DNA-based catalysis

Modular Assembly of Cyclodextrin Containing Bio-mimetic Catalysts

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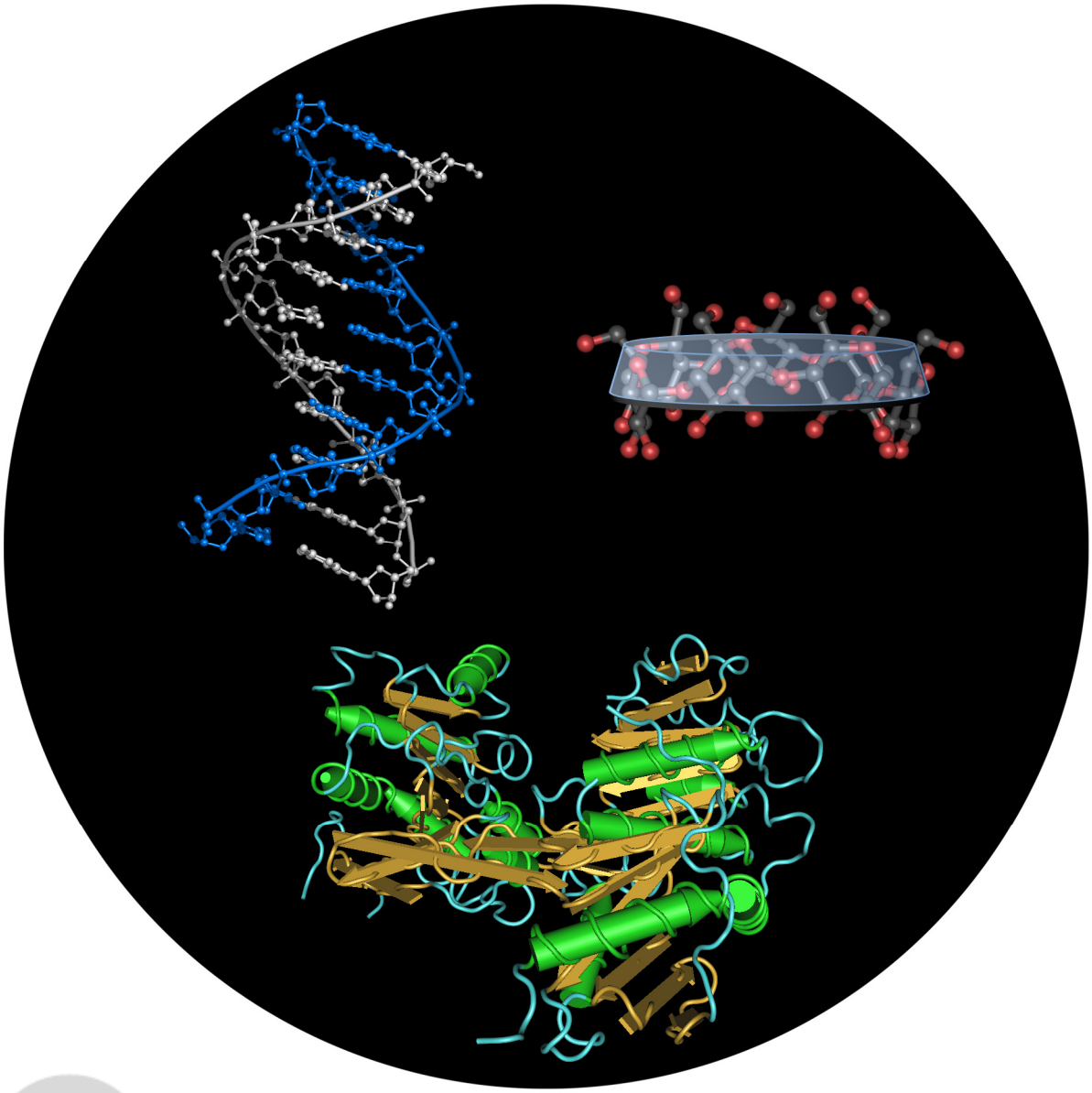
DNA-based catalysis · artificial enzyme · β-cyclodextrin · modular assembly · click chemistry



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Attempts to design and synthesize artificial enzymes with modified specificity and activity have already come a long way. Herein, we report a modular strategy towards the assembly of a DNA-based catalyst in which a bipyridine-copper(II) complex was covalently anchored to a 16-mer oligonucleotide module at the 5'and a β-cyclodextrin covalently anchored to another 16-mer oligonucleotide module at the 3'. Hybridisation of both modules with a template strand containing complementary bases to both the modules, will result in formation of a bio-mimetic DNA-based enzyme, in which the metal complex and the β-cyclodextrin are in close proximity to each other, which should result in rate enhancement of copper(II)-catalysed ester hydrolysis reactions of apolar molecules, due to formation of inclusion complexes with β-cyclodextrins. Both modules were successfully synthesised and analysed using rp-HPLC and MALDI-TOF mass spectrometry. The β-cyclodextrin anchored unit could only be obtained in low yield. Optimization of the process is therefore needed. In addition, a different approach in which click chemistry was used, looks very promising. We are however still awaiting the results. After optimization the system can be completed and kinetic studies can be performed.

From the Contents

1. Introduction: DNA-based Catalysis

In the past, many chemists thought of DNA as solely being a carrier of genetic information, playing a key role in the synthesis of enzymes and other peptides that regulate important processes in cells of living organisms.[1] In the last decade however, scientists discovered ways to make use of the special properties of biomolecules, including DNA, giving birth to a whole new field of research.[2] [3] Two interesting examples that show that DNA is more than an important molecule for carrying genetic information, are given by Rothemund[4] and He *et al.*[5] Rothemund demonstrated that the specificity of hydrogen bonding between bases of nucleotides can be used to fold DNA to create nanoscaled two-dimensional structures and patterns in a simple 'one-pot' method which he termed 'scaffolded DNA origami' (Figure 1). He *et al.* used a similar approach to create three-dimensional polyhedra by self-assembly, to form tetrahedra, dodecahedra and even buckyballs (Figure 2).

A totally different use of DNA, on which this report will focus, is DNA-based catalysis (DBC). One of the first demonstrations of DBC in which no direct modification of the nucleobases is done, was given by our group.[6] In nature, chirality is almost exclusively transferred by enzymes which are encoded by DNA. In our system, the chirality, which is inherent to the right-handed double helix shape of the DNA molecule, is transferred directly from the DNA molecule to a metal catalysed reaction, forming a chiral product.

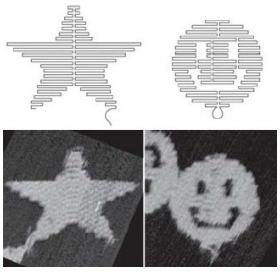

Figure 1. DNA origami structures. Top row, folding paths. Left, star; right, smiley. Second row, AFM images.[4]

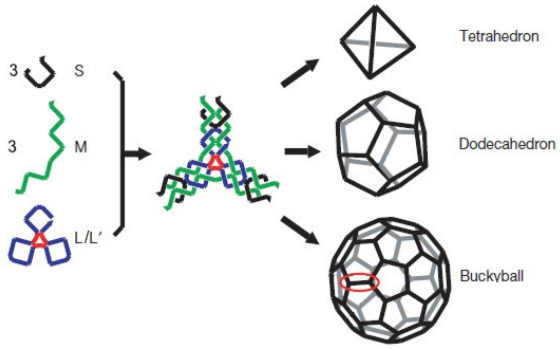

Figure 2. Self-assembly of DNA polyhedra. Three different types of DNA single strands stepwise assemble into symmetric three-point-star motifs, eventually resulting in polyhedra.[5]

The copper(II)-catalysed Diels-Alder reaction was the first reaction to be studied in detail. The system comprises an intercalator and a copper-chelating moiety. In the first generation, these moieties were linked via a spacer[6] [7] [8] whereas in the second generation both sites are integrated into a single bipyridine moiety (Scheme 1).[7] [8] [9] This brings the reactive copper centre into closer contact to the DNA molecule, giving very high endo selectivity and high enantioselectivity. Furthermore, it was demonstrated that this type of reaction could be performed on a synthetically relevant scale.[10] Later on, the concept was applied to the catalytic asymmetric Michael reaction in water[11] and to fluorination reactions,[12] both with good to excellent enantioselectivities.

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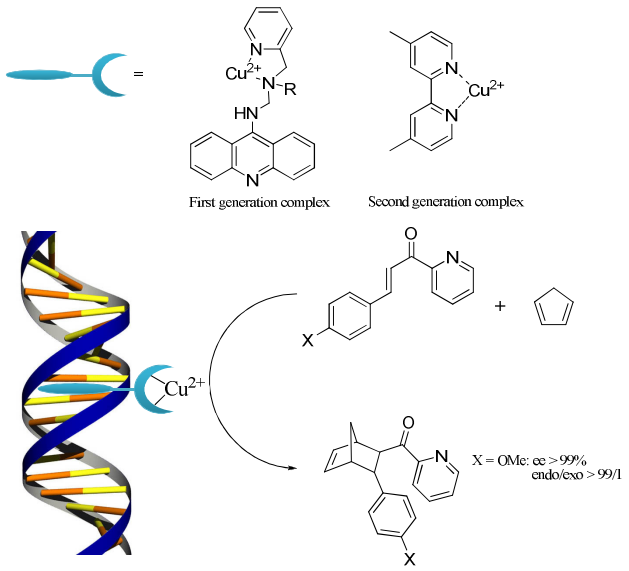


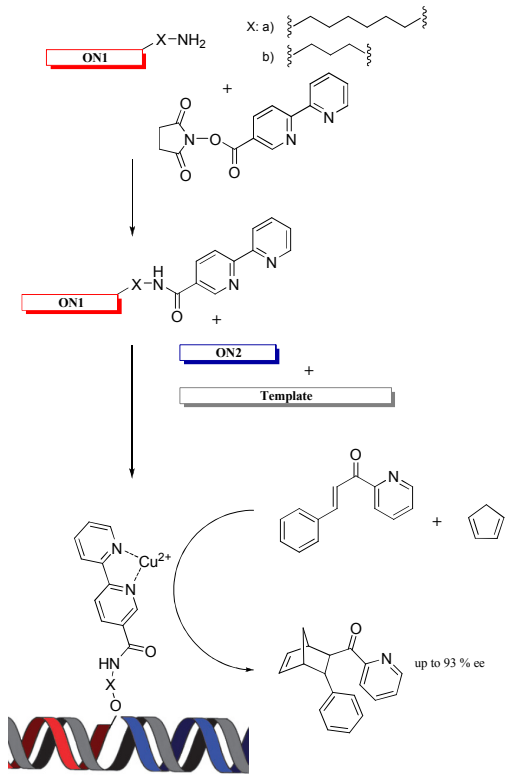
Scheme 1. Schematic representation of the asymmetric Diels-Alder reaction of cyclopentadiene with different aza-chalcones, catalysed by a copper complex in the presence of DNA. Examples of a first- and second generation complex are shown at the top of the scheme.[6] [8] [9]

