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Lubbe, Anouk S.; Szymanski, Wiktor; Feringa, Ben L.

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REVIEW ARTICLE

Wiktor Szymanski, Ben L. Feringa *et al.*
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Recent developments in reversible photoregulation of oligonucleotide structure and function

Anouk S. Lubbe,^a Wiktor Szymanski^{*ab} and Ben L. Feringa^{*a}

There is a growing interest in the photoregulation of biological functions, due to the high level of spatiotemporal precision achievable with light. Additionally, light is non-invasive and waste-free. In particular, the photoregulation of oligonucleotide structure and function is a rapidly developing study field with relevance to biological, physical and material sciences. Molecular photoswitches have been incorporated in oligonucleotides for 20 years, and the field has currently grown beyond fundamental studies on photochemistry of the switches and DNA duplex stability, and is moving towards applications in chemical biology, nanotechnology and material science. Moreover, the currently emerging field of photopharmacology indicates the relevance of photocontrol in future medicine. In recent years, a large number of publications has appeared on photoregulation of DNA and RNA structure and function. New strategies are evaluated and novel, exciting applications are shown. In this comprehensive review, the key strategies for photoswitch inclusion in oligonucleotides are presented and illustrated with recent examples. Additionally the applications that have emerged in recent years are discussed, including gene regulation, drug delivery and materials design. Finally, we identify the challenges that the field currently faces and look forward to future applications.

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^a Centre for Systems Chemistry, Stratingh Institute for Chemistry, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands. E-mail: b.l.feringa@rug.nl

^b Department of Radiology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands. E-mail: w.szymanski@umcg.nl

1 Introduction

1.1 General introduction

Poly- and oligonucleotides carry the genetic information of all known living beings and are involved in the key processes of life. Ever since the elucidation of the double stranded helical

Anouk S. Lubbe

Anouk S. Lubbe studied chemistry at the University of Groningen, where she received her MSc in 2012 after working with Prof. Ben L. Feringa on molecular motors. In 2012, she worked with Prof. Jean-Marie Lehn at the University of Strasbourg on dynamic covalent metallocsupramolecular grids. She is currently pursuing her PhD under the supervision of Prof. Ben L. Feringa and Dr Wiktor Szymanski. Her research focuses on the application of molecular motors in biological settings and the study of molecular motors in new media.

Wiktor Szymanski

Wiktor Szymanski received his PhD degree from The Warsaw University of Technology in 2008, under the supervision of Prof. Ryszard Ostaszewski. He spent two years working on the use of biotransformations in organic chemistry with Prof. Ben L. Feringa and Prof. Dick B. Janssen at the University of Groningen. Since 2010 he has been working on the construction of photoactive protein-, peptide- and DNA-bioconjugates and photopharmacology in the Feringa Labs. In 2014, he joined the Department of Radiology, University Medical Center Groningen, where he was appointed in 2015 as tenure track assistant professor, working on molecular medical imaging.

Scheme 1 Overview of the most commonly used photoswitches discussed in this review and their switching characteristics.

structure of DNA by Watson and Crick, based on the X-ray data provided by Franklin in 1953,¹ scientists have aspired to control the properties of oligonucleotides in order to manipulate biological processes at the molecular level. Many disorders affecting living organisms have an underlying genetic cause; mutations in DNA are closely related to cancer, and even ageing is thought to be associated to naturally occurring DNA damage.² The ability to code and produce DNA has already lead to fascinating applications in nanotechnology and materials design, since by use of the unique recognition pattern almost any structure can be formed by self-assembly.^{3,4} This recognition pattern is also the basis for the coding ability of oligonucleotides, which can be used for information storage.⁵

Reversible control of oligonucleotide structure and function would allow for *in situ* manipulation and study of biological processes in the living cell. Light is an ideal tool for non-invasive manipulation, because it is orthogonal to most of the

processes in the cell. The use of light offers a high level of spatial and temporal control and is completely waste-free. Although UV light-induced DNA damage is a leading cause of skin cancer,⁶ it should be noted that the use of wavelengths up to 320 nm is easily avoided. Additionally, the intensity and wavelength of the light can be precisely controlled. These advantages are chiefly responsible for the recent appearance and exponential growth of both optogenetics and photopharmacology.^{7,8} Installing a molecular photoswitch is one of the most widely used methods to introduce light sensitivity in biological compounds, including oligonucleotides, proteins and ligands.^{9–11}

A comprehensive overview of the introduction of molecular photoswitches in nucleic acids up to mid-2011 can be found in the eponymous chapter of a review published previously by our group.⁹ Since then, the field has grown significantly and a large number of publications has appeared in the past five years. This review provides a comprehensive and critical overview of the principles and recent developments in the application of molecular photoswitches for reversible control over oligonucleotide structure and function. For more information regarding alternative reversible triggers for oligonucleotides or irreversible light-regulated triggers, the reader is referred to other recent reviews.^{12,13}

1.2 Overview of photoswitches used for oligonucleotide regulation

A molecular photoswitch is a chromophore that can interconvert between two or more forms.¹⁴ The isomerization is triggered by light, and in some cases one of the isomerization processes may also be induced by thermal energy. Scheme 1 shows the most common photoswitches that have been used for photoregulation of oligonucleotides. Azobenzenes and stilbenes undergo *cis-trans* isomerization, while diarylethenes and spiropyrans interconvert between open and closed forms. Relevant differences between these switches with respect to oligonucleotide incorporation include wavelengths used for switching, thermal stability, geometry, solubility, switching efficiency and change in dipole moment upon switching. Therefore, different switches may suit

Ben L. Feringa

Ben L. Feringa obtained his PhD-degree in 1978 at the University of Groningen in the Netherlands under the guidance of Prof. Hans Wynberg. After working as a research scientist at Shell he was appointed full professor at the University of Groningen in 1988 and named the distinguished Jacobus H. van't Hoff Professor of Molecular Sciences in 2004. He was elected foreign honorary member of the American Academy of Arts and Sciences and member

of the Royal Netherlands Academy of Sciences. His research interests include stereochemistry, organic synthesis, asymmetric catalysis, molecular switches and motors, photopharmacology, self-assembly and nanosystems.

different applications. A short summary of the four classes of switches is depicted in Scheme 1. Other switches, such as hemithioindigos¹⁵ and thiophenefulgides,^{16,17} have been applied for peptide modifications⁹ and may be promising candidates for oligonucleotide regulation, but are not included here as they have not been used in any of the studies discussed in this review.

1.2.1 Azobenzenes. Azobenzenes^{10,18} are by far the most extensively used photoswitches for oligonucleotide modifications and biological applications in general. This is likely due to their easy synthesis, high quantum yields, high efficiency and fast switching. *trans*-Azobenzene is a planar molecule¹⁹ with zero dipole moment. Irradiation with near-UV light causes isomerization to the *cis* isomer, which is nonplanar and has a dipole moment of ~ 3 D. The *cis/trans* ratio in the photo-stationary state (PSS) is approximately 80/20 for unmodified azobenzene.²⁰ Back-isomerization can be achieved with visible light (>460 nm) to give a *cis/trans* ratio of approximately 20/80.²⁰ Alternatively, the *trans* isomer can be fully regenerated *via* thermal isomerization. The half-life of unmodified *cis*-azobenzene is 2 d.¹⁸ These properties can be influenced by substitution of the azobenzene core structure and the choice of solvent.²¹ For example, red-shifting of the absorption maxima could be achieved by several methods, among others installation of *ortho*-methoxy or *ortho*-fluoro substituents or an ethylene bridge, after which both isomerization processes could be induced by irradiation with visible light.^{22–24} These developments are important for future *in vivo* applications, since UV light is unable to penetrate tissue as well as being harmful to living systems. Electron-poor azobenzenes may suffer from reduction under physiological conditions.²⁵ However, photo-switching *in vivo* yielded excellent results even in the presence of glutathione.²⁶ Recently, red-light switching of azobenzenes was also achieved *in vivo*.²⁷

1.2.2 Stilbenes. Stilbenes²⁸ are closely structurally related to azobenzenes and exhibit similar photochemistry.²⁸ However, the *cis* isomer of stilbene shows enhanced thermal stability compared to *cis* azobenzene and thermal *cis/trans* isomerization at room or body temperature is negligible. While the geometric change upon photoisomerization is similar to that of azobenzenes, the change in dipole moment is much smaller. The major disadvantage of using stilbenes is their tendency to undergo irreversible cyclization/oxidation of the *cis* isomer.²⁹ Stilbenes have been used for oligonucleotide photoregulation in the past,⁹ but are mentioned here mainly in older examples as only one application has been developed in the last five years (see Section 7).

1.2.3 Diarylethenes. Diarylethenes^{30–32} (depicted in Scheme 1 as containing thiophene moieties and a cyclopentene ring) are a special class of stilbene-type structures in which the *ortho* hydrogens are substituted to suppress irreversible oxidation after photocyclization of the *cis* isomer. Typically, the incorporated aryl rings are replaced by heterocycles to elongate the lifetime of the closed form, and the ethene moiety is often embedded in a small ring to prohibit *cis/trans* isomerization.³³ Diarylethenes are highly fatigue resistant and switching cycles can typically be repeated hundreds of times.³⁰ The change in

dipole moment upon switching between the open and closed forms is small. However, substituents can be used to move absorption wavelengths into the visible light region *via* extension of the conjugated system³⁴ or alternatively through attachment of a triplet sensitizer.³⁵

1.2.4 Spiroprans. Spiroprans^{36–38} contain a quarternary carbon atom and are therefore nonplanar. Irradiation with 360–370 nm light leads to heterolytic cleavage of the C_{spiro}–O bond, thereby forming the zwitterionic, planar merocyanine form. This ring opening causes a large change in dipole moment ($\Delta\mu = 7\text{--}15$ D).^{39,40} The closed form can be regenerated by thermal energy or upon visible light irradiation. The equilibrium in the PSS can be tuned both by the nature of the substituents or by the solvents used.^{41,42}

1.3 Overview of the photoswitch incorporation strategies

Fig. 1 gives a schematic overview of the six different approaches to modifying oligonucleotides with molecular photoswitches discussed in this review. Fig. 1a shows oligonucleotide photo-regulation *via* noncovalent interactions. In this method, a photoswitch is usually functionalized with a cationic tail to improve binding *via* electrostatic interactions to the negatively

Fig. 1 Schematic illustration of the six different methods of photoswitch inclusion in oligonucleotides discussed in this review. Photoswitches are represented by green barrels. (a) Noncovalent interactions between switch and oligonucleotide, (b) photoswitch attached to a nucleoside *via* covalent bond, (c) inclusion of nucleoside where the nucleobase is part of a photoswitch, (d) photoswitch used as a backbone linker between two nucleosides, (e) nucleoside replaced by photoswitch on a linker, (f) end-capping of oligonucleotide with photoswitch. For a more detailed description of each methods, see the main text.

charged oligonucleotide backbone. The switch itself may increase binding to the nucleic acid by intercalating or placement in the major or minor groove. The supramolecular approach has up to 2011 mainly been used for photoreversible DNA/RNA condensation⁴³ and influencing duplex stability with intercalating switches.^{44,45}

Fig. 1b illustrates the concept of photoswitches attached to nucleotides. The structure of the oligonucleotide remains largely unaltered, save for the inclusion of one or more switches by connection through a bridging group. The connecting bridge can be used to influence positioning of the switch, for example, attachment of a substituent to the C5 position of a pyrimidine base usually places the substituent in the major groove.^{46–48} Up to 2011, this attachment of photoswitches to nucleic acids was achieved using amidation of the ribose moiety⁴⁹ and alkylation of the phosphate backbone.⁵⁰

Fig. 1c shows inclusion of photochromic nucleotides into oligonucleotides. Contrary to attachment of switches to nucleotides, in this approach the nucleotide itself is incorporated in a photoswitch, typically by exchanging an aromatic ring in the switch with the π -system of a nucleobase. This fusion forces the photoswitch to reside within the duplex in all conformations which may lead to very large effects upon isomerization. Before 2011, both stilbenes⁵¹ and diarylethenes⁵² have been fused with nucleobases and the formed photochromic nucleotide was included in oligonucleotides. However, this approach has so far been only moderately successful.

Fig. 1d illustrates the inclusion of molecular photoswitches in the phosphate backbone. Typically, a self-complementary sequence is used, causing hairpin formation. This method was pioneered using stilbene in the 1990's by Lewis⁵³ and Letsinger⁵⁴ and has subsequently also successfully been applied using azobenzenes.⁵⁵ The main goal of this approach has been to reversibly control hairpin formation. However, while the ΔT_m 's (see Section 1.4) obtained can be large, the hairpins are often unusually stable due to stacking of the switch. Therefore, unfolding of longer hairpins often requires high temperatures and applications of this strategy have been limited.

