and the micelles are stable in these conditions for at least 4h (fig. 5, black).

Upon addition of an equimolar amount of the complementary oligonucleotide the stability of the $C_{18:1}$-OL1 micelles is decreased, as can be observed by the change in the fluorescence emission spectra (fig. S6b). The fluorescence intensity at 575 nm significantly decreased, while the emission of the donor at first slightly decreased and then increased. The initial decrease could be related to the burst release of the dyes located at the hydrophobic-hydrophilic interface in the micelles, as observed in similar studies[26]. Moreover, the maximum of the emission is shifting from 510 nm to 505 nm, indicating the change in the local environment of the probe. The FRET ratio, $I_{575}/(I_{575} + I_{510})$, was calculated to monitor the relative peak shift between I_{510} (the emission of DiO) and I_{575} (the emission of DiI). Upon addition of the complement, the FRET ratio of $C_{18:1}$-OL1 showed a significant decrease (fig. 5, purple). This confirms that the presence of the G-4 in the system could be used to tune micelle stability. In fact, when the oligonucleotide headgroups are not able to assemble in a G-4 due to hybridization, the micelles release the encapsulated dyes. To support this, we tested whether the incubation with a non-complementary strand would affect the FRET ratio (fig. 5, orange), but in this case no change is observed and the behaviour is similar as in the control experiment (fig. 5, black).

We envisioned the incorporation of this design into a more

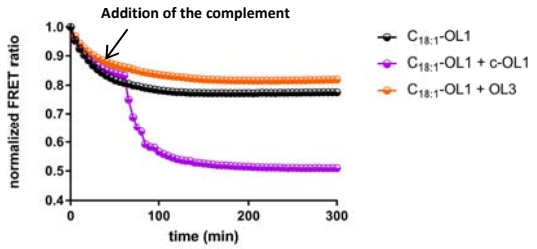

Fig. 5 Normalized FRET ratio upon addition of 1 equivalent of antisense oligonucleotide. An oligonucleotide that can hybridize with the hydrophilic headgroup of the micelle, allowing duplex formation (purple), leads to a change in FRET efficiency. On the contrary an oligonucleotide that doesn`t interact with the G-4 (orange) doesn`t affect stability of the micelles and the FRET signal resembles the one of $C_{18:1}$-OL1 alone (black).

OL3: 5'-GACATGTCTGACCTTG-3'

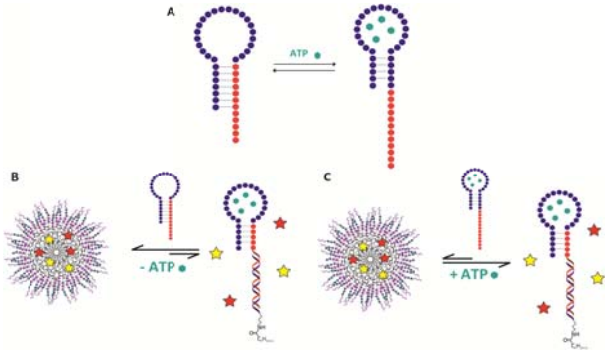

Fig. 6 A) Design of the DNA hairpin composed of the ATP-binding domain (in blue) and a responsive domain (in orange). B) In the absence of ATP the responsive domain is locked and the interaction with the complementary strand in the G-4 micelles is disfavored. C) When ATP binds to the recognition domain, the structure of the hairpin rearranges, exposing the responsive domain. This promotes the hybridization with its complementary strand and leads to micelle disruption and release of cargo.

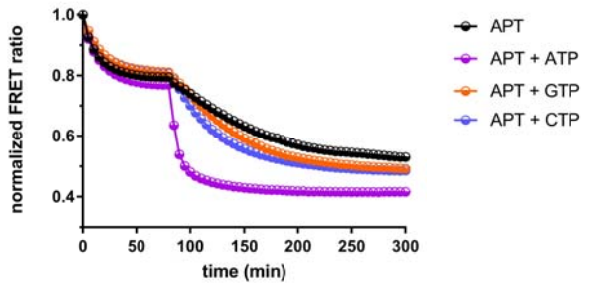

Fig. 7 Normalized FRET ratio over time measured for the DNA G-4 micelles responsive to ATP. The system in the presence of the aptamer (APT, black) is destabilized but with lower efficiency and slower rate than in the presence of 5 mM ATP (purple). In the latter case, an immediate decrease of the FRET ratio is observed upon addition of ATP, indicating dye release. The response is specific for ATP, since the addition of GTP (orange) or CTP (blue) does not affect the stability of the micelles.

complex system, i.e. a DNA G-4 micelles responsive to the presence of a small molecule. The ATP-binding DNA aptamer sequence[28,29] was engineered to obtain a DNA hairpin that assumes two different conformations, depending on the presence of its ligand (fig. 6). The hairpin is composed of: (a) the ATP-recognition domain and (b) the responsive domain consisting of a DNA sequence complementary to the G-4 forming sequence. In the absence of ATP, the hairpin is preferentially in a locked conformation that inhibits the responsive domain to interact with the G-4 micelles. Conversely, upon binding of ATP the hairpin structure is rearranged, liberating the responsive domain, which can hybridize with the G-4 micelles, thus leading to cargo release.

The FRET experiment was performed on G-4 micelles composed of $C_{18:1}$-OL4 (fig. 7 and table S1 for the sequence). Upon addition of ATP the micelles were immediately disrupted and the dyes were released. Destabilization of the micelles does occur in the presence of the aptamer (APT) without ATP (fig.7 black) but at a much slower rate and lower efficiency compared to the system in the presence of ATP (fig. 7, purple).

The selectivity of the system for ATP was determined by using ATP analogues, such as 5'-guanosine triphosphate (GTP, fig. 7 orange) and 5'-citosine triphosphate (CTP, fig.7 blue). The results show that in this case the decrease in the FRET ratio is not different compared to the system with the aptamer alone, indicating that the response of the system is selective for ATP.

In conclusion, we present a novel and versatile design of DNA G-4 micelles. Cryo-TEM studies and CMC determination show how the assembly of the oligonucleotide headgroup into a G-4 plays an important role in determining micelle formation. We demonstrate that modulation of the stability of the micelles is possible, by introduction of a complementary strand that leads to disassembly of the G-4 micelles and release of encapsulated dyes. The system proved to be suitable for the development of novel DNA-based nanodevices as was demonstrated by the DNA G-4 micelles that release their cargo selectively in the presence of a target molecule, such as ATP. This DNA aptamer based approach to G4-micelle disassembly is highly versatile and we envision its application to different kind of targets or stimuli.

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Notes and references

‡ Determination of the CMC for $C_{18:1}$-TTTTT did give a larger variation in the results than with the other conjugates, albeit that the CMC was always significantly higher than that of $C_{18:1}$-GGGTT. The variation could be due to the low stability of the aggregates formed.

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