Because of the many advantages of this method (i.e., rapid structural variation and optimization for use in other reactions,[6] easy isolation of product,[6] simple and readily available ligands and very high regioselectivity and enantioselectivity[9]), this supramolecular anchoring method is still a major subject of investigation in our group. Supramolecular anchoring however, results in a heterogeneous mixture of complexes that are all located in different parts of the sequence, and hence, all catalyse the reaction with different selectivity.[2] In order to study how the surroundings of the metal complex affect the selectivity of the reaction, a novel modular strategy towards (enantioselective) DBC was presented by our group, containing a covalently anchored metal complex.[2]

Earlier reported enantioselectivities obtained by use of covalent anchoring, were generally quite modest (ee < 82 %).[13] [14] We reported ee values as high as 93 %.[2] The system is outlined in Scheme 2 and comprises three oligonucleotide (ON) components: ON1 is functionalised with a 2,2'-bipyridine (bipy) ligand at the 5' or 3' terminal phosphate by use of a spacer (X in Scheme 2), ON2 is non-functionalised and the template ON has a sequence complementary to ON1 and ON2. Hybridising these oligonucleotides in the presence of copper(II) ions gives the active system in which the copper(II) complex is positioned at the interface of ON1 and ON2.[2]

The covalent system allows for precise positioning of the complex in the DNA. Hence, all complexes have the same selectivity, because control over the structure and geometry of the catalytic site is gained.[2] Interestingly, when the copper(II) bipy complex of the supramolecular and covalent approaches is compared, a higher ee for the covalent approach is observed.[2] Furthermore, terminally modified oligonucleotides are commercially available and prepared routinely via solid phase synthesis.[2] The ligand is attached easily using well-established procedures.[2]

Above all, the covalent system as shown in Scheme 2 has one very important property. ON1 and ON2 can be considered as two different modules which are assembled on a template ON, forming the DNA-based catalyst.[2] The advantage of this modular assembly approach is that, in principle, every module is readily exchanged by another.[15] Each module can be functionalised differently, which



Scheme 2. General procedure for coupling of bipyridine derivatives to amino-functionalised DNA and a general method for modular assembly of the DNA-based catalyst. The asymmetric Diels-Alder reaction of cyclopentadiene with aza-chalcones catalysed by the copper(II) complex is shown.[2]

makes it possible to assemble new catalysts of different nature and complexity, simply by putting different modules together on a template strand. We will go into more detail about modular assembly in the third section.

2. Artificial Enzymes

Evolution over millions of years has provided us with a vast number of enzymes which perform a variety of catalytic reactions. The scope of reactions that enzymes catalyse became even broader when nature incorporated metal ions and cofactors, forming metalloproteins.[7] Due to our increased understanding of chemical and enzymatic reactions during the last decades, both the interest and possibility to design artificial (metallo) enzymes with modified specificities and activities increased significantly. This laid the foundation for the field of hybrid catalysis, in which a metal-complex with catalytic activity is introduced into a molecular host, such as a protein, antibody or DNA, to induce selective and enantioselective catalysis.[2] [7] [16]

In an active site of an enzyme, a substrate molecule becomes oriented in such a way as to undergo catalysis in the most efficient way. The orientation of the substrate is to a large extent influenced by different residues of the peptide chain situated at or near the active site. In order to make an artificial enzyme, organic molecules can be used to mimic the interactions the peptide chain residues have with the substrate. An excellent example of a group of molecules that can do this, is known as cyclodextrins.

Cyclodextrins (CyDs) are cyclic oligosaccharides consisting of D-α-glucopyranose residues. The cyclisation occurs during the catalysed degradation of starch by cyclomaltodextrin glucanotransferase (E.C. 2.4.1.19; CGTase).[17] In nature, CyDs with

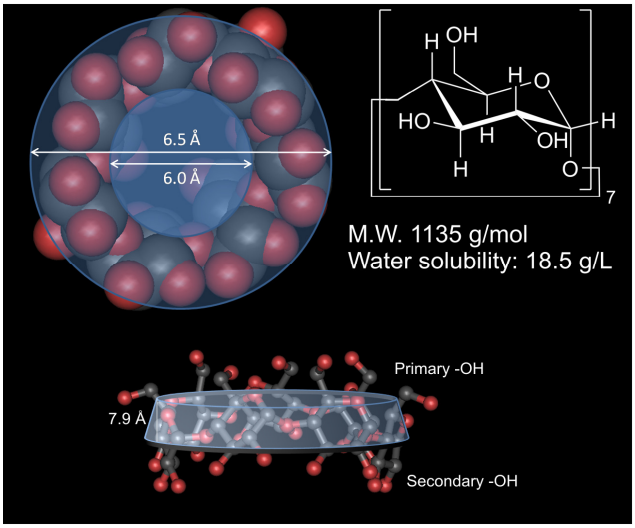


Figure 3. Structure of β-cyclodextrin.

six (α), seven (β) and eight (γ) glucose units exist. Schematic representations of a β-CyD are given in Figure 3. As can be seen, the molecule is cone-shaped, with a smaller upper side containing seven primary alcohol groups and a larger lower side, with fourteen secondary alcohols. All hydroxyl groups are situated on the outside of the ring, causing the inside of the ring to be apolar.[18] The polar outside makes the molecule soluble in water. The water solubility of β-CyD however, is relatively low compared to the other two types. This appears to be due to strong intramolecular hydrogen bonding between the hydroxyl groups of the lower ring, which prevents hydration by water.[17] This, together with the low entropy of hydration makes the skeleton of the cone much more rigid.[19] CyDs are known as host-guest compounds, in which apolar molecules can form inclusion complexes with guest molecules of appropriate size.[17] [18]

3. Goal: Designing a DNA-based Bio-mimetic Catalyst

3.1. Project Details

In the remainder of this report, we present a bio-mimetic catalyst which is based on the modular assembly method of our group, described in the first chapter.[2] The system of Scheme 2 consists of only one functionalised oligonucleotide, which we will refer to as the catalytic module, because of the attached copper(II) bipyridine complex. The goal of our project is to mimic an active site of an enzyme, which is covalently attached to double stranded DNA. In order to do this, we also need a host to accomodate a substrate in place while catalysis occurs. In other words, we need a second module which functions as a substrate binding unit. Therefore we have to attach a certain molecule to a single DNA strand that has good affinity to organic substrates. Our choice fell on cyclodextrins. The rigidity of the cone together with the possibility to form inclusion complexes make β-CyDs ideal to use as part of the substrate binding unit. The β-CyD can be covalently bond to a DNA strand via the upper side of the cone, forming the substrate binding unit.

The system of our project is schematically represented in Figure 4. It consists of two modules: the catalytic moiety with a covalently attached copper(II) bipyridine unit at the 5'end of a 16-mer oligonucleotide and the substrate binding unit, which consists of a β-CyD covalently attached to the 3' end of another 16-mer oligonucleotide, non-complimentary to the first. Both modules are hybridized with a complementary 32-mer template strand assembling the metal complex-cyclodextrin supramolecular catalyst. The metal complex and the CyD are now in close proximity to each other. Because the β-CyD can form inclusion complexes with organic apolar molecules,[17] the catalytic system can now bind a substrate molecule in the cavity of the β-CyD, were it can be subsequently transformed into product by the metal complex. In nature, enzymes catalyse reactions often due to proximity effect and high effective molarity of both the catalytic group and the substrate.[20] In our system both effects are also present which should result in enhanced reactivity. Hence, the term bio-mimetic catalyst is used. In addition, CyDs should be able to improve the solubility of the apolar substrates in the water-based system,

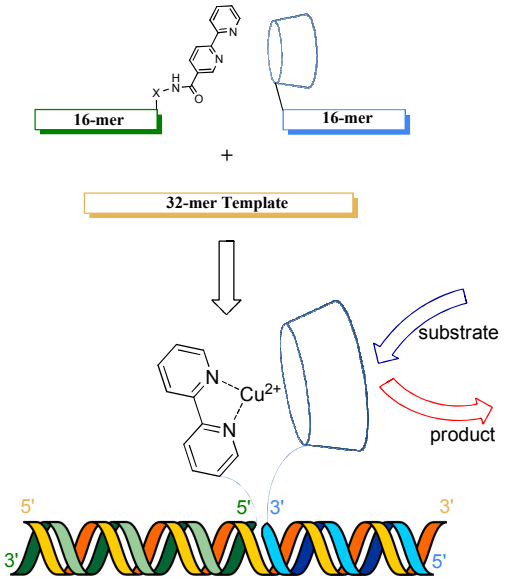


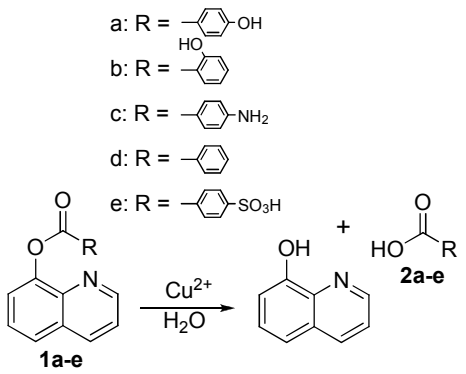
Figure 4. Schematic representation of the bio-mimetic catalyst based on modular assembly. The catalyst consists of a 16-mer catalytic module (green) containing a covalently attached copper(II) bipyridine complex, a substrate binding module (blue) containing a covalently attached β-CyD and a template strand (orange) with 32 complementary bases.

improving in that way the rate of the reaction.

3.2. Finding and Analysing a Suitable Model Reaction

The behaviour of the system depends in part on the type of substrate that is being examined. In literature, examples are known in which inclusion complexes with β-CyDs causes strong inhibition instead of rate enhancement.[21] This is attributed to the polar functional groups of the substrate interacting with hydroxyl groups at the rim of the CyD, which forces the substrate into an unfavourable orientation for undergoing reaction.

The substrates we want to use in a model reaction are the 8-quinolinol benzoic esters **1a-e** in Scheme 3,[22] which will undergo copper(II) catalysed hydrolysis in water. To the best of our knowledge, up till date, no strong inclusion complexes of 8-quinolinol esters with β-CyDs are known. This suggests that the substrate might be suitable for this system, because a too strong binding affinity of a substrate would not release the product fast enough, therefore decreasing the rate of hydrolysis.

