Chapter 4

Photoswitching of DNA Hybridization using a Molecular Motor

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4.1 Introduction

DNA carries the genetic information of all known organisms. In the more than 60 years since Watson, Crick and Franklin unraveled the double helix, immense advances have been made in our understanding of DNA structure and function. Moreover, the programmable nature of DNA has led to its use in nanotechnology, genetic engineering, information storage, and a range of other applications. In the ongoing search to understand and control the key processes of life, the ability to modulate DNA structure and function is highly desired. Various triggers, such as pH change, small molecules, short primers, biological signals, heat, metal ions and light, have been applied to achieve this goal. The use of light has distinct advantages over the other triggers. Light is non-invasive to living tissue, and a high level of spatial and temporal control over its application is possible. Therefore, light-responsive molecular switches (photoswitches) are considered particularly attractive for reversible control over poly- and oligonucleotide structure and function.

In photoregulation of oligonucleotides, extensive use has been made from hairpin structures which comprise short loops of self-complementary DNA or RNA. They can form naturally and are frequently found in RNA secondary structure, where, among a variety of functions, they guide folding, protect mRNA from degradation and act as recognition sites or substrates for enzymatic reactions. Hairpins are short oligonucleotides and are therefore relatively easy to synthesize while their self-hybridization is a small-scale model for double stranded DNA hybridization. Typically for preparing photoresponsive hairpins, the bridging nucleotides of the loop are replaced by a molecular photoswitch. The photoswitch is usually incorporated into the phosphate backbone of the oligonucleotide. In one state, the switch stabilizes the double stranded helix structure. Irradiation causes a conformational change in the structure of the switch, which leads to destabilization of the helix and a lower melting temperature ($T_m$). Ideally, in a certain temperature range, the oligonucleotide can be fully switched between double and single stranded structures. As a result, in that specific temperature range, the structure can exist as a ‘closed’ double stranded form, or as an ‘open’ single stranded form, which may engage in interactions with other biomolecules.

Backbone incorporation of photoswitches was pioneered by Letsinger and Wu, using stilbenes as photoactive bridging units at one terminus of the double-stranded nucleic acid. Subsequently, this method was expanded to the use of azobenzenes by Yamana and co-workers. Both trans-stilbene and trans-azobenzene stabilize the hairpins through π-π interactions with neighboring nucleobases as shown by...
enhanced $T_m$. Upon switching to nonplanar cis isomers, the extra stabilization is lost and the duplex structure of the stem is partially disrupted, leading to a lower $T_m$. This effect was enhanced by Sugimoto and co-workers, by precise engineering of the azobenzene backbone linker length.\(^{22}\) In their design, the cis isomer of the photoswitchable backbone linker is too short to function as a bridgehead for the hairpin. Therefore, the hairpin is distorted upon trans to cis isomerization, leading to additional destabilization and lowering of the $T_m$. The difference in $T_m$ ($\Delta T_m$) between the two isomers was found to be 20 °C for a 5 base pair (bp) hairpin. The $\Delta T_m$ is highly dependent on hairpin length, and drops to 17.3 °C when the base pair adjacent to the bridgehead is changed from C-G to A-T and to 13.9 °C for a 6 bp hairpin.\(^{23}\) Regardless, by the use of an ingenious linker design, Sugimoto and co-workers were able to achieve an unusually high $\Delta T_m$ by the incorporation of only a single molecular photoswitch.\(^{22}\)

At the same time, overcrowded alkene-based rotary molecular motors offer novel opportunities in the field of photoregulation of biologically active molecules due to their unique dynamic properties. The first of this type of responsive molecules was reported in 1999 and was of particular interest because it exhibited repetitive, photochemically driven unidirectional rotation around a carbon-carbon double bond.\(^{24}\) In recent years, however, molecular motors have found a vast range of applications as multistate switches.\(^{25}\) The rotary cycle of an overcrowded alkene-based molecular motor consists of four steps and therefore features four different isomers. The large geometrical change upon cis-trans isomerization in rotary molecular motors, accompanied by the structural rigidity, are particularly suited to induce a significant structural change in a DNA hairpin upon irradiation. Moreover, the four-state switching cycle and the change in helicity of the motor in each rotary step offer potential for new functionalities and a high degree of photoregulation.