Fig. 1e shows the inclusion of photoswitches as nucleoside surrogates. This method is by far the most frequently applied and most successful of the six approaches discussed here. A photoswitch is tethered to a linker which is included in the oligonucleotide backbone. In contrast to photochromic nucleotides and photoswitches attached to nucleotides, there is no nucleobase present on the backbone linker. Depending on switch conformation and linker flexibility, the switch can be intercalating in the duplex or dangling out of it. This method is mainly applied to photoregulate duplex stability. The stabilization/destabilization effect can be quite large and has up to 2011 been applied to influence enzyme regulation,⁵⁶ RNA cleavage using a DNase,⁵⁷ and peptide nucleic acid activity.⁵⁸

Finally, Fig. 1f shows a nucleic acid modified with a photo-switchable end-cap. This method does not necessarily differ from the others at the synthetic level, since a photochromic nucleotide, a photoswitchable nucleoside surrogate or a photoswitch attached to a nucleobase may be used. However, all of

the above methods of switch inclusion typically force the switch to be in close proximity to the duplex, often leading to intercalation and/or groove binding. With end-capping, the switch may reside completely separate from the duplex, leading to very distinct applications.

1.4 Analytical methods

Most research into photoregulation of oligonucleotide structure and function aims at introducing a measure of control over duplex stability. If, at a given temperature, the photoswitch stabilizes the relatively inert double-stranded oligonucleotides in one form and favours single strands in the other form, switching will induce reversible unwinding of the helix. The single stranded oligonucleotide is then free to bind a different complementary strand, undergo replication or transcription, or bind any other target that will inhibit or set the desired process in motion. The melting temperature (T_m) of an oligonucleotide is defined as the temperature at which 50% of the duplexes are disassociated. For effective photocontrol over oligonucleotide duplex stability, the T_m 's of the two states of oligonucleotide/switch complex should be above and below the application temperature, which is typically either 25 °C or 37 °C. The ΔT_m is defined as the difference between the T_m 's of the two states that are observed. Because the helix unwinding proceeds over a certain temperature gradient, a large ΔT_m (>10 °C) ensures that there is a working temperature at which all oligonucleotides are double stranded in one state and single stranded in the other. The melting temperature can be measured by UV/Vis spectroscopy. Since the UV/Vis absorption of double stranded nucleic acids is markedly different from single strands, the change in the UV/Vis spectrum over a temperature gradient will indicate the melting temperature.⁵⁹ More recently, the T_m is often determined by measuring the temperature dependence of the fluorescence spectrum of an intercalating dye.⁶⁰ Additionally, FRET⁶¹ can provide a useful insight in the degree of unwinding by the attachment of a FRET pair to the two separate strands.

The binding mode of the switch to the oligonucleotide is an equally informative parameter. Especially for supramolecular approaches, the binding mode may be hard to predict. LD and CD spectroscopy can give a good indication of the binding mode. These methods are discussed in more detail in Section 6.2.

In addition to the methods described above, a range of techniques is used to study interactions between photoswitches and oligonucleotides. The most prevalent among these are UV/Vis spectroscopy, fluorescence spectroscopy (notably of GFP expression) and Atomic Force Microscopy (AFM). Molecular dynamics and other computational methods are also applied.

2 Molecular photoswitches as nucleoside surrogates

The inclusion of photoswitches as nucleoside surrogates is the most widely used approach towards photoregulation of oligonucleotides (Fig. 1e). In this method, a photoswitch is

connected to a diol linker. In the past, spiropyrans have been used without much success,^{62,63} but in recent years, this approach has been almost exclusively focussed on azobenzenes.

The diol linker is installed in the phosphate backbone of an oligonucleotide, typically *via* standard solid-phase synthesis. Because a whole (deoxy)ribose unit is replaced by the functionalized linker, there is space for the photoswitch to fit inside the helix. The general method is to use a switch that is planar in one configuration and nonplanar in the other. The planar isomer therefore stacks between neighbouring base pairs, interacting favourably with its surroundings by π - π interactions. By irradiation with light of the appropriate wavelength, the switch isomerizes to its nonplanar geometry. This isomer either directly destabilizes the duplex by steric interactions or flips out of the helix entirely, thus leading to the decrease in double strand stability and a lower T_m value. The structural difference between the two isomers can be seen in Fig. 4. The functionalized linkers may be introduced in both DNA and RNA, and in any secondary structure.

2.1 Design

The inclusion of azobenzenes as a nucleoside surrogates was pioneered by Komiyama, Asanuma and co-workers.⁶⁴ They introduced an azobenzene moiety to an 8-base DNA strand using a 2,2-bis(hydroxymethyl)propionic acid linker and showed that the azobenzene could still be switched using UV and visible light. The same group later refined their design and developed the widely used tAzo (Fig. 2), *i.e.* an azobenzene moiety attached to a threoninol linker, which is readily synthesized and allows for facile inclusion to nucleic acids.⁶⁵ Either enantiomer of tAzo may be used, although in DNA the stabilization effect of *trans*-azobenzene attached to a D-threoninol unit is larger than when it is attached to L-threoninol. This can be rationalized by considering that D-threoninol prefers a clockwise winding, as does the DNA double helix.⁶⁶ NMR analysis has shown that the skew angle of D-threoninol in a DNA helix is 35–36°, which is very close to the skew angle of naturally occurring DNA (36°), while for L-threoninol this value is 30–31°. ⁶⁷ Additionally, the destabilization effect of *cis*-azobenzene on L-threoninol is smaller than that of *cis*-azobenzene linked by D-threoninol. The destabilization effect of the *cis*-tAzo was observed by various research groups and

for several different azobenzene nucleoside surrogates,^{56,57,68} yet the exact nature of this conformational response has not been experimentally determined. In contrast to effect of tAzo on DNA, the RNA helical structure is destabilized by both *trans*- and *cis*-forms, although this effect is very strong for *cis* and only minor for the *trans* isomer.⁶⁹ While D-tAzo is mainly used, alternative linkers are still being developed. Fig. 2 provides an overview of the azobenzenes and backbone linkers discussed in this section.

2.2 Computational studies

In order to elucidate the structural changes in oligonucleotides upon tAzo isomerization in both DNA and RNA, molecular dynamics studies were applied.^{70,71} Biswas and Burghardt⁷¹ concluded that inclusion of L-tAzo into dsDNA (Fig. 3) leads to destabilization of the duplex, most likely due to the anti-clockwise winding of L-threoninol which disturbs the clockwise winding of B-DNA (*vide supra*). However, *trans* azobenzene may flip in and out of the duplex, providing some extra stabilization *via* intercalation when stacked at the inside. *cis*-Azobenzene is not able to stack between the base pairs once it has flipped out of the duplex, due to its nonplanar configuration. While these results provide useful insight into the structural changes in DNA upon L-tAzo isomerization, L-threoninol is rarely used as a linker due to its small effect upon the T_m compared to D-threoninol. Molecular dynamics studies on the isomerization of D-tAzo in DNA might therefore be of more interest and are eagerly awaited.

D-tAzo was used for molecular dynamics studies on RNA by Rastädter, Biswas and Burghardt,⁷⁰ who focused on a hairpin

Fig. 3 Molecular dynamics simulation of a DNA oligomer with *trans* L-tAzo (magenta) incorporated in the backbone.⁷¹ Reproduced with permission from ref. 71. Copyright 2014, Biophysical Society.

Fig. 2 Overview of azobenzenes and backbone linkers for inclusion of a photoswitch as a nucleoside surrogate.

Fig. 4 Influence of *D*-tAzo on a RNA hairpin.⁷⁰ Simulated structures of azobenzene–RNA complexes, displaying the structural changes induced by both *trans*- and *cis*-azobenzenes (major conformations only). *trans*-Azobenzene stacks between neighbouring base pairs, while *cis* is mainly dangling outside the hairpin. Reproduced with permission from ref. 70. Copyright 2014, American Chemical Society.

of A-form RNA, an abundant secondary structure (Fig. 4). The minor groove, in which tAzo partly resides, is much wider and shallower in the A-helix than in the B-helix,⁷² which leads to less favourable binding of the azobenzene moiety to the oligonucleotide. This is thought to be the reason that tAzo destabilizes RNA in both the *cis* and the *trans* form.⁶⁹ The molecular dynamics study confirms this hypothesis.⁷⁰ It was shown that the *cis* isomer in fact mainly dangles outside of the hairpin, while the *trans* isomer flips in and out. Surprisingly, when the azobenzene is in the *cis* configuration, the DNA helix is the closest to its native structure. However, π - π stacking interactions between the *trans* isomer and the neighbouring base pairs lead to higher hairpin stability, manifested by a higher T_m value.

Finally, the influence of linker flexibility was examined in detail using molecular dynamics simulations by Mondal, Burghardt and co-workers.⁷³ By comparing *L*-tAzo, *D*-tAzo, β -deoxyribose-azobenzene (β -dAzo) and α -deoxyribose-azobenzene (α -dAzo) (Fig. 2), they were able to establish a set of design principles for azobenzene modified RNA. It was found that minimizing steric hindrance by placing an abasic site or no nucleotide at all opposite the azobenzene provides extra stabilization. A rigid linker will keep the azobenzene inside the duplex, which is favourable both for stabilization in the *trans*-isomer and destabilization in the *cis*-isomer. Finally, minimal duplex distortion is found for linkers such as *D*-threoninol and β -deoxyribose, due to the fact that these linkers have the same orientation as the RNA bases. Based on these findings, a β -deoxyribose linker is recommended for both high photoisomerization efficiency and large ΔT_m .

So far, no quantitative results were obtained through molecular dynamics simulations. However, due the high level of agreement with experimental results, this method is a promising approach towards design of new oligonucleotide-switch hybrids and understanding of the structural changes that accompany isomerization processes.

2.3 Photochemistry of azobenzenes as nucleoside surrogates

In addition to simulations, spectroscopic methods have been used to shed light on the mechanism of photoisomerization in azobenzene-modified oligonucleotides. Yan, Ginger and co-workers showed that quantum yield of tAzo photoisomerization in oligonucleotides is dependent on local sequence.⁷⁴ In case of a mismatch close to the tAzo moiety, the quantum yield is increased compared to the complementary sequence. This effect is more pronounced when an abasic site is present next to the azobenzene, and is therefore attributed to a larger amount of free volume available for the isomerization. These results are to be taken into account in the design of switchable oligonucleotides, as azobenzene photoisomerization quantum yield is dramatically decreased in dsDNA compared to solution.

D-Threoninol is commonly the backbone linker of choice for the inclusion of azobenzene as a nucleoside surrogate due to practical reasons (*vide supra*) and, presumably, the large body of literature already available on this compound. However, considering *in vivo* applications, a serious disadvantage to incorporating an azobenzene using this linker is the low photoisomerization efficiency at lower temperatures. Under the T_m of the double stranded oligonucleotides, the *trans*-azobenzene is efficiently stacked between neighbouring base pairs. Therefore, the amount of free volume available for the isomerization process is far less than required and the efficiency of the isomerization can be significantly decreased. For example, Liu and Asanuma observed the formation of only 30% of *cis* isomer upon UV irradiation at 37 °C.⁷⁵ Larger amounts of *cis* isomer can only be obtained if irradiation is performed at a temperature above the T_m , which is highly impractical for biological applications. This disadvantage can be overcome by using an unusually high number of *D*-tAzo moieties.⁷⁶ In complementary strands, containing two *trans*-*D*-tAzo groups per base pair, a *cis*-*trans* isomerization of as little as 40% is still enough to completely disassociate the duplexes at 37 °C. However, due to the rather extreme structural modification, this material may no longer be suitable for biological applications.

Kou, Liang and co-workers hypothesized that a more flexible glycerol linker would allow the azobenzene to flip more easily in and out of the DNA helix, without the necessity of heating the oligonucleotide to a temperature above the T_m (gAzo, Fig. 2).⁷⁷ Indeed, at human body temperature a PSS of 80% *cis* isomer could be reached, and after irradiation with blue light, 96% of the azobenzene moieties was found to be in the *trans* configuration.

Complementing the findings of Mondal, Burghardt and co-workers (*vide supra*),⁷³ β -dAzo was installed in RNA.⁷⁸ As expected, high PSS's (80–98%) were reached within 1 min of irradiation at 20 °C, for photoisomerizations in both directions. Identical strands were synthesized with *D*-tAzo, which showed a PSS of merely 50% *cis* isomer upon 10 min of irradiation at 20 °C. Equally good results of photoswitching were obtained for β -dAzo in DNA.⁷⁹ This high photoswitching efficiency at low temperatures makes β -dAzo especially attractive for *in vivo* application.

2.4 Effect of azobenzenes on duplex stability

The effect of switch isomerization on duplex stability (as clearly visualized in Fig. 4) is commonly assessed by the ΔT_m . Ideally, the difference in T_m of the duplexes containing *cis* and *trans* isomers should be large, and for biological and medical applications the two T_m values should lie below and above the human body temperature. For some applications, especially in the field of nanotechnology, the amount of switch modifications introduced in the oligonucleotide may be less relevant, but in general for applications under physiological conditions a small number of switch inclusions is desired.