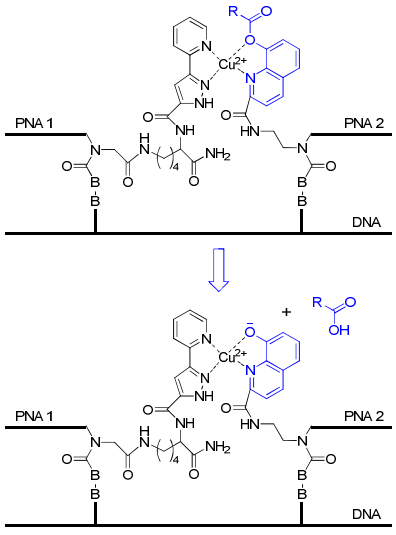


Scheme 3. Copper(II) catalysed hydrolysis in water of 8-quinolinol benzoic esters.

Zelder *et al*,[23] reported a kinetic study of copper(II) catalysed hydrolysis of covalently attached 8-quinolinol esters to PNA using quantitative MALDI-TOF MS spectroscopy.[24] [25] Scheme 4 depicts their system. They reported an initial rate of ester hydrolysis which is 146 times higher than the same reaction without the presence of a DNA template strand (i.e. PNA 1 and PNA 2 strands in solution without hybridisation). This suggests that proximity effect and high effective molarity indeed play an important role in catalysis.[20]

In the case of our system, the substrate is not covalently attached to a DNA strand, making quantitative MALDI-TOF MS spectroscopy unsuitable for kinetic studies. Instead, UV-Vis absorption spectroscopy will be used. The UV absorption of the benzoic acid derivative (**2a-e** in Scheme 3) that is present in the reaction mixture after ester hydrolysis will be monitored.

The maximum absorption at different solvent conditions are summarized in Table 1.[26] [27] [28] [29] [30] Which benzoic acid derivative is used, depends on the absorptions of the other compounds present in the reaction mixture. Preferably, no other compounds (e.g. DNA) absorb in the region of the spectrum we want to use for detection. Luckily, the compounds of Table 1 show that λ_{max} differs considerably depending on compound and conditions. Furthermore, some compounds show two or even three absorption bands, giving us many possibilities to observe the ester hydrolysis in a clean part of the spectrum.[26] [27] [29]



Scheme 4. System used by Zelder *et al* for quantitative MALDI-TOF MS kinetic studies, containing (3-pyrid-2-yl)pyrazolyl and 8-quinolinol ester moieties attached to the C-terminus of PNA 1 and the N-terminus of PNA 2 respectively.[22]

Table 1. λ_{max} (nm) and ε_{max} (M^{-1} cm^{-1}) for substances **2a-e** in numerous solvents.

Entry		0.1 M HCl	0.1 M NaOH	MeOH	H_2O
2a[a]	λ_{max}	255	279	252	-
	ε_{max}	14890	17810	14750	
2b[b]	λ_{max}	303/237	296	302/234	-
	ε_{max}	3620/8470	3510	3940/7550	
2c	λ_{max}	270/225[c]	275[d]	288[d]	272
	ε_{max}	1300/11710	14630	18300	[e]$9.4\cdot10^{-4}$
2d	λ_{max}	-	-	226[f]	225[f]
	ε_{max}			12500[g]	[e]$6\cdot10^{-4}$
2e	λ	-	-	-	225[h]

Data of **2a-c** (except for H_2O column) from [25],**2c-d** H_2O from [29], **2d** MeOH from [26], **2e** from [27]. [a] 0.5 mg/100 mL, T= rt. [b] 1 mg/100 mL, T= rt. [c] 1 mg/100 mL, T= rt. [d] 0.5 mg/100 mL, T= rt. [e] Numbers represent specific absorbance ($(g/100\ mL)^{-1}cm^{-1}$), concentrations unknown. [f] Only intramolecular CT band shown, two other bands appear around 280 nm and 200 nm. [g] ε_{max} of ethanol, no value for methanol known. [h] λ_{max} unknown, λ_{225} used in [27], 25 mM ammonium citrate/acetic acid buffer (pH 5.5)

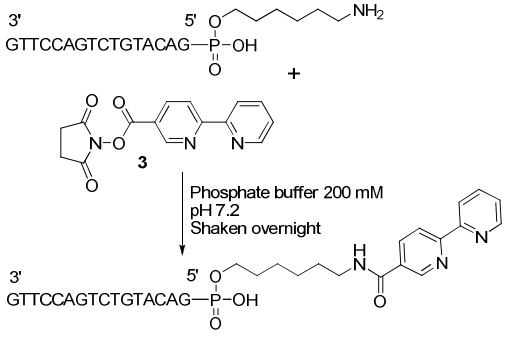
4. Results and Discussion

4.1. Synthesis of the catalytic DNA module

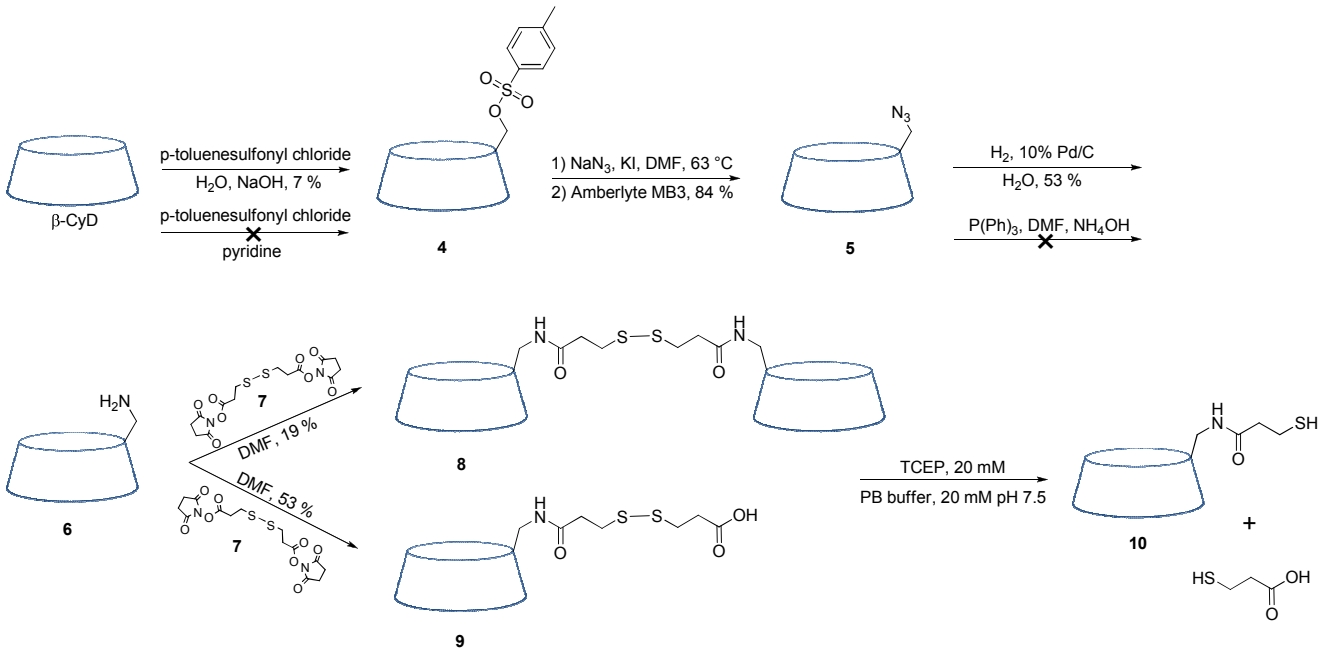
The bipy-oligonucleotide conjugate was prepared by reaction of a 5' amino-modified oligonucleotide with the *N*-hydroxysuccinimide activated ester of bipyridine (**3**, Scheme 5) The oligonucleotide consists of 16 bases with a GC content of 50 % and is 5' terminally modified with a C6-amine linker. All oligonucleotides used in our research were obtained from commercial sources via optimized solid-phase synthesis. The bipy-oligonucleotide conjugate was purified by size exclusion chromatography and analysed by rp-HPLC and MALDI TOF.[2] The rp-HPLC chromatogram showed full conversion and MALDI-TOF analysis confirmed that the bipy-oligonucleotide was formed ($(M-H)^-$ = 5249.58; calcd. 5250.34).

4.2. Synthesis of 3-mercaptopropane amide functionalised β-cyclodextrins

In order to attach β-CyDs to DNA, the CyDs have to be functionalised. Our first approach was to synthesise 3-mercaptopropane amide functionalised β-CyDs. The proposed five step synthesis is presented in Scheme 6.



Scheme 5. Synthesis of the 5' functionalised bipy-oligonucleotide conjugate.



Scheme 6. Synthesis of thiol functionalised β-CyD **10** in five steps.

The first step was to monofunctionalise the β-CyD with a good leaving group at the smaller upper ring side, for which a tosylate group was chosen. Because the primary alcohols are more reactive than the secondary alcohols, the tosylate group could in principle be easily introduced into the β-CyD, without the need of protecting groups. The first attempt however, in which p-toluenesulphonyl chloride was used under water free conditions with pyridine as solvent, gave a negligible yield of the tosylate functionalised β-CyD, as concluded from the ^{1}H-NMR spectrum. It was thought that a more basic environment was needed for the primary hydroxyl groups in order to react with the p-toluenesulphonyl chloride. Indeed, when the reaction was carried out in water with addition of NaOH, compound **4** was obtained in 7 % yield (lit. 11 %[31], 4.4 %[32]). The poor yield is mainly a consequence from the conditions that prevents polyfunctionalisation of the β-CyD. Furthermore, the highly basic environment is assumed to cause hydrolysis of the p-toluenesulphonyl chloride, therefore deactivating it.

In the next step, the tosylate group was substituted for an azide group by addition of NaN_3 to compound **4**. An anionic ion exchange resin, Amberlyte MB3, was used to purify the reaction mixture from the excess of azide ions present. Compound **5** was obtained in good yield (84 %, lit. 88 %[31] and 74 %[32]). Analysis of the compound using IR spectroscopy showed a clear band of the azide at 2038 cm^{-1} (asymmetric stretch). A small band of the azide salt was observed at 2104 cm^{-1}. The symmetric stretch, expected around 1200-1300 cm^{-1} was too weak to be observed.[33]

Compound **5** was reduced by hydrogenation with activated Palladium on charcoal, resulting in the amine functionalised β-CyD which was obtained in moderate yield (53 %, lit. 87 %[31]). The IR spectrum showed the disappearance of the band at 2038 cm^{-1}, indicating that the azide was indeed reduced to the amine (**6**). Although **6** was eventually successfully synthesised, the reduction caused some problems. To begin with, the reaction had to be done twice each time in order to obtain full disappearance of the azide band in the IR spectrum, indicating completion of the reaction. This resulted in product loss, because work-up had to be done twice. Secondly, and most importantly, after the second attempt, the obtained product had a greyish colour, indicating that a rather large amount of Palladium was still present. It is assumed that the Palladium has a large affinity for the hydroxyl groups of the β-CyD, making it difficult to fully purify the compound by filtration over Celite.