With this in mind, we set off to evaluate the possibility of using a molecular motor to reversibly control the hybridization of a DNA hairpin. Both stable isomers of the motor were synthesized and subsequently incorporated into DNA hairpins using a DNA synthesizer separately. $T_m$ of both isomers modified DNA was determined using SYBR Green I in a fluorescence assay. Unlike previously reported systems, it was found that both motor isomers could greatly stabilize DNA hairpins, as demonstrated by the enhanced $T_m$. The trans-motor showed better stabilization of the DNA hairpin, however, Density Functional Theory (DFT) simulation showed otherwise. Gel electrophoresis revealed that both isomers adopted a hairpin structure. Besides, we demonstrated the photoisomerization of the motor in the DNA scaffold was not hindered even under hybridization condition.
4.2 Result and Discussion

Before starting the synthesis of the target motor-hairpin hybrid, computational calculation was performed to ensure that the design would be optimal for our envisioned application. By analogy to the azobenzene reported by Sugimoto and co-workers,\textsuperscript{22} we designed a double-primary-alcohol-functionalized motor, which could be incorporated in the DNA strand through standard solid phase DNA synthesis. Ideally, the cis isomer should have an O-O distance of 13.3 Å, which is the optimal bridgehead length. Rigid side chains are necessary to enforce sufficient distortion of the hairpin upon photochemical switching. We chose to use first generation motor which is symmetrical, has limited conformational flexibility and therefore maximizes geometrical change, as shown in Figure 4.1A. The xylene-based core structure of these designs has excellent photochemical properties and can be readily synthesized.\textsuperscript{26}

![Figure 4.1. (A) Structure of proposed motor linker. The molecule has conformational freedom around the bonds indicated in red\textsuperscript{22} and the structure is designed to bring the primary alcohol moieties closer together upon trans to cis isomerization. (B) PES scan of the O-O distance in the proposed motor, plotted against the Self Consistent Field (SCF) energy.](image)

Density Functional Theory (DFT) was employed to investigate the design computationally. From the calculation, the potential energy surface (PES) scan of the O-O distance was used to estimate the effectiveness of the linker. Figure 4.1B shows the PES scan for the O-O distance for the proposed motor. This structure has limited conformational freedom, which is reflected in a much steeper PES. Cis (blue) has a global minimum at \( \sim 15 \) Å. However, the extra energy required to reorganize to an O-O distance of 13.3 Å (green) is only 1.7 kJ/mol. The trans isomer (red) has a global minimum at 17.9 Å. Reorganization to 13.3 Å would require an energy input of 22.9 kJ/mol. Therefore, to accommodate trans as a bridgehead in a hairpin, partial disruption of the B-form helical structure by breaking hydrogen bonding between one or more base pairs seems much more likely than distortion of the motor. Based on the computation, we envision that switching from cis to trans in a DNA hairpin
containing motor as a bridgehead unit will lead to a significant destabilization of the hairpin.

**Figure 4.2.** (A) Synthesis of trans-motor phosphoramidite building block and trans-motor modified DNA hairpin (the synthesis of cis-motor modified DNA followed the same route); (B) MALDI-TOF mass spectra of 8T-cis-8A (left) and 8T-trans-8A (right) (Calculated mass for both: 5361g/mol).

