

SHEDDING LIGHT ON NUCLEIC ACIDS AND DNA UNDER CONSTRUCTION

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ABSTRACT

The first part of our research deals with the spatiotemporal regulation of biological processes. We use light as addressing mechanism and modify nucleic acids in a way to make them light-responsive. Light has the advantage that it can be easily generated and manipulated with well-established (laser and microscope) technologies and many of the currently investigated model organisms are light-accessible. On the other hand nucleic acids are the base of powerful techniques such as for example RNA interference for the regulation of genes and aptamers for the regulation of the function of proteins.

In the second part of our research we are exploring new ways to assemble nanometer-scaled objects from DNA but instead of only relying on the Watson-Crick interaction we are using alternative – orthogonal – interaction strategies like for example “Dervan-type polyamides” which can sequence-selectively bind to double-stranded DNA. Two of these polyamides – combined via a linker – form a “DNA strut” which can sequence-selectively “glue” together two DNA double helices.

LIGHT-ACTIVATABLE (“CAGED”) NUCLEIC ACIDS – PHOTOCHEMISTRY IN LIVING CELLS

Most of the processes in living organisms are exquisitely spatiotemporally regulated – and this is true at every level of organization. A cell is more than just the sum of its individual (non-interacting) constituents, a tissue is more than just an assembly of individual cells and an organism is more than just a statistic assembly of tissues. This aspect of complicated but well-choreographed systems is for example especially important in developmental biology or neurology. If one wants to understand the systems involved or diseases resulting from perturbations thereof, a prerequisite is that we can ask nature our questions in equally spatiotemporally well-defined manners – hence the need for tools that allow us to set biological stimuli with exact control of the location, the point in time and the magnitude. This requirement calls for a broadly applicable addressing mechanism and light is ideally suited in this respect. Light is an orthogonal trigger signal because only a minority of biological systems is already light-responsive by themselves. Light is also a “harmless” trigger signal if one chooses the right wavelength. Additionally many model organism or even tissues are light-accessible and the technologies to generate and manipulate light are very well established. For example a confocal microscope (with appropriate lasers) contains already everything one needs: The laser light sources, the scanning system with mirrors to position the beam in arbitrarily regions of the sample and the confocal setup to detect what is happening upon stimulation. Using two-photon excitation it is even possible to irradiate three-dimensionally well-defined volume elements – as if a “light cursor” were moving through the object of interest [1–2]. These are the reasons why we got interested in doing “photochemistry in living cells”.

The question now arises how to couple a trigger signal such as light to a biological process so that for example genes or proteins can be switched on or off. There are several ways to realize this. All of them involve either using photolabile “protecting” groups or bi- or multistable photoswitchable systems [3]. The latter approach of attaching “photoswitches” to biologically active molecules has the appealing benefit of reversible switchability but it is certainly very difficult to realize. In this case both the ON- and the OFF-state are modified compounds and this makes it difficult to obtain a clear “binary” switching behavior which is needed for most of the subsequent experiments. The approach of attaching a photolabile group to the active site of a biologically active substance is much easier to realize. The price to pay is that these compounds can only be irreversibly activated but in many cases this is already enough. The general concept of temporarily blocking the biological activity of a compound has already been realized thirty years ago when Hoffman at Yale prepared a derivative of ATP which he called “caged ATP” [4]. Caged ATP has up to now been used in a vast number of studies and is also commercially available nowadays. On the other hand only very few studies existed with caged nucleic acids until some years ago. This is how we got interested in this field.

Our approach of making nucleic acids light-responsive is to attach photolabile caging groups to the nucleobases (Fig. 1). These cages act both as steric block and also perturb the hydrogen bonding capabilities of the nucleobases. Hence they can be seen as temporary mismatches. This can for example be demonstrated by melting temperature studies. Before photolysis the melting temperature of a duplex is reduced significantly if caged residues are introduced whereas after photolysis the melting temperature of an unmodified duplex is recovered [5].