A simple way to get rid of the Palladium would be to use Fast Protein Liquid Chromatography (FPLC), but unfortunately β-CyDs have no significant chromophores.[34] Therefore, no significant UV absorption could be observed. Because full purification seemed almost impossible without significant loss of product, a different approach was tried. A Staudinger reduction seemed a reliable choice,[32] because purification is in principle much easier. Triphenylphosphine (PPh_3) is insoluble in water and therefore easy to filter off. Indeed, no traces of PPh_3 were observed after work-up. However, analysis of the IR spectrum showed almost no disappearance of the band at 2038 cm^{-1}, not even after two attempts of reduction. It was concluded that the PPh_3 had too little reductive power. A possible cause is that the PPh_3 has binding affinity for the β-CyD cavity. It was decided to proceed to the next step with the impure product of the hydrogenation.

3,3'-dithiobis(propionic acid *N*-hydroxysuccinimide ester) (**7**) reacted with compound **6** to give either the dimer in which the CyD moieties are attached via a disulfide bond (**8**), or the monomer, with a loose mercaptopropanoic acid chain connected to the β-CyD via a disulfide bond (**9**). Dimer **8** was formed when half an equivalent of compound **7** was used. Two equivalents of compound **7** resulted in mainly the formation of compound **9**. Compound **8** was obtained in low yield (19 %, lit. 99 %[32]) because a lot of product was not recovered during filtration. Compound **9** was obtained in moderate yield (40 %, lit. 89 %[32]) for the same reason as compound **8**. Characterisation of both molecules turned out to be a challenge. In general, characterisation of all functionalised β-CyDs proved to be so.

Characterisation by ^{1}H and ^{13}C NMR is inconclusive, because of the broad peaks in the proton NMR and low intensity in the ^{13}C NMR, which makes small differences in structure difficult to be observed. Even 2D HSQC experiments gave too low intensity. The broadness of peaks in the ^{1}H NMR spectrum can partly be assigned to the large amount of water molecules a β-CyD can bind (in a non-functionalised β-CyD: 10-13%). Because β-CyDs are relatively

spectroscopically inert, techniques that use UV/Vis absorption, like FPLC and HPLC, are not suitable for characterisation. In addition, thin layer chromatography (TLC) turned out to be difficult as well. Spots only appeared at high concentration of product, making column chromatography impossible due to dilution, even when colouring agents like *p*-anisaldehyde with concomitant use of sulphuric acid were used. The best evidence (still, non-conclusive) was obtained by MALDI-TOF mass spectrometry, which gives the mass of a molecular ion with a discrepancy of only 0.1 mass percentage. However, this technique has also its limitations, as can be nicely seen for the characterisation of compounds **8** and **9**. Although the quasi molecular ion of both could be easily identified (**8**: $(M+Na)^+$ = 2464.50; calcd. 2465.23 **9**: $(M+Na)^+$ = 1348.63; calcd. 1348.37), assignment of the other peaks proved to be rather difficult. Figure 5 depicts the MALDI-TOF spectrum obtained from compound **9** (a similar pattern is obtained for compound **8**, data not shown). The peak of the molecular ion is easily identified together with a fragmentation pattern which seems to be the rupture of hydroxyl groups. The peak at m/z 1244.69 was assigned to the rupture of the disulfide bond and the peak at m/z 1157.71 could be the rupture of the amide bond. However, if starting material (**6**) is still present, it will appear at the same m/z value ($(M+Na)^+$ = 1157.71 calcd. 1156.38). So although MALDI-TOF seems the best way to characterize, even this method cannot tell us for sure if the reaction went to completion. The presence of compound **6** could have undesirable consequences as will be further discussed in chapter 4.4.

The final reaction step consists of the reduction of the disulfide bond of compounds **8** and **9** This step is done *in situ*, during the coupling of the β-CyD with DNA and is therefore also further discussed in chapter 4.4.

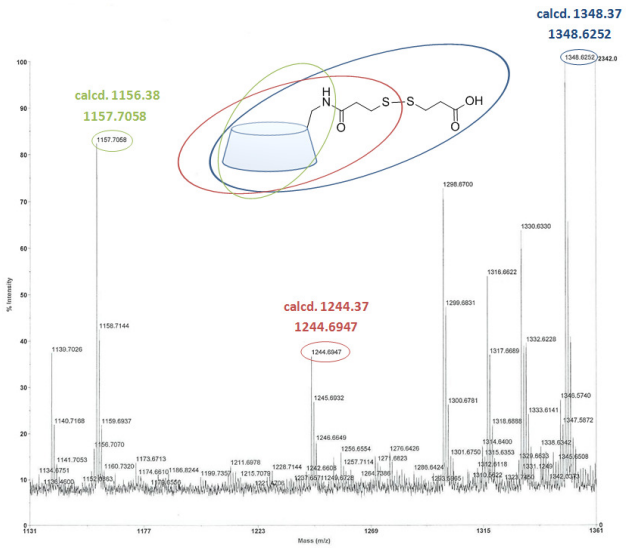
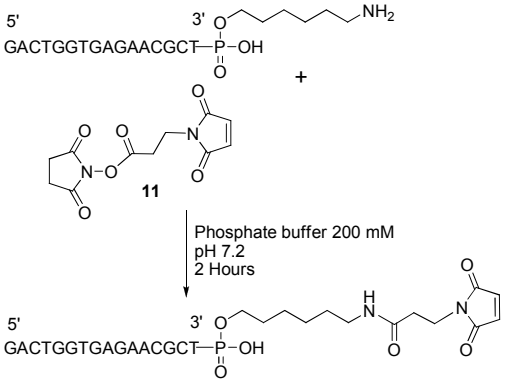


Figure 5. MALDI-TOF spectrum of compound **9**, obtained in positive reflector mode. Matrix: 2,5-dihydroxybenzoic acid (10 mg/mL) with 0.25 % TFA. Number of laser shots: 250. Acquisition mass range: 700-5000 Da. Accelerating voltage: 25,000 V.

4.3. Synthesis of maleimide functionalised oligonucleotides

Before a cyclodextrin compound can be nucleophilically bound to an oligonucleotide, the oligonucleotide itself must be functionalised with an electrophilic group, such as a maleimide group. The maleimide-oligonucleotide conjugate was prepared by reaction of a 3' amino-modified oligonucleotide with the *N*-hydroxysuccinimide ester of 3-maleimidopropionic acid (**11**, Scheme 7). The oligonucleotide consists again of 16 bases with a 50 % GC content and a 3' terminally modified C6 amine linker. The maleimide-oligonucleotide conjugate was purified by size exclusion chromatography and analysed by rp-HPLC, to give almost full conversion.



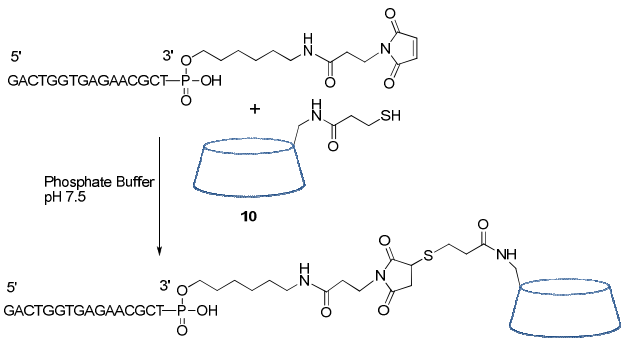
Scheme 7. Synthesis of the 3' functionalised maleimide-oligonucleotide conjugate.

4.4. Synthesis of β-cyclodextrin-oligonucleotide conjugates

It is known from literature that the nucleophilic thiol functional group can easily attack an electrophilic maleimide group in a 1,4-fashion, even at neutral pH.[35] [36] [37] In order to let a β-CyD attack a maleimide functionalised oligonucleotide, the disulfide bond of **8** or **9** has to be reduced, to give thiol **10**. The coupling reaction is shown in Scheme 8.

For the reduction of **9**, a typical protocol for the reduction of disulfide bonds in peptides was followed,[38] using tris(2-carboxyethyl)phosphine (TCEP). The reaction was done *in situ*, just prior to the coupling to the oligonucleotide, limiting the time for oxidation of the disulfide bond to occur.

TCEP was deliberately chosen above a sulfhydryl containing reductor like dithiothreitol (DTT), because of its lack of reactivity towards other functionalities and the stability of the oxidized form of the phosphine, preventing reversal of the reaction. TCEP can therefore be used without removal of an excess added, unlike DTT, which could act as a nucleophile for the attack on the maleimide group, resulting in side products. In addition, phosphorus atoms are in general less nucleophilic than sulphur atoms.



Scheme 8. Nucleophilic attachment of the thio functionalised β-CyD to the maleimide group of the oligonucleotide, should give a cyclodextrin-oligonucleotide conjugate.

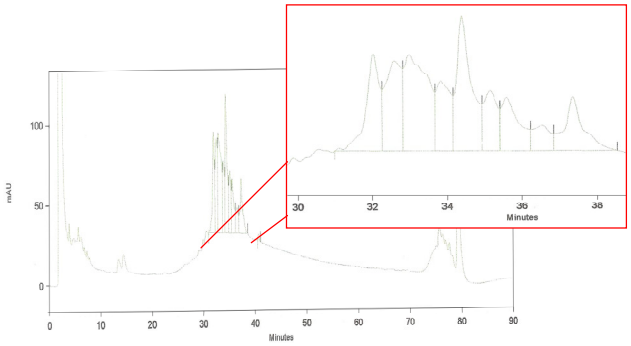


Figure 6. rp-HPLC chromatogram of the coupling of **10** to the maleimide functionalised oligonucleotide. t = 19 hours.

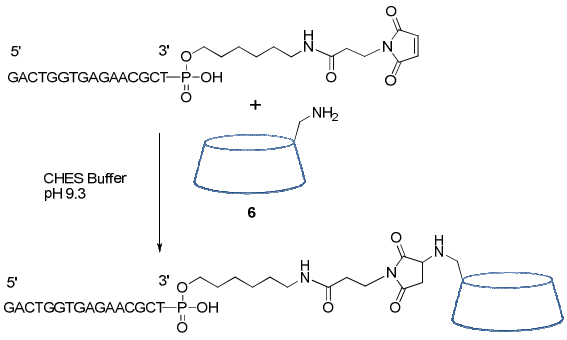
After incubation for two hours at 30 °C, the maleimide functionalised oligo was added. The ratio of DNA : **10** was approximately 1 : 50 and the coupling was analysed using rp-HPLC and MALDI-TOF mass spectrometry. rp-HPLC analysis were done at t = 0, 1.5 hours, 2.5 hours and 19 hours. In all chromatograms, eleven peaks were observed with significant overlap between each peak (Figure 6). During time, only little changes in peak intensities were observed. It was not possible to obtain any useful information about the coupling from the HPLC traces. Also, from the MALDI-TOF spectrum, no coupling of the β-CyD to the oligonucleotide could be concluded.