The maximum effect of *D*-tAzo on duplex stability was explored by engineering a heavily modified tAzo oligonucleotide, containing twice as many azobenzenes as base pairs.⁷⁶ According to the authors, this design should not even be considered as modified DNA anymore, but rather as a novel material for nanotechnology. They observed a high recognition ability for the complementary strands, driven by the nucleobases, as well as a strong stabilization effect due to the *trans*-azobenzene moieties. A T_m of 55.9 °C was measured for a duplex containing 7 base pairs and 14 *D*-tAzo units, 25.4 °C higher than the T_m of the native 7 bp duplex. After irradiation with UV light, all of the duplexes disassociate even at temperatures as low as 20 °C. Since only 5 of the 14 azobenzene units were required to switch for duplex disassociation, the low photoisomerization efficiency of *D*-tAzo was circumvented using this method. The T_m of the photoproduct was too low to be measured, resulting in an exceptionally high ΔT_m (>36 °C).

Recently, *D*-tAzo modified DNA duplex association and dissociation was studied in exceptional detail by Terazima and co-workers.⁸⁰ It was discovered that dissociation of the duplexes proceeds on the microsecond scale, while the *trans*-*cis* isomerization occurs in 20 ps. Therefore it is indicated that photoisomerization does not cause an immediate dissociation, but rather increases the overall strain in the helix, leading to a gradual disassociation. Additionally, the duplex association rate was found to be higher for *cis* azobenzene modified DNA than for the *trans* isomer even though the T_m decreases upon *trans*-to-*cis* isomerization. This rather unexpected result may be explained by assuming that the intercalation of the azobenzene into the DNA is associated with the main energy barrier of hybridization. Detailed computational studies may further rationalize these findings. However, these experimental results are already invaluable additions to our understanding of the association/dissociation equilibrium.

Although *D*-tAzo has been used successfully in many applications, there is an ongoing search for linkers that combine the high ΔT_m induced by *D*-tAzo with a higher photoisomerization efficiency at lower temperatures. Promising results were obtained by isomerizing gAzo modified dsDNA (Fig. 2) at 37 °C.⁷⁷ However, after the incorporation of a single *R*-gAzo in 12 base pair dsDNA a ΔT_m of ~6 °C was measured, whereas Nishioka, Asanuma and co-workers report a ΔT_m of 14.6 °C for the incorporation of 2',6'-dimethyl-*D*-tAzo at the same position in the same sequence.⁸¹ This is to be expected, since the more flexible linker allows the *cis* isomer to flip out of the duplex

(thus increasing the isomerization efficiency below the T_m), where it has a much smaller destabilization effect.⁷³ When five *R*-gAzo moieties are included, the ΔT_m rises to 45 °C (or 9 °C per azobenzene unit), but such a heavy modification is less desirable.

β -dAzo (Fig. 2) contains a β -deoxyribose linker and may therefore induce only minimal disturbance to the oligonucleotide backbone. Although β -dAzo-modified RNA duplexes can be efficiently isomerized at 37 °C, irradiation was also performed at higher temperatures to enable accurate comparison with the analogous tAzo RNA strands.⁷⁸ In these experiments, the ΔT_m was consistently larger for the tAzo modified RNA duplexes. However, in DNA, 5 out of 7 14 bp duplexes showed a slightly higher ΔT_m for β -dAzo than for *D*-tAzo.⁷⁹ Taking also the photochemistry results into consideration, it seems that β -dAzo is a very promising candidate for photo-regulation of DNA, where the rigidity of the linker, forcing the switch to stay inside the duplex, balances perfectly with the relative flexibility of the helix compared to RNA, allowing the *trans*-azobenzene to stack efficiently between the neighbouring base pairs and the *cis*-azobenzene to flip out of the duplex.

As noted, the extent of duplex stabilization by isomerization of a photoswitch is usually assessed by measuring the ΔT_m (*vide supra*). Recently, however, Sengupta, Ginger and co-workers performed dynamic force spectroscopy measurements to measure the force associated with complete disassociation of the two strands.⁸² An Au-coated surface was functionalized with ssDNA containing two *D*-tAzo moieties, while a complementary strand was connected to an AFM tip, lined up in such a way that the surface connection and the AFM tip were on the same end of the hybridized duplex. Pulling experiments show a rupture force of 34.6 pN before, and 24.2 pN after UV irradiation. Subsequent irradiation with blue light leads to a rupture force of 32.3 pN, which is very close to the value obtained for the non-irradiated sample. Although the exact extent of isomerization could not be measured, due to the nature of the experiment, the results indicate a high PSS for the *trans*-*cis* isomerization and a near complete *cis*-*trans* isomerization. Moving the *D*-tAzo moiety further towards the unmodified end of the duplex and away from the pulling site leads to a smaller decrease in rupture force, indicating that destabilization through switching is a very local effect and does not apply to the complete duplex. More recently, the same group performed some additional studies on *D*-tAzo-ssDNA functionalized surfaces, photochemically controlling hybridization and dehybridization of a fluorescently labelled target sequence in solution.⁸³ Although only a single label is analysed, the authors anticipate that this technique may be used in information storage by constructing multilabel arrays on a chip.

2.5 Alternative molecular photoswitches for use as nucleoside surrogates

While the azobenzene has become the switch of choice for oligonucleotide modifications, in particular as a nucleoside surrogate, addition of other switches to the molecular toolbox would be most welcome. Spiropyran has been included in DNA as a nucleoside surrogate without much success.⁶² Bridged azobenzenes

Fig. 5 Alternative photoswitchable moieties applied as nucleoside surrogates: bridged azobenzene **1**,⁸⁴ anthracene **2**⁸⁵ and styrylpyrene **3**,⁸⁷ each functionalized with a D-tAzo linker.

(**1**, Fig. 5) have recently been proven to undergo reversible photoisomerization.²⁴ These compounds are of theoretical interest because their *cis*-isomer is thermally stable while the *trans* is not, as opposed to regular azobenzenes, and because they can be addressed with visible light. However, incorporation in DNA has not yet led to successful results, since at elevated temperatures *trans*–*cis* isomerization is too fast to determine the ΔT_m .⁸⁴

Manchester, Tucker and co-workers have used reversible anthracene dimerization to create a dual-mode gated photochromism system.⁸⁵ Two anthracene moieties (**2**, Fig. 5) were incorporated in a short ssDNA strand by D-threoninol linkers. The anthracenes can be effectively dimerized by UV light, and subsequently reverted to the monomer form by prolonged heating. The strand can be hybridized with complementary ssDNA, after which photodimerization is heavily suppressed, while the dimerized form does not form a duplex with the complementary strand.

A similar approach based on cyclization reactions was used by Doi, Asanuma and co-workers. This group previously reported irreversible cyclization of two stilbazole nucleoside surrogates, leading to crosslinking of complementary DNA strands.⁸⁶ By changing the pyridine unit of the stilbazole to a pyrene group (**3**, Fig. 5), the [2+2] photocycloaddition could be triggered with visible light.⁸⁷ Irradiation of the cyclized strands with $\lambda = 340$ nm light lead to almost complete ring-opening. A small diastereomeric excess between the two cycloaddition products was also observed, suggesting the possibility to use the helical duplex as a chiral scaffold for asymmetric photochemical transformations.

While bridged azobenzenes and dimerising anthracenes and styrylpyrenes are interesting additions to the range of possible oligonucleotide functionalizations for photochemical control of shape and function, traditional azobenzene remains superior for application as a nucleoside surrogate. Several studies indicate that the choice of backbone linker requires a fine balance between sufficient rigidity to induce a large ΔT_m and some flexibility to allow for high photoisomerization yields at lower temperatures and efficient stacking of the *trans* isomer. Based on these considerations, β -dAzo appears to be a valuable alternative to D-tAzo. Additionally, since helix destabilization is a local effect and photoisomerization efficiency is dependent on local sequence, the exact position of an azobenzene in the oligonucleotide should be carefully considered. Although impressive results have been obtained by inclusion of a large number of azobenzenes into one system, it is clear that large effects may be induced by small, well-placed modifications.

2.6 Biological applications

The application of photoswitches as nucleoside surrogates in a biological setting has been relatively limited so far. This may be caused by several factors. First of all, most photoswitches undergo isomerization under UV irradiation, which is harmful to living organisms. Additionally, as discussed in detail above, photoisomerization efficiency is usually poor under physiological conditions. At temperatures below the T_m of the oligonucleotide, the free volume available for isomerization is limited, leading to low photoisomerization efficiencies upon irradiation. Finally, the approach is so far limited to short modified oligonucleotides. Inclusion of any non-natural backbone linker in long strands of RNA or DNA can be challenging, because the modification can block primer extension by DNA polymerase. Therefore, the possibilities for tAzo inclusion in genes *via* PCR are limited.

2.6.1 Transcription. Only a small window of the light spectrum is suitable for irradiation under physiological conditions. UV light is harmful to living organisms and most light from the visible spectrum is heavily absorbed by haemoglobin, lipids and water.⁸⁸ Asanuma and co-workers found previously that the introduction of a thiol group in the *para* position of D-tAzo shifts the absorption maximum towards the visible light,⁸⁹ while two methyl substituents in the *ortho* positions greatly improve thermal stability of the *cis* isomer.⁸¹ Combining these two modifications led to (S-DM)-tAzo (Fig. 2), which was used for orthogonal dehybridization of a dsDNA strand containing two different domains.⁹⁰ In earlier work, this group introduced several tAzo moieties in the side chain of a promoter involved in transcription *via* RNA polymerase (Fig. 6).⁷⁵ This modification allowed them to control melting of the unwinding region by irradiation, thus influencing transcription rate. By changing the tAzo units in this promoter to (S-DM)-tAzo, photoswitching of GFP (green fluorescent protein) expression was achieved using $\lambda = 400$ nm light.⁹¹ Notably, in this example only two azobenzene units were incorporated in the T7 promoter region, which is not enough to significantly influence hybridization sign. Instead, the small structural deformation resulting from *trans* azobenzene intercalation inhibited binding of RNA polymerase. As usual in D-tAzo modified oligonucleotides, heating to above the T_m was required to obtain satisfactory photoisomerization yields. The authors note that GFP production remains constant after irradiation for 1 hour, whereas their UV-light regulated system shows significant decrease in expression, which is attributed to UV light damage to enzymes in the expression system. However, the difference in GFP production between *cis*- and *trans*-azobenzenes is larger for the UV regulated system.

Fig. 6 Photochemical control of transcription. Upon irradiation with $\lambda = 400$ nm light, (S-DM)-tAzo (right) isomerizes to the *cis* configuration (left). The unwinding region is destabilized, allowing RNA polymerase (green oval) to start transcription. mRNA, the ribosome and the new GFP are also depicted. Reproduced with permission from ref. 91. Copyright 2014, American Chemical Society.

Fig. 7 Photoinduced DNA strand displacement. (a) In the *cis* configuration, hybridization of the two complementary strands is unfavourable. Under visible light conditions, the *trans* isomer is generated and the duplex is formed. (b) When the azobenzene-modified strand is in the *cis* configuration, the native strand prefers complexation to a shorter complementary strand, leaving a small toehold region. Upon isomerization to the *trans* isomer, hybridization becomes favourable and the shorter strand is displaced. This process may be reversed with UV light irradiation. Fluorescence of the FAM group is quenched in close proximity to the DABCYL, allowing for quantification of hybridization degree using fluorescence spectroscopy. Reproduced with permission from ref. 92. Copyright 2015, Royal Society of Chemistry.

2.6.2 Strand displacement at physiological temperature.

As shown previously, gAzo (Fig. 2, *vide supra*) has potential for applications where switching at lower temperatures is required. Kou, Xiao and co-workers use this property in a light-switchable DNA strand displacement (Fig. 7).⁹² Two strands can indeed be very successfully hybridized and dehybridized in the temperature range between the T_m 's of the two isomers, although 37 °C is below the T_m of the UV photoproduct and at this temperature only 11% of the DNA strands can be disassociated. However, in a competition experiment, displacement of a native DNA strand with the analogous *trans*-azobenzene-modified strand is very efficient (57% at 37 °C and up to 86% at higher temperatures, quantified by fluorescence quenching measurements). Such a high degree of

strand displacement could only be achieved by the incorporation of 16 gAzo moieties in a 35 bp strand of dsDNA. This heavy modification is presumably required in order to achieve a large ΔT_m , but in consequence, the use of gAzo instead of D-tAzo does not lead to much better results. The high photoisomerization efficiency of gAzo at low temperatures might be better utilized in a system that, potentially by introducing base-pair mismatches, would have been engineered to have a T_m close to 37 °C. The small ΔT_m induced by the introduction of a much smaller number of gAzo moieties would then still be sufficient to perform hybridization switching at physiological temperature.