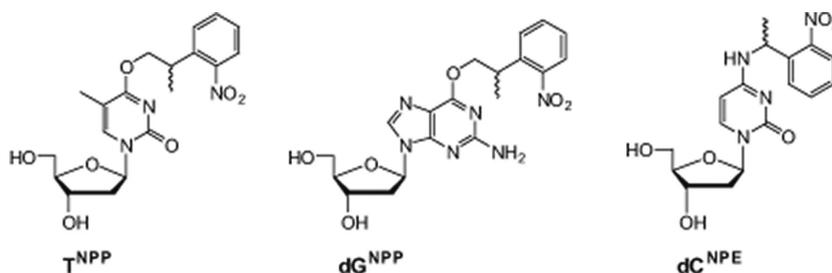


Figure 1. Examples of our nucleobase-caged residues for light-activatable DNAs and RNAs. The photolabile NPP group had been introduced by Pfleiderer *et al.* [6] and is an interesting alternative to the NPE group. Instead of generating a nitroso compound upon photolysis this group yields α -methylnitrostyrene.

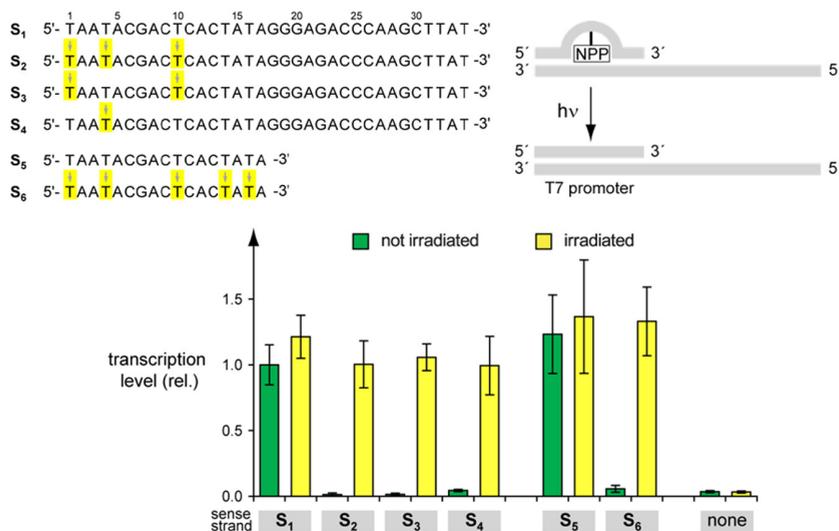


Figure 2. An example for light-induced transcription. Caged residues act as local perturbations in a T7 promoter and prohibit transcription until the photolysis event in which they are fully removed [5].

In a first attempt to demonstrate the usefulness of this type of caged nucleic acids we began to study light-induced transcription (Fig. 2). Therefore we used a luciferase gene which was under the control of a T7 promoter. Normally the T7 RNA polymerase would recognize this promoter and start transcription. However, caged residues in the double-stranded promoter region should result in a local perturbation which prevents the T7 RNA polymerase from recognizing its target binding site and hence transcription should not occur until – after irradiation – the perturbation is removed and the unmodified double-stranded promoter is regenerated. Figure 2 shows the results we obtained: It can be seen that no matter where the cage was introduced in the sense strand no transcription could be observed before irradiation within error limits. However, after irradiation in every case as much transcript was produced as if there had never been any modification. In particular one cage was enough to completely prohibit transcription but even five cages could still be completely photolyzed.