Unfortunately, it is not very easy to point out the exact problem for the failure of the thiol coupling to the maleimide. Because the reduction was done *in situ*, many variables determined the outcome of the experiment. First, it could be that the conditions and concentrations of reactants for the reduction of the disulfide bond were too mild, therefore leading to incomplete formation of thiol functionalised β-CyD (**10**). Second, the ratio of DNA : **10** was approximately 1 : 50, which could mean that the excess of **10** was insufficient. In addition, the concentration of DNA in the reaction solution could be too low, decreasing the probability of coupling. Third, and most importantly, the HPLC trace consists of many peaks, indicating that side reactions could play an important role. For example, although TCEP is known for its relative inertness towards other functionalities, some of the TCEP molecules could have attacked the maleimide group of the oligonucleotide. In addition, reduction of compound **9** gives 3-mercaptopropanoic acid as a side product. The thiol group of this molecule can act as a nucleophile and also attack the maleimide group.

In short, because of the many variables, optimization of this procedure seems unlikely within a reasonable amount of time. Therefore, we focused the research on finding different and preferably easier ways to couple the β-CyD to the oligonucleotide. A second approach was soon considered.

It is known that in addition to thiol groups, also amines can attack a maleimide group, especially under basic conditions (pH 9-9.5). [36] [37] [39] This procedure could be interesting for two reasons. In the first place, direct usage of **6** considerably simplifies the synthetic route and reduces the chances of side reactions. Secondly, if the coupling of the amine would be unsuccessful, we can conclude that traces of **6** will not cause significant side reactions during the thiol coupling. This would mean that other side reactions play a more important role. In addition, although the attempt of amine-maleimide coupling cannot tell us whether the m/z value 1157.7058 from the MALDI-TOF spectrum of **9** (Figure 5) comes from **6** or is a fragment of **9**, it does tell us that an incomplete conversion and thus presence of **6** would not cause significant problems. Either result would therefore help us better understand the system.

Amine functionalised β-CyD **6** was first centrifuged in order to



Scheme 9. Nucleophilic attachment of amino functionalised β-CyD to the maleimide group of the oligonucleotide, gives a cyclodextrin-oligonucleotide conjugate.

get rid of the insoluble impurities present from the hydrogenation step. The supernatant was freeze dried and then added to an N-cyclohexyltaurine (CHES) buffer solution of the maleimide functionalised oligonucleotide in a one to one fashion at pH 9.3 (Scheme 9). The reaction was analysed using rp-HPLC and MALDI-TOF. A low intensity peak of the coupled product was observed in the MALDI-TOF spectrum ($(M-H)^-$ = 6413.90; calcd. 6410.62; Figure 7, green). A second larger peak was ascribed to the hydrolysed maleimide oligonucleotide ($(M-H)^-$ = 5287.04; calcd. 5294.61). The hydrolysis of the maleimide group occurs readily at high pH.[35] [36] [37] [39] Therefore the reaction was retried with 120 equivalents of **6**. This gave a strong intensity peak at the m/z value of the coupled product (Figure 7, purple). The rp-HPLC spectra in both experiments showed almost full disappearance of the peak corresponding to the maleimide functionalised oligonucleotide and appearance of two new peaks, a large broad one and a smaller narrower one, with considerable overlap between the two. After a preparative rp-HPLC experiment of the reaction mixture containing 120 equivalents of **6**, MALDI-TOF spectra of all fractions made clear that the smaller peak in the rp-HPLC chromatogram corresponded to the coupled product of **6** with the oligonucleotide (Figure 7, orange). The larger peak corresponded to the hydrolysed maleimide oligonucleotide.

The data shown, suggests that optimization of this coupling might be possible. As discussed, the maleimide hydrolysis and **6** coupling peaks from the rp-HPLC spectra are overlapping considerably. In addition, the coupling peak has an area of about half the peak of hydrolysis, suggesting the CyD coupling is the minor product.

Optimization of peak separation and peak width in HPLC measurements, could be obtained by either changing the pH of the running buffer used (currently 6.8), or changing the column material. Reducing the rate of hydrolysis could be done by increasing the concentration of the reaction mixture or addition of more equivalents of **6**. Indeed, when the experiment was carried out with 600 equivalents of **6** and a twice as high concentration of the reaction mixture (150 mM instead of 75 mM), two peaks were again observed in the rp-HPLC chromatogram, but this time with almost equal intensity. This suggested that the amount of hydrolysed product was about equal to the CyD coupled product under these conditions. However, because MALDI-TOF measurements could not be obtained (due to malfunctioning of the machine), it is not certain yet if an increase in amount of coupled product was indeed obtained.

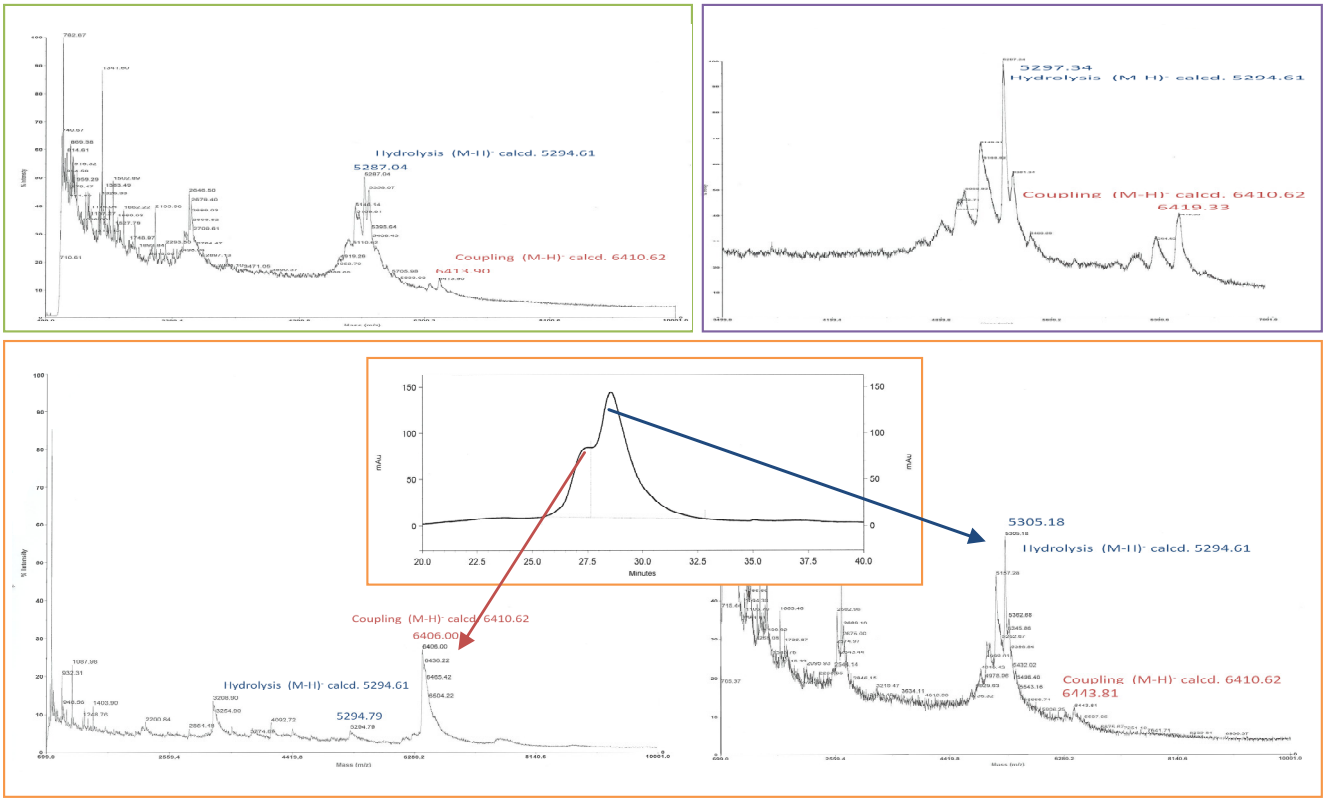
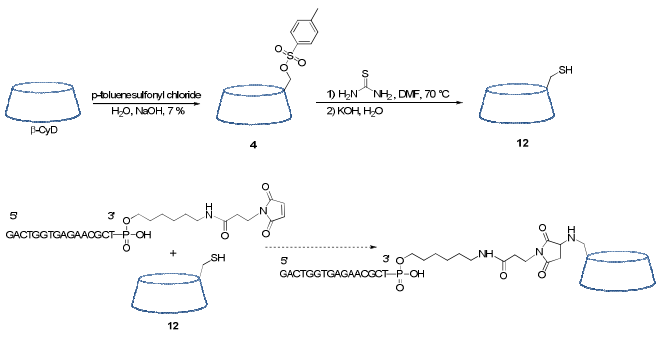


Figure 7. Green: MALDI-TOF spectrum of coupling **6** to the maleimide functionalised oligonucleotide in a 1 : 1 ratio. Matrix: 2,4,6-trihydroxyacetophenone 0.5 M in EtOH with ammonium citrate 0.1 M in H_2O. Purple: MALDI-TOF spectrum of the same reaction as green, 120 fold excess of **6**. Same matrix was used. Orange: two MALDI-TOF spectra of the corresponding two peaks in the preparative rp-HPLC experiment. The smaller left peak in the HPLC chromatogram matches the β-CyD-oligonucleotide conjugate. Matrix: saturated solution of 3-hydroxypiccolinic acid in water/ACN. The right peak matches the hydrolysed maleimide functionalised oligonucleotide. Matrix: 2,4,6-trihydroxyacetophenone 0.5 M in EtOH with ammonium citrate 0.1 M in H_2O. ‡ All MALDI-TOF spectra were obtained in linear negative mode.