The motor was synthesized and provided by our collaborator, starting from previously reported dibromo-functionalized motor. The as synthesized motor was firstly converted into the corresponding phosphoramidite building block (Figure 4.2A) and incorporated into a self-complementary DNA sequence, 5'-TTTTTTTTT-motor-AAAAAAA-3' (8T-motor-8A), on an ÄKTA Oligopilot Plus DNA synthesizer using standard β-cyanoethylphosphoramidite coupling chemistry on polystyrene solid support. Both trans and cis isomers modified DNA were synthesized. The products were confirmed by MALDI-TOF mass spectrometry (Figure 4.2B).
Gel electrophoresis confirmed that both isomers formed hairpins (Figure 4.3). The positions of 8A8T (HY) (lane 5) is slightly lower than the 10bp band from the ladder, which means that this self-complementary DNA forms an intramolecular hairpin structure in buffer instead of hybridization between 2 strands. Meanwhile, for both cis and trans motor modified DNAs (lane 6 and lane 7), the electrophoretic mobility of the major bands is lower than the unmodified 8A8T DNA (lane 5) while much higher than the 15bp band from the ladder, which means the majority of both cis and trans motor modified DNAs form an intramolecular hairpin structure as well. Their slightly decreased mobility compared to 8A8T can be attributed to the introduction of the motor. When DNA is modified with hydrophobic polymer units, mobility is also reduced compared to pristine DNA. The minor bands for both motor DNAs could be attributed to intermolecular DNA duplexes.

The melting temperature of each hairpin was determined using a SYBR Green I fluorescence assay (Figure 4.4). \( T_m \) for 8T-cis-8A was determined to be 59 °C, and...
for 8T-\textit{trans}-8A to be 65 °C. The $\Delta T_m$ is therefore 6 °C, which is comparable to the achievement of Sugimoto and co-workers (20 °C/17.8 °C for 5 bp (depending on base pair adjacent to bridgehead), 13.9 °C for 6 bp).\textsuperscript{22, 23} Comparison with an 8 bp DNA hairpin containing an azobenzene or stilbene linker is not possible. Only three such hybrids were previously reported, and a $\Delta T_m$ was not reported for any of them.\textsuperscript{29-31} Notably, the $T_m$ of the native hairpin 8T8A was determined to be 51.5 °C. The observation that the $T_m$ of the native hairpin is lower than the $T_m$ of the hybrids can be partly attributed to the fact that the loop in this hairpin consists of a few bases, which are therefore not engaging in base pairing. Typically, a four nucleotide loop is found to be most stable.\textsuperscript{32} The loss of two base pair interactions is expected to decrease the $T_m$ a few degrees, while the $T_m$ of 8T-\textit{cis}-8A and 8T-\textit{trans}-8A are, respectively, 7.5 °C and 13.5 °C higher than the $T_m$ of 8T8A. It seems therefore that for both isomers, the motor has a significant stabilizing effect on the hairpin. A similar stabilizing effect is observed for \textit{trans} azobenzenes and stilbenes, where it has been attributed to $\pi$ stacking interactions.\textsuperscript{12, 33}

\begin{figure}
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\caption{(A) Changes of the absorption spectrum of 8T-\textit{trans}-8A upon irradiation with 312 nm light for 10 min. Insert shows the region 290-450 nm. (B) 8T-\textit{trans}-8A (black line), the sample after irradiation with 312 nm light for 10 min (red line) and the sample after incubation at 67 °C for 6 h (blue line). All spectra recorded in Milli-Q, 67 °C, at ambient atmosphere. (C) First derivative curves of melting temperatures analysis of different DNA samples. (HY) = DNA was hybridized prior to recording melting curves.}
\end{figure}

