DOI: 10.1002/anie.201208460

Non-natural Translation

Reactions Templated by Nucleic Acids: More Ways to Translate Oligonucleotide-Based Instructions into Emerging Function

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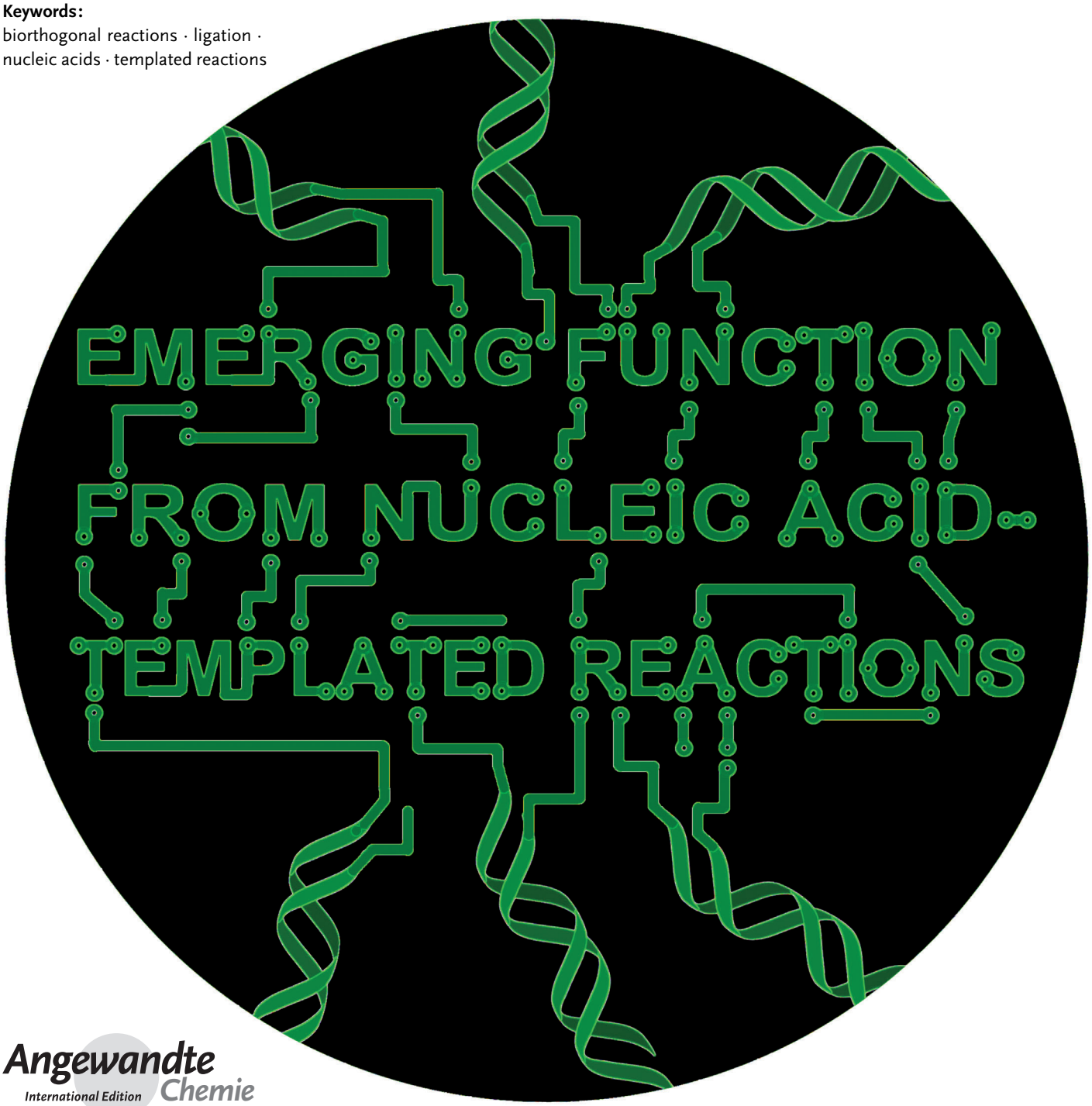
Keywords:
biorthogonal reactions · ligation · nucleic acids · templated reactions



The programmability of oligonucleotide recognition offers an attractive platform to direct the assembly of reactive partners that can engage in chemical reactions. Recently, significant progress has been made in both the breadth of chemical transformations and in the functional output of the reaction. Herein we summarize these recent progresses and illustrate their applications to translate oligonucleotide instructions into functional materials and novel architectures (conductive polymers, nanopatterns, novel oligonucleotide junctions); into fluorescent or bioactive molecule using cellular RNA; to interrogate secondary structures or oligonucelic acids; or a synthetic oligomer.

From the Contents

1. Introduction

Templated chemical reactions have a long history dating back to prebiotic chemistry. Interest in the origin of life[1] led pioneers, such as Gilham[2] and Orgel,[3] to demonstrate that an oligonucleotide could serve as the template for the ligation of complementary strands bearing activated phosphodiesters. Other landmark achievements in nucleic acid-templated reactions include the demonstration that an amide ligation templated by RNA could be achieved between two peptide nucleic acid (PNA) strands;[4] the ligation of a 3′-phosphorothioate with 5′-tosylate;[5] templated formation of a dynamic imine surrogate of the native phosphodiester linkage followed by irreversible trapping through reduction;[6,7] and the demonstration that a peptide acyl chain could be transferred from a thioester at the 5′ position of one strand to the amino group at the 3′ position of an adjacent strand.[8] All of these examples take advantage of the very high level of preorganization that results from the reactions mimicking the geometry of the native bond joining the adjacent strand in the templated reaction. The 21st century marked the development of new nucleic acid-templated reactions that are unrelated to backbone mimicking and that focus on using the template to instruct diverse functions ranging from small-molecule synthesis to nucleic acid sensing and nucleic acid-based assemblies.

The specific bonding of base pairs is the foundation of genetic information. This highly programmable supramolecular system, whether it uses DNA, RNA, or their synthetic analogues, provides a convenient framework with which to engineer assemblies that are capable of translating the underlying instructions encoded within sequences into structures, molecules, and functional outputs (fluorescence, bioactivity, movement, and logic operations, for example). At the structural level, DNA has proven to be an incredibly versatile platform for rationally designing nanoscale structures from the bottom up. The programmable and specific bonding between DNA base pairs has been used in this way to direct the self-assembly of two- and three-dimensional objects.[9–13] To this end, the ability to template reactions leading to novel architectures may become important. In terms of small molecule synthesis, a broad array of reactions have been reported by Liu and co-workers in the context of DNA templated synthesis (DTS) of small molecules and have been reviewed.[14,15] The potential of using templated reactions for RNA and DNA sensing[16–18] has gathered a lot of attention, starting with ligation reactions between two strands labeled with interacting dyes to more recent advances which use a transfer reaction to convert a pro-fluorescent molecule in the presence of catalytic template.

Templated reactions are designed and performed under conditions in which the spontaneous reaction does not proceed until the reactive moieties are organized by a template, thereby increasing their effective concentration and accelerating the rate of reaction. As illustrated in Figure 1, nucleic acid-templated reactions can be broadly divided into three categories: interstrand crosslinking, templated strand ligation, and templated transformation without ligation. This classification reflects important differences in the behavior of the reactions. In interstrand crosslinking, the reaction covalently traps the supramolecular complex and thus requires stoichiometric amounts of both partners. In templated strand ligation, a template aligns the reactive functionalities on two complementary strands, causing the ligation of the fragments, although the resulting duplex can still dissociate. The product tends to have higher affinity for the template than either starting material, but some turnover of the template can be achieved at low conversion through strand displacement or thermal cycling. Finally, in template transformation without ligation, a reaction occurs because of the proximity of the reactive moieties within the supramolecular assembly, although there is no ligation. Thus, this reaction design has

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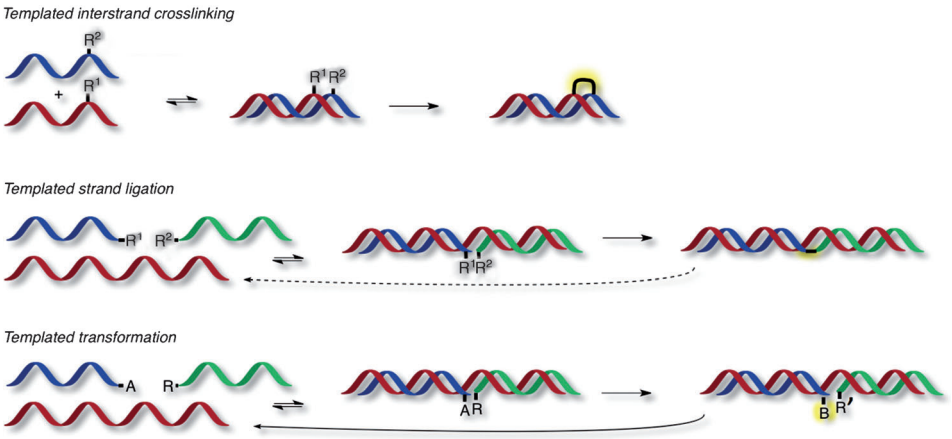

Figure 1. General concept of a templated reaction. Hybridization to a template locally increases the effective molarity of substrates, thereby accelerating the reaction.

the highest potential for turnover and the successful amplification of the nucleic acid instructions. The reactions are generally performed at or below 1–10 μM micromolar concentration. At this concentration, the bimolecular reactions are typically negligible. The rate of the reaction depends on the level of preorganization in the system and the effective concentration achieved within the supramolecular assembly; however, depending on the reaction and experimental conditions, the hybridization step may be rate-limiting.[19]

The aim of this Review is to summarize recent developments in nucleic acid-templated reactions, with an emphasis on the chemical reactions and their applications. Most of the in vitro methods were performed with synthetic DNA using either DNA or PNA[20] probes. Based on the comparable duplex stability between DNA:DNA and DNA:RNA, or PNA:DNA and PNA:RNA, templated reactions generally perform comparably well with RNA templates. Several reactions have now been performed using cellular RNA as the template.

2. Interstrand Crosslinking

Interstrand crosslinking is achieved using a reaction that forms a covalent bond between complementary strands. Early applications used such crosslinking reactions to study RNA conformation and binding. Using a phenyl azide, photocrosslinking was achieved via a short-lived and indiscriminate nitrene electrophile that reacted with nearby nucleobases.[21,22] More recent efforts have focused on reactions with greater selectivity, with the goal of chemoselectively joining complementary strands. As shown in Figure 2, this covalent capture can be achieved either reversibly or irreversibly and triggered by two different external stimuli, namely light and oxidation. Fujimoto and co-workers extensively used photocrosslinking between annealed DNA strands for single nucleotide polymorphism (SNP) genotyping. Their first interstrand photocrosslinking strategy achieved a [2+2] cycloaddition between a *p*-carbamoylvinyl phenol nucleoside (excited by 366 nm light) and an adenosine on the complementary strand (Figure 2.1), thus providing an irreversible link between the two strands.[23] Using a capture probe incorporating the modified nucleoside, which was immobilized on the surface of a glass slide, the analyte strand (labeled with biotin) could be captured on the surface. Using oligonucleotide sequences derived from H-Ras and its oncogenic mutants, they demonstrated that the kinetics of the reaction was highly dependent on a perfectly matched duplex between the immobilized strand and the analyte. Reactions reached 50% conversion after 45 min of irradiation using the perfectly matched duplex, whereas conversion was largely or completely attenuated after the introduction of a point mutation into the duplex. From a diagnostic and analytical perspective, the specificity achieved with the crosslinking procedures surpassed the sensitivity of simple hybridization methods. The same group reported an alternative system that reversibly photocrosslinked a complementary target RNA (Figure 2.2).[24,25] A 3-cyanovinylcarbazole nucleotide surrogate (Figure 2.2) reacted with pyrimidines

Nicolas Winssinger received his BS from Tufts University before joining K. C. Nicolaou at the Scripps Research Institute for his PhD. He remained at Scripps as a postdoctoral fellow in the group of P. G. Schultz. In 2002, he began his independent career at the Institut de Science et Ingénierie Supramoléculaires, University of Strasbourg (formerly University Louis Pasteur) and was later elected to the Insitut Universitaire de France (IUF). In 2012, Winssinger moved to the University of Geneva, where he currently is a professor in the department of organic chemistry. An important theme in this research is the use of oligonucleotides to encode molecules and to program spatial organization and reactions.

Katarzyna Gorska obtained chemistry and biotechnology degrees in 2007 from the University of Warsaw, Poland. She then joined the group of Professor Nicolas Winssinger at the University of Strasbourg as a Boehringer Ingelheim Fonds fellow and completed her PhD in 2011. Her work on RNA visualization and programmable biomolecule release using templated chemistry was recognized with the thesis prize at the University of Strasbourg and by a DSM Science & Technology Award. Currently she is a postdoctoral researcher in the group of Professor Kai Johnsson at École Polytechnique Fédérale de Lausanne in Switzerland.