4.5. Change of Plans: A Simpler Approach

As discussed, the couplings of thio and amino functionalised β-CyDs to maleimide functionalised oligonucleotides are difficult to control and optimize. Therefore a new type of coupling was considered (Scheme 10). The β-CyD was functionalised with a thiol group (**12**) in only two steps, in principle reducing the chance of impurities being able to give side reactions during coupling. The first step, tosylate functionalisation, was already described (chapter 4.2, Scheme 6). **4** was therefore used for the second step, in which thiourea was added under a nitrogen atmosphere at 70 °C. After 48 hours, addition of water and KOH at room temperature, gave **12**. The compound was contaminated by a large amount of inorganic salts. MALDI-TOF analysis indicated however, that **12** was formed $(M+Na)^+$ = 1173.49; calcd. 1174.00). The m/z value of **4** was also observed, indicating incomplete conversion. Because this is a relatively simple approach, coupling of **12** to a maleimide functionalised oligonucleotide could be a very effective way of coupling β-CyDs to DNA, as long as the right reaction conditions can be obtained to give complete conversion and **12** can be obtained as a pure compound.

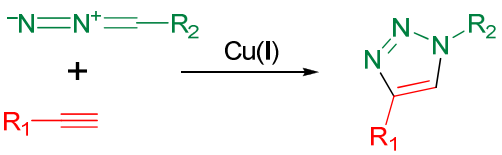


Scheme 10. Two-step synthesis of **12** via **4** and subsequent addition of thiourea (upper half). The nucleophilic attack of **12** at the maleimide group of the oligonucleotide which should give a β-CyD-oligonucleotide conjugate, is also represented (lower half).

4.6. Go With the Flow: Introduction of 'Click' Chemistry

4.6.1. The Properties of 1,3-Dipolar Huisgen Reactions

Although the 1,3-dipolar cycloaddition reaction of azides and alkynes – the Huisgen reaction – has been known for quite a long time, it has only recently found an enormous number of novel applications.[40] This can be ascribed to the discovery that the reaction can be efficiently catalysed by copper(I) (Scheme 11). The copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) is a prominent example of a group of reactions named 'click' reactions.



Scheme 11. General scheme of a copper(I) catalysed azide-alkyne cycloaddition (CuAAC), also known as a copper(I) catalysed Huisgen reaction.

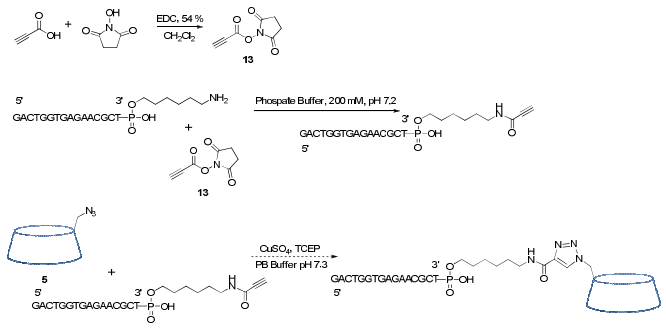
This particular reaction has had a major impact on biomolecular research for various reasons.[40] First of all, the CuAAC reaction is often characterised by high yields and mild reaction conditions. In addition, the reaction is known for its high tolerance of a broad range of functional groups. Furthermore, in most cases, only simple product workup is required. Most importantly, the CuAAC reaction is known for its unique orthogonality, meaning that the functional groups present in biomolecules are not sensitive to attack of the reagents.

The CuAAC reaction seemed an excellent choice for the coupling of CyDs to oligonucleotides, since the previously discussed coupling reactions seem to suffer from side reactions that make optimization of the reactions quite hard.[40] [41] [42] [43] In addition, the azide functionalised CyD was already synthesised (**5**). Most obviously, the reaction was therefore first tried with **5** and an alkyne functionalised oligonucleotide (Scheme 12).

4.6.2. Preparation of the Alkyne Modified Oligonucleotide

Propargylic acid reacted with *N*-hydroxysuccinimide to which 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added as a carboxy-activating reagent to give succinimidyl-3-propiolate (**13**) in 54 % yield. The *N*-hydroxysuccinimide activated ester **13** could subsequently be attacked by a 3' amino-modified oligonucleotide. After size exclusion chromatography, the product was analysed using preparative rp-HPLC and MALDI-TOF. Unfortunately, an unexpected side reaction was observed. Before the coupling reaction was started, only one peak in the HPLC chromatogram was observed at t_R = 25.0 min (**A**, Figure 8). After one hour, a second peak appeared at t_R = 28.2 min (**B**, Figure 8) with about half the area of **A**. From determination of the average mass of all collected fractions from a preparative HPLC experiment using MALDI-TOF, it could be concluded that **B** was indeed the alkyne functionalised oligonucleotide ($(M-H)^-$ = 5175.84 calcd. 5176.40). Because the 3' amino-modified oligonucleotide was still present in a significant amount, the reaction was set overnight. Analyses after 19 hours revealed an unexpected peak at t_R = 26.0 in the HPLC chromatogram, with a large peak area (**C**, Figure 8). MALDI-TOF measurements revealed the presence of a product with a mass of 5308.19. No reasonable structure could be drawn that is consistent with this mass number. The chromatogram which was obtained after 19 hours, also shows that a considerable amount of starting material is still present (**A**, Figure 8).

Because the formation of the side product could not be explained, the final step of coupling the azide functionalised β-CyD (**5**) with the alkyne functionalised oligonucleotide (Scheme 12), was not carried out. However, since for a CuAAC reaction it does not matter which functionality (alkyne or azide) is attached to which molecule, the reaction could be retried by switching the functional groups, i.e. the β-CyD could be functionalised with a terminal alkyne and the oligonucleotide with an azide group (Scheme 13).



Scheme 12. Preparation of the alkyne modified oligonucleotide and subsequent coupling of the azide functionalised β-CyD to the alkyne via a CuAAC reaction, which should result in the formation of the substrate binding module.

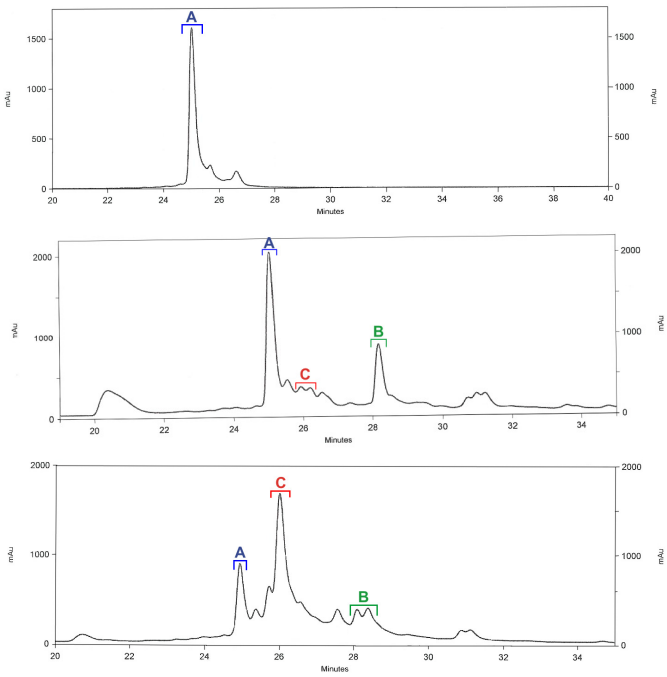
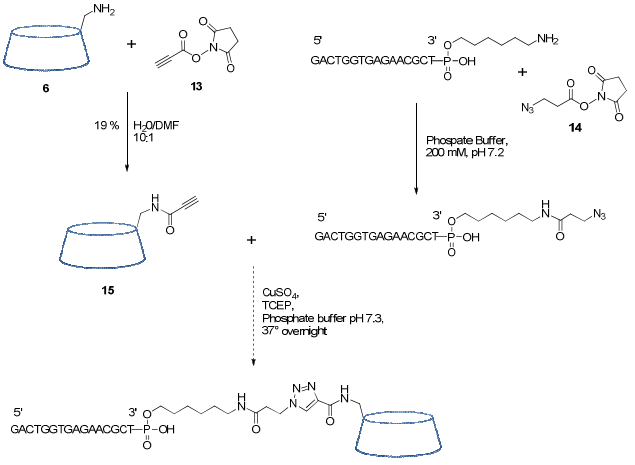


Figure 8. rp-HPLC chromatograms for the preparation of the alkyne modified oligonucleotide. Peaks appear at t_R = 25.0 min (A), 28.2 min (B) and 26.0 min (C).

4.6.3. Reversal of functionalities in the CuAAC reaction

Succinimidyl-3-propiolate (**13**) reacted with the amine functionalised β-CyD (**6**) to give the alkyne functionalised β-CyD (**15**). Analysis with MALDI-TOF gave the quasi molecular ion ($(M+Na)^+$ = 1211.10 calcd. 1207.36). The 3' amino-modified oligonucleotide reacted with the *N*-hydroxysuccinimide activated ester of 3-azidopropanoic acid (**14**) to obtain the azide functionalised oligonucleotide. HPLC experiments confirmed full



Scheme 13. Preparation of the alkyne functionalised β-CyD (**15**) and the azide functionalised oligonucleotide. A CuAAC reaction of **15** with the azide functionalised oligonucleotide in the presence of copper(I) ions, should in principle yield the substrate binding module.

conversion.

The azide functionalised oligonucleotide reacted with **15** under a nitrogen atmosphere in the presence of Cu(I) ions, that were formed by reduction of $CuSO_4$ with TCEP. Formation of the 1,2,3-triazole five-membered ring should give the substrate binding module. Analysis however, was not possible because the rp-HPLC experiments failed due to pollution of the column and the MALDI-TOF machine was broken at the time of writing. Further research is needed to see whether the coupling works or not and if so, if the reaction is selective without formation of major side products.

In the case that the coupling was successful but only little material has coupled, the yield can possibly be improved by using a copper(I)-stabilizing ligand. Cu(I) is generally thermodynamically unstable, which results in easy oxidation to Cu(II) or disproportionation to Cu(0) and Cu(II).[44] In addition, Cu(I) is known to produce HO• radicals that can damage DNA which can result in strand breaks.[45] A well known Cu(I)-stabilizing ligand is tris-(benzyltriazolylmethyl)amine, TBTA. Its tetradendate binding ability is believed to completely envelope the copper(I) center, leaving no free binding site available for potential destabilising interactions. [40] [44] [46]

Something that should be kept in mind when using the CuAAC reaction, is that copper can bind strongly to 1,2,3-triazoles. Since the catalytic module uses Cu(II) ions to coordinate to the bipyridine ligand, the copper affinity for the 1,2,3-triazole group must be taken into account for the kinetic measurements.

5. Summary and Outlook

Although in the past DNA used to be seen as solely being a carrier of genetic information, the properties of this impressive molecule soon made clear that it could be used in many other types of research, including DNA based catalysis. This field has certainly come a long way now, ever since our first publication in 2005. The possibility to use many different modules in our modular assembly catalyst approach is particularly appealing for the future design of DNA-based catalysts, in which the ultimate goal could be to catalyse multiple reaction steps successively. The formation of the bio-mimetic system is again a step further into this direction. The results presented clearly demonstrate that still some hurdles have to be taken in order to make the next step forward.