We then proceeded to check if it was also possible to put RNA interference under the control of a light trigger signal. The central players in RNAi are the siRNAs. However, upon introduction of mismatches siRNAs become miRNAs which act in a different mechanism but are certainly still active. However, it is known that there are regions in which mismatches in siRNAs or miRNAs are not tolerated [7]. Understandably, so this is true for the central region in the so-called guide strand of the double-stranded siRNA which is the one that is incorporated into the RISC complex and guides it as to which mRNA to cut. This point of scission of the mRNA is exactly opposite of the center of the guide strand. Interestingly the same study showed that at this position deoxyresidues are well tolerated. Even though we had already prepared caged RNA it turned out not to be necessary in this case. Thus, we prepared hybrids of siRNAs with one caged deoxyresidue in the middle and thus circumvented several synthetic steps addressing the regiochemical aspects of RNA phosphoramidite synthesis [8]. As can be seen in Figure 3 our caged siRNAs were completely inactive within error limits before irradiation but the activity could be restored very well upon photolysis. In this assay HeLa cells were used which were transiently transfected with plasmids coding for EGFP and RFP. By its sequence siRNA only targeted EGFP expression. It is important to note that the reduction of fluorescence upon photolysis is not due to photobleaching as can be seen from the bars corresponding to the experiments in which (caged) nonsense RNA (nsRNA) targeting neither EGFP nor RFP was used with or without irradiation.

Shedding Light on Nucleic Acids and DNA under Construction

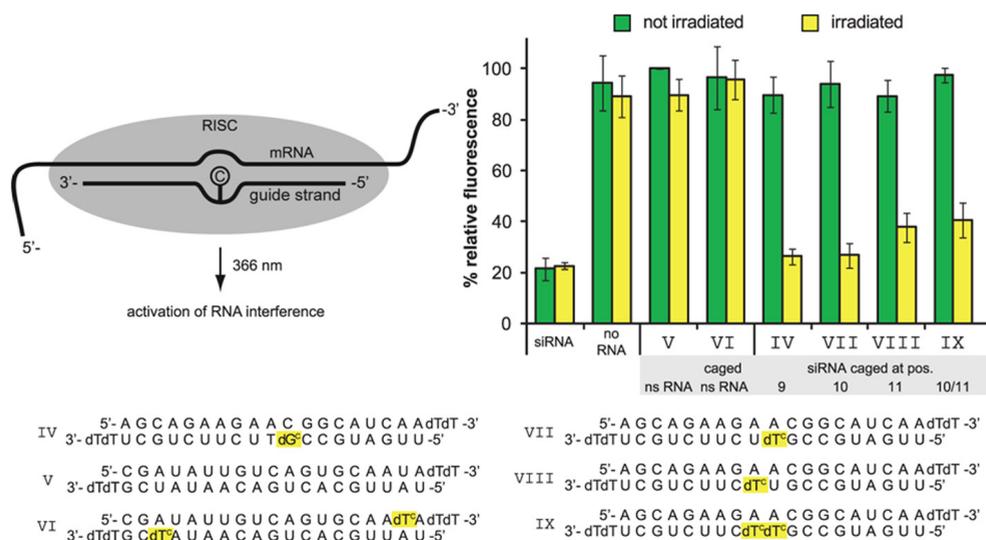


Figure 3. Overview of RNA interference experiments using caged siRNAs [8].

Another interesting question is whether it is not only possible to regulate gene expression by light but also to regulate protein activity – using the same approach based upon caged nucleic acids! At first this might seem like a contradiction but the link is the aptamers technology. Aptamers are single-stranded DNAs or RNAs which can be obtained in a selection process (that does not involve rational design). Using this “SELEX” process it is possible to generate aptamers against very many molecular targets or supramolecular target structures up to even cells [9]. One such aptamers is for example a 15mer DNA sequence which can fold into a G-quadruplex structure and bind and inhibit thrombin which acts as factor IIa of the blood clotting cascade (Fig. 4) [10]. Thus, this aptamers can act as an anticoagulant. From a crystal structure it was known how this aptamers interacts with thrombin (shown as cartoon representation of the PDB structure 1HAO in the upper left corner of Figure 4). This crystal structure identifies four T residues as mediating the interaction. Thus our rationale was that we block the access to one of them and thus make the aptamers temporarily inactive [11]. Figure 4 also shows the result of blood clotting assays using the caged aptamers variants and the wild type version. As can be seen the caged aptamers variant A₂ was indeed completely inactive and its activity could be regenerated by irradiation – albeit not to 100% in this case. We could show that this is due to a pH-dependent side reaction which is described in a mechanistic paper on the NPP group [6]. This reaction generates a photostable byproduct which is still modified on the nucleobase. Even though this is undesired using a higher amount of caged aptamers this problem can easily be circumvented.