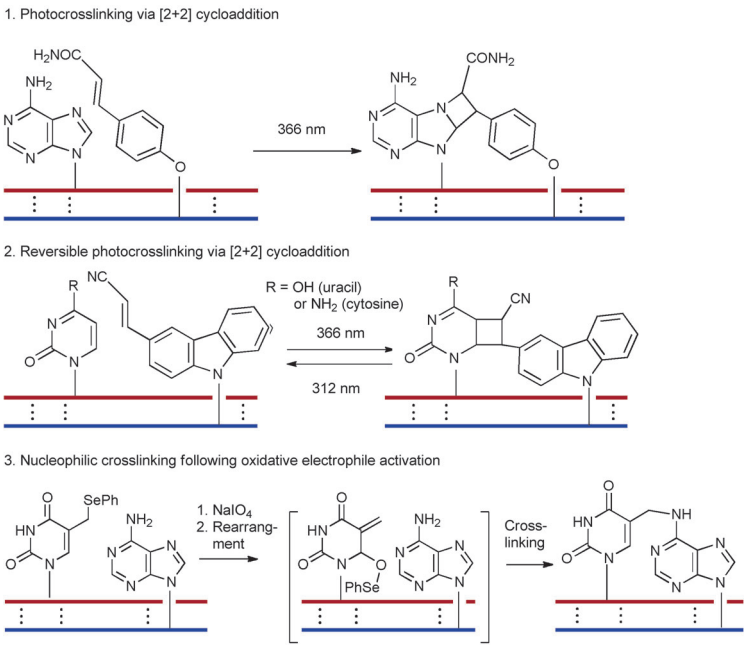

Figure 2. Templated interstrand crosslinking.

on the complementary strand upon excitation at 366 nm, and retrocycloaddition proceeded with 312 nm light. This method was shown to be suitable for capturing a target microRNA (miRNA) on a polymer bead from a complex mixture with high degree of specificity and subsequently releasing it using the retrocycloaddition reaction.[25]

Peng and Greenberg reported an alternative crosslinking strategy (Figure 2.3) using a phenyl selenium derivative of thymidine. Upon mild oxidative treatment ($NaIO_4$), the phenyl selenoxide underwent a [2,3]-sigmatropic rearrangement to reveal a powerful electrophile capable of reacting with an adenosine nucleobase on the complementary strand.[26] The sequence specificity achieved through crosslinking 16 nucleotide probes with their targets was tested in a model based on exon 7 of the p53 gene; in cancer cells, a G→A transition in codon 248 of this exon is often observed. Reactions with perfectly matched and mismatched templates (with mutations two bases away from the expected crosslink) were analyzed using polyacrylamide gel electrophoresis (PAGE). At the optimal reaction temperature (45 °C), 24–214-fold selectivity for a perfect match relative to the mismatch was obtained at more than 20 % overall conversion. Performing the reaction at lower temperature resulted in a higher rate of conversion but the selectivity was compromised. The conditions validated for mismatch sensing were then applied for detection of SNPs in plasmid DNA. Using biotin-tagged DNA, the chemically crosslinked product could be detected in a microplate format using an enzymatic amplification with a sensitivity threshold of 250 fmol. The same procedure was applicable to crosslink two strands within a triplex DNA.[27] This [2,3] selenoxide rearrangement was later reported with templated catalytic photoactivation to generate singlet oxygen ($^1O_2$) as an oxidant (see Figure 11.12 in Section 4.4).

3. Templated Strand Ligation

Templated strand ligation was the first nucleic acid-templated reaction to gain attention because of its implications for the origin of life; this reaction was the focus of the landmark publications discussed in the introduction. A number of reactions with different levels of orthogonality, biocompatibility, and efficiency now have been shown to be suitable for this purpose.

3.1. Photoligations

The use of a photoligation is a natural choice for ligation chemistry because ultraviolet (UV) light can promote [2+2] cycloaddition reactions between pyrimidines. However, the short wavelength (>260 nm) and high intensity required damage nucleic acids and curb the utility of this reaction in templated processes. Taylor and co-workers have replaced a thymidine at the end of a DNA fragment with a thiothymidine and used 366 nm light to enhance a photoreaction between the thiothymidine and a T on an adjacent fragment.[28] Fujimoto's group pioneered the use of 5-vinyldeoxyuridine for photoreversible ligation with an adjacent strand terminating with a pyrimidine that was produced by a [2+2] photoaddition.[29] The use of 5-carboxyvinyldeoxyuridine (Figure 3.1) was found to improve reaction performance (reducing the reac-

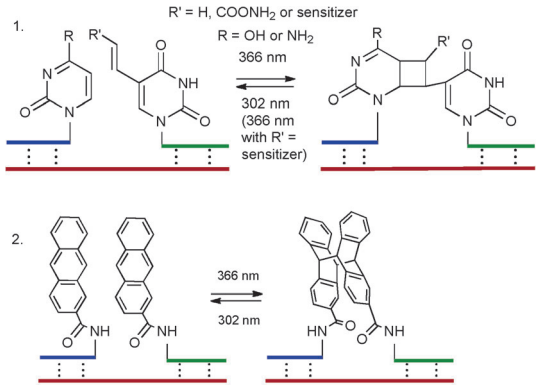

Figure 3. Templated photoligation.

tion time from 6 h to 15 min relative to the 5-vinyldeoxyuridine) and afforded remarkable selectivity for a single nucleotide mismatch in the template (with selectivity increased by up to 10^3-fold). This system was shown to be suitable for both DNA[30,31] and RNA[32] targets, and the reaction performance was maintained if genomic DNA was used instead of a synthetic template.[33] This templated reaction was also performed in a microarray format wherein an analyte nucleic acid templated the reaction between the surface-immobilized strand and the different carboxyvinyl strands labeled with spectrally resolved fluorophores (Cy3 and Cy5) allowing two-

color detection of SNP within the human aldehyde dehydrogenase 2 gene (also in heterozygous sample).[34] More recently, the reaction was used to link multiple fragments with overhangs along a template to generate a comb architecture.[35] A further improvement in the performance of the reaction was achieved with the addition of a sensitizer covalently linked to vinyldeoxyuridine, thereby decreasing the decomposition of the oligonucleotides and making the system reversible with 366 nm light.[36] Jyo and co-workers proposed an alternative method for the templated photoligation of oligodeoxynucleotides using the photodimerization of appended anthracene tags (Figure 3.2). When templated using a perfectly matched DNA strand and with the optimal anthracene geometry, this reaction reached a high rate of conversion within minutes.[37] This system was also shown to be useful for SNP detection. Furthermore, the reaction is compatible with thermocycling and an approximately five-fold signal amplification could be achieved after 10 rounds of thermocycling. A thorough investigation of the fidelity of templated photoligation showed that the yield of the ligation was strongly affected by the position of the mismatch, suggesting that local structure is very important for the performance of the reaction.[38]

3.2. Azide–Alkyne Cycloaddition (The Click Reaction)

The success of copper-catalyzed azide–alkyne cycloaddition (CuAAC) in a broad array of settings[39–42] has inspired several groups to evaluate this reaction in the context of templated reactions. Because the triazole resulting from a CuAAC is an isostere of the native amide bond in PNA, the ability of the reaction to ligate two PNA fragments to produce a surrogate PNA linkage was investigated (Figure 4.1).[43] It was shown that the click linkage 1 produced an oligomer with comparable affinity and specificity to an unmodified PNA, whereas click linker 2 generated an oligomer with reduced affinity. The reaction was found to proceed in a DNA-templated fashion with good sequence specificity. The same reaction was used to join PNA and DNA fragments by a longer linker bridging an abasic site on the complementary strand (Figure 4.2).[44] By incorporating an alkyne on one end and an azide at the other, this strategy can be used to access extended PNA oligomers and without cyclic byproducts. However, a limitation of CuAAC is the fact that the catalyst (copper) can generate reactive oxygen species that result in nucleic acid oxidation reactions. Furthermore, the remaining traces of copper may be problematic for future applications, such as the production of nanodevices or biological applications. A metal-free version of the reaction promoted by the strain of a cycloalkyne substrate[45] was thus adapted for templated conditions[46] and offers an interesting alternative (Figure 4.3), even if the junction that is formed is significantly bulkier, which may affect the resulting nucleic acid structure.

Because of its robustness, the CuAAC ligation has applications both in nucleic acid sensing and in the covalent capture of nucleic acid-based assemblies.[47] Jentzsch and Mokhir reported a naphthaleneimide–alkyne profluorophore

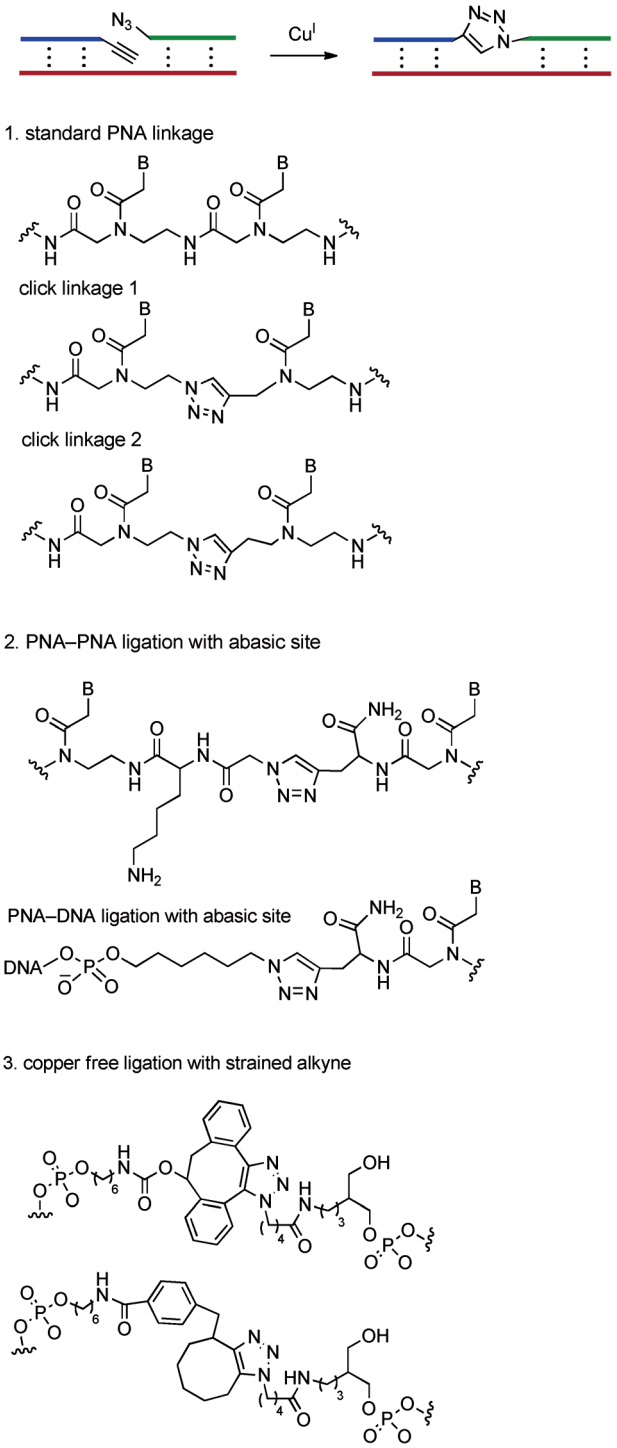

Figure 4. Templated alkyne–azide cycloaddition. 1–3 depicts the reaction product of different templated azide–alkyne reactions.

that is activated after its conversion to a triazole (Figure 5.1). Using DNA fragments derivatized with the alkyne profluorophore and an azide at the 3′ and 5′ ends, respectively, the reaction proceeded in a sequence-specific templated fashion at template concentrations as low as 20 nM.[48] The use of excess tris(3-hydroxypropyltriazolylmethyl)amine ligand helped to prevent the copper-induced degradation of reac-

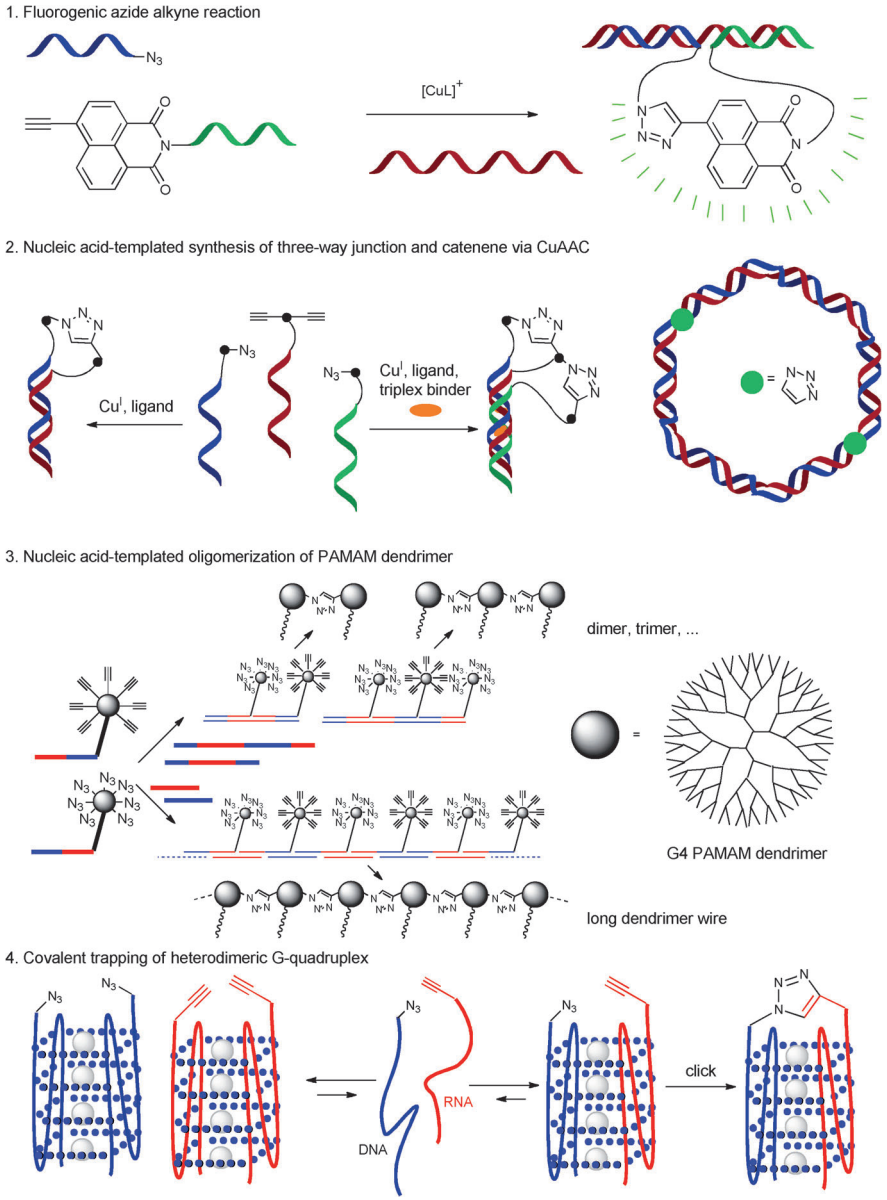

Figure 5. Application of templated CuAAC. PAMAM = poly(amidoamine) dendrimer (4th generation).

tants, but also interfered with the fluorescence readout. Reliable results were obtained after thermal denaturation or copper removal with sodium sulfide.