In particular, the substrate binding module for which a β-CyD has to be coupled to the DNA, is still considered a challenge. The coupling of amine functionalised β-CyDs to maleimide functionalised oligonucleotides was successful and optimization of the process in order to obtain higher yield should be possible by changing the reaction conditions or the way of characterisation. Many options are still open to be further examined. The approach in which we used 'click' chemistry is preferably used, because the CuAAC reactions are known for its orthogonality. Side reactions are thus not very likely, although we have to await the results in order to be certain.

Once the substrate binding module is synthesised in sufficient yield, it is just a matter of putting the module together with the catalytic module and the template strand in order to obtain the first DNA-based bio-mimetic enzyme, based upon our modular assembly principle.

The support of Núria Sancho Oltra is gratefully acknowledged. A.G. thanks Dr. Gerard Roelfes for his many helpful discussions and for writing this interesting research proposal.

Experimental Section

Materials. β-Cyclodextrin (β-CyD) was purchased from Sigma-Aldrich and used without further purification. The β-CyDs were dried before use by stirring overnight under vacuo at RT. The exact amount of β-CyD used for the reactions was determined after drying. 3' or 5' C6 amino-modified oligonucleotides were commercially prepared using optimized solid-phase synthesis and purchased from Biotez (Berlin, Germany). Ultra high purity water from a Millipore Milli Q purification apparatus containing a 0.22 µm filter was used for all biochemical experiments. Biograde DMF 99.8 %, DNAse, RNAse and protease free, was purchased from Acros for use in biochemical experiments. Compound **11** was purchased from Alfa Aesar (Ward Hill, MA, USA). Anionic exchange resins Amberlite MB3 and IWT TMD-8 with colour indicator were purchased from Merck and Sigma-Aldrich respectively. Size-exclusion chromatography for purification of oligonucleotides was done using a prepacked NAP-10 column from GE Healthcare, containing DNA-grade Sephadex G-25 gel filtration material. Size-exclusion chromatography for purification of β-CyDs was done with a Sephadex G-10 gel material. All buffer solutions were prepared with Milli Q water and filtered before use with a 0.2 µm disposable filter. All other chemicals were purchased from Acros, Sigma-Aldrich, Fluka or Merck and were used as received.

Equipment. rp-HPLC analysis were performed on a Shimadzu LC-10AD VP system equipped with a Shimadzu SPD-M10A-VP diode array detector. Analysis was done with a Waters Xterra MS C18 column (3.0 x 150 mm, particle size 3.5 µm) using a gradient of CH_3CN/TEAA buffer 50 mM pH 7; gradient: 05/95 0 to 10 min, to 35/65 at 60 min, to 70/30 at 65 min. Flow: 0.5 mL/min. For preparative experiments, fractions of 0.3 mL or 0.5 mL were collected. Flow: 1 mL/min.

MALDI-TOF measurements were done on a Voyager-DE Pro apparatus, with an accelerating voltage of 20,000 or 25,000 Volts (grid voltage 77 % or 95 % respectively). Number of laser shots is 500/spectrum, laser repetition rate 20 Hz and low mass gate at 500 Da. Calibration was done both externally and internally, with non-functionalised β-CyD for all β-CyD derivatives (1134.37 Da) and a 16-mer oligonucleotide (5051.33 Da) for all samples containing DNA. Matrix A: 20 µL of a solution of 2,5-dihydroxybenzoic acid 10 mg/mL in ACN with 2.5 µL TFA + 2 µL saturated sample solution in Milli Q water. Matrix B: 20 µL of a solution of 2,4,6-trihydroxyacetophenone 0.5 M in EtOH + 10 µL of a solution of ammonium citrate dibasic 0.1 M in Milli Q water + 2 µL sample solution in Milli Q water. Matrix C: 5 µL of a saturated solution of 3-hydroxypicolinic acid in 50% Milli Q water/ACN + 2.5 µL of a solution of ammonium citrate 50 mM + 5 µL sample solution in Milli Q water 100 µM. Matrix A and C were used for measuring β-CyDs in positive reflector mode, Matrix B was used for measuring samples containing DNA in negative linear mode. ESI mass spectra (HRMS) were recorded on an LTQ Orbitrap XL (ESI+).

The NMR spectra were recorded on a Varian Gemini 200, Varian VXR-300, Varian Mercury Plus 400 and Varian Unity Plus 500 spectrometer operating at ^1H frequencies of 199.97, 299.97, 399.93 and 499.86 MHz respectively, at ambient temperature. Chemical shifts are reported in δ units (ppm) and referenced to the residual deuterated solvent signals of CD_3Cl (^1H: δ 7.26 ppm ^{13}C: δ 77.16 ppm), D_2O (^1H: δ 4.79) or (CD3)2SO (^1H: δ 2.50 ppm ^{13}C: δ 39.52 ppm) Coupling constants are indicated with: *J*=, followed by the frequency given in Hz. Splitting patterns are abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), broad (broad band) and app. (apparent). For ^{13}C the carbons are designated as follows: p (primary), s (secondary), t (tertiary), q (quaternary).

IR spectra were recorded on a Perkin-Elmer 2000 FTIR ATR spectrophotometer (2 cm^{-1} resolution). Melting points were uncorrected and recorded on a Büchi B-545 melting point apparatus. Optical rotations were measured on a Schmidt and Haensch Polartronic MH8. UV-Vis spectra were recorded on a Jasco V-570 spectrophotometer. Fast measurements for oligonucleotides were

done using a NanoDrop ND-1000 UV-Vis spectrophotometer with a 1 mm path length, Xenon lamp and 2 µL samples. TLC was performed with silica plates 60 Å with F_{254} fluorescing indicator, purchased from Merck. Eluent used: 7:7:5 EtAc/2-propanol/H_2O. TLC spots were visualised with 1.5 % H_2SO_4 along with UV-light of λ = 254 nm. TLC spots of β-CyDs were only detected at very high concentrations.

Compounds **3**[2] and **14**[47] were prepared by Núria Sancho Oltra according to literature procedures.

Mono-6-deoxy-6-(*p*-tolylsulfonyl)-β-cyclodextrin (4). A procedure from the literature[31] was modified as follows: β-cyclodextrin (100 g, 88.1 mmol) was suspended in 820 mL of water. NaOH (11.0 g, 275 mmol) dissolved in 33 mL of water was added dropwise over 80 min. The suspension became homogeneous and slightly yellow before addition was finished. *p*-Toluenesulfonyl chloride (16.6 g, 86.6 mmol) dissolved in 50 mL of ACN was added dropwise over 30 min, causing immediate formation of a white precipitate. After 2h of stirring at RT, the precipitate was removed by suction filtration and the filtrate was set for 48h at 4 °C. The resulting second precipitate was recovered by suction filtration and dried overnight under vacuo at RT to give 8.2 g (6.36 mmol, 7 %) of a white solid: mp 179.3 (lit. 179 slow dec); TLC (SiO_2) one spot at R_f = 0.58 (lit. 0.60), slightly UV-positive; $[\alpha]^{20\,°C}_D$ +110° ±4°; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.73 (d, *J*= 7.69 Hz, 2H), 7.42 (d, *J*= 7.69 Hz, 2H), 5.82-5.58 (br m, 21H) 4.81-4.75 (m, 9H), 4.60-4.10 (br m, 7H), 3.76-3.10 (br m, overlaps with HOD), 2.42 (s, 3H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 144.9 (q), 132.8 (q), 129.8 (t), 126.2 (t), 102.1 (m), 81.2 (m), 74.0-72.0 (m), 70.2 (m), 69.8 (m), 60.2 (m), 21.1 (p); IR (ATR): 3680-3000, 3000-2800, 1710-1560, 1365, 1170 and 1025 cm^{-1}; UV-Vis (H_2O): 257, 266 nm; positive ion MALDI-TOF MS (Matrix A) *m/z* 1314.60 for $[M+Na]^+$, calcd ($C_{49}H_{76}O_{37}S$) 1288.38.

Mono-6-deoxy-6-azido-β-cyclodextrin (5). A procedure from the literature[31] was modified as follows: powdered **4** (2.02 g, 1.56 mmol) was suspended in 6.5 mL of dry DMF under N_2 flow. After warming to 64 °C, the solution became homogeneous. Crystalline KI (0.13 g, 0.77 mmol) and NaN_3 (1.01 g, 15.5 mmol) were added and the reaction mixture was stirred for 18h at 63 °C. The mixture was then allowed to cool to room temperature and treated with Amberlite MB-3 and IWT-TMD-8 resin to remove salts. The resin was separated by filtration and the filtrate concentrated to 2 mL. Addition of acetone resulted in a white precipitate, which was filtered and dried for 48h at 37 °C under vacuum, yielding 1.69 g (1.32 mmol, 84 %) of a white solid: mp 204.1 (lit. 206 slow dec); TLC (SiO_2) one spot at R_f = 0.73 (lit. 0.71), slightly UV-positive; $[\alpha]^{20\,°C}_D$ +131° ±3°; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 5.80-5.58 (m, 14H), 4.87-4.81 (m, 7H), 4.55-4.40 (m, 7H), 3.80-3.45 (m, 28H), 3.43-3.22 (m, overlaps with HOD); ^{13}C-NMR (125 MHz, D_2O) δ 102.1, 101.9, 82.3, 81.3, 73.3, 73.1, 72.0, 70.8, 60.3, 51.3; IR (ATR): 3680-3000, 3000-2780, 2038 (N_3 assym st), 1670, 1390, 1170 and 1020 cm^{-1}; positive ion MALDI-TOF MS (Matrix A) *m/z* 1182.85 for $[M+Na]^+$, calcd ($C_{42}H_{69}N_3O_{34}$) 1159.38.

Mono-6-deoxy-6-amino-β-cyclodextrin (6). A procedure from the literature[31] was modified as follows: powdered **5** (0.81g, 0.70 mmol) was dissolved in 50 mL of water. 111 mg of 10% Pd/C was added under vigorous stirring and the resulting suspension was subjected to H_2 by means of a balloon. After 48h at RT the suspension was filtered through a bed of Celite and the slightly grayish filtrate was evaporated under reduced pressure. This gave a grayish solid (316 mg, 279 µmol, 53 %): mp 197.4 °C (lit. 201 °C), ^{1}H-NMR (400 MHz, D_2O) δ 5.18 (app s, 7H), 4.00-3.80 (m, 28H), 3.68-3.55 (m, 14H); ^{13}C-NMR (125 MHz, D_2O) δ 102.0 (m), 101.8 (t), 83.0 (t), 81.3 (m), 73.2 (m), 72.2 (m), 71.9 (m), 61.4 (s), 60.4 (m); IR (ATR): 3700-3000, 3000-2800 and 1020 cm^{-1} (disappearance of azide assym st at 2038 cm^{-1}); positive ion MALDI-TOF MS (Matrix A) *m/z* 1156.91 for $[M+Na]^+$, calcd ($C_{42}H_{71}NO_{34}$) 1133.39.