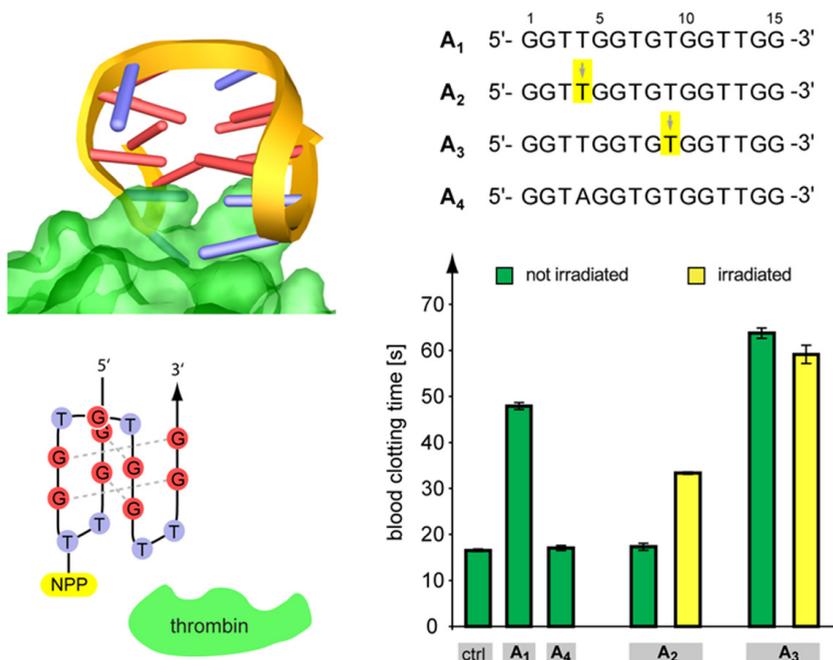


Figure 4. Overview of a DNA aptamers which binds to thrombin (cartoon representation of the PDB crystal structure 1HAO shown in the upper left corner) and a strategy to sterically block its activity with a cageing group [11].

While the previous example shows how aptamers can be switched on with light, the strategy is not yet generally applicable because detailed information about the aptamers-target interaction must be present. However, while it is not easy to obtain for example crystal structures of protein-nucleic acid complexes it is much easier to predict secondary structure formation of DNA or RNA. Since aptamers are only active if they are present in the required conformation a more general approach is to use the cageing technology to trigger the formation of this active conformation with light. The predominant structural feature of the above-mentioned aptamers is the G-quadruplex. Hence, we got interested in the light-triggered formation of G-quadruplex structures. Therefore we chose again the previous aptamers but also a sequence that folds into a three-layer G-quadruplex structure (Fig. 5) [12]. This sequence is derived from human telomers. The presence or absence of the G-quadruplex structure was assayed by CD spectroscopy. Figure 5 shows the CD spectra of the wild type sequence T_1 both in buffer and in ion-free water. The first shows the presence of the G-quadruplex. In the second case no central ions which are required for the formation of the G-quadruplex are present so that the corresponding spectrum can serve as a positive control for the absence of the G-quadruplex. As can be seen the caged sequence T_2 yields the same spectrum even in the presence of buffer salts. Upon irradiation the secondary structure formation is triggered and the same spectrum as the one of the wild type is obtained.