2.6.3 DNA ligation and cleavage. Liang, Fujioka and Asanuma developed a method to include non-natural backbone linkers in oligonucleotides by incorporating a D-tAzo moiety or another intercalator into one of the primers.⁹³ PCR using such primers leads to the generation of dsDNA with a single stranded overhang starting from the modification. Subsequent DNA ligation to a short ssDNA strand complementary to the overhang afforded the full dsDNA (Fig. 8). Interestingly, the rate of ligation was

Fig. 8 DNA ligation between the PCR generated complementary strand and a short ssDNA strand complementary to the overhang. Reproduced with permission from ref. 93. Copyright 2011, Wiley-VHC.

Fig. 9 Schematic illustration of the mechanism of DNA nanoscissors. Under visible light irradiation, hairpin H2 is stabilized and the regulatory domain is unavailable for binding. Upon UV irradiation, the tAzO units isomerize, destabilizing the hairpin. The regulatory domain can bind the substrate and cleavage is activated. Reproduced with permission from ref. 94. Copyright 2013, Royal Society of Chemistry.

much higher with the azobenzene in the *cis*-configuration, most likely because it is pushed out of the duplex and does not interfere with the ligation. Although the dsDNA synthesized in this study is only 42 base-pairs long, this ligation could potentially be used to synthesize azobenzene-modified genes.

Complementary to ligation, light-regulated DNA cleavage has also been reported (Fig. 9), dubbed “DNA scissors”.⁹⁴ A previously reported DNAzyme which mediates DNA cleavage⁹⁵ was extended with a *D*-tAzO-modified strand that is complementary to the regulatory domain for substrate binding. Hairpin formation between the regulatory domain and the extended strand is stabilized by the *trans*-isomer, easily competing out the substrate binding and suppressing DNA cleavage. UV-light irradiation leads to the formation of the *cis*-isomer, in which 85% of the hairpins open at room temperature. This activates the DNA cleavage with a 90% efficiency compared to the unmodified DNAzyme. Low photoisomerization yield of *D*-tAzO at room temperature is negated by using three *D*-tAzO moieties in the 7 bp hairpin. Using this approximate ratio of azobenzenes to base pairs, it has previously been shown that switching is efficient at 37 °C, while by using a higher concentration of azobenzenes no additional stabilization effects are generated.⁵⁶

2.7 Oligonucleotides for nanotechnology and materials

The idea of using oligonucleotides as a material stems from the 1980's³ and has been most successfully been applied in DNA origami.^{96,97} A variety of 2D and 3D objects can be made by self-assembly of programmed DNA; external control over formation and function of these structures is a desirable research target, as it would lead to responsive materials. Recent investigations have focused on using such external triggers as primer addition,⁹⁸ chemical adducts⁹⁹ and pH.¹⁰⁰ Control by light is advantageous because of the high level of spatiotemporal control and no waste is generated. Efficient functioning at low temperatures is usually not required for photoswitchable materials and nanostructures. Therefore, *D*-tAzO is used almost exclusively in such designs.

2.7.1 Photoswitchable annealing. The most straightforward method of introducing photocontrol to DNA origami and other nanostructures is to use short, azobenzene-modified strands as “photocontrolled sticky ends” (Fig. 10a). Complementary strands on two different objects can be used to anneal and disassociate these objects, taking advantage of the typical *D*-tAzO regulated duplex stabilization and destabilization. This process was visualized with AFM using a DNA origami “tile”, which has a cavity containing two dsDNA strands, each functionalized with a complementary strand of photoresponsive ssDNA (Fig. 10b).¹⁰¹ In the *trans* configuration, these domains hybridize, distorting the dsDNA and creating a distinctive X-shape. Isomerization to the *cis*-isomer using UV light was efficient and could be reversed with visible light. The concept was further developed to an orthogonal chemical/photochemical switching system, where an X-shape can also be formed from one of the two strands to a third strand by reversible, K⁺-induced G-quadruplex formation.¹⁰² The photoresponsive sticky ends were combined with DNA origami to induce larger quarternary structures from hexagonal DNA origami tiles (Fig. 10c).¹⁰³ Synthesis of the *D*-tAzO modified origami tiles is facile, since *D*-tAzO only needs to be present in a few ssDNA strands, which are tethered on the outside of the structures by disulphide bridges. Several 2D structures could be reversibly formed using irradiation and thermal annealing cycles, and were observed using fluorescence measurements and AFM. The disassembly and assembly of the larger structures could even be followed in real time using high-speed AFM, showcasing the potency of the photoresponsive sticky ends.¹⁰⁴ By using strand recognition of different nucleobase sequences,¹⁰⁵ a measure of selectivity might be included. The photoreversible construction of more intricate structures using this method is eagerly awaited.

2.7.2 Cargo release. The “photoresponsive sticky ends” principle was applied in a more direct functional manner as a method for triggered release of cargo.¹⁰⁶ Mesoporous silica nanocontainers were fitted with 6 base ssDNA strands on the surface (Fig. 10d). A 21 bp ssDNA strand, containing 2 complementary domains and 6 *D*-tAzO modification, was added to

Fig. 10 "Photoresponsive sticky ends" for reversible association of nanoscale objects such as DNA origami tiles or nanoparticles. (a) General concept. Both objects are functionalized with complementary ssDNA, containing several tAzo units. Upon irradiation with UV light, the azobenzenes isomerize to the *cis* form and destabilize the double strands, allowing the objects to move away from each other. By irradiation with visible light the azobenzenes isomerize back. DsDNA is allowed to reform, inducing association of the objects again. Adapted with permission from ref. 103. Copyright 2012, American Chemical Society. (b) Schematic representation of AFM visualization of photoreversible annealing. The two dsDNA strands in the cavity are pulled together upon photoinduced hybridization of the two ssDNA strands, creating an X-shape. Adapted with permission from ref. 101. Copyright 2012, Wiley-VHC. (c) Schematic representation of "photoresponsive sticky ends" for creating larger quarternary structures from hexagonal DNA origami tiles, and AFM images of several of these aggregates. Adapted with permission from ref. 103. Copyright 2012, American Chemical Society. (d) "Photoresponsive sticky ends"-induced cargo release via pore functionalization with tAzo containing complementary ssDNA. Adapted with permission from ref. 106. Copyright 2012, American Chemical Society.

act as a photoresponsive cap. Irradiation with UV light lead to the formation of the *cis* isomers and subsequent disassociation of the cap, which enabled gradual release of a rhodamine dye used as a model cargo (91% release in 25 h). The containers have a low *in vitro* cytotoxicity, and preliminary results using release of a chemotherapeutic agent are promising.¹⁰⁶ As a result, these containers may in the future be successfully applied for targeted drug delivery. In a similar investigation, a nanocontainer was constructed entirely by DNA origami, which could be opened and closed by the same D-tAzo system, thereby releasing an Au nanoparticle.¹⁰⁷ For more information regarding azobenzene-induced drug delivery using mesoporous silica nanoparticles, the reader is referred to a different review.¹⁰⁸

The use of short D-tAzo modified complementary ssDNA strands as photoresponsive sticky ends has recently also been used to control Au nanoparticle aggregation,¹⁰⁹ a DNA-based rotaxane system,¹¹⁰ scissoring motion between two Au nanorods¹¹¹ and hydrogel cross-linking.^{112,113} Additionally, using a similar approach, shape change in DNA origami structures was induced using a D-tAzo modified ssDNA strand.¹¹⁴ These applications are a very relevant addition to the respective fields, because a highly efficient measure of photocontrol is introduced. However, in the scope of this review, these results are of less interest because the D-tAzo system is already previously discussed in detail, and is in all these examples applied in a manner similar to previously discussed research.

2.7.3 DNA walkers. DNA walkers are mechanical devices based on transporter proteins such as kinesin and myosin.¹¹⁵ Inclusion of tAzo units allows for a measure of control *via* an external trigger, *i.e.* light. You, Tan and co-workers designed a molecular walker that moves along a track in a reversible manner (Fig. 11).¹¹⁶ The track contains three anchorage sites;

Fig. 11 Molecular walker. S1, S2 and S3 have an increasingly longer toehold region, which induces a movement towards S3 under visible light. Upon UV irradiation, the azobenzenes switch to the *cis* configuration. Since S2 has less azobenzenes units than S3, and S1 has none at all, irradiation with UV light induces a movement towards S1. Adapted with permission from ref. 116. Copyright 2012, American Chemical Society.

a starting site S1, a second anchorage site S2 containing a D-tAzo-modified toehold region for binding the searching leg of the walker, and a third anchorage site S3 containing a longer toehold region with more azobenzenes moieties. Under visible light irradiation, the azobenzenes moieties are in the *trans* configuration. The increasingly longer toehold regions mediate movement of the walkers towards S3. Upon irradiation with UV light, the D-tAzo moieties switch and consequently destabilize binding between the walker and S3. The walker therefore moves to S2 which contains less D-tAzo moieties, and subsequently moves to S1. Several locomotion cycles could be completed, as demonstrated by a FRET assay. Due to the conservation of the track and the reversibility of the motion, this work provides real improvement of earlier, non-reversible light-regulated DNA walkers.¹¹⁷ More recently, an exceptionally fast DNA walker was modified with D-tAzo.¹¹⁸ Irradiation with UV light arrested the movement along a track, while motion was restored upon visible light irradiation. For an excellent perspective on the recent developments in light-driven DNA nanomachines, the reader is referred to an account by Kamiya and Asanuma.¹¹⁹

2.8 Concluding remarks

The incorporation of molecular photoswitches as nucleoside surrogates is unquestionably the most successful approach towards photoregulation of oligonucleotide structure and function. Although a few attempts at photoregulation have been made, using among others spiropyrans and anthracene dimers, azobenzene remains the preferred photoswitch. Due to the intercalation of *trans* azobenzene-based nucleosides between their neighbouring base pairs, all studies discussed here rely on photoinduced double helix destabilization. A large number of experimental and computational studies has provided a deep understanding of the influence of the structure of the nucleoside on the stability of the duplex. As a result, it has become possible to engineer the ΔT_m of a modified strand around the application temperature. This development has led to impressive results in photoregulation of key biological processes such as ligation and transcription. Additionally, advances were made in the fields of DNA nanotechnology and drug delivery.

3 Molecular photoswitches attached to nucleosides

Photoswitches may be attached to nucleosides, either on the nucleobase or on the ribose unit (Fig. 1b). The coupling reaction between the switch and the oligonucleotide can be performed post synthesis, which offers the possibility of easy diversification and inclusion of molecules that may be difficult to convert to phosphoramidite building blocks for solid phase synthesis. This approach requires the inclusion of a modified nucleoside containing a synthetic handle into an oligonucleotide. In this method, the natural nucleobase is still present in the modified nucleoside. Therefore, the switch has a very different binding mode to the oligonucleotide than in the systems from the other approaches described herein. Previously, only azobenzenes

have been incorporated in oligonucleotides *via* this method.⁹ Although very efficient switching behaviour has been observed for these modified oligonucleotides, T_m shifts have not been large. No molecular modelling has been performed on these systems yet. Therefore, the exact mode of interaction between the azobenzenes and the oligonucleotide is largely unknown, making it difficult to engineer improvements. The lack of computational results may be the reason that only a handful of examples utilizing this method have appeared in the past few years.

3.1 Design

In recent years, several photoswitches other than azobenzenes have been ligated to nucleosides. Wagenknecht and co-workers previously described spiropyrans ligated to uridine, either by an acetylene bridge to the 5-carbon of uracil or by a copper-catalysed click reaction to an acetylene functionality tethered to the 2'-hydroxy group of the ribose unit.⁶³ However, they found isomerization of the spiropyran in a DNA environment to be quenched and chose not to pursue inclusion into DNA any further.¹²⁰ Recently, the same group reported the inclusion of a diarylethene functionalized deoxyuridine **4** into ssDNA (Fig. 12a).¹²¹ For this switch, the photochemistry is retained in the nucleic acid environment. The diarylethene is connected to the uracil moiety *via* an acetylene bridge to the C5 carbon. This connection typically places the substituent (such as the photoswitch) in the major groove.¹²² However, creation of this link requires a Sonogashira coupling, and to avoid possible synthetic complications, the switch is installed before the solid phase synthesis. Wang, Guo and co-workers constructed a similar nucleoside with a diarylethene switch ligated to deoxyadenosine *via* an acetylene bridge (Fig. 12b, **5**).¹²³ Because the diarylethene is ligated to a different position than in the previous example, the switch might interact differently with a nucleic acid duplex. However, nucleoside **5** has not yet been included in an oligonucleotide. Using the C5 acetylene bridge, azobenzenes can also be ligated to uridine (**6**, Fig. 12c).¹²⁴

Locked nucleic acids (LNAs) contain a methylene bridge between the 2'-O and the 4'-C position of the ribose ring (**7**, Fig. 12d). Oligonucleotides containing LNAs show enhanced binding affinity towards complementary RNA. Additionally, LNA-modified oligonucleotides containing C5 modifications exhibit improved resistance against nucleases. Combining these features, Morihiro, Obika and co-workers synthesized a locked uridine with an azobenzene connected to the C5 position *via* an alkynyl linker (Fig. 12d).¹²⁶ Unfortunately, after inclusion in an oligonucleotide, the stabilizing effect of the bridge appeared to be completely neutralized by the azobenzene inclusion. Nuclease resistance was also measured, and although 3'-phosphate scission proceeded at the same rate as for the native strand, 5'-phosphate scission relative to the LNA was 300-fold decreased. This enhanced resistance may be very relevant for future application in biological systems, when combined with a different method for photoswitch inclusion. For example, β -dAzo (Fig. 2) provides an extra stabilizing effect to oligonucleotides and could therefore be a prime candidate for functionalization with an extra bridge.