3,3'-Dithiobis(propan-(*N*-mono-6-deoxy-β-cyclodextrin)amide) (8). A procedure from the literature[32] was modified as follows: powdered **6** (40,0 mg, 35.3 µmol) was suspended in 5 mL of dry DMF under a N_2 atmosphere. 3,3'-Dithiobis(propionic acid *N*-hydroxysuccinimide ester) (7.26 mg, 18.0 µmol) in 10 mL of dry DMF was added dropwise over 45 min under vigorous stirring. The slightly yellowish mixture was stirred for 48h at RT and subsequently evaporated under reduced pressure to obtain 16.5 mg of a slightly yellow solid (6.70 µmol, 19 %): ^{1}H-NMR (400 MHz, D_2O) δ 7.94 (br s, 2H), 6.00-5.60 (br m), 4.91-4.72 (br m), 4.58-4.40 (br m), 3.82-3.50 (app s, overlaps with HOD), 2.95-2.80 (app s, overlaps with D_2O), 2.18-2.00 (app s); IR (ATR): 3700-3000, 3000-2800 and 1020 cm^{-1}; positive ion MALDI-TOF MS (Matrix A) *m/z* 2464.50 for $[M+Na]^+$, calcd ($C_{90}H_{148}N_2O_{70}S_2$) 2440.75.

3,3'-Dithiopropionic acid mono(*N*-mono-6-deoxy-β-cyclodextrin) amide (9) Following the procedure for the synthesis of **8**, starting from **6** (102 mg, 90.0 µmol) in 7.5 mL of dry DMF and 3,3'-dithiobis(propionic acid *N*-hydroxysuccinimide ester) (73.0 mg, 180 µmol) in 10 mL of dry DMF, compound **9** was obtained as a slightly yellow solid (47 mg, 35 µmol, 39 %): ^{1}H-NMR (400 MHz, D_2O) δ 5.00-4.83 (app s), 4.80-4.20 (br m, overlaps with D_2O), 3.90-3.60 (br m), 3.60-3.38 (br m), 3.25-2.22 (br m); ^{13}C-NMR (125 MHz, D_2O) δ 179.0 (q), 177.6 (q), 102.1 (m), 81.2 (m), 73.3 (m), 72.3 (m), 72.0 (m), 60.3 (m), 36.0 (s), 32.5 (s), 27.7 (s), 25.3 (s); positive ion MALDI-TOF MS (Matrix A) *m/z* 1348.63 for $[M+Na]^+$, calcd ($C_{48}H_{79}NO_{37}S_2$) 1325.38.

3-Mercaptopropionic acid (*N*-mono-6-deoxy-β-cyclodextrin) amide (10) A procedure from the literature[38] was modified as follows: 4.56 mg (3.46 µmol) of **9** was dissolved in 459 µL of phosphate buffer (20 mM, pH 7.2) to give a 7.5 mM solution of **9**. 2.5 mg of tris(2-carboxyethyl)phosphine (TCEP) was added to the solution with a total TCEP concentration of 22 mM. The yellow mixture was incubated at RT for 1.5h after which the temperature was risen to 30 °C for 1h. This mixture was subsequently used to form DNA conjugates. No product analysis was done. TCEP was checked for purity: ^{31}P-NMR (121 MHz, D_2O) δ 17.2 (t).

Per-6-thio-β-cyclodextrin (12). A procedure from the literature[48] was modified as follows: powdered **4** (0.515 g, 400 µmol) was dissolved in dry DMF under a N_2 atmosphere. Thiourea (160 mg, 2.11 mmol) was then added and the reaction mixture was heated to 70 °C for 48h. The mixture was then allowed to cool down and removal of DMF under reduced pressure gave a yellowish oil, which was dissolved in 42 mL of H_2O. NaOH (0.25 g, 6.25 mmol) was added and the white solution was refluxed for 2h. The mixture was then acidified with $NaHSO_4$ (pH 1) and subsequently evaporated under reduced pressure to obtain crude **12** (7.53 g): ^{1}H-NMR (300 MHz, D_2O) δ 6.25-6.15 (app s), 5.00-4.75 (m, overlaps with D_2O), 3.70-3.42 (m), 3.40-3.20 (m), 2.90-2.82 (m), 2.75-2.60 (m), 2.35-2.25 (m).
A concentrated solution of **12** in 1 mL Mili Q water was further purified by size exclusion chromatography with a NAP-10 column. After lyophilisation, crude **12** was obtained as a yellow solid: positive ion MALDI-TOF MS (Matrix A) *m/z* 1173.49 for $[M+Na]^+$, calcd ($C_{42}H_{70}O_{34}S$) 1150.35.

Succinimidyl-3-propiolate (13). To a stirred solution of 0.70 g (95 %, 0.67 mL, 9.5 mmol) propargylic acid and 14 mL of CH_2Cl_2, 1.06 g (9.5 mmol) of *N*-hydroxysuccinimide and 1.82 g (9.5 mmol) of 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide (EDC) were added. The dark brown mixture was stirred at RT for 18h and subsequently washed with 20 mL of water, 40 mL of saturated $NaHCO_3$ and 20 mL of water, after which the organic layer was separated, dried over Na_2SO_4 and filtrated. The filtrate was then evaporated under reduced pressure to give a brown solid (128 mg, 0.766 mmol, 5.4 %): ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.82 (s, 1H), 2.77 (s, 4H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 171.2 (q), 162.5 (q), 95.5 (q), 95.6 (t), 26.5 (s); HRMS (ESI+) calcd for $C_7H_5NO_4$ 167.022, found 168.029 (MH^+) low intensity.

Propargylic acid (*N*-mono-6-deoxy- β-cyclodextrin)amide (15). 50.1 mg (0.044 mmol) of **6** was dissolved in 5 mLof H_2O. 29.4 mg (0.176 mmol) of **13** was dissolved in 400 µL of DMF and the solution was added to the brownish solution of **6**. The mixture was stirred for 18h at RT and subsequently evaporated to give a brownish solid. The solid was dissolved in 1 mL of Milli Q water for purification by size exclusion chromatography with a NAP-10 column. After lyophilisation,

crude **10** was obtained as a brownish solid (10 mg, 8.4 µmol, 19 %): ^{1}H-NMR (200 MHz, D_2O) δ 5.15 (app s), 5.00-4.82 (m), 4.82-4.40 (m, overlaps with D_2O), 3.85-3.25 (m), 2.85-2.52 (m); positive ion MALDI-TOF MS (Matrix A) *m/z* 1211.25 for $[M+Na]^+$, calcd ($C_{45}H_{71}NO_{35}$) 1208.37.

Synthesis of bipyridine-DNA conjugate, representative procedure. 250 µL of a stock solution of amino-modified oligonucleotide (200 µM in H_2O) was mixed with 200 µL of phosphate buffer (200 mM pH 7.2) and 50 µL of DMF. To this solution, 125 µL of a stock solution of *N*-hydroxysuccinimide 2,2'-bipyridine-5-carboxylate (20 mg/mL in DMF) was added in fractions of 25 µL over a period of 4 hours with continuous shaking. The mixture was shaken overnight and the coupled product was purified by size exclusion chromatography (NAP-10 column, Triethylammonium acetate 50 mM pH 7). The product was analysed by rp-HPLC (one peak, t = 39.4 min) and MALDI-TOF MS: negative ion (Matrix B) *m/z* 5249.58 for $[M-H]^-$, calcd 5251.34.

Synthesis of maleimide-DNA conjugate, representative procedure. 183 µL of a stock solution of amino-modified oligonucleotide (200 µM in H_2O) was mixed with 236 µL of phosphate buffer (200 mM pH 7.2). To this solution, 59 µL of **14** (30 mg/mL in DMF) was added and the solution was let stand over a period of 2.5h. The coupled product was purified by size exclusion chromatography (NAP-10 column, Triethylammonium acetate 50 mM pH 7) and subsequently analysed by rp-HPLC (one peak, t_R = 34.6 min).

Synthesis of β-cyclodextrin-DNA conjugate, representative procedure via amine functionalized β-CyD 6. 230 µL of a solution of maleimide-modified oligonucleotide (120 µM in CHES buffer, 100 mM, pH 9.2) was prepared. To this solution, 240 µL of a stock solution of compound **6** (30 mg/mL in CHES buffer) was added in fractions of 120 µL over a period of 1h. The coupled product was purified by size exclusion chromatography (NAP-10 column, Triethylammonium acetate 50 mM pH 7) and subsequently analysed by preparative rp-HPLC (two peaks: t_R = 27.5 min, 22.8 %; t_R = 28.6, 77.2 %). The isolated fractions were further analysed by MALDI-TOF MS: (for t_R = 27.5 min) negative ion (Matrix B) *m/z* 6406.00 for $[M-H]^-$, calcd 6410.62.

Synthesis of azide-DNA conjugate, representative procedure. 183 µL of a stock solution of amino-modified oligonucleotide (200 µM in H_2O) was mixed with 236 µL of phosphate buffer (200 mM pH 7.2). To this solution, 118 µL of a stock solution of compound **14** (30 mg/mL in DMF) was added in fractions of 59 µL over a period of 3 hours with continuous shaking. The mixture was then purified by size exclusion chromatography (NAP-10 column, Triethylammonium acetate 50 mM pH 7). The product was analysed by rp-HPLC (one peak, t_R = 43.7 min).

Synthesis of β-cyclodextrin-DNA conjugate, representative procedure via alkyne functionalized β-CyD (15). All solutions were prepared under oxygen- and moisture-free conditions. 100 µL of a solution of azide-modified oligonucleotide (200 µM in phosphate buffer, pH 7.2) was mixed with 86 µL of a solution of compound **15** (30 mg/mL in phosphate buffer, pH 7.2). 40 µL of a 1.5 M solution of $CuSO_4 \cdot 5H_2O$ was prepared as well as a separate 50 µL 1.6 M solution of tris(2-carboxyethyl)phosphine (TCEP). From both stock solutions, 27 µL and 38 µL respectively were mixed together to give a green solution. After five minutes, the full 65 µL solution was added to the solution containing azide-modified oligonucleotide and compound **15**. This total mixture was then placed in the oven at 37 °C and mixed overnight. The product was purified by size exclusion chromatography (NAP-10 column, Triethylammonium acetate 50 mM pH 7) and subsequently lyophilised and kept in the freezer at -20 °C.

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