The construction of sophisticated nanostructures would benefit from structural elements affording junction topologies beyond the simple ligation of two linear fragments. A three-way junction is the simplest example of a branching element. Gothelf and co-workers demonstrated that such a branch point could be obtained between a DNA fragment terminated with two alkynes and two other DNA fragments bearing azides (Figure 5.2). The required ternary assembly was induced using a strong triplex DNA binder (naphthylquinoline) promoting the double CuAAC.[49] In the absence of the triplex DNA binder, ligation occurred strictly between the complementary strands. Generation of catenane structures with multiply entwined strands was also achieved with templated alkyne–azide cycloaddition. The formation of a six-fold entwined double-stranded structure (pseudohexagon) 4 nm in length was demonstrated using the circular oligonucleotide-directed ligation of multiple bifunctional probes (Figure 5.2).[50]

DNA-assisted assembly and the subsequent covalent ligation of more complex functional elements also have been reported. Gothelf's group employed DNA-templated click chemistry for the preparation of organized structures from PAMAM dendrimers (Figure 5.3).[51] DNA-tagged dendrimers functionalized with azides and alkynes were assembled on different DNA templates, including DNA origami, and then crosslinked by CuAAC. The DNA-controlled self-assemblies of alternating azide and alkyne dendrimers were shown to be fast and reliable and should be useful in the production of nanoscale patterns.

Templated CuAAC reactions also were used successfully by Komiyama and co-workers to probe G-quadruplex structures and to obtain information about the different available topologies. To this end, sequences bearing either an alkyne or an azide were assembled under different conditions and exposed to copper catalysts, to probe their spatial proximity and gain information about their assembly (Figure 5.4). This approach led to the discovery that the DNA–RNA-hybrid G-quadruplex structures that have been predicted for human telomeric DNA and RNA sequences are indeed possible.[52]

3.3. Nucleophilic Substitution, Condensation, and Cycloaddition Reactions

Because of their potential implications in prebiotic chemistry, templated nucleophilic displacement and condensation reactions are amongst the first studied templated reactions; they would be required for enzyme-free amplification of genetic material. The pioneering efforts discussed in the introduction inspired the first application of templated ligation for nucleic acid sensing. Kool and co-workers used phosphorothioate–iodide (Figure 6.1) to ligate two strands derivatized with fluorophores that were capable of Förster resonance energy transfer (FRET) following the ligation of

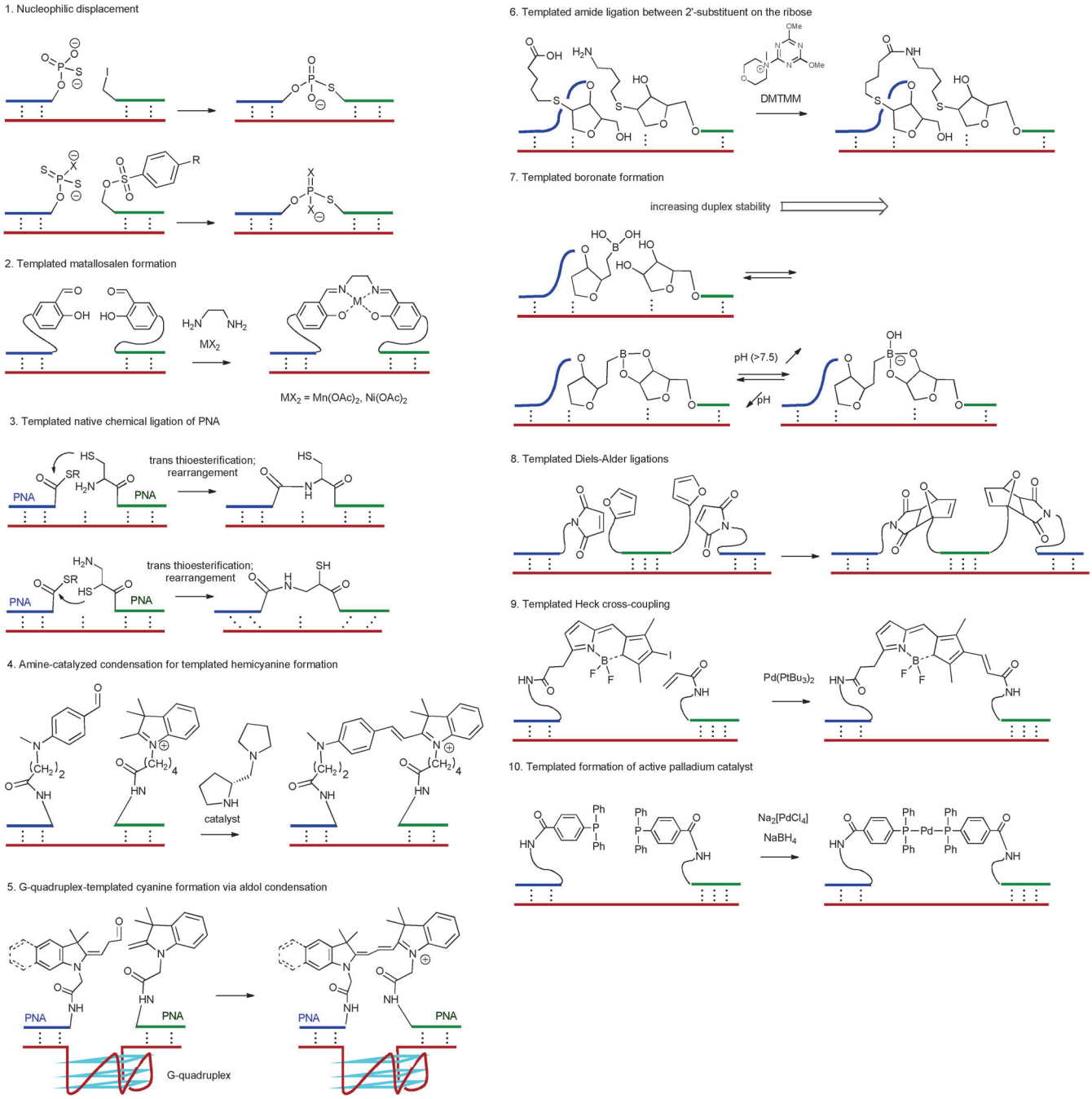

Figure 6. Templated ligation by nucleophilic substitution, condensation, cycloaddition, and cross-coupling.

the DNA fragments.[53] Kool and co-workers later reported that the reaction can be further accelerated by the use of more nucleophilic phosphoroditioate and phosphorotrithioate relatively to the initial phosphoromonothioate nucleophiles, resulting in reduced detection time of ribosomal RNA in bacteria (20 min vs. 1–2 h).[54]

Czlapinski and Sheppard demonstrated efficient nucleic acid-templated metallosalen synthesis using DNA fragments functionalized with 2-salicylaldehydes in the presence of ethylenediamine and Ni^{2+} or Mn^{2+} (Figure 6.2).[55] This pioneering reaction subsequently has been used to synthesize conjugated structures (discussed in Section 3.4, Figure 7).[56]

Seitz and co-workers developed a powerful method to ligate PNA fragments in a templated fashion[57] (Figure 6.3) using native chemical ligation[58] (NCL). A rapid and reversible trans-thioesterification followed by an irreversible S- to N-acyl shift could take place between two PNA fragments bearing a cysteine at the N-terminus and a glycine thioester at the C-terminus, respectively. The newly formed peptide bond provided a linkage geometry that matched one abasic site on the template. The reaction was reported to be fast (55% conversion in 22 min), proceeded with single nucleotide resolution, and could be performed directly from double-stranded DNA (dsDNA) in the presence of additional PNA

fragments that blocked the reannealing of the dsDNA template. The reaction specificity was compromised if the PNA fragments were contiguously hybridized onto the template but performed with comparable efficiency to an enzymatic ligation in the optimal alignment.[59] A further development of this system was reported using isocysteine rather than cysteine at the N-terminus of the PNA in the ligation. The resulting product had a less favorable linkage geometry that aided template turnover compared to the cysteine ligation product (greater than four-fold higher turnover was achieved) without compromising reaction specificity. To simplify the analysis of the ligation product, the two PNA fragments were also labeled with fluorophores yielding a FRET signal.[60] Impressively, greater than 100-fold selectivity was observed between the perfectly matched template and the template with a single mismatch.[61]

Although the near-neutral conditions typically used for hybridization in templated reactions are not conductive to aldol condensations, Huang and Coull reported that hemicyanine dye could be formed from two DNA fragments derivatized with *p*-aminobenzaldehyde and indolinium, respectively, in the presence of a diamine catalyst (Figure 6.4).[62] This reaction is particularly appealing because neither of the starting materials have fluorescent properties whereas the product is highly fluorescent. Thus, the templated reaction can be followed in real time through fluorescence monitoring, which can indicate the presence of a given nucleic acid template. The use of a catalyst with two amines was important to achieve a fast reaction, suggesting that both iminium and base catalysis are involved in the reaction. Conversions of 25–50% were obtained after 2 h using 10 mM catalyst and 200 nM each of template and probes. Importantly, the reaction was shown to be compatible with serum, suggesting that it would be useful in diagnostic settings. In a conceptually related approach, Ladame and co-workers reported a similar strategy to obtain a cyanine dye from a PNA fragment derivatized with a non-fluorogenic aldehyde and an enamine (Figure 6.5). An important distinction is that this reaction did not require an external catalyst to promote the aldolization/condensation. The reaction was templated by a parallel-stranded G-quadruplex DNA in a sequence- and structure-specific manner (the same DNA template did not promote the reaction in the absence of G-quadruplex induction).[63] The use of different indoline derivatives produces cyanine dyes with spectrally resolved fluorescence.

It has been shown by Liu and co-workers that DMTMM (see Figure 6.6 for structure) can be used for DNA-templated amide bond formation in DTS.[19] More recently, Seeman, Canary, and co-workers used this reaction to efficiently ligate (>95% yield) DNA fragments bearing an amine and an acid tethered at the 2′ position of the ribose (Figure 6.6).[64] At room temperature, the reaction proceeded with equal efficiency between adjacent DNA fragments as well as fragments separated by three nucleotides. However, at 4 °C, ligation was restricted to adjacently hybridized DNA fragments. It is worth noting that such a bridging strategy does not engage the terminal 3′ and 5′ positions, allowing them to stay available for the additional modifications that may be necessary to create more complex junctions and thus to form nanostructures.

All previous ligation reactions relied on irreversible transformations. Smietana, Vasseur, and co-workers reported an alternative system that afforded a covalent yet highly reversible templated ligation. This was achieved by replacing the terminal phosphate at the 5′ position with a boronic acid that can form a boronate ester with an adjacent nucleic acid fragment terminating at the 3′ end with a ribose (Figure 6.7). It was shown that the equilibrium of this reaction could be shifted by varying temperature and pH. A more basic pH led to the formation of a hydroxyboronate linkage (rehybridization of boron from sp^2 to sp^3) with higher duplex stability than the corresponding boronate ester.[65] A similar effect was observed in the presence of cyanides. The ligated system was insensitive to a large excess of an external diol (fructose). The responsiveness of boron linkages within nucleic acid strands to external stimuli may be useful in the construction of biologically relevant dynamic systems.[66]

For the ligation of DNA fragments, the Diels–Alder reaction between furan and a maleimide is notable because of its orthogonality to the functionality present in nucleic acids and because of its efficiency. Brown and co-workers reported an extremely fast and efficient templated ligation of oligonucleotide fragments derivatized with these two functionalities (Figure 6.8). Using this method, it was possible to ligate three oligonucleotides simultaneously in a controlled manner; the reaction proceeded within 1 min at room temperature, whereas nontemplated reactions were slow and incomplete.[67]

Liu and co-workers demonstrated that palladium-catalyzed cross-coupling could be performed in a DNA-templated fashion.[68] More recently, Herrmann and co-workers reported an original application of this reaction to modify an iodo-boron-dipyrromethene fluorophore through a Heck reaction, resulting in a higher-fluorescence quantum yield for the product of the Heck coupling (Figure 6.9).[69]

Hermann and co-workers also demonstrated that a DNA template could be used to align phosphine groups and direct the formation of a catalytically active palladium complex (Figure 6.10).[70] The template-directed formation of the catalyst was validated with a deiodination reaction of profluorescent iodo-BODIPY. Impressively, the catalyst could convert nearly 1000 equiv of the profluorophore, thus affording a very powerful signal amplification.

3.4. Application of Templated Ligation

Although it is clear that the condensation reactions discussed can be a powerful addendum in DNA-based nanoassemblies and to translate nucleic acid instructions into defined materials, most of the reports thus far have focused on the methodological aspects of the templated reactions. Gothelf and co-workers reported exciting applications of the metallosalen condensation (Figure 6.2), showing that, for example, molecular modules containing from one to three salicylaldehydes tagged with unique oligonucleotides could be self-assembled by hybridization and then covalently linked following diamine and Mn^{+2} addition to afford a bottom-up assembly of predetermined nanomatrices.[56] More recently, the same chemistry was used to link nanorod

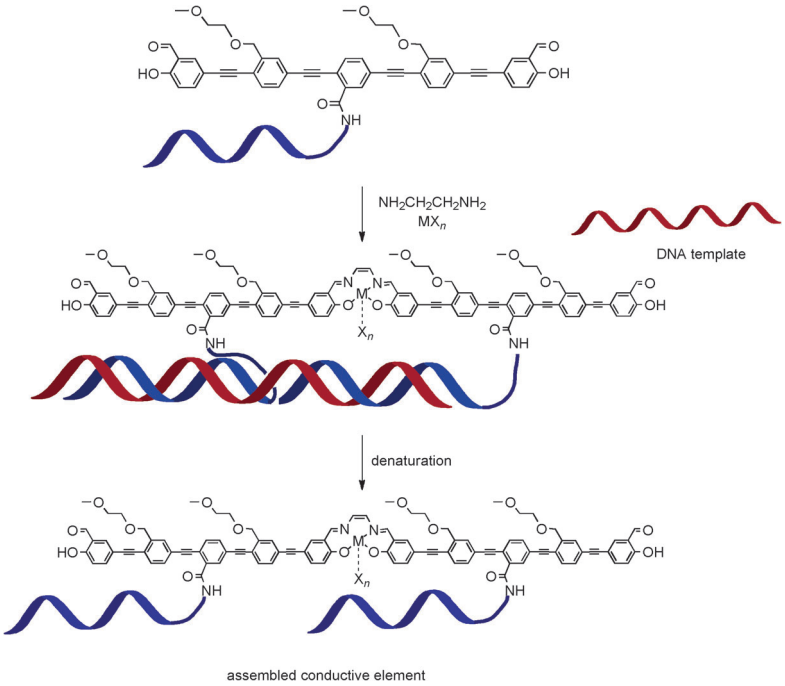

Figure 7. Templated synthesis of conducting polymers by metallosalen condensation.

fragments according to the instructions of a DNA template (Figure 7).[71] The programmability of this approach coupled to the high level of interest in modular monodispersed macromolecular structures makes it appealing in nanoscience.

Displacement reactions were also the first non-enzymatic reactions to be used in nucleic acid sensing. Kool and co-workers used the templated phosphorothioate–iodide ligation chemistry for the detection and identification of both RNA and DNA sequences.[53] The first-generation system, inspired by Lestinger's work,[5] relied on a high level of preorganization to generate a $-OP(O_2^-)S-$ surrogate of the native phosphodiester linkage.[72] Combining ligation specificity with the hybridization specificity of the ligated product afforded a high discrimination against mutations in the template sequence ($>10^4$-fold). In contrast to enzymatic ligation, the ligation was equally productive with DNA and RNA templates. Using different fluorophores yielding a FRET signal on the respective ligation fragments, the reaction could be monitored by fluorescence. The second generation of this method modified the leaving group to a tosylate substitute containing a dabsyl group, which quenched the fluorescence of an adjacent fluorophore. In this way, upon nucleophilic ligation, the quencher was removed and the product became fluorescent.[73] This quenched autoligation (QUAL) probe system was used for direct sequence-specific detection of RNA in bacteria and whole cells,[74,75] allowing ribosomal sequences and β-actin mRNA to be detected in eukaryotic cells[75] (probes were introduced by streptolysin O pore-forming peptides). Furthermore, rapid discrimination of pairs of similar bacterial strains based on a single nucleotide difference in their rRNA was achieved with two-color detection by fluorescence microscopy.[76] A further modification of this system was reported using two quencher residues per fluorophore-tagged DNA probe (Figure 8).[77,78] As a result, the background fluorescence level in such a double-displacement system was decreased together with the influence of the non-templated loss of the quencher owing to its hydrolysis by external nucleophiles. Complete unquenching of the "sandwich probes" containing reactive groups on the 3′ and 5′ termini is possible only after reaction and using two different phosphorothioate probes hybridizing to the same template, up- and downstream. This system was reported to give a specific templated reaction even in the presence of 50 μM dithiothreitol (DTT), whereas the single quencher probe suffered from high background reactions under these conditions. In a clever extension of the templated displacement reaction for nucleic acid sensing, Kool and coworkers used miRNA to template the cyclisation

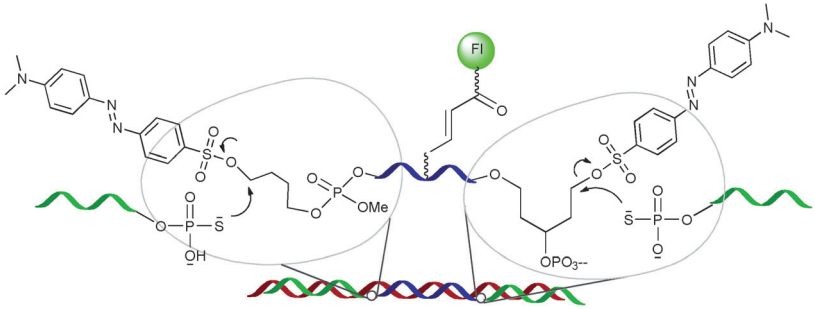

Figure 8. Sandwich-quenched auto-ligation (QUAL) reaction (Fl = fluorescein); the ligation displaces the quencher group (Dabsyl).

of a linear precursor containing a 5′-iodide and 3′-phosphorothioate. The cyclized product could then be amplified through rolling-circle amplification with a polymerase and detected by a fluorogenic templated reaction. miRNA are a class of RNAs that play an important regulatory role in the cell and their abnormal expression has been linked to numerous pathologies. The combination of the enzymatic amplification and templated fluorogenic reaction afforded a detection threshold of 200 pM in solution.[79]

The condensation of fluorophore precursors into a cyanine dye developed by Ladame and discussed in Section 3.3 (Figure 6.5)[63] was originally reported to detect the formation of G-quadruplexes through templated condensation. This strategy was more recently extended to investigate other secondary structures.[80] Beyond the highly abundant double-stranded helical form of DNA, secondary DNA structures have been proposed that may be part of the transcription regulation mechanism.[81] For example, partially self-complementary or palindromic sequences can form stem–loop

hairpins whereas fragments rich in guanine clusters can assemble monovalent cation-stabilized guanine quartets stacked into G-quadruplexes. Theoretical analysis of the genome indicates the enrichment of such sequences with alternative folding potential in important regulatory regions such as gene promoters or replication start sites. Thus, there is a high level of interest in the development of methods for sensing the formation of specific secondary structures in biological systems. Ladame and co-workers have reported a fluorescent readout to discriminate between similar DNA templates differing in their quadruplex-forming ability, using probes targeting the flanking regions of a potential four-stranded assembly. Several patterns of probe binding were tested for sensing the presence of hairpin motifs (Figure 9) and were found to yield a specific fluorescence signal in the

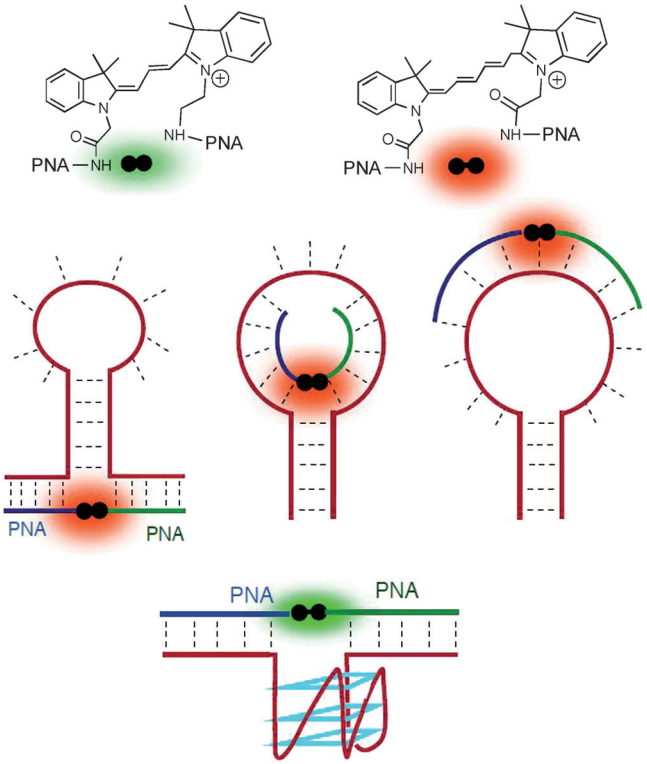

Figure 9. Templated synthesis of cyanine fluorophores using different DNA template architectures.

presence of the DNA loop structure. However, an architecture in which the reaction takes place in the vicinity of the hairpin stem was found to give the highest fluorescence. The reaction was also extended to longer emitting cyanine dye reaching into the near infrared, which offers the possibility of detecting different DNA architectures with a two-color detection system.

3.5. Turnover in Templated Ligation using Thermal Cycling

The examples discussed clearly illustrate that oligonucleotide templates assist the designed reactions by aligning functional groups in close proximity and providing a high effective concentration, thus accelerating conversion. However, an intrinsic limitation of templated ligations is that the products of the different reactions have longer oligonucleotide sequences and tend to have higher affinity for the template than either of the starting material fragments. Amplification of nucleic acid instructions requires template turnover.[82] Although some template turnover may occur at low conversion yield through strand displacement of the product by the starting material fragments, which are still present in large excess, this process is rapidly attenuated as the reaction progresses. Achieving high conversion in a ligation reaction using a catalytic amount of template remains challenging. The simplest approach to achieving catalytic turnover of the template employs thermal cycling to trigger dissociation of the template–product duplex, analogous to polymerase chain reaction (PCR). However, this beneficial turnover must be balanced with the fact that high temperatures may also promote an untemplated reaction. Kool and co-workers investigated the benefit of thermal cycling in the ligation of a 3′-phosphorothioate-5′-iodide probe (13-mer and 7-mer, respectively) and found only a marginal benefit (1.5-fold increase).[53] Abe and Ito used the ligation of phosphorothioate–iodoacetate,[83] which is known to proceed within minutes in a templated fashion, to investigate the benefit of thermal cycling (Figure 10).[84] Although the product of this reaction does result in a slightly longer linker compared to the native linkage, it is not significantly destabilizing. Using RNA templates, the ligation was found to proceed to 70% in 5 s with a stoichiometric template at 35 °C. By carefully optimizing the cycling conditions (10 s ligation at 35 °C and 10 s denaturation at 60 °C; 99 cycles in 100 min), a 60-fold amplification was obtained using 0.1% template (1 nM; 6% conversion), whereas no amplification was observed without thermal cycling. Compared to PCR, these amplifications remain modest because the amplification is not exponential. Nonetheless, thermal cycling could be achieved easily in a regular PCR thermal cycler. Using an equally efficient templated reaction (photodimerization of anthracene-tagged DNA fragments, Figure 3.2), Ihara and co-workers also successfully achieved amplification by thermal cycling. The

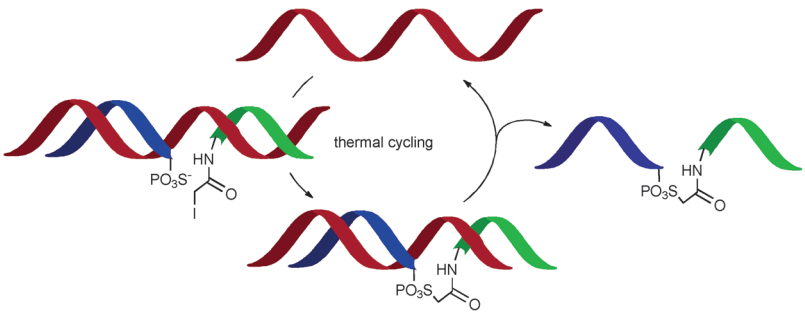

Figure 10. Templated ligation amplified through thermocycling.

template reaction proceeds to near completion following irradiation for 1 min, making it a good candidate for fast cycling. Using 10 cycles of irradiation and denaturation, a five-fold amplification was achieved, whereas no amplification was observed in the absence of thermal cycling.[85] The fast kinetics coupled to the potential amplification makes these methods an interesting alternative to enzymatic ligation strategies, in which efficiency can vary substantially.[86]

4. Templated Transformation without Ligation

Although templated ligations are required for enzyme-free amplification of genetic material, templated transformations are necessary to translate the nucleic acid information into a functional molecule, such as protein synthesis. In pioneering work, Joyce and co-workers reported the templated transfer of a peptide chain from a 5′-thio ester to the 3′-amino group on an adjacent nucleic acid fragment using an acyl transfer reaction.[8] This reaction was inspired by the rapid acyl transfer observed in the native chemical ligation following the trans-thioesterification of cysteine. The templated acyl transfer of a peptide was found to proceed to 70% within 15 h. In a series of landmark publications, Liu and co-workers reported several new reactions in DTS that enable the translation of nucleic acid sequences into small molecules and polymers. A significant difference between nucleic acid-templated transfer reactions and ligation is that the former should be less sensitive to product inhibition, because the product of the reaction does not gain in affinity for the template (Figure 1).

4.1. Templated Hydrolysis and Acyl Transfer

Taylor and Ma reported the first example of a reaction designed to achieve turnover with a nucleic acid template under isothermal conditions. The hydrolysis of a nitrophenyl ester tethered to the 3′-end of an 8-mer DNA fragment by an imidazole tethered to the 5′-end of a 15-mer DNA fragment was achieved in the presence of a catalytic DNA template (Figure 11.1). This reaction was developed as a model system for the hydrolysis of prodrugs in response to a specific mRNA sequence corresponding to a disease state.[87] The reaction afforded 50% conversion after 5 h using 10% template–imidazole conjugate and was found to be sensitive to a single mismatch. Subsequently, the reaction was performed with profluorescent coumarin ester to allow nucleic acid sensing in diagnostic applications.[88] However, such phenolic esters were found to be labile to hydrolases present in plasma, precluding the application of this method in more complex biological settings. Kraemer, Mokhir, and co-workers reported nucleic acid-templated hydrolysis using a metal catalyst (Figure 11.1) inspired by the facile copper-catalyzed hydrolysis of picolinate. In this system, a copper(II) chelating pyridylpyrazolyl is linked to the N-terminus of a PNA fragment and a pyridine-2-carboxylic ester to the C-terminus of a second PNA fragment. The hydrolysis was reported to proceed with catalytic DNA template (20%) and achieved over 80% conversion after 50 min.[89] This approach was found to discriminate amongst templates bearing a single nucleotide mismatch with >100-fold selectivity (better than that of T-4 DNA ligase) with a detection limit down to 10 fmol using single-stranded DNA (ssDNA)[90] and could also be used with dsDNA.[91] An alternative strategy in which the catalyst is trapped in an inactive bidentate complex involving a cyclic probe and then decomplexed (activated) upon hybridization also has been reported. The reaction proceeded under mild conditions with favorable kinetics but with significant background reaction (less than a three-fold difference in response to the presence of a single mismatch).[92]

The templated NCL discussed in Section 3 (Figure 6.6) has been adapted to an acyl-transfer reaction displaying high turnover frequency (Figure 11.2).[93] In this configuration, a PNA strand derivatized with a thioester serves as the acyl donor. Attack of the N-terminal isocysteine residue of an acceptor PNA forms a new thioester with the acyl moiety from a donor PNA probe. This intermediate rapidly rearranges following the S- to N-acyl transfer. The high performance of the reaction has enabled a number of applications in nucleic acid sensing and nucleic acid-triggered bioactive molecule synthesis (see discussion in Section 4.5 and Figure 12). In parallel studies, Stulz and co-workers screened several other nucleophiles along with amines that could participate in acyl-transfer reactions with thioester. Both the thioester and nucleophile were linked to DNA fragments by a fairly long linker (>10 atoms), providing a large degree of freedom to the reagent upon templated alignment. Generally, none of the nucleophiles that were examined afforded more than 20% conversion after 48 h at 38 °C using conditions ranging from pH 6.5 to 9.5, attesting to the importance of the high level of preorganization in the system reported by Joyce and co-workers[8] and to the benefit of the NCL-based strategy. Notably, a moderate acyl transfer (12% yield) was observed at pH 6.5 with a hydrazide, whereas an amine and a hydroxylamine had negligible reactivity under these conditions. This observation can be correlated to the lower pK_a of the hydrazide.[94]

Using more activated acyl donors (DNA-NHS-ester conjugates; Figure 11.3), nucleic acid-templated amide bond formation has been reported recently in multistep DTS (see also Figure 13). The reaction generally afforded more than 75% yield within minutes.[95,96]

4.2. The Templated Wittig Reaction and other Transfer Reactions with Nucleophilic Displacements

DNA-templated Wittig reactions (Figure 11.4) were first reported by Liu and co-workers and have been used extensively in DTS reactions.[68,97,98] More recently, Turberfield, O'Reilly, and co-workers used this reaction in multistep one-pot DNA synthesis (Figure 14.2 and 14.3).[99–101] This reaction has also been used with PNA probes to yield a fluorophore (Figure 14.3).[102]

Marx and co-workers reported a DNA-templated cross-aldol reaction using a DNA strand derivatized with proline

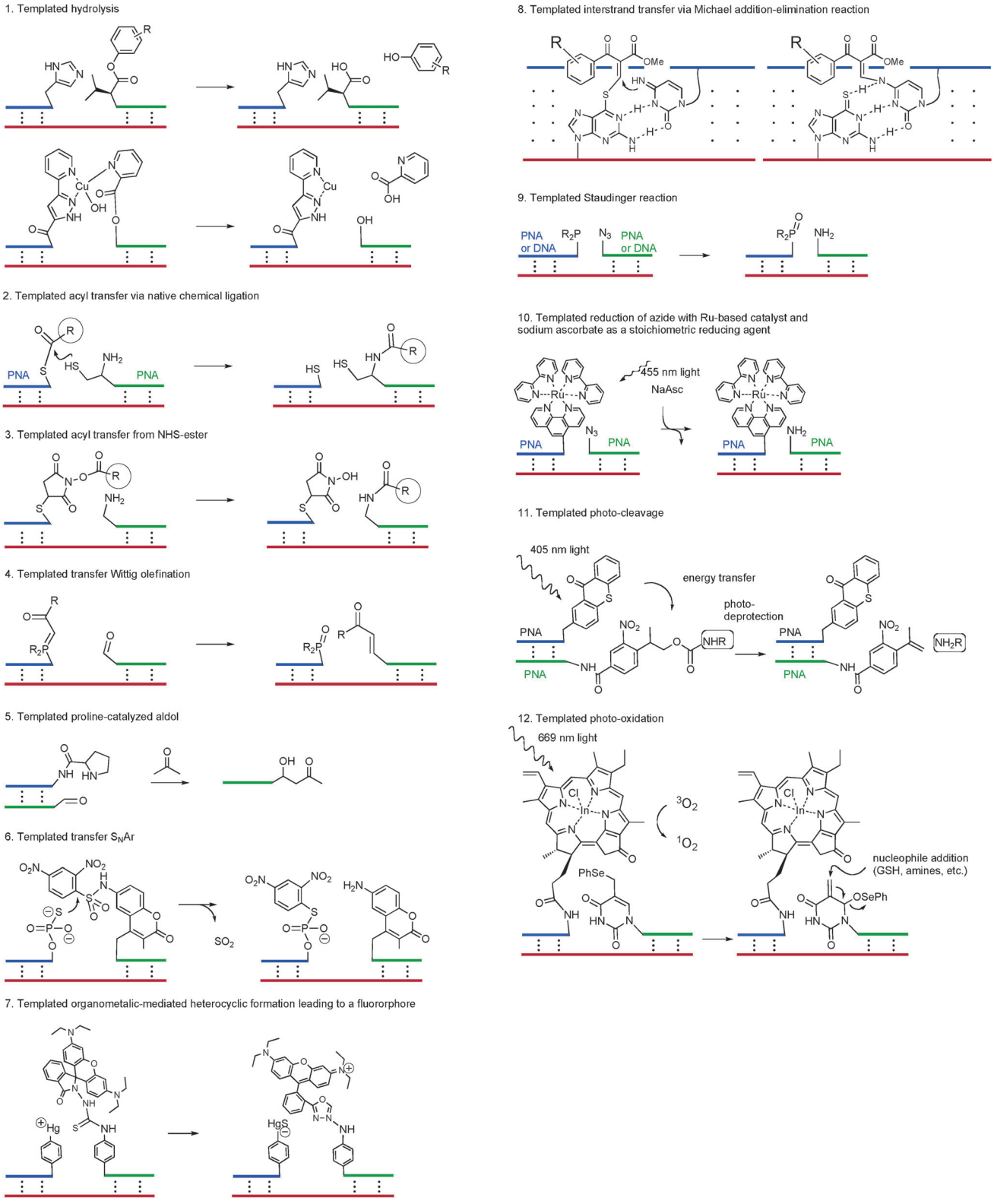

Figure 11. Templated transfer reactions.

that reacted with a ketone in solution, thus forming an enamine; the enamine, in turn, reacted with a complementary strand bearing an aldehyde (Figure 11.5).[103] The reaction was found to proceed nearly to completion within 2 h and the proline-DNA conjugate was able to catalyze multiple condensations, particularly when thermocycling was used.

DNA fragments bearing a phosphorothioate have been used extensively by Kool and co-workers in nucleophilic

ligation reactions (Figure 6.1 and Figure 8). Ito and co-workers harnessed the nucleophilicity of the phosphorothioate to achieve a nucleophilic aromatic substitution (S_NAr) on a 2,4-dinitrosulfonamide (Figure 11.6).[104] The dinitrosulfonamide used was coupled to coumarin, thereby reducing its fluorescence quantum yield by 70-fold. Importantly, this new fluorogenic coumarin substrate was found to be stable at pH values ranging from 5–10 and at temperatures up 90 °C. The templated reaction offered good discrimination of a single nucleotide mismatch; however, the rate of reaction was slow compared to the ligation reaction, and potential template turnover was not investigated.

Kool and co-workers also explored organometallic-templated activation of profluorescent rhodamine B (Figure 11.7).[105] In the presence of a matching template, the DNA conjugated profluorescent phenylthiocarbazide derivative of rhodamine reacted with the *p*-mercuriobenzoate-DNA conjugate and the spirolactam moiety was unlocked by the resulting cyclisation. However, although this novel setup potentially has a high turn-on ratio (fluorescence increase upon reaction), it did not perform as well as other reactions that were developed by the same group to discriminate between mismatched templates. Nevertheless, the reactions of thiols with organomercury are fast and reversible, and may find utility in different applications.

Another potentially useful strategy, developed by Sasaki and co-workers, uses the precise modification of a selected site in nucleic acid assemblies. Specific covalent modification of a given point along the nucleic acid strand can be achieved by hybridization of a complementary probe bearing a reactive moiety that can then be transferred to a complementary nucleobase position in a target strand. Although this reaction takes place across the duplex, which is similar to the interstrand crosslinking method previously discussed (Section 2), the system reported by Sasaki and co-workers relies on a nucleophilic addition/elimination mechanism. The reaction occurs between the amino group of a cytosine and the α,β-unsaturated system of an S-functionalized 6-thioguanosine donor (Figure 11.8).[106] Efficient functionality transfer is promoted by the close proximity of the reactants in the duplex, and the reaction proceeded to $>50\,\%$ in 10 h. In this way, various tags (R) conjugated to the vinyl phenyl carbonyl unit were selectively coupled to RNA in physiological conditions. Such an approach offers an alternative to the direct incorporation of modified phosphoramidate during oligonucleotide synthesis.

4.3. Templated Azide Reduction

The orthogonality and biocompatibility of the Staudinger reaction[107] have inspired several groups to explore the utility of this reaction in templated processes (Figure 11.9).[79,108–121] The reaction has been found to be highly efficient using both DNA and PNA strands and generally proceeds within 30 min with high conversion. A major focus has been the use of this reaction in nucleic acid sensing. The reaction displays the turnover expected from unligated fragments and thus provides significant and useful signal amplification. The first report[108] used a fluorescein that was esterified with 2-carboxytriphenyl phosphine, thereby quenching its fluorescence. Upon initiation of the templated reaction, a Staudinger ligation[122] took place, leading to a transfer of the phosphine moiety, thus revealing fluorescein fluorescence. A limitation of this design is the undesired air-oxidation of phosphine, which precludes full conversion of the profluorophore. Alternatively, several groups have reported new profluorophores that are activated by an azide reduction (Figure 15.1, see discussion below).[109–111,118–121] The major advantage of this strategy is that an excess of phosphine can be employed to overcome the partial oxidation of phosphine under aerobic conditions. Analogously, azide-based immolative linkers have been used to dissociate a fluorophore and its quencher or release a profluorophore or other bioactive molecules (Figure 15.2).[112,114]

A notable limitation of the Staudinger reaction remains the sensitivity of phosphine to oxidation when the templated reaction is open to air. This has generally been circumvented by using an excess of the phosphine probe relative to its azide partner. This solution is not ideal, however, because an excess of the phosphine probe increases the untemplated bimolecular reaction. Liu and co-workers recently reported the photocatalyzed reduction of aryl and alkyl azides using catalytic $[Ru(bpy)_3]^{2+}$ and stoichiometric ascorbate or NADPH as a reducing agent.[123] Our group implemented this new method to the templated reduction of azide. Using commercially available 5-isocyanatephenanthroline ($[Ru(bpy)_2(5\text{-}NCOphen)]^{2+}$) as a surrogate for the ruthenium catalyst, a facile conjugation to the amino group of a nucleic acid was achieved (Figure 11.10).[124] By comparison to the templated Staudinger reaction, this catalyzed version can be performed with just 2 % of the Ru-derivatized probe rather than an excess of the phosphine probe. This catalytic system was highly efficient at reducing azidocoumarin as well as in triggering the cleavage of an azide-based immolative linker (Figure 15.2).

4.4. Templated Photodeprotection and Photo-oxidation

Photolabile groups offer a unique orthogonality profile that is routinely used to cage bioactive molecules or reporters in biological systems. Inspired by the fact that triplet sensitizer thioxanthenone had been reported to significantly increase the rate of deprotection of such photolabile groups, we explored templated energy transfer from the sensitizer to a photolabile group (Figure 11.11).[125] The sensitizer was excited using a 405 nm LED and an electron-deficient 2-(2-nitrophenyl)propyloxycarbonyl (NPPOC) moiety was selected based on its poor direct photocleavage yield with light with a wavelength of more than 300 nm. The templated reaction had a greater than 20-fold rate acceleration over background. This templated reaction was used in conjunction with strand displacement to design four-component systems that respond with different behavior to DNA analytes (Figure 17).

Mokhir and co-workers reported a templated catalytic photochemical reaction using an indium-based photosensi-

tizer that is excited with red light (669 nm).[126] Excitation of the sensitizer resulted in the production of $^1O_2$ that oxidized the adjacent phenyl selenium thymine derivative, leading to a [2,3] sigmatropic rearrangement affording a reactive electrophile (Figure 11.12).[127] This electrophile could, in turn, be trapped by various nucleophiles such as amines or thiols. The reaction proceeded nearly to completion in 30 min, whereas the absence of a template gave a marginal reaction with a 1 μM concentration of reactants. Importantly, the reaction showed up to 45% conversion of the phenylselenium thymine conjugate using only 10% of the template and photosensitizer. Although the electrophile formed in the reaction is known to react with the complementary strand (Figure 2.3), this interstrand crosslinking was found not to occur with nucleic acids bearing the phenyl selenium at the terminal residue, reflecting the faster dynamics of a terminal residue in the duplex formation. Nucleic acid-triggered formation of $^1O_2$ was also reported to convert fluorophore in solution, allowing nucleic acid sensing (Figure 16).[128,129]

4.5. Application of the Templated Acyl Transfer Reaction

Templated native chemical ligation has proven to be a robust and fast reaction. Initial efforts focused on ligation, most notably using an isocysteine (Figure 12.1) that produces a ligation product bearing a linkage geometry that destabilizes the duplex and allows template turnover.[60,61] The progress of the reaction was followed by FRET between fluorophores (fluorescein and tetramethylrhodamine) on the individual ligation probes. To further enhance the amplification of signals generated by the templated reaction, transfer reactions were investigated (Figure 12.2 and 12.3). To this end, a fluorescence quencher (dabsyl) was transferred from

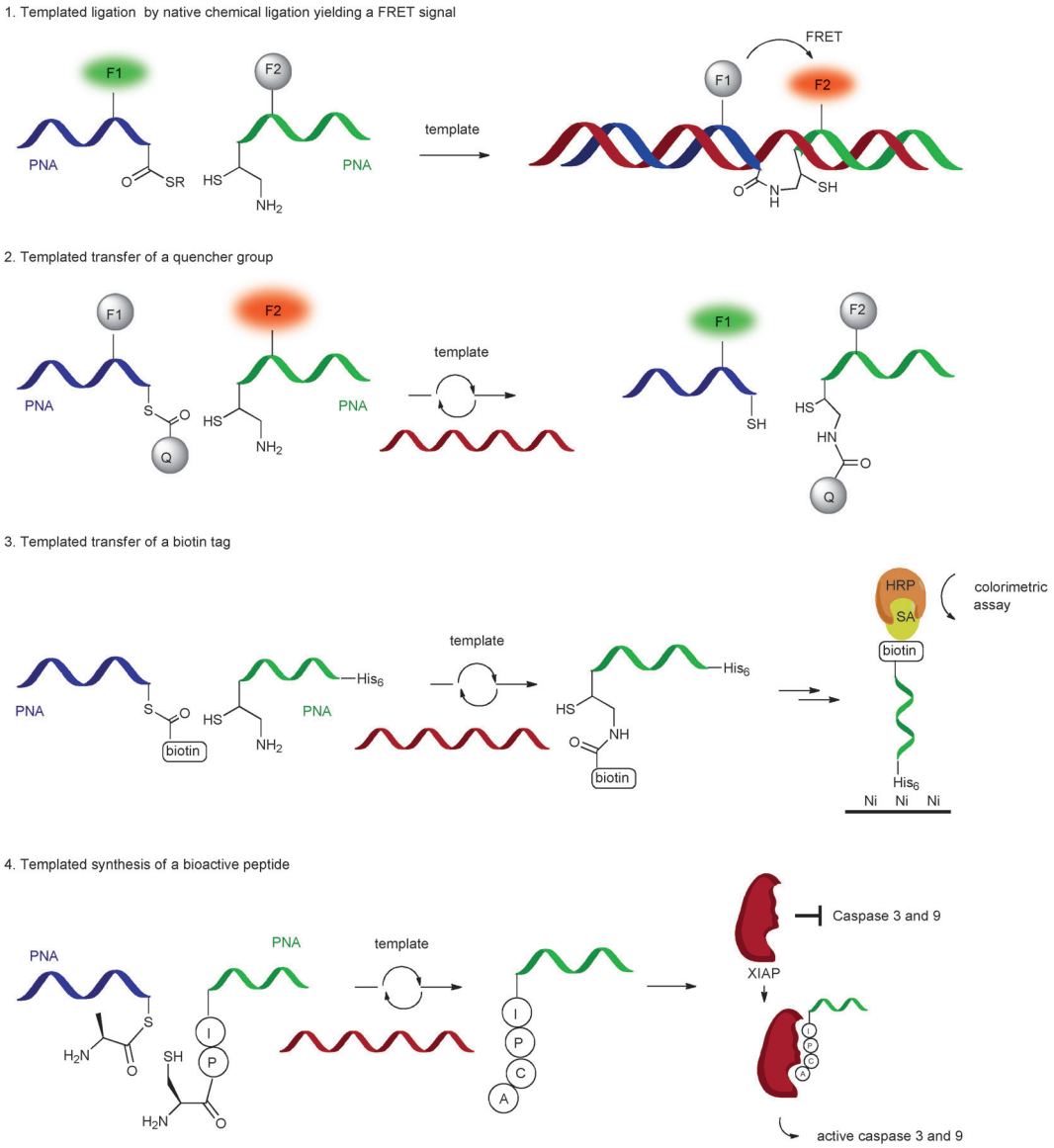

Figure 12. Application of NCL-templated reactions. HRP = horseradish peroxidase, SA = streptavidin.

one strand to the second strand by NCL (Figure 12.2) while both strands were derivatized with a spectrally resolved fluorophore.[93,130,131] This quencher transfer allowed the monitoring of both the hydrolysis of the thioester and the desired transfer of the quencher group. Using one equivalent of template, the reaction reached near-completion after 90 min. Importantly, the reaction turned over with significant signal amplification. Using 10% template produced seven turnovers in 1 h. At very low template load (0.0001 equivalent; 10 pM), the reaction was calculated to give 402 turnovers after 24 h (two-fold over background). However, in this system, template-independent hydrolysis of the thioester led to significant background fluorescence. In an alternative strategy to follow the reaction, a pyrene moiety was transferred to a pyrene-labeled PNA strand producing an pyrene excimer that can be monitored at 340 nm.[132] In this format, signal generation is restricted to the desired ligation.

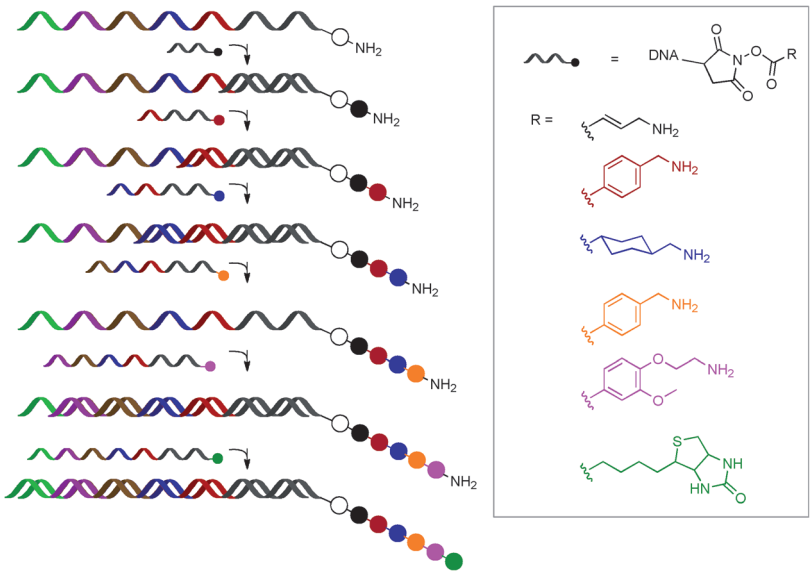

Figure 13. One-pot, six-step, DNA-templated synthesis using sequential strand displacements.

It was reported that the sensitivity and sequence specificity of the transfer reaction is strongly influenced by subtle changes in the structure of the thioester. Benzoyl-type thioesters or long thiol tethers give slower transfer but with better discrimination between matching and mutated templates.[132] RNA templates are as effective as DNA in these reactions. The RNA-catalyzed transfer of a biotin reporter to a His_6-tagged PNA allowed the preamplification of viral RNA for an enzyme-linked immunosorbent assay readout (Figure 12.3).[130] This tandem amplification allowed for a detection threshold of 500 attomol of human immunodeficiency virus-I RNA using a simple colorimetric readout.

Seitz and co-workers also reported the use of templated NCL to synthesize a bioactive peptide (Figure 12.4).[133] X-linked inhibitor of apoptosis protein (XIAP) is frequently overexpressed in tumor cells conferring resistance to apoptotic stimuli by inhibiting caspase-9. Using a peptide motif that directly competes for this interaction, the antiapoptotic activity of XIAP can be reduced. A template-controlled peptide-coupling reaction was designed to provide the bioactive peptide in response to a template harboring an oncogenic mutation in the gene encoding Ras. Using crude cell lysates, the PNA probes by themselves had no significant impact on apoptosis; however, in the presence of added DNA template, a recovery of the apoptotic signal was observed (45% with 1.0 equiv, 23% with 0.5 equiv). As could be anticipated based on the different levels of preorganization, the length and nature of the peptide affected the rate of the reaction, with longer peptides generally showing slower reaction rates.[134]

Liu and He used a sequential strand-displacement strategy to perform a DNA-templated six-step synthesis in one pot with remarkable efficiency (83% average yield, Figure 13).[96] This synthesis took advantage of sequential toehold displacement—the ability of an ssDNA to invade and displace a shorter fragment off a dsDNA by hybridizing on the edge of the duplex. Using six different DNA-NHS ester-linked amines that were added sequentially (30 min cycle), the DNA template was translated into a peptide containing six residues. An important point is that the product remains associated with the DNA template containing the instructions for its synthesis, which provides a convenient tag for identifying the structure of the product following its selection from a library. An implicit requirement in this chemistry is that the NHS ester-linked amine must be geometrically constrained so that it will not cyclize.

4.6. Application of Templated Wittig Transfer Reactions

The first study of DNA-templated reiterative oligomerization with bifunctional reagents was reported by Turberfield, O'Reilly, and co-workers using templated Wittig olefination with bifunctional ylide–aldehyde (Figure 14.1).[99] Ordered oligomerization was achieved through the sequential addition of reagents followed by the addition of a "waste sequence" that sequestered spent reagent and liberated the template for the next reagent addition, in a step-wise fashion. The templated couplings were performed using the same hybridization site ("adapter region"), whereas displacement of spent reagent by the waste sequence was achieved by strand displacement with a unique toehold region corresponding to the reagent used. A drawback of this approach is that the sequence of the product oligomer is determined by the sequence in which reactants are added, not by the template. This excludes potential applications such as molecular evolution that rely on the retention of a DNA-coded sequence record attached to the product. Performing the templated Wittig reaction for 2 h produced an overall yield of 72% for a tetramer (90% average yield). This method was used to perform longer oligomer synthesis yielding decamers with 23–31% yield.[101] The same group reported an alter-

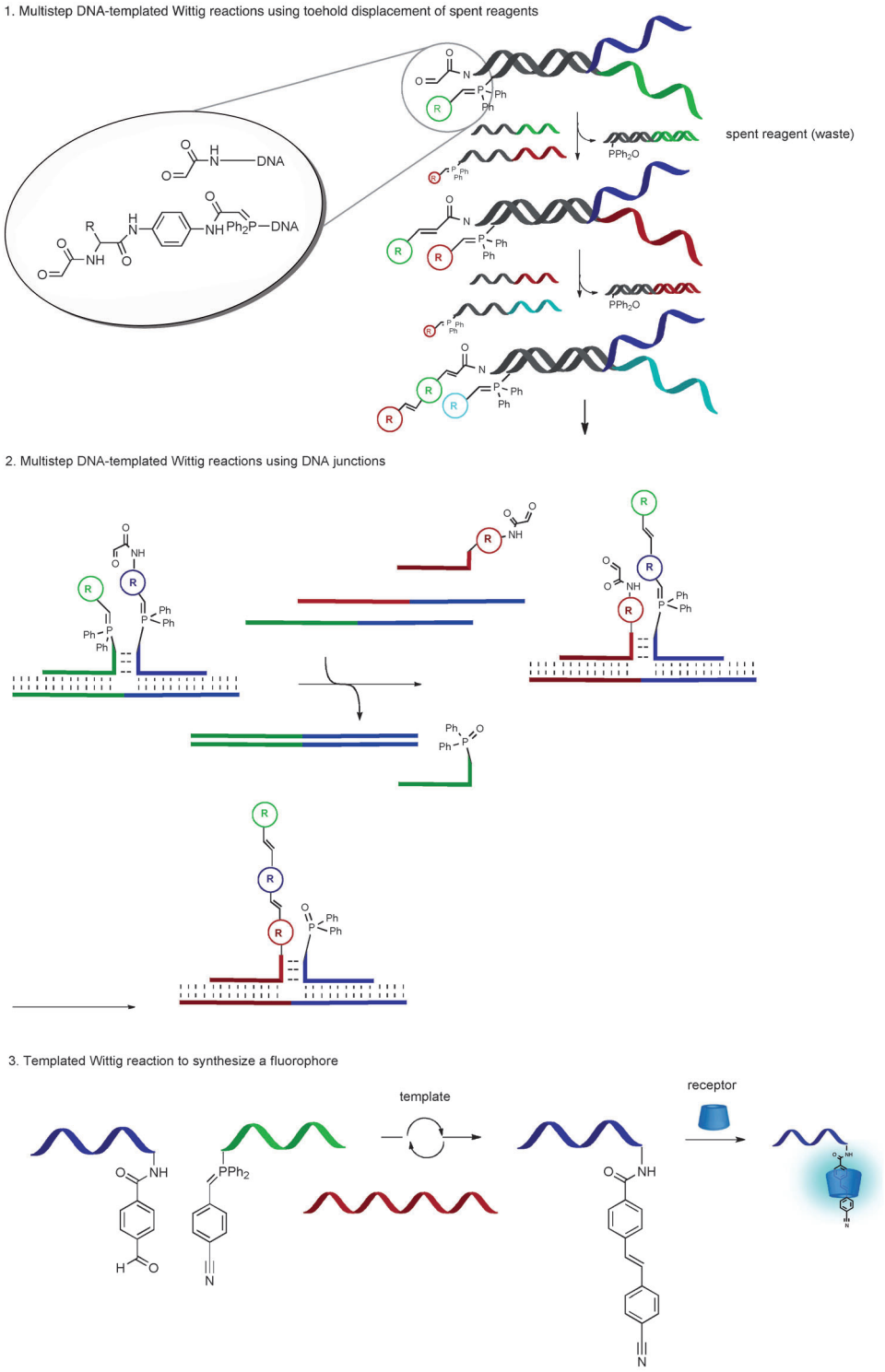

Figure 14. Application of Wittig-based transfer reactions in DNA templated synthesis and nucleic acid sensing.

native strategy using different template architectures (Figure 14.2).[100] This strategy allowed a back-and-forth transfer that is compatible with convergent segment couplings, thus reducing the overall number of steps required to produce an oligomer and maintaining a constant reaction environment. As a proof of principle, a 6-mer olefin was prepared in three reaction steps.

Seitz and co-workers reported a conceptually new approach for nucleic acid sensing based on consecutive signal amplifications, first through a templated reaction and then through a host–guest interaction of the product.[102] A templated Wittig reaction was used to access a stilbene that fluoresces at 380 nm and is recognized by α-cyclodextrin (Figure 14.3). The optimal temperature for the reaction was 65 °C, producing 50 % yield after 2 h and 84 % after 24 h. Using 0.02 % of template, 1.7 % yield (84-fold turnover of the template, uncorrected for background reaction) was achieved. At 10 % template loading, four-fold turnover was reported. The tight binding with α-cyclodextrin stabilizes the planar conformation of the *trans*-stilbene, resulting in enhanced fluorescent emission. Using 100 mM of α-cyclodextrin, up to a 30-fold increase in fluorescence was obtained. Using this double amplification approach, a detection limit of 40 pM of template was reported (1.6-fold above background).

4.7. Application of Templated Azide Reductions

The Staudinger reaction is notable in chemical biology because of its bio-orthogonality and compatibility with cellular processes. Several applications in live cells using Staudinger ligations have been reported.[107] Inspired by the success of this chemistry, several groups have developed strategies to sense nucleic acids based on a Staudinger reaction with the ultimate goal of performing the reaction in cells. Two strategies have been the subject of intense focus: the development of azide-quenched profluorophores (Figure 15.1), and triggering the cleavage of a linker following azide reduction (Figure 15.2).

The first azide-based profluorophore to be reported was an azidocoumarin (Figure 15.1).[109] Upon reduction of the azide, a 22-fold increase in fluorescence was observed. The

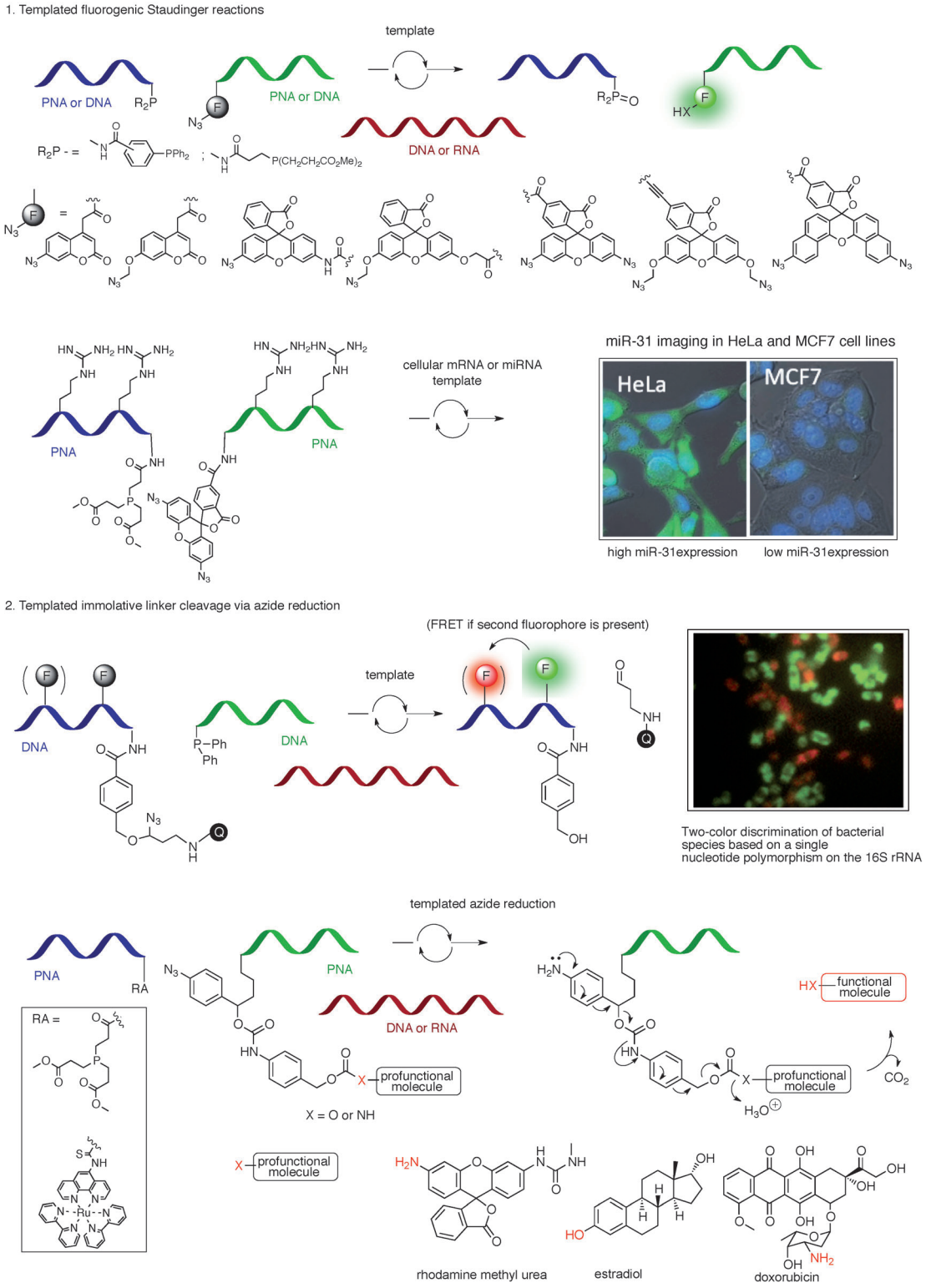

Figure 15. Application of templated azide reduction.

templated reaction between two PNAs derivatized with 4-carboxy-triphenylphosphine and azidocoumarin, respectively, produced a fast reaction (35% conversion in less than 15 min). It was noted that the addition of formamide further accelerated the reaction and enhanced its performance. The template acted catalytically, affording over 20 turnovers within 30 min (1% catalyst loading) with detection at levels as low as 1 nM of template (lower concentrations were not investigated). The reaction produced marginal fluorescence if a single nucleotide mismatch was present in the template.

Shortly thereafter, azidomethoxycoumarin[113] was reported to have a superior increase in fluorescence upon reduction (62-fold increase). A templated reaction using DNA probes afforded similar kinetics, turnover frequency, and sequence specificity, as observed with the azidocoumarin and the PNA probes. This system was further combined with a second fluorophore (fluorescein) to achieve a longer wavelength emission through FRET. In these first examples, the fluorescence of coumarin was greatly reduced by converting the electron-donating group, the amino or phenol, into an azide or azidomethyl ether group, respectively. The same logic was applied to fluorophores with red-shifted emission: azidorhodamine,[110,118] azidomethylfluorescein,[119] bis-(azido)rhodamine,[111] bis(azidomethyl)fluorescein,[121] and bis(azido)naphthorhodamine (Figure 15.1).[120] The reaction of azidorhodamine with triphenylphosphine was found to generate a surprisingly stable aza-ylide that did not hydrolyze over the time course of the templated reaction (templated reaction was complete within 30 min and the half-life of aza-ylide decomposition was approximately 24 h).[118] The aza-ylide intermediate was fluorescent (emission at550 nm vs. 520 nm for rhodamine), although the templated reaction led to a ligation that slowed down template turnover. The reaction was nevertheless found to be useful in detecting rRNA 23 s in paraformaldehyde-fixed bacteria (*E. coli*). The azidorhodamine could also be reduced with dithiols, such as DTT, and a templated version of the thiol reduction produced the desired rhodamine product, albeit with a slower reaction rate. Importantly, the azidorhodamine was stable at a pH range of 5–10 and at high temperature (90 °C). A subsequent report showed that a phosphine reduction of azidorhodamine was indeed possible but required a more reactive trialkyl phosphine rather than a triphenylphosphine conjugate.[110] The templated reaction was very fast (reaching completion within 6 min using one equivalent of template) and turned over to produce seven-fold signal amplification within 20 min using 2 % template.

A long-standing goal of the use of templated reactions is to image cellular nucleic acids; however, because of the challenge of cellular delivery of the nucleic acid probes, membrane permeabilization has been necessary. The use of guanidinium-functionalized peptide nucleic acid (GPNA)[135] endowed with cellular permeability permitted the detection of mRNA in live cells. To further improve the signal-to-noise ratio of azidorhodamine, bis(azido)rhodamine was prepared.[112] Reduction of the bis(azido)rhodamine led to a 120-fold increase in fluorescence versus 32-fold increase for the azidorhodamine. Although this bis(azido)rhodamine fluorophore requires two equivalents of phosphine to achieve full unquenching, the reduction of the second azide is faster than the reaction of the first; with no partially reduced product observed. This strategy was found to be suitable to detect and quantify miRNAs across different cell lines (Figure 15.1) using both fluorescence microscopy and flow cytometry (six-fold gain in fluorescence for the perfectly matched probes versus mismatch probes). In parallel, mono- and bis(azidomethyl)fluorescein were reported. Bis(azidomethyl)fluorescein was found to have the highest increase in fluorescence upon unquenching (>300-fold) and shown to yield a fast templated reaction with good turnover (8 turnovers with 10 % template; 53 turnovers with 0.1 % template). Detailed kinetic analysis of the reaction revealed that the apparent first-order rate was comparable to the rate of hybridization, suggesting that at the studied concentration (50 nM), the rate-limiting step is hybridization rather than reduction. This is in agreement with earlier observations that the distance between the reactants (and thus the level of preorganization and effective molarity) in templated Staudinger reactions is only moderately important, with an optimal distance of 2–4 nucleobases between the hybridization sites.[109–110] Templated reduction of bis(azidomethyl)-fluorescein was found to be suitable for detecting ribosomal and messenger RNA. Permeation of the membrane with streptolysin O (SLO), a pore-forming peptide, was used to introduce the DNA-based probes and detection was achieved both by fluorescence microscopy and flow cytometry (two-fold gain in fluorescence for mRNA and tenfold gain in fluorescence for rRNA). The same chemistry was used to detect Lariat RNA (LaRNA), a 2′,5′ branched linkage implicated in Group II introns and spliceosomes.[121] The branching geometry brings into proximity nucleic acid segments that would otherwise be distant. Using probes targeting these segments in conjunction with templated Staudinger reduction, LaRNA could be detected in vitro with higher accuracy than using the analogous approach with fluorophores yielding a FRET signal; detection at levels as low as 50 pM was achieved because of template turnover-induced signal amplification. The templated Staudinger reaction that yields the most red-shifted fluorophore uses bis-(azido)naphthorhodamine and gives rise to a fluorescence signal at 655 nm. In contrast to the azidorhodamine fluorophore, reduction with triphenylphosphine conjugate was not hindered by the formation of a stable aza-ylide intermediate, although the potential signal amplification resulting from template turnover was not investigated with this fluorophore.[120] The combination of azidorhodamine and bis-(azido)naphthorhodamine-templated reduction was used to provide a dual-color readout for two templates differing by a single nucleotide.

As an alternative to the conversion of profluorophores through azide reduction, two linkers that are cleaved upon azide reduction have been reported (Figure 15.2).[112,114] The first report used a benzylic α-azidoether linkage that, upon reduction, cleaves to yield benzyl alcohol and an aldehyde. This linker was used to connect a quencher (Dabsyl) to a DNA strand labeled with fluorescein that was efficiently cleaved in a templated reaction with a second DNA strand derivatized with triphenylphosphine. The performance of the reaction in terms of rate and turnover was comparable to the reactions using azide-based profluorophores discussed above. Incorporation of a second fluorophore (TAMRA) that yields a FRET with fluorescein allowed the development of a two-color detection system that was used to differentiate two strains of bacteria based on their rRNA sequence, as had been demonstrated previously using quencher-displacement ligation chemistry (Figure 6.1).[76] This method was also used to detect the product of a rolling-circle PCR reaction that was, in turn, enabled by a miRNA-templated nucleophilic cyclisation

of a linear probe containing a 5′-I and a 3′-OPO_2S^-.[79] This method has also been applied to the detection of dsDNA using sequences of a polypurine–pyrimidine duplex known to engage in triplex structures.[115] A limitation of the benzylic α-azidoether linkage is that prolonged incubation in buffer leads to partial hydrolysis, particularly at temperatures above 50 °C. A further advance of this method was reported using DNA probes derivatized with two azide-linked quenchers. Using this approach, two successive template reactions were required to unquench the fluorophore; this improved the signal-to-background ratio and sequence specificity of the reaction, analogous to that achieved through displacement chemistry (Figure 8).[116] In parallel, further increases in the signal-to-background ratio were achieved using a fluorophore with red-shifted emission (Quasar 670) that emits in the near infrared (670 nm) in conjunction with a black hole quencher (BHQ), producing a more than 200-fold gain in the signal-to-background ratio.[117] The utility of this new fluorophore/quencher pair was demonstrated through the imaging of rRNA in bacteria.

The second strategy to link the reduction of an azide to the cleavage of a linker involved the 1,6-elimination of 4-azidobenzyl carbonate or carbamate (Figure 15.2), leading to the release of an alcohol, phenol, amine, or aniline.[112] Using this strategy, templated azide reduction could unleash the function of a broad diversity of small molecules, such as estradiol (a transcription-factor agonist), doxorubicin (a cytotoxin), and rhodamine (a fluorophore). In all cases, the biological or fluorogenic function of the molecule had been caged by the carbonate or carbamate linker. Cleavage of the linker proceeded rapidly ($t_{1/2} < 21$ min) and a nearly quantitative templated uncaging of estradiol was reported after 30 min incubation with 50 % template.

Despite the impressive achievements in templated chemistry reported using the Staudinger reaction, a limitation of this reaction is the propensity of phosphine to oxidation, particularly at the dilutions required in templated chemistry, at which small quantities of dissolved oxygen become significant. Practically, this issue has been circumvented by the use of an excess of the phosphine conjugates. The demonstration that the templated azide reduction can be performed catalytically using a ruthenium-photocatalyzed reduction offers an attractive alternative (Figure 11.10).[124] In comparison to the templated Staudinger reaction, this photocatalyzed version was reported to proceed with just 2 % of the Ru probe and template, leading to the cleavage of the azide-based linker or the reduction of azidocoumarin with a reaction rate that is comparable to that of the Staudinger reaction (Figure 15.2). Furthermore, the fact that the reaction is activated by photoexcitation of the Ru probe provides temporal control of the reaction and allows both probes to be mixed at high concentration, without a background reaction. Recently, this reaction has been implemented in a conceptually related setting to report on protein oligomers.[136] To this end, ligands targeting oligomeric proteins were derivatized with the immolative linker and ruthenium reagent respectively and brought within reacting distance upon protein binding. Importantly, the reaction could be used to report on protein oligomers in cells.

4.8. Application of Templated $^1O_2$ Generation and Photodeprotection

Gothelf's and Mokhir's groups studied the nucleic acid-controlled generation of $^1O_2$. The first report used a $^1O_2$ sensitizer (pyropheophorbide) quenched by a complementary strand derivatized with a BHQ (Figure 16). In the presence of a DNA analyte, the sensitizer was liberated and could photocatalyze the generation of $^1O_2$.[137] The general concept has potential applications both as a therapeutic and an imaging agent. The cytotoxicity of $^1O_2$ is used in photodynamic therapy. The reported method could confine photodynamic therapy to cells possessing a given nucleic acid sequence. This was demonstrated in a proof-of-principle study introducing the nucleic acid probes targeting rRNA into cells permeabilized with SLO. In this study, a more stable indium-based sensitizer was used and the generation of $^1O_2$ was visualized using a fluorescent scavenger.[128] In vitro experiments revealed more than 6000-fold concentration of $^1O_2$ relative to the sensitizer. Using a short incubation time (> 30 min), probes targeting rRNA led to $^1O_2$ generation, whereas a scrambled sequence did not. However, the metabolic instability of the probes led to indiscriminate $^1O_2$ generation with a longer incubation time. It was subsequently reported that eosin conjugates could also be used to sensitize

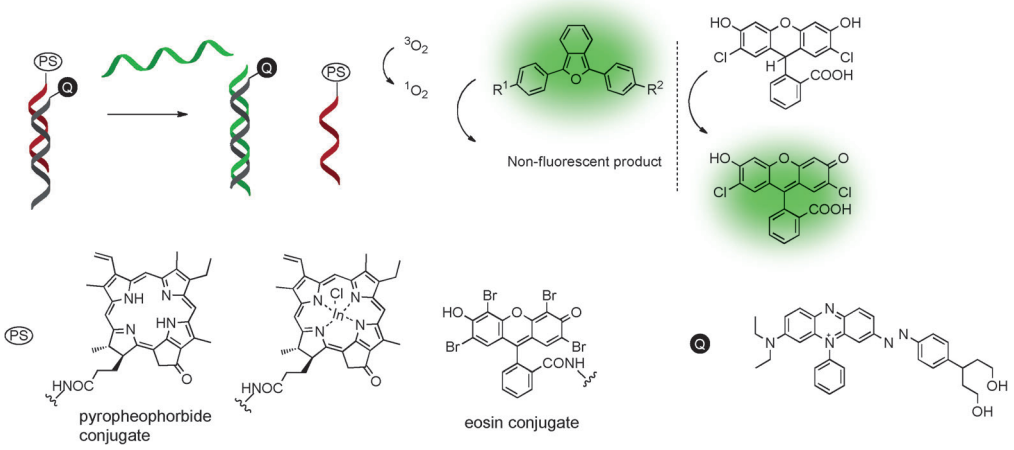

Figure 16. DNA-controlled generation of singlet oxygen. PS = photosensitizer, Q = quencher.

$^1O_2$ generation, which in turn oxidized dichlorofluorecin to its fluorescent product (dichlorofluorescein).[138] Because this product can photocatalyze the oxidation of its precursor, the reaction has the potential for autocatalysis.

Beyond nucleic acid sensing, the programmability of hybridization offers a unique platform to design dynamic systems with unique behavior. Strand-displacement strategies have been used extensively to engineer logic-gate operations. The templated photorelease reaction (Figure 11.11) was used to investigate the behavior of system with multiple components that can compete for mutual interactions (Figure 17).[125] A system composed of up to four compounds with a predefined hierarchical interaction was investigated using the release of profluorescent rhodamine as a readout.

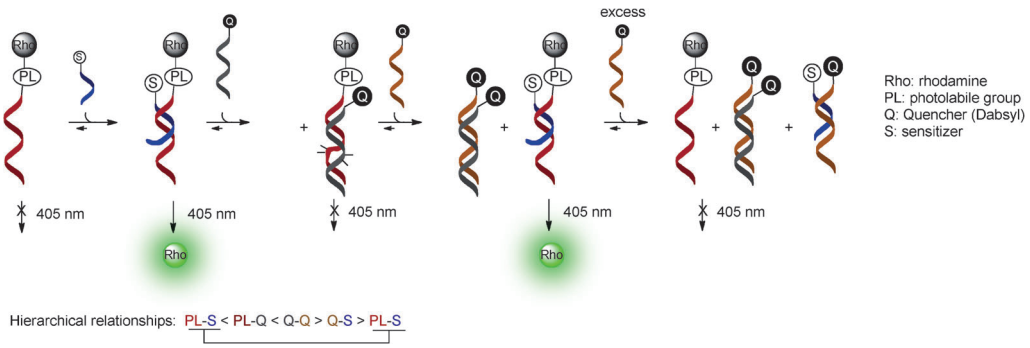

Figure 17. Application of templated photocleavage in dynamic systems with a nonlinear response. (see Figure 11.11 for chemical structures).

Used alone, the first two components (the photolabile and sensitizer strands) yielded a positive readout. The addition of a third component (a quencher strand) with higher affinity to the photolabile strand than to the sensitizer shuts down photocleavage. Addition of a fourth strand that is fully complementary to the third strand sequesters the quencher and restores photocleavage. However, the addition of an excess amount of this fourth strand displaces the sensitizer from its interaction with the photolabile strand, and shuts down the reaction. Overall, the system first responds positively to the addition of the fourth strand and then negatively. The unique behavior of this system makes it amenable to four different logic operations, namely AND, NOT, XNOR, and NOR.

5. Translating DNA into a Non-natural Polymer

The remarkable success of in vitro selection methods, such as phage display[139] and SELEX,[140] takes advantage of iterative cycles of selection and amplification. Until recently, this approach was limited to biopolymers because of the lack of methods to amplify synthetic polymers. Liu and co-workers have developed two complementary strategies to address this limitation: a DNA-templated condensation of PNA fragments through reductive amination,[141–143] and a DNA-templated base filling of the PNA backbone through acylation or reductive amination[144] (Figure 18.1 and 18.2).

The first approach builds on Lynn's pioneering work with templated reductive amination[6,145] to condense PNA aldehyde tetramer and pentamer building blocks bearing one or multiple side chains at various positions within the building block. The initial imine condensation is highly reversible and should drive the assembly of the fully complementary synthetic oligomer that is subsequently reduced, thus locking the sequence (Figure 18.1). This reaction was used to assemble a PNA up to 40 mer in which 24 PNA nucleotides contained side-chain modifications with an overall yield of 66–90%.[141] This work stands as the first example of non-enzymatic translation of a nucleic acid sequence into a synthetic polymer functionalized with different side-chains and raises the possibility of discovering synthetic polymers with functional properties through cycles of selection and amplification. To demonstrate the feasibility of this approach, 4.3×10^8 DNA templates were translated into their synthetic polymers and displaced from their templates to allow the synthetic polymers to adopt unique secondary and tertiary structures; the polymers were then selected for streptavidin binding. The cycle was repeated six times resulting in a 10^6-fold enrichment of a template encoding a biotinylated PNA.[142]

The second approach developed by Liu and co-workers relied on base filling. In pilot studies, PNA duplexes with one to two abasic sites were incubated with the four nucleobase aldehydes or activated acids under reductive amination conditions or DMTMM (carboxylic acid activation), respectively (Figure 18.2).[144] The reductive amination of a single abasic site proceeded with higher yield and sequence fidelity affording more than 87% yield and greater than 7:1 selectivity for the complementary nucleobase. Multiple abasic or single abasic sites at the edges of the sequence afforded poorer yield and less specificity. The higher performance of reductive amination compared to acylation may result from the benefit of the reversible formation of an iminium intermediate in reductive amination, as opposed to the unfavorable steric congestion of the activated ester in acylation chemistry.

Ghadiri and co-workers concurrently reported an alternative strategy for base filling that makes use of the trans-thioesterification of thioester PNA (tPNA, Figure 18.3).[146] The tPNA efficiently self-assembled by means of the reversible covalent anchoring of nucleobases onto the oligodipeptide backbone and underwent dynamic sequence modification in response to changing templates. This novel PNA oligomer self-pairs with its complementary sequence and cross-pairs with DNA and RNA through Watson–Crick hybridization. It was demonstrated that a pentameric tPNA