Fig. 12 Structures of several switches attached to nucleosides discussed in this chapter: (a) diarylethene **4**,¹²¹ (b) diarylethene **5**,¹²³ (c) azobenzenes **6a–f**,^{124,125} and (d) locked nucleic acid azobenzene **7**.¹²⁶

Freeman, Vyle and Heaney explored the possibility of covalently linking an azobenzene to an oligonucleotide *via* a solid-phase, catalyst-free nitrile oxide–alkyne cycloaddition (Fig. 13).¹²⁷ A commercially available 2'-O-propargyl uridine phosphoramidite building block was incorporated *via* solid-phase synthesis. Using a chloramine-T-mediated click reaction in an ethanol/water mixture, the ssDNA was coupled to an oxime-containing azobenzene in near quantitative yield, after which the oligonucleotide could be cleaved from the resin under standard conditions. Coupling of photoswitches after solid phase synthesis would allow for diversification without the synthesis of multiple complicated building blocks.

3.2 Interactions between oligonucleotides and attached molecular photoswitches

The exact mode of interaction between photoswitches and oligonucleotides determines the effect of photochemical modulation. When photoswitches are attached to oligonucleotides, this interaction depends not only on the choice of switch, but also on the choice of connecting bridge. It is well known that substituents linked to the C5 position of a pyrimidine nucleobase commonly reside in the major groove of oligonucleotide duplexes,^{46–48} although introduced mismatches can lead to stacking of the acetylene bridge, causing the substituent to be placed in the minor groove or to intercalate.¹²² Additionally, destabilization has been observed for aromatic substituents in the C5 position,¹²⁸ which is attributed to disturbance of groove hydration and interstrand cation bridges by the hydrophobic bulk of the substituent. These results give an indication which position azobenzenes attached to the C5 position occupy in the groove, but have not been confirmed by any experimental or computational data. Obika and co-workers hypothesize that destabilization would be less for *cis*-azobenzene, because it is

more compact.¹²⁴ The predicted relative stability of the *cis* isomer is confirmed by experimental results, although it remains unclear if the resulting ΔT_m originates from the difference of size between *cis*- and *trans*-azobenzene or a difference in other parameters. Barrois and Wagenknecht¹²¹ connected a diarylethene switch to uridine C5 *via* an acetylene bridge. They speculate that the closed form of the switch may intercalate, but this theory is based on a non-covalently attached diarylethenes, which have a vastly different orientation towards the oligonucleotide than the rigidly bound switch of the present example.¹²⁹ In the current design, placement in the major groove is more likely, unless mismatches are present in the dsDNA. However, the authors do not provide the sequence of the complementary strand. Therefore, any comments on the binding interaction are speculative.

Finally, Heaney and co-workers used a click reaction to attach azobenzenes to oligonucleotides containing a single propargyl functionality (Fig. 13).¹²⁷ Due to the position on the ribose 2'-position and the relatively flexible linker, the *trans*-azobenzene could potentially stack in the duplex, similar to the nucleoside surrogate azobenzenes. However, the authors focus on synthetic methodology and the binding mode of the modified oligonucleotides was not explored further.

3.3 Photochemistry of molecular photoswitches attached to nucleosides

When photoswitches intercalate into the oligonucleotide duplex, photoswitching ability may be reduced due to lack of available space. When C5-acetylene bridge attachment is used, the switch is placed in the major groove. Therefore, higher photoisomerization efficiencies are expected. Azobenzene installed *via* this method can be isomerized to the *cis*-isomer with 60% efficiency, under 365 nm light irradiation at room temperature.¹²⁴

Fig. 13 Chloroamine-T (Ch-T) mediated click reaction towards azobenzenes functionalized nucleic acids. *meta*-Functionalized azobenzene could be coupled as well. The clicking reaction can be performed with good yield both mid-solid-phase synthesis or after completion of the full sequence.

Isomerisation to the *trans*-isomer proceeds with 80% conversion, and several cycles could be performed to illustrate the high fatigue resistance of the system.¹²⁴ Functionalization of the azobenzenes (Fig. 12c) did not lead to improved photochemical properties. Electron-rich azobenzene **6b** can be isomerized to the *cis* configuration in 79% yield, but irradiation back to the *trans* isomer proceeded with only 59% conversion. Electron-poor azobenzenes **6d** and **6e** can be isomerised to 91–96% *trans* isomer, but only 14–28% *cis* can be obtained under UV irradiation. A methyl substituent (**6c**) does not affect photoswitching behaviour, while pyridyl azobenzene (**6f**) is found exclusively as the *trans* isomer and has either lost switching ability or thermally switches back too fast to be measured.

When a LNA is used as a backbone linker (Fig. 12d), the photochemistry remains unaffected.¹²⁶ Diarylethenes attached to oligonucleotides retain their switching ability and are fatigue resistant.¹²¹ However, no *cis/trans* ratios of the photostationary states were reported.

Using the click-chemistry approach of Heaney and co-workers,¹²⁷ azobenzenes can be attached to the ribose unit *via* a flexible isooxazole linker (Fig. 13). The interaction between these azobenzenes and the oligonucleotide duplex are unknown, but *trans*-to-*cis* isomerization is already heavily impaired (17–27%) in a single stranded oligonucleotide. As intercalation seems to most probable mode of azobenzene–oligonucleotide interaction, isomerization may be completely suppressed in a duplex. However, this hypothesis remains speculative until such a duplex is actually constructed.

3.4 Effect of photoswitches attached to nucleosides on duplex stability

The effect of switching of an azobenzene or a diarylethene switch, attached to uridine *via* an acetylene bridge to C5 (Fig. 12a and c), on duplex stability, expressed in ΔT_m , has generally been small. Because C5-acetylene attached photoswitches reside in the major groove and any theory on the structural effect of photoswitching on the oligonucleotide remains purely speculative, the effect of photoswitching on the duplex stability is difficult to predict. For azobenzenes, ΔT_m is 5 °C for a 12 bp DNA/RNA hybrid, and only 2 °C for the corresponding dsDNA.¹²⁴ Diversification of the azobenzenes did not lead to a significant increase in ΔT_m .¹²⁵ Using LNAs, a ΔT_m of 17 °C could be achieved, but only with the inclusion of three azobenzene-modified locked uridine units into a 12 bp DNA/RNA hybrid. Installation of a single azobenzene modified LNA led to a ΔT_m of 3 °C. It has to be noted that in these

examples the *trans* isomer is destabilized relative to the *cis* isomer, which means this modification could potentially be used for inverse photocontrol. In diarylethenes, the T_m of 17 bp dsDNA decreases with 3.8 °C upon switching to the closed form.¹²¹

3.5 Concluding remarks

The majority of the publications discussed in this chapter concerns photoswitches attached to nucleosides *via* a rigid C5 linker. This orientation places the switch in the major groove, where it may influence duplex stability by disturbing groove hydration and interstrand cation bridges. Compared to other strategies, switches attached to nucleotides induce ΔT_m 's of average magnitude. This method has the advantage that switching may be performed at low temperatures with high conversion. However, the precise mode of interaction between the switch and the oligonucleotides is not known. Computational simulations to elucidate structural changes upon switching are highly anticipated, as this would allow for knowledge-based design of improvements.

4 Photochromic nucleosides

Photochromic nucleosides are photoswitches where one of the aromatic groups of the switch is replaced by the purine or pyrimidine unit of a nucleic acid (Fig. 1c). Inclusion of photochromic nucleosides in oligonucleotides is a relatively new methodology and has been applied in only a handful of examples. Pioneering research in the groups of Maeda and Spada focused on stilbene–guanosine hybrids (Fig. 14a),^{51,130} which display highly efficient *E-Z* isomerization and induce a ΔT_m of up to 7.9 °C in 12 bp dsDNA. Additionally, an adenosine-based diarylethene (Fig. 14b) was reported by the group of Jäschke.⁵² For this compound, photoisomerization proceeded with low efficiency in aqueous solvents. Recent developments in the field of photochromic nucleosides all come from the group of Jäschke, and focus on the improvement of diarylethene–nucleobase hybrids.

Two new designs have been presented, based on deoxyguanosine¹³¹ and deoxyuridine (Fig. 14c and d).¹³² Deoxyguanosine diarylethene switch **8** can be ring-closed with 86% and 81% conversion for **8a** and **8b**, respectively. Ring opening could be induced by visible light irradiation or exposure to 60 °C for 20 min. The absorption maximum of the closed form could be significantly red-shifted by protonation with

Fig. 14 Photochromic nucleotides (a) older stilbene-based design,⁵¹ (b) older diarylethene-based design,⁵² (c) diarylethene-based **8** and its switching behaviour¹³¹ (d) diarylethene-based **9**^{132,133} and its switching behaviour.

trifluoroacetic acid or complexation to Cu^{2+} . However, this complexation causes a destabilization of the closed form, leading to ring opening at room temperature. Additionally, severe degradation was found over several switching cycles. Therefore, inclusion of photochromic nucleoside **8** in oligonucleotides was not further pursued.

Photochromic nucleoside **9a** (Fig. 14d) is based on deoxyuridine.¹³² The diarylethene unit has just one alkyl group attached to the carbon atoms that form the new bond in the ring closing reaction. The missing alkyl group may influence photochemistry, but simplifies the synthesis, as the 5-iodosubstituted deoxyuridine precursor is commercially available. Suzuki–Miyaura cross-coupling to the corresponding boronic acid yields **9a**. This reaction can also be performed post solid-phase synthesis in yields of up to 36%.¹³² The analogous deoxycytidine–diarylethene switch was also synthesized in even better yields, but the closed form of this switch was highly thermally unstable. Photochromic nucleoside **9a**, however, displayed only very little degradation even when stored at 90 °C for 1 hour. Irradiation with UV light led to formation of 83% of closed-ring isomer, while ring opening with visible light was nearly quantitative, showing that the missing alkyl group does not significantly influence the photochemistry. Effects on the melting temperature were found to be small but highly dependent on position of the switch within the oligonucleotide. One internal modification in 15 bp ds DNA caused a decrease in T_m of 2.3 °C, both in the

closed and open form. Inclusion of closed-form **9a** near the end of the duplex caused an increase in T_m of 0.9 °C and 1.6 °C for the 5' and the 3' position, respectively, and no change for the open form of **9a**. A small effect of switch isomerization on transcription rate was also observed.

The photochemical properties of **9** were further explored using ultrafast time-resolved spectroscopy.¹³³ Three derivatives of **9a** were synthesized, in which the phenyl ring was changed to a naphthalene moiety (**9b**), a *para*-pyridyl group (**9c**) or a *ortho*-pyridyl group (**9d**). The authors take a fundamental spectroscopic approach towards elucidating the photochemical properties of the novel diarylethene switch. Therefore, other than the observation that the quantum yield of the ring opening is highest for **9b** and lowest for **9d**, these results are outside the scope of this review.

4.1 Concluding remarks

Photochromic nucleosides present the option for different interaction modes between oligonucleotides and photoswitches. However, the research is still in a very early stage. There is however very little evidence for the structural effect of diarylethene–nucleic acid hybrids. Although the approach seems promising at first sight, in the past few years there have been few advances, and it remains to be seen whether photochromic nucleosides will become an established method for photoregulation of oligonucleotides.

5 Photoswitches as phosphate backbone linkers

Photoswitches may be introduced into the phosphate backbone of oligonucleotides (Fig. 1d). The distinct difference between this and the other approaches discussed in this review is that the photoswitch forms an integral part of the backbone, as opposed to being tethered to it. Duplex formation of an oligonucleotide containing a switchable phosphate backbone linker with a native DNA strand was found to be energetically unfavourable.⁵⁴ As a result, oligonucleotides with photoswitchable backbone linker are typically designed to form hairpin structures, so that the switch forms a hydrophobic cap connecting both halves of the duplex. The position of the switch on top of the base pairs can lead to very different structural effects of the switch inclusion compared to other methods discussed in this review. Both stilbenes and azobenzenes have been incorporated into the phosphate backbone.^{55,134} Typically, the *trans* isomer provides extra stabilization by stacking on top of the nucleobases in the hairpin, which is lost upon isomerization to the nonplanar *cis* isomer. The change in melting temperature upon isomerization of a single switch can be quite large; ΔT_m values of 28 °C have been reported for a 4 bp stilbene-linked hairpin.¹³⁵ However, this approach is mostly limited to hairpin structures.