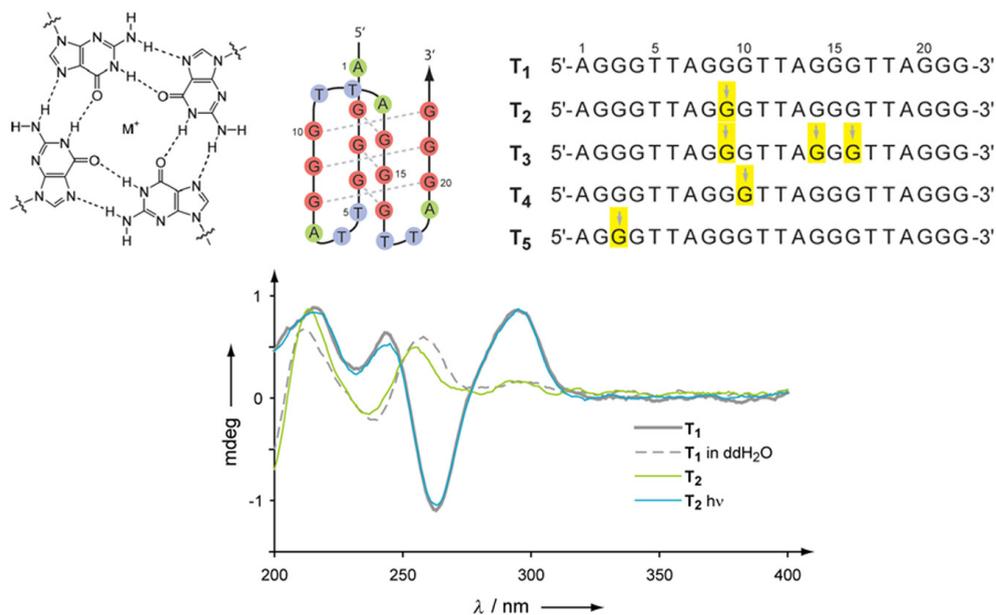


Figure 5. Light-triggered formation of nucleic acid secondary structures [12]. CD spectroscopy shows that only upon irradiation the sequence T₂ forms a G-quadruplex structure.

While the two above examples have shown that it is possible to turn aptamers activity ON with light it might also be desirable to turn aptamers activity OFF. This should also be possible: By attaching an antisense sequence for example to the 5'-end of the anti-thrombin aptamers (cf. Figure 4) the aptamers will not be present in its active conformation any more but rather exist as an inactive hairpin. If the antisense sequence is caged, however, this will not happen until the moment of irradiation (Fig. 6) [13]. It turned out that in addition to the required turn it was sufficient to use only four antisense residues to completely prevent any anticoagulatory activity. As can be seen in Figure 6 aptamer A₄ with the caged antisense-region was active before irradiation but could be efficiently switched off upon irradiation.