For any application under biologically relevant conditions, and in this case to achieve photocontrol over DNA secondary structure, it is very important that the switching ability of the motor in the hybrid is retained. To investigate the action of the motor without interaction between the two substituent DNA strands we started our experiments under non-hybridizing conditions: in Milli-Q water and at 67 °C, above the $T_m$ of either isomer. We subjected a 2.65 μM solution of 8T-\textit{trans}-8A in Milli-Q to the standard UV-vis experiment used to follow the isomerization processes of a molecular motor (Figure 4.5). In the initial absorption spectrum (Figure 4.5A, B, black line) both components of the hybrid can be clearly
distinguished. The major absorption band can be attributed to DNA ($\lambda_{\text{max}} = 262$ nm), while above ~300 nm, only the motor units contribute to absorption. The band with two maxima at $\lambda_{\text{max}} = 330$ and 345 nm is characteristic for the stable trans conformation of xylene-based first generation motors$^{26,34,35}$ and is also observed in the UV-vis spectrum of the motor. Because the DNA does not absorb above 300 nm, the motor unit can be irradiated without affecting the DNA part of the hybrid. Irradiation with 312 nm at 67 °C leads to the appearance of a new absorption band at a higher wavelength ($\lambda_{\text{max}} = 385$ nm), which typically results from the formation of a higher energy motor isomer ($8T$-unstable-\textit{cis}-8A, Figure 4.5A). The clear isosbestic point indicates the absence of photodamage or side reactions. After 10 min, a photostationary state was reached and the irradiation was halted (Figure 4.5B, red line). Subsequently, the sample was left at 67 °C for several hours to induce thermal helix inversion. As expected, the new band disappeared and an absorption at a lower wavelength ($\lambda_{\text{max}} = 347$ nm, Figure 4.5B, blue line) appeared, most likely corresponding to $8T$-\textit{cis}-8A. MALDI-TOF analysis showed that the hybrid does not undergo degradation (Figure 4.8). Although the UV-vis spectra alone clearly indicate a photoisomerization followed by thermal helix inversion (THI), the sample used in this experiment was subjected to a melting temperature analysis by a fluorescence assay. We hypothesized that a mixture of the two hairpins ($8T$-\textit{trans}-8A and $8T$-\textit{cis}-8A) should lead to two maxima in the differentiated curve of the fluorescence spectrum, corresponding to the two different $T_m$. In fact, the main maximum in this curve was found at 59 °C, which corresponds to the $T_m$ of $8T$-\textit{cis}-8A (Figure 4.5C). This result, in combination with the UV-vis spectra results, leads us to conclude that an efficient photoisomerization and subsequent THI have taken place. To determine the kinetics of the THI, the absorption of the sample was measured at regular intervals (Figure 4.9). The half-life of the unstable \textit{cis} isomer of $8T$-motor-8A is determined to be ~51 min at 67 °C, about 2.5 x slower than for the motor 3 itself (\textit{vide supra}, 19.5 min at 67 °C). Therefore, it appears that motor rotation is slightly slowed down but otherwise unhindered when integrated in the backbone of a biomolecule and operated in aqueous non-hybridizing conditions. Because these experiments are performed in bulk solution, irradiation had to be performed for 10 min and we were unable to look at the structural dynamics of the hairpin. However, it may be possible that reconfiguration of the hairpin occurs on a slower timescale than photoswitching of the motor, similar to responses observed in peptides.$^{36-38}$ Future investigations may include ultrafast IR studies to elucidate the dynamics of hairpin reconfiguration upon photoswitching.
Photoswitching of DNA Hybridization using a Molecular Motor

Figure 4.6. UV-vis spectra of analysis of the photochemical isomerization of stable 8T-trans-8A. (A) Changes of the absorption spectrum of 8T-trans-8A upon irradiation with 312 nm light. (B) 8T-trans-8A (black line), the sample after irradiation with 312 nm light for 14 min (red line), the sample after incubation at 37 °C for 3 h (blue line) and the sample after incubation at 70 °C for 5 h (turquoise line). All spectra were recorded in Tris buffer, pH = 8.0, at ambient atmosphere.