5.1 Design

Recently, mainly azobenzenes have been applied as phosphate backbone linkers. From a synthetic viewpoint, an ether linker

Fig. 15 Azobenzene backbone linkers **10** and **11**.^{136,137}

between the switch and the backbone is preferred, since the corresponding azobenzene diol may be converted to a phosphoramidite and included in the hairpin *via* solid-phase synthesis. Sugimoto and co-workers previously included azobenzene linkers **10** and **11** (Fig. 15) in a DNA hairpin, where they observe an unusually high destabilization effect for *cis*-**10**.¹³⁶ The authors attribute this to precise engineering of the linker length, reasoning that *trans*-**10** isomer fits perfectly between the two strands while the *cis* isomer is too short and distorts the backbone. Because linker **11** is longer and more flexible, this extra destabilization effect is lost and the ΔT_m derives only from the loss of stacking interactions. This approach was extended by Wu, Tang and co-workers, who synthesized several different azobenzene-linked hairpins ranging from 4 to 6 base pairs, using backbone linkers **10** and **11** (Fig. 15).¹³⁷

Amide bonds between the azobenzene linkers and the phosphate backbone have been utilized in the past, but are synthetically less accessible than ether bonds. For their computational studies, McCullagh, Schatz and co-workers have chosen for amide bonds (Fig. 17b), however the authors do not comment on their choice of connection units.^{138,139}

Spiropyrans are rarely used for oligonucleotide inclusion, since they are quickly hydrolysed in aqueous environments and can lose switching ability when incorporated into DNA.^{62,121} Brieke and Heckel partly circumvented these problems by incorporating a phosphoramidite derivative of 6-nitrospiropyran (NitroBIPS) in the middle of 15 base DNA strand *via* a ether linker (Fig. 16).¹⁴⁰ Because the strand is not self-complementary, no hairpin was formed, nor was the complexation to a complementary strand attempted. However, the photoswitchable linker is designed to keep the switch in the duplex in both forms, in order to maximize destabilization caused by the nonplanar spiropyran form. Additionally, the stereogenic carbon atom, which might cause duplex destabilization due to its relative bulkiness, is kept out of the duplex at all times. After backbone inclusion, over 40%

of NitroBIPS was shown to degrade in 5 h in PBS buffer at 25 °C. Post-synthetic diversification gave access to PyBIPS (Fig. 16), which could not be obtained through regular synthesis. PyBIPS has previously been proven to be highly resistant to hydrolysis,¹⁴¹ however, the degree of degradation of PyBIPS backbone linker was not quantified.

5.2 Computational studies

McCullagh, Schatz and co-workers performed molecular dynamics simulations and free-volume calculations to gain understanding of the changes in hairpin stability upon switching of an azobenzene backbone linker.¹³⁸ A DNA hairpin is proposed, consisting of two C-G base pairs linked by an azobenzene backbone linker *via* amide bonds. The hairpin is connected to the surface on one end, and to an AFM tip on the other end (Fig. 17). This setup can be used to estimate the work generated by a switching cycle. This work may be generated in two different modes. Mode 1 is based on the unfolding of the hairpin upon isomerization to the *cis* isomer, while in mode 2 the work is generated by the photoinduced contraction and elongation of the azobenzene in the unfolded hairpin. In mode 1, the *trans* isomer stabilizes the hairpin and results therefore in the shortest configuration, whereas in mode 2 the *trans* isomer is the longest. Therefore, the two modes cannot be used in a complementary fashion. The authors propose that an experimental setup according to their design could be used to generate 3.4 kcal mol⁻¹ of extractable work per cycle in mode 1, with a potential maximum efficiency of 2.4% energy regained from the optical energy required. This efficiency drops to ~0.4% when energy waste due to inherent inefficiencies is taken into consideration. Using an experimental setup similar to the one created by Sengupta, Ginger and-workers,⁸² this design could be implemented to construct a working DNA-nanomachine. Additionally, the computational studies show that, similarly to when azobenzenes are used as nucleoside surrogates (Section 2),⁸² destabilization induced by the *cis*-azobenzene backbone linker is a highly localized effect. The authors explore this effect further in follow-up research using the same hypothetical hairpin.¹³⁹ More detailed computational analysis shows that structural DNA damage induced by the *cis*-azobenzene in the backbone is limited to two base pairs. However, the amide bond used in this

Fig. 16 Spiropyran backbone linkers NitroBIPS, PyBIPS and the aldehyde exchange reaction.¹⁴⁰ The exchange to PyBIPS is depicted here as an example, but several other 2-hydroxybenzaldehyde derivatives could also be used. Excess aldehyde has to be used and RP-HPLC purification is required.

Fig. 17 Schematic overview depicting the single-molecule pulling experiment. (a) Experimental setup. The hairpin is attached between the surface and the AFM tip. Slow pulling of the AFM tip allows for precise calculation of the applied force versus the molecular end-to-end distance. (b) Structure of the hairpin. Reproduced with permission from ref. 138. Copyright 2011, American Chemical Society.

research is uncommon and it remains unclear whether a different linker could induce further ranging structural changes.

5.3 Photochemistry of molecular photoswitches as phosphate backbone linkers

When azobenzenes are included as phosphate backbone linkers, they reside on top of the hairpin, forming a hydrophobic cap. In this position on the outside of the duplex, a relatively large amount of free volume is available for the isomerization process, even below the melting temperature. Wu, Tang and co-workers calculate 79.8% conversion to the *cis* isomer after UV irradiation (20 min) of a hairpin containing linker **10** (Fig. 15).¹³⁷ NitroBIPS and PyBIPS (Fig. 16)¹⁴⁰ reside in the middle of the backbone and may not benefit from more free volume. However, no dsDNA is formed using these two linkers. In a single strand, for NitroBIPS, reversible switching between nearly pure spiropyran and 25% merocyanine form can be obtained by alternative 530 nm light irradiation and heating to 60 °C.¹⁴⁰ PyBIPS is more thermally stable, but switching between ~95% spiropyran and ~35% merocyanine could be achieved by alternating irradiation with visible and UV light.¹⁴⁰ The authors contribute the successful photoisomerization to the placement of the spiropyrans between the neighbouring bases as opposed to positioning in the minor groove. This may be supported by formation of double stranded DNA containing these linkers or by detailed computational analysis.

5.4 Influence on hairpin stability

Change in melting temperature upon isomerization of a switchable backbone linker is only reported for the azobenzene-linked hairpins reported by Wu, Tang and co-workers.¹³⁷ Linker **10** performs invariably better than linker **11** (Fig. 15). ΔT_m 's for hairpins containing backbone linker **10** range from 12.1 °C for a 6 bp strand to 24.1 °C for a 4 bp strand. These results are similar to those reported by Sugimoto and co-workers, who used the

Fig. 18 RNA cleavage mediated by isomerization of two azobenzene backbone linkers **10**. In the *trans* configuration, the azobenzene moieties (red bars) stabilize hairpin formation with the RNA binding site. Upon UV irradiation, the switches are isomerized to the *cis* configuration and the hairpins are destabilized. This causes the binding site to be available for binding to the target RNA strand (pink line), leading to RNA digestion by RNAase (orange oval). Reproduced with permission from ref. 142. Copyright 2015, American Chemical Society.

same linker.¹³⁶ Both values are quite large compared to ΔT_m 's obtained with inclusion of photoswitches *via* other methods.

5.5 Applications of oligonucleotides with molecular photoswitches introduced as phosphate backbone linkers

Wu, He and Tang used linker **10** (Fig. 15) for the construction of a series of light-switchable dumbbells for the photoregulation of RNA digestion (Fig. 18).¹⁴² The dumbbells are formed from a

binding site linked to two short complementary strands *via* two linkers **10**. Under ambient conditions, the linkers are in the *trans* configuration and hairpin structure is stabilised. Upon isomerization to the *cis* configuration, the hairpins are destabilized and the T_m is lowered by up to 21.3 °C. Therefore, in the *cis* configuration, binding between the dumbbell and a target RNA strand is much more favourable. Upon binding of the target RNA, its digestion can occur. By irradiation with UV light, RNA digestion can be increased 4.2-fold or 7.5-fold, depending on the exact sequence. This relative increase is comparable to that obtained by the photoregulated DNA-scission reported by Zou, Yang and co-workers, who need inclusion of three azobenzene nucleoside surrogates to achieve this result.⁹⁴ However, in more precise numbers, for the earlier-reported DNA scission an increase from less than 10% cleavage to 85% upon activation is reported. In the current example, either the deactivated dumbbell has a relatively high cleavage rate (19.9%, with 83.5% for the activated dumbbell) or, for a different sequence, the activated dumbbell has a low cleavage rate (36.7%, with 4.9% for the deactivated dumbbell). Therefore, the current system¹⁴² does not improve much on the photoregulated oligonucleotide cleavage reported by Zou, Yang and co-workers.⁹⁴

5.6 Concluding remarks

The use of molecular photoswitches as phosphate backbone linkers can result in large ΔT_m 's, but is of limited use because of the formation of hairpin structures. The role of the photochromic linker has been explored extensively, since linker length is crucial to successful destabilization. Although stilbenes and spiropyranes have been used with moderate success, this method remains largely limited to the use of azobenzenes. Introduction of other photoswitch architectures could possibly broaden the scope of this approach. Applications are still scarce, and the number of publications reporting on photochromic backbone linkers is declining. Therefore it seems that in future research this method will mainly be used for select applications taking advantage of hairpin structures, but less and less to influence duplex stability.

6 Noncovalent interactions between oligonucleotides and molecular photoswitches

A supramolecular approach towards photoregulation of oligonucleotides (Fig. 1f) has the considerable advantage that no modification to the nucleic acids is required. Comparable to photopharmacology, using noncovalent DNA binding photoswitches allows for a high selectivity due to the on/off reversibility. Furthermore, considering possible *in vivo* applications, the transport of these relatively small compounds through the cell membrane may be easier than of large modified oligonucleotides. Reversible DNA/RNA condensation has been investigated extensively¹⁴³ and light has recently drawn attention as an interesting new trigger. Most other noncovalent approaches

are in an early stage and focus initially on binding studies, before potential regulation of oligonucleotide stability can be achieved.

6.1 Design

Azobenzenes, diarylethene switches and spiro compounds are all used for the studies on photocontrolled binding to DNA or RNA and they are functionalized according to the desired mode of interaction. In general, quaternary ammonium groups are attached for solubilisation and to provide electrostatic interaction with the negatively charged phosphate backbone.

To influence duplex stability, intercalators are usually applied, similar to the approaches based on the inclusion of photoswitches as nucleoside surrogates (Section 2). For example, AzoDiGua, an azobenzene functionalized with guanidinium groups, was designed for this purpose (Fig. 19a).¹⁴⁴ Linear dichroism studies show that planar *trans* AzoDiGua intercalates with calf thymus DNA, while the guanidinium groups bind the phosphate backbone. Intercalation was also proven for thiazole orange-modified diarylethene **12** (Fig. 19b).¹⁴⁵ The thiazole orange groups were installed to be used for DNA-gated photochromism, but are also very likely to be involved in binding to the backbone. However, intercalation is not the only binding mode for **12**. At different **12**/DNA ratios a different binding mode was observed by CD spectroscopy. While there is no conclusive evidence for the nature of this second binding mode, a dimer formation in the minor groove appears most likely. The conditions under which either of the binding modes is preferred are sequence-dependent.

Merocyanine, the open form of spiro-switches, is a planar aromatic molecule and may therefore be applied as an intercalator. Andréasson and co-workers designed several substituted spiropyranes for this application.¹⁴⁶ Of these, the authors selected spiropyran **s13** (Fig. 19c) for further studies, because it displayed three different modes of binding.¹⁴⁷ The closed form **s13** does not bind, or only very weakly. The open form, merocyanine **m13**, binds relatively weakly, most likely at the outside of the helix ($K = 9.9 \times 10^2 \text{ M}^{-1}$). Upon protonation to **m13H**⁺, which occurs upon addition to DNA, depending on pH value, binding is increased 35-fold to $3.4 \times 10^4 \text{ M}^{-1}$ and the binding mode changes to intercalation. While the relative increase of binding constant is impressive, these binding constants are all relatively low compared to established DNA intercalators.¹⁴⁸

Ihmels, Thomas and co-workers attempted to increase the binding constant by incorporating *n*-methylated phenanthroline, a known intercalator, in a spirooxazine to create switch **14** (Fig. 19d).¹⁴⁹ However, the results were similar to the work of Andréasson, with no significant binding for the spiropyran form and a binding constant of $2.6 \times 10^4 \text{ M}^{-1}$ for the merocyanine form. Similar to most spirooxazines, protonation of the merocyanine lead to immediate ring closure.