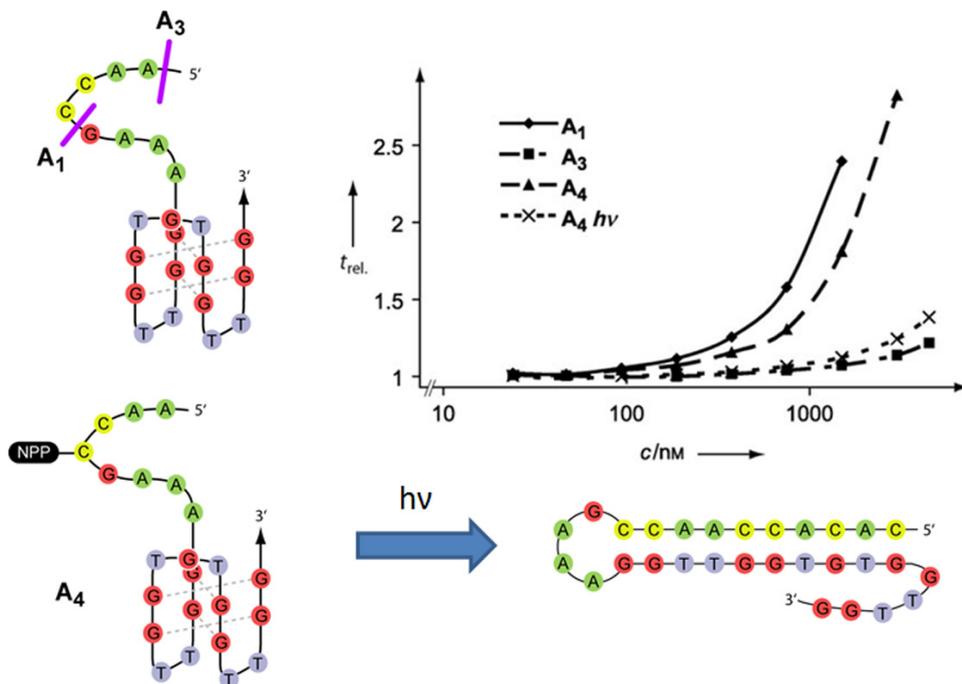


Figure 6. A strategy to turn aptamers OFF with light using a caged antisense-strand. The graph shows dose-dependent anticoagulatory behavior [13].

The above examples demonstrate the broad applicability of the concept of using nucleobase-caged nucleic acids. Ongoing research on all levels of the approach will even improve its versatility and make it suitable for the next step which will be addressing spatiotemporal questions in entire biological systems with all their inherent complexity.

USING DNA AS BUILDING MATERIAL

The second part of my group addresses an entirely different question. Throughout the last decades computers have become tremendously more powerful. This development has profound implications on society. Examples for these implications can be found everywhere and include among others also the broad availability of the internet which has indeed changed our everyday lives. Still ten years ago mobile phones were far from being common. The steep increase in computing power has also left its footprints in the world of science and made things possible that were still unthinkable several years ago. However, this miniaturization trend cannot go on indefinitely. The reason is that photolithography – the process used for generating the computers' hearts – is using light to transfer the information of a mask onto the silicon surface and even with ideal optics the size of the smallest feature that can be generated is about the wavelength of the light that is used.

This has already been realized over a century ago by Ernst Abbé. Reducing this wavelength indefinitely is also no viable solution. But instead of constantly trying to make things smaller in a “top-down” approach an alternative is to try and use the power of molecular self-assembly (“bottom-up” approach) to generate structures beyond the diffraction limit. This idea is not new and Richard Feynman was one of the most prominent advocates for this idea for example when he gave his famous lecture entitled “There’s plenty of room at the bottom” about 50 years ago [14].

Proofs that nano-machineries are possible exist amply inside of living organisms and cells. The ATP synthase or multidrug efflux pumps are beautiful examples of efficient nano-machineries. However, while we have come very far in understanding how these nanoscopic miracles work we are still far from being able to construct similar objects from scratch. This is for example due to the fact that the prediction of protein folding is still very difficult. On the other hand proteins are not the only material from which functional or structural nanoscopic objects can be constructed. Nucleic acids offer many advantages here: They form well-known structures, have predictable, “programmable” interactions (via the Watson-Crick base pairings), can be synthesized in automated fashion and manipulated with established protocols and have exactly the right dimensions in the nanometer range.

While the usefulness of DNA and RNA as nano-material has already been nicely demonstrated [15] there remains a problem; nucleic acids are linear, “soft” and do not form significant tertiary interactions. The latter is what makes them different from proteins and peptides which offer a richness of possible interactions. To compensate for this we have decided to add other interaction principles to the world of nucleic acid nano-architectures. What this means is that we want to add orthogonal structural elements – orthogonal to the Watson-Crick base pairing.