The experiment was also performed under hybridizing conditions at physiological temperature (37 °C, 20 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl2, pH 8.0). 8T-trans-8A was again readily photoisomerized without the occurrence of side reactions. However, after several hours at 37 °C, only a slight decrease of the absorption band corresponding to the unstable cis isomer was observed (Figure 4.6). Potentially, helix inversion is hindered by the hybridized DNA strands. When the sample was heated to 70 °C (above the Tm), THI occurred in a similar manner in aqueous buffer as in water. For full spectra and MALDI-TOF analysis of the irradiated sample, see Figure 4.8. Melting temperature analysis revealed a Tm of 59 °C, indicating efficient conversion to the stable cis isomer. Combining the results of the UV-vis and melting temperature experiments, Figure 4.7 gives a schematic overview of the operation of 8T-motor-8A. Stable 8T-trans-8A (left) forms a hairpin structure with a Tm of 65 °C. Upon irradiation with 312 nm light, photoisomerization to unstable 8T-cis-8A (middle) occurs with high conversion. Upon heating, THI can be induced and stable 8T-cis-8A (right) is formed. The Tm of this isomer is 59 °C, indicating a destabilization of the hairpin structure.
Chapter 4

Figure. 4.7. Schematic overview of the functioning of 8T-motor-8A. Trans-cis isomerization leads to a destabilization of the helix, as is apparent from the decrease in $T_m$. For clarity, the destabilization is represented by partial helix unwinding in this figure.

4.3 Conclusion

Aided by computational studies, we have designed a first generation molecular motor-based linker that can function as a photoswitchable bridgehead for an 8 base pair DNA hairpin. Both cis and trans isomers of a bifunctional linker were prepared and, they were incorporated into a 16-mer strand of self-complimentary DNA via solid phase synthesis. Hairpin formation was confirmed, and the DNA-motor hybrid was shown to be able to undergo both photoisomerization and thermal helix inversion processes. The $T_m$ of 8T-trans-8A was determined to be 65 °C, and the $T_m$ of 8T-cis-8A was 59 °C. An unexpected observation was the destabilization due to trans-cis isomerization, since DFT calculations suggested the opposite. The results and structural insights of this study are very important for the design of even more potent molecular motor-backbone linkers. The measured $\Delta T_m$ of 6 °C (for an 8 bp hairpin) represents a very promising value which ranks this investigation among the most successful attempts to influence DNA hybridization through the incorporation of a photoswitchable backbone linker. Moreover, the isomerization process was highly efficient, and the bistable switching mode provides a real advance over azobenzenes, for which the thermal cis-trans reisomerisation limits possible applications. This study marks the first time that a molecular motor has been used to control the secondary structure of DNA, and in fact one of the first examples of a molecular motor being applied under physiological conditions, demonstrating the ability to regulate a key biological process such as DNA hybridization.
Finally, it must be noted that molecular motors do not just rival conventional photoswitches in efficiency and power. They also offer a much higher degree of control and precision due to their four state switching cycle and helicity inversion. This investigation has only begun to uncover the vast range of new possibilities that may be accessed in photoregulated biohybrid systems. It is apparent that the motor unit by itself is powerful enough to significantly influence hybridization behavior of short oligonucleotide hairpins. Moreover, our results showcase the potential of rotary molecular motors and consolidate their position among the most effective photoswitches for use in biological surroundings.

4.4 Experimental Section

4.4.1 Materials

Chemical were purchased from Sigma Aldrich, Acros or TCI Europe N.V.; solvents were reagent grade and distilled and dried before use according to standard procedures, if required. Melting points are taken on a Büchi B-545 melting point apparatus. UV-vis absorption spectra were measured on a Jasco V-630 spectrometer. Irradiation was performed using a Spectroline ENB-280C/FE lamp (312 nm). All solvents and reagents for oligonucleotide synthesis were purchased from Novabiochem (Merck, UK) and SAFC (Sigma-Aldrich, Netherlands). Solid supports (Primer Support™, 33 μmol/g) from Glen Research were used for the synthesis of motor-DNA. The motor-DNA were characterized by MALDI-TOF mass spectrometry using a 3-hydroxypicolinic acid matrix. Spectra were recorded on an ABI Voyager DE-PRO MALDI TOF (delayed extraction reflector) Biospectrometry Workstation mass spectrometer. All unmodified oligonucleotides were purchased from Biomers.net in HPLC purification grade.

4.4.2 Synthesis and characterization of the motor-DNA sequences

The motor was firstly converted into the corresponding phosphoramidite building block as depicted in Figure 4.2A. The obtained motor phosphoramidite was dissolved in CH₃CN to a final concentration of 0.0255 M, in the presence of 3 Å molecular sieves, and the prepared solution was directly connected to a DNA synthesizer (ÄKTA oligopilot plus, GE Healthcare (Uppsala, Sweden)). Oligonucleotides were synthesized on an 8 μmol scale using standard β-cyanoethylphosphoramidite coupling chemistry. Deprotection and cleavage from the PS support were carried out by incubation in concentrated aqueous ammonium hydroxide solution for 5 h at 55 °C. Following deprotection, the oligonucleotides
were purified by reverse-phase chromatography (RPC), using a C15 RESOURCE RPC™ 3 mL reverse phase column (GE Healthcare) through a custom gradient elution (A: 100 mM triethylammonium acetate (TEAAc) and 2.5% acetonitrile, B: 100 mM TEAAc and 65% acetonitrile). After RPC purification, collected peaks were subjected to anion-exchange chromatography (AIEX), using a Resource™ Q 1 mL column (GE Healthcare) through a custom gradient elution (A: 10mM Tris-HCl, pH 8.0; B: 10mM Tris-HCl, 1M NaCl, pH 8.0). Fractions were desalted using centrifugal dialysis membranes (MWCO 3000, Sartorius Stedim). Oligonucleotide concentrations were determined by UV absorbance using extinction coefficients. Finally, the identity and purity of the oligonucleotide hybrids were confirmed by MALDI-TOF mass spectrometry.

![MALDI-TOF mass spectra](image)

**Figure. 4.8.** MALDI-TOF mass spectra of 8T-motor-8A after irradiation with 312 nm and subsequent THI. (A) Trans, water, 67 °C, (B) Trans, buffer, irradiation at 37 °C, THI at 70 °C. (C) Cis, buffer, irradiation at 37 °C, THI at 70 °C.

Native polyacrylamide gel electrophoresis (PAGE) was performed using a 20% gel (Invitrogen life technologies), and run at 100 V for 100 min in TBE buffer (90 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). After electrophoresis, the gel was stained with SYBR Gold (Life Technologies Europe BV) and photographs of the gels were taken with a LAS-3000 Image Reader (Fuji Photo Film S3 (Europe) GmbH, Dusseldorf, Germany).

The thermal helix inversion (unstable cis to stable cis) of the DNA-motor hybrid was followed over time using UV-vis spectroscopy. Figure 4.9 depicts the absorbance of the sample at 330 nm and 390 nm, measured at regular intervals. Exponential decay curves were fitted and the mean lifetime was calculated to be 4572 s (based on 330 nm) and 4336 s (based on 390 nm). On average this measurement leads to a half-life of the unstable cis isomer of 51 min.
Photoswitching of DNA Hybridization using a Molecular Motor

**Figure 4.9.** Change in absorbance over time of 8T-trans-8A. The sample (8T-trans-3-8A, 2.65 μL, Milli-Q) was first irradiated with 312 nm for 10 min at 67 °C. Subsequently the sample was left to undergo THI. Depicted here is the absorbance at 330 nm (black squares) and 390 nm (red circles) and their respective fitted exponential decay curves. A small time window is taken out due to artifacts.

**Author Contribution**

In this chapter, Anouk S. Lubbe from the group of B. L. Feringa performed the synthesis and investigation of the photo-switching behavior of molecular motor. Moreover, she carried out the optical characterization of the motor-DNA hybrid. Qing Liu synthesized, purified and characterized the motor-DNA conjugate. Moreover, he performed the melting point analysis of the motor-DNA hybrid.
Reference