Paramonov, Fedorova and co-workers attempted to use a new type of switch for intercalation in oligonucleotides and studied a chromene derivative.¹⁵⁰ Due to poor solubility and an extremely short lifetime of the open form, this compound does not seem promising for further application. Two amine

Fig. 19 Structures of photoswitches: (a) AzoDiGua,¹⁴⁴ (b) thiazole orange modified diarylethene **12**,¹⁴⁵ (c) spiropyran **s13** and the corresponding merocyanine form **m13**,^{146,147} (d) spirooxazine **s14** and its open form **m14**.¹⁴⁹

functionalized azobenzenes were used by the group of Matczyszyn for intercalation experiments.¹⁵¹ CD experiments suggest that switching of the azobenzene could induce structural changes in the DNA helix.

DNA/RNA condensation is the change of the macrostructure of an oligonucleotide from worm-like coils to a more compacted state. In this state, the oligonucleotide is more resistant to nucleases and may potentially be transported into the cell *via* endocytosis.¹⁵² However, in the condensed state nuclear uptake is reduced. Therefore, external control of oligonucleotide condensation would greatly improve the applicability, allowing for reversible cycles of condensation for cell membrane crossing and expansion to enter the nucleus. Condensation can be achieved by reducing electrostatic repulsion between strands and/or creating unfavourable interactions between the oligonucleotide and the solvent.¹⁵³ There are many different methods to achieve these effects, such as multivalent cations,¹⁵⁴ neutral polymers in combination with monovalent salts¹⁵⁵ and cationic surfactants.¹⁵⁶ AzoTAB (an azobenzene trimethylammonium bromide surfactant, Fig. 20a) is an extensively studied photo-switchable compacting agent.⁴³ By photoisomerization, the hydrophobicity can be altered, leading to a much more effective condensation for the hydrophobic *trans* AzoTAB than for the more hydrophilic *cis* AzoTAB. Recently, some variations of AzoTAB were investigated. Zakrevskyy, Santer and co-workers performed extensive studies on the effects of varying tail length,^{157–160} while Venancio-Marques, Baigl and co-workers studied polycationic variations (Fig. 20b).¹⁶¹ Research was exclusively limited to

Fig. 20 Structures of 3 azobenzene based photoswitchable condensation agents.

cationic surfactants; the bolaamphiphiles AzoEt, AzoPr and AzoBu were investigated by Zinchenko, Tanahashi and Murata (Fig. 20c)¹⁶² and Li, Liu and co-workers developed an azobenzene-containing polycationic polymer.¹⁶³ The results are discussed in the following sections.

Electrostatic interactions may also be used for oligonucleotide binding without causing compaction. Mammana, Feringa and co-workers presented a diarylethene switch **15**,

Fig. 21 Structure of photoswitches: (a) diarylethene **15**,¹²⁹ (b) azobenzene **16a–d**.¹⁶⁴

functionalized with two primary amines, which are readily protonated under physiological conditions (Fig. 21a).¹²⁹ Although this switch is structurally very similar to AzoDiGua (Fig. 19a), no intercalation could be demonstrated and binding appears to occur entirely through nonspecific electrostatic interactions. As a result, only a small or no difference (depending on sequence) between the binding constants of the open and closed forms could be determined.

Xing, You and co-workers developed azobenzene-containing dications **16a–c** for photoregulation of G-quadruplexes (Fig. 21b).¹⁶⁴ While the results are interesting (*vide infra*), the binding mode of the dication remains unclear. In the authors' earlier report on a photoswitchable quadruplex regulator with a very similar design (**16d**), computational results are presented.¹⁶⁵ However, at the start of these computations, the compound is manually docked in a specific position without a rationalization of the choice of placement. It therefore seems that the explanation regarding the binding mode is not conclusively supported at present.

6.2 Photochemistry of photoswitches complexed to oligonucleotides

The photochemistry of photoswitches noncovalently bound to oligonucleotides is highly dependent on the binding interaction. Most switches reported in this paragraph bind *via* nonspecific electrostatic interactions. As a result, these switches reside outside the duplex and have ample free volume for isomerization. Therefore, it is generally assumed that the switching behaviour is unaltered compared to solution.

Spiro compounds **s13** and **s14** (Fig. 19c and d) do not bind to oligonucleotides, but their open forms **m13** and **m14** do.^{147,149} For switch **14**, the open/closed ratios of the photostationary states were not determined. However, when **m14** was irradiated with 590 nm light in the presence of calf thymus DNA, the characteristic merocyanine absorption around 575 nm almost completely disappeared. Irradiation with UV light ($\lambda = 350$ nm),

Fig. 22 CD and LD spectra of AzoDiGua in the presence of calf thymus DNA (50 μ M (phosphate groups) in 10 mM phosphate buffer, pH = 7.0) at AzoDiGua : nucleobase ratios of 0 (black line), 0.5 (red) and 2 (blue), with and without UV irradiation. Dashed lines indicate the absorption maxima of *trans* AzoDiGua (without irradiation) and *cis* AzoDiGua (with irradiation). Reproduced with permission from ref. 144. Copyright 2016, American Chemical Society.

caused partial regeneration of the absorption band. Circular dichroism studies showed that after this cycle the complex is regained and the DNA is undamaged. Spiropyran **s13** was also switched in the presence of dye-modified DNA. Again, the PSS was not reported, but a series of switching cycles was performed. The complex undergoes $\sim 40\%$ degradation after 10 cycles. This degradation appears to be mainly due to photocleavage of the linker between the DNA and the dye, while the stability of the spiropyran under these conditions is unknown.

As mentioned previously, linear dichroism (LD) and circular dichroism (CD) spectroscopies are useful tools for elucidating the nature of binding interactions between oligonucleotides and small molecules. These methods are particularly well-described in the publication regarding AzoDiGua (Fig. 19a) from the group of Baigl (Fig. 22).¹⁴⁴ CD spectroscopy shows that binding to DNA occurs for both isomers, since there is in both cases a signal in the absorption range of the azobenzene ($\lambda = 300\text{--}450$ nm). In the CD spectrum of DNA-bound *trans* AzoDiGua it can clearly be observed that the binding mode depends on the switch:DNA ratio. After UV irradiation however, the shape of the spectrum is similar for both ratios, indicating that *cis* AzoDiGua binds to DNA in one major binding mode. LD spectroscopy reveals more details of the exact binding mode, since it is known that LD signals of complexes of DNA bases with intercalators are negative, while groove binders cause a weakly positive absorption.¹⁶⁶ The LD spectrum of *trans* AzoDiGua shows a clear negative signal around the absorption maximum of the switch. Upon irradiation with UV light, this band significantly decreases, yet no new absorptions appear at the absorption maxima of the *cis* isomer. These data indicate

that part of the switch remains intercalated in the *trans* configuration, while a part isomerizes and is expelled from the duplex. However, the negative CD signal around 450 nm indicates that the expelled *cis* isomer remains bound to the DNA *via* nonspecific electrostatic interactions.

Diarylethene switch **12** was modified with thiazole orange groups to build a gated photochromic device (Fig. 19b).¹⁴⁵ However, **12** cannot be switched in an aqueous environment and its fluorescence is quenched, presumably due to a folded state induced by strong interaction between the thiazole moieties. As mentioned previously, several different binding modes for **12** were identified. In all of them fluorescence was restored, but to a different degree. Additionally, the switching ability of **12** was found to be dependent on the binding mode. Therefore, the switch exhibits a type of gated photochromism, where the DNA acts as a trigger to control photochemical properties, and the photochromic process itself reflects the binding mode to the DNA.

6.3 Influence of supramolecular binding of photoswitches on duplex stability

Most switches discussed in this paragraph bind to the outside of the oligonucleotide helix. While an influence on duplex stability may not be ruled *a priori*, melting temperature studies are usually not performed as exemplified by the series of spiro compounds synthesized by the groups of Andréasson^{146,147} and Ihmels,¹⁴⁹ which are intercalators in their merocyanine forms and may therefore influence duplex stability. Since these spiro switches all exhibit excellent photoswitchable properties, future research into the effect of switching on duplex stability would be a welcome addition. The only recent attempt at influencing duplex stability is the use of AzoDiGua intercalator (Fig. 19a) presented by Bergen, Baigl and co-workers.¹⁴⁴ The *trans* isomer of AzoDiGua was shown to significantly increase the T_m of both > 10 kbp calf thymus DNA ($T_m = +22$ °C) and 10 bp oligonucleotides ($T_m = +14$ – 15 °C). Isomerization caused a 4–13 °C decrease of the T_m , depending on sequence, buffer and AzoDiGua:DNA ratio. For a 33 bp self-complementary DNA hairpin, a ΔT_m of 18 °C was measured. The hairpin disassociation upon switch isomerization was followed by FRET and proven to be almost quantitative.

6.4 Oligonucleotide condensation

AzoTAB (Fig. 20a) is an extensively studied oligonucleotide compaction agent. The group of Baigl previously proved that AzoTAB could be used for reversible inhibition of RNA production, leading to a photoreversible control of GFP production.¹⁶⁷ This work was recently followed up by a publication in which the authors show that AzoTAB can also be used for gene silencing, parallel to gene silencing by small RNAs, creating an orthogonally triggered system (Fig. 23).¹⁶⁸ Condensation by AzoTAB is not sequence-specific. However, a measure of selectivity could be induced by silencing several mRNAs with small RNAs. Condensation with AzoTAB caused all gene expression to be silenced under dark conditions, while UV irradiation induced synthesis of only those proteins that were not expressed by the small RNA-silenced genes. Therefore, using this method, two orthogonal triggers, *i.e.* UV light and small RNAs, are introduced

Fig. 23 Gene silencing by small RNA and/or AzoTAB. In presence of AzoTAB, all mRNA's are compacted and gene expression is silenced. UV irradiation causes decompaction, after which some genes are no longer silenced, and some still are being silenced due to small RNA complexation. Reproduced with permission from ref. 168. Copyright 2011, American Chemical Society.

in the process of gene expression, leading to a valuable increase of the level of control over such processes.

Zakrevskyy, Santer and co-workers published a series of papers on the exploration of the tail length of AzoTAB, based on findings of Diguët, Baigl and co-workers,¹⁶⁹ who showed that compaction is more efficient when the hydrophobic tail is longer. Therefore, Zakrevskyy *et al.* extended the aliphatic chain between the hydrophilic head group and the azobenzene to 6 carbons, while the ethoxy group on the other side of the azobenzene was exchanged for a butyl group.¹⁵⁷ These modification improved the binding efficiency between the surfactant and DNA. DNA condensation by addition of the *trans* azobenzene surfactant was studied at 50 μM concentration in solution of calf thymus DNA. At azobenzene:DNA charge ratios Z of less than 1.2, the DNA was found in solution in an extended coil shape. Increase of surfactant concentration to $1.2 < Z < 2.4$ caused precipitation of compacted DNA globules. At higher charge ratios, the surfactant starts to form a second layer around the globule, causing the formation of stable colloids. It was also demonstrated that the globules are predominantly triggered by the *trans* isomer, and that isomerization to the *cis* isomer leads to an effective decrease of Z , allowing for photoreversible DNA condensation at certain charge ratios. A series of follow-up publications showed that the binding efficiency could be increased more by a longer linker¹⁵⁹ and decreased by a high salt concentration.¹⁵⁸ Extensive phase diagrams were constructed of the interaction between DNA and the surfactants under various conditions.¹⁶⁰

Polyamines are natural compaction agents. Since a higher charge ratio leads to a more efficient condensation, polycationic surfactants may be used to achieve compaction at low concentration. Venancio-Marques, Baigl and co-workers developed several photosensitive polyamines, the most effective of which is AzoTren (Fig. 20b).¹⁶¹ Using studies on concentration-dependence it was shown that at a 0.1 μM T4 DNA (166 kbps) phosphate concentration, 5 μM *trans* AzoTren causes condensation, whereas 5 μM *cis* isomer does not. Using AFM imaging, the gradual condensation of DNA by an increasing concentration of AzoTren was visualized, as was the subsequent unfolding by UV irradiation (Fig. 24). In this example shorter (4.5 kbps) DNA was used in 2 μM phosphate concentration, and 5 μM AzoTren was found to be sufficient for photoreversible condensation. Comparison with AzoTAB showed about a 100-fold increase in condensation efficiency.

Fig. 24 AFM study of 4.5 kbps DNA condensation using AzoTren. Images A to E show DNA condensation with an increasing concentration of AzoTren (0, 0.5, 2, 4 and 5 μM , respectively). Images E to H show the gradual unfolding of these globules by irradiation with $\lambda = 365$ nm light (for 0, 30, 60 and 180 s, respectively). DNA concentration in phosphate groups is 2 μM (10 mM Tris-HCl, pH = 7.4). Reproduced with permission from ref. 161. Copyright 2014, American Chemical Society.