An example of such orthogonal structural elements – but by far not the only solution – are Dervan-polyamides [16]. This is a set of compounds which binds and sequence-selectively recognizes the DNA minor groove. Dervan-polyamides can be generated to bind to almost any possible DNA sequence. In the simplest realization of our approach to shape the tertiary structure of DNA we have therefore combined two of these polyamides with a flexible linker and constructed what we called a “DNA strut” (Fig. 7) [17]. Such a strut can bind to double-stranded DNA with both its ends and hence hold them together. For this reason one could think of this system as “sequence-selective glue” for DNA-architectures. This new structural element will allow whole new ways of assembling DNA objects.

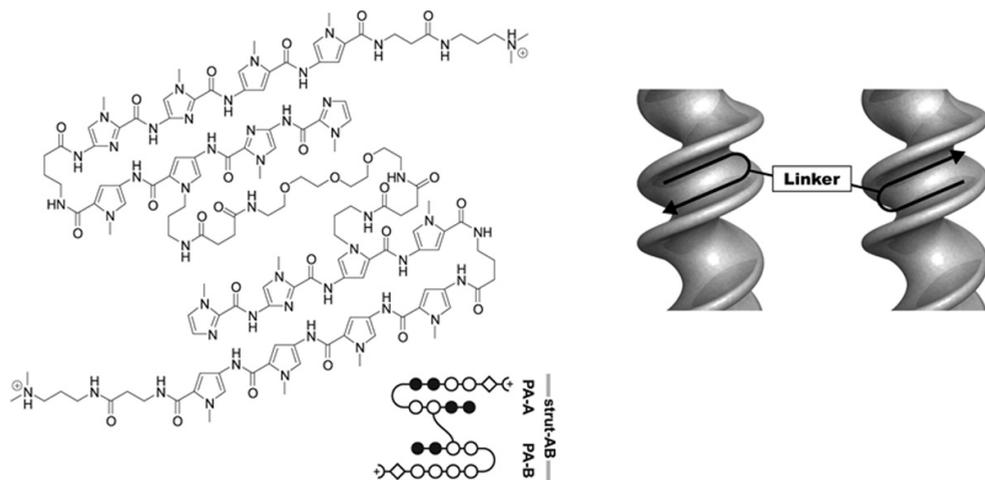


Figure 7. Joining two Dervan-type polyamides (PA-A and PA-B, also shown as cartoon representation) via a linker a DNA strut is obtained which is capable of holding (“gluing”) two DNA double strands sequence-selectively together [17].

To prove and quantify that the expected ternary complex between two (different) DNA double helices and the DNA strut is formed we had to explore new techniques because none of the established ones for the characterization of Dervan-polyamides was suitable. Using fluorescence cross-correlation spectroscopy (FCCS) [18] and small DNA hairpins we were able to prove that the expected ternary complex is indeed formed and under the given conditions a dissociation constant of 20 nM was measured (for more details see the original paper [17]). What is equally important is that upon a slight change in DNA sequence no binding could be observed any more.

The next question was to see if DNA objects bigger than hairpins could also be held together using the DNA strut. Therefore, we started constructing small double-stranded DNA rings containing 168 base pairs. These rings are relatively small and shape-persistent compared to common DNA plasmids. The rings could be obtained using repetitive A-tract sequences which are known to be inherently bent. Six constituting oligodeoxynucleotides were ligated and objects with incomplete ring closure were digested using an exonuclease. Again by FCCS we studied whether the ternary complex between two DNA rings and the strut was formed and obtained 30 nM as dissociation constant. This is already a complex of about 208 kDa to which the strut only contributes with about 1%. Interestingly, after replacing three base pairs in the binding site of one polyamide no interaction could be detected any more by FCCS. Encouraged by these results we will now proceed and use this strategy to assemble more complicated objects from nucleic acids as well as new alternative orthogonal approaches for the interaction with double-stranded DNA.

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