Photoreversible DNA compaction was also achieved by Zinchenko, Tanahashi and Murata using bolaamphiphilic azobenzenes diAzoEt, diAzoPr and diAzoBu (Fig. 20c).¹⁶² Concentrations required were up to 100 times lower than for AzoTAB. However, due to the nature of the switch, the proposed mechanism is entirely different. For these dicationic azobenzenes, the *cis* isomer is the stronger condensation agent. The authors hypothesize that *cis* diAzoEt can interact with adjacent phosphates on the backbone. The minimum possible distance between the ammonium groups in *trans* diAzoEt is too large for binding adjacent phosphates, leading to a much less effective charge neutralization. For diAzoPr and diAzoBu, binding of adjacent phosphate groups is possible in the *trans* isomer. However, the bulky shape of the *trans* azobenzene makes binding of a second switch immediately adjacent to the first impossible, again leading to less efficient charge neutralization (Fig. 25). This publication describes the only example of photoreversible DNA condensation where the *cis* isomer induces compactations instead of the *trans*. The half-life of the *cis* isomers is not reported in this publication. However, this system could potentially be used to induce slow thermal decompaction for *in vivo* applications.

Li, Liu and co-workers attempted to use an azobenzene-containing polycationic polymer for photoreversible pDNA condensation.¹⁶³ Photoisomerization efficiency in the polymer was not quantified, but was clearly lower than for regular azobenzene, which might be explained by the decrease in free

Fig. 25 Scheme illustrating the less effective backbone binding of *trans* diAzoPr and diAzoBu. In the *cis* isomer, the loop between the ammonium groups is smaller and neighbouring binding can occur. Reproduced with permission from ref. 162. Copyright 2012, Wiley-VHC.

volume available.^{170,171} Condensation of pDNA with these polymers was successful, and UV irradiation caused some degree of decompaction. Gene transfection of the pDNA/azo-polymer complexes was shown to be more efficient than that of the DNA/unmodified polymer, but intercellular decompaction of the complexes by UV-light irradiation was only minimal. Although the photoreversibility of this system is low, the authors show the first example of through-membrane transport of DNA complexed to an azobenzene containing condensation agent.

6.5 Other applications for molecular photoswitches complexed to oligonucleotides

Spiropyran intercalator **s13** (Fig. 19c) was used for the photo-control of energy transfer between two dyes attached to DNA.¹⁴⁷

Fig. 26 The three different structures of human telomeric G-quadruplexes. Reproduced with permission from ref. 164. Copyright 2011, Royal Society of Chemistry.

Pacific Blue (PB) and Alexa488 (A488), an established donor-acceptor pair for FRET, were tethered on opposite ends of a 20 bp dsDNA. Addition of **s13** does not lead to a significant change in the FRET process. However, the absorption spectrum of the open form **m13** exhibits a significant overlap with the emission spectrum of PB. Upon irradiation of the mixture, **m13** is formed, which intercalates and thus interferes with the FRET. The authors show that the process is reversible and note that by tuning UV intensity and exposure time, the FRET quenching can be tuned to intermediate values.

Xing, Zhou and co-workers present a series of dicationic azobenzenes **16a–c** (Fig. 21b) that can be used to regulate the conformation of telomeric G-quadruplexes (G4A's).¹⁶⁴ Human telomeric G4A's are involved in gene expression and telomerase activity, and are therefore of great interest. The three azobenzenes were based on a previous design (**16d**) which could be used to induce photoregulated folding and stretching of G4A's, but could not be used in the presence of metal ions.¹⁶⁵ Human telomeric G-quadruplexes exist in three different conformations: parallel, anti-parallel and hybrid (Fig. 26), which can be distinguished by CD spectroscopy. The three *trans* azobenzenes were all shown to induce G4A formation; a parallel conformation was formed upon addition of **16b** and **16c**, while **16a** induces antiparallel G4A formation. Upon switching to the *cis* isomer, in all cases an unfolding of the quadruplexes was observed. Hybrid-type quadruplex formation of the telomere was observed under K^+ -rich conditions. However, addition of **16a**, **16b** or **16c** caused a refolding into an antiparallel conformation. Irradiation of the *cis* isomer regenerated the hybrid-type G4A's. This supramolecular approach provides a possible future opportunity for control of G4A's in a biological setting.

6.6 Concluding remarks

Noncovalent interactions between photoswitches and oligonucleotides provide the opportunity to regulate nucleic acid structure and function in an elegant way. Since no modification of an oligonucleotide is required, this method is attractive from a synthetic point of view. Additionally, the need for covalently attaching a modified oligonucleotide to a biomolecule is eliminated, which may be attractive for *in vivo* applications. Using noncovalent intercalators, large differences in stability of oligonucleotide duplexes can be induced, making this method a real

competitor for the more established method of introduction of photoswitches as nucleoside surrogates. Considering binding modes outside of the duplex, photoswitchable DNA condensation is of particular interest. Especially the possibility of decompacting oligonucleotides at a specific location (such as inside the nucleus or another specific location in the cell) can offer a real improvement to the field. In addition, regulating G-quadruplexes might offer a viable approach to regulating gene expression.

7 End-capping of oligonucleotides with molecular photoswitches

The covalent attachment of molecular photoswitches to the end of oligonucleotide strands (Fig. 1f) can be performed using any of the covalent methods of photoswitch inclusion discussed in this review. Due to the position of the switch relative to the oligonucleotide (at the end instead of in the middle), the switch is not likely to intercalate or reside in a groove. Therefore, switching of the photochromic group does not result in a change in oligonucleotide structure or stability, but is instead often utilized in supramolecular interactions. This leads to entirely different possible applications than for the other methods of switch inclusion discussed above, and consequently end-capping is discussed separately.

Ogasawara and Maeda used a photochromic nucleotide as a 5'-endcap for GFP mRNA.¹⁷² This nucleotide consists of a guanine–stilbene hybrid (Fig. 27). The authors showed that for the most effective cap, translation was significantly inhibited by the *trans*-photochromic nucleotide. Isomerization to the *cis* isomer lead to a 26-fold increase of translation, to an efficiency comparable with normal-capped RNA. This difference is attributed to the shape of the active site of the eIF4E protein, which directs the ribosome to the RNA cap, where translation begins. The authors also report molecular modelling studies which support this explanation.

The azobenzene/cyclodextrin complex is an established host–guest system and was recently utilized for the controlled release of DNA in living cells.¹⁷³ Gold nanoparticles were functionalized with mercapto- β -cyclodextrin moieties, while single stranded DNA was end-capped with azobenzene. Because

Fig. 27 Reversible photoregulation of translation. The structure of both isomers of the photochromic nucleotide-capped RNA is shown. The *trans* isomer inhibits translation, whereas for the *cis* isomer the efficiency is unaltered compared to normal-capped RNA. Adapted with permission from ref. 172. Copyright 2011, Elsevier Ltd.

the binding constant of *cis* azobenzenes is much lower than of *trans* azobenzenes, this system could be used for photo-triggered release of the functionalized DNA from the cyclodextrin. The release process was rather slow (>1 h), and it was shown that HeLa cells were prone to massive cell death under these irradiation conditions. The process could be significantly sped up by the addition of ferrocene, a competitive guest molecule, which did entirely inhibit reformation of the azobenzene/cyclodextrin complex under visible light conditions. Some release of azobenzene-modified DNA was also observed in HeLa cells incubated with the complex.

Micelles are promising candidates for drug delivery and tumour targeting,¹⁷⁴ and stimulus-responsive self-assembly is of particular interest. Gu, Brittain and co-workers have developed a DNA-azobenzene conjugate which can be used for photoregulated micelle formation.¹⁷⁵ The azobenzene end-caps aggregate in the *trans* configuration, while the *cis* conjugates are more hydrophilic. Upon irradiation with UV light to form the *cis* isomers, no complete micelle disassociation was observed. However, the micelles formed by the *cis* conjugates were much smaller and the percentage of unassembled DNA-azobenzenes was increased. While this concept is certainly intriguing, no application can realistically be envisioned until full photo-triggered micelle dissociation can be demonstrated.

7.1 Concluding remarks

Noncovalent interactions between an end-capping photoswitch and the nucleic acid are minimal, and therefore the photoswitch

is free to engage in self-aggregation or complexation to a host molecule. This potential for reversible supramolecular interactions of a moiety covalently attached to an oligonucleotide opens up a range of new applications, which are not accessible with any other of the methods discussed in this review. Since the stability of end-capped duplexes cannot be influenced using the photoswitch there is not a major interest for end-capping, but due to its versatility this method is a valuable addition to the chemists' toolbox.

8 Summary and outlook

The use of photoswitches for reversible photoregulation of oligonucleotide structure and function has in recent years moved from a mere curiosity to a potentially powerful method for oligonucleotide control and has already found real, promising applications. Although six different strategies for switch incorporation in oligonucleotides are identified in this review, the supramolecular approach (Section 6) and the inclusion of photoswitches as nucleoside surrogates (Section 2) stand out both regarding results and number of studies. Especially the incorporation of photochromic units in nucleosides has been extensively studied using computational methods and experimental linker optimization. The ability to modulate duplex stability using intercalating switches as nucleoside surrogates has been refined and has led to reversible control of key biological processes such as transcription and DNA cleavage. In the application of DNA as a programmable material, reversible duplex formation in the form of photoresponsive sticky ends has been utilized for the reversible bottom up construction of larger structures.

The use of noncovalent interactions between molecular photoswitches and oligonucleotides (Section 6) has the considerable advantage of not requiring a synthetically challenging modification to the oligonucleotide. Relatively large changes in stability can be achieved using noncovalent intercalators, but the most promising application of this method lies in photo-reversible oligonucleotide condensation. Reversible condensation has been achieved using several different photochromic surfactants and polycations. Through-membrane transport of the globular state and subsequent light-controlled unfolding is eagerly awaited.

Reversible duplex destabilization is the preferred method of regulating oligonucleotide structure and function, and is most successfully achieved using photoswitches as nucleoside surrogates (Section 2). Therefore, various approaches including photochromic nucleosides, photochromic backbone linkers, photochromic end-caps and photoswitches attached to nucleosides have been used less in recent years. However, as each of these methods can provide a unique advantage, such as hairpin regulation, placement of the switch in the major groove or supramolecular interactions with other biomolecules, a more specialized application for each of these methods in the coming years is expected.

Over the course of twenty years of studies towards photoregulation of oligonucleotides, azobenzene keeps surfacing as the ideal photoswitch. In recent years, spiropyran, diarylethenes

and stilbenes have been incorporated into oligonucleotides using various methods, but results rarely surpass those obtained for azobenzenes. Although the search for new and efficient photoswitches for oligonucleotide photoregulation continues, it is likely that future application will be exceedingly limited to azobenzenes. As impressive as the development in this field in the previous years has been, some important challenges remain. The most urgent of these, considering future *in vivo* applications, is the wavelength range used for irradiation. Wavelengths below 320 nm are easily avoided, but DNA damage may also arise from irradiation in the 320–400 nm regime and UV light is scattered and absorbed by the skin. Additionally, light absorption by haemoglobin and water limits the ideal window of irradiation to 650–900 nm.⁸⁸ Recently, a range of new and modified switches has been reported that are switchable with visible light.^{10,23,176–179} Incorporation of these switches in an oligonucleotide is eagerly awaited.

Cytotoxicity and photoswitch stability are important factors to consider. Azobenzenes can be prone to reduction under physiological conditions.¹⁸⁰ Woolley and co-workers demonstrated that photoswitching ability of their red-light switchable azobenzenes in developing zebrafish was retained for days.^{26,181} This is an important observation as such resistant photoswitches are required for future *in vivo* applications.

Photoregulation of biological processes is highly dependent on transmembrane transport of photoresponsive agent. This barrier will have to be overcome, or even better, be subject to photoregulation itself. Important steps in this direction are being made using photoresponsive condensation agents (Section 6.4). If photoresponsive oligonucleotides are to be delivered to the cell using photoresponsive condensation agents, orthogonal photochemistry is required. Such systems may be designed using visible light and/or infra-red switches (*vide supra*) or multiphotochromic molecular systems.^{182,183}

Precise regulation of biomolecular processes, ranging from individual oligonucleotides and proteins to cellular networks, takes advantage of responsive functions at the molecular level and is one of the key challenges for chemical biology and future medicine. Although various triggers are extensively investigated, light remains an unsurpassed external regulation tool due to the combination of high spatiotemporal control and minimal tissue damage. The recent rise to prominence of the field of photopharmacology shows an increasing scientific interest in photoregulation of cellular systems, as well as its relevance for modern medicine. In the past few years synthetic procedures have been optimized and the success of the first *in vitro* studies make us believe that the road to real interference with biological processes stands wide open. Photoregulation of oligonucleotide structure and function offers particularly fascinating opportunities to control a myriad of biological processes, as well as nano-based materials. Bringing this exciting field to the next level of sophistication will require a combination of various expertises and creative thinking, providing a challenge that will no doubt inspire chemists for years to come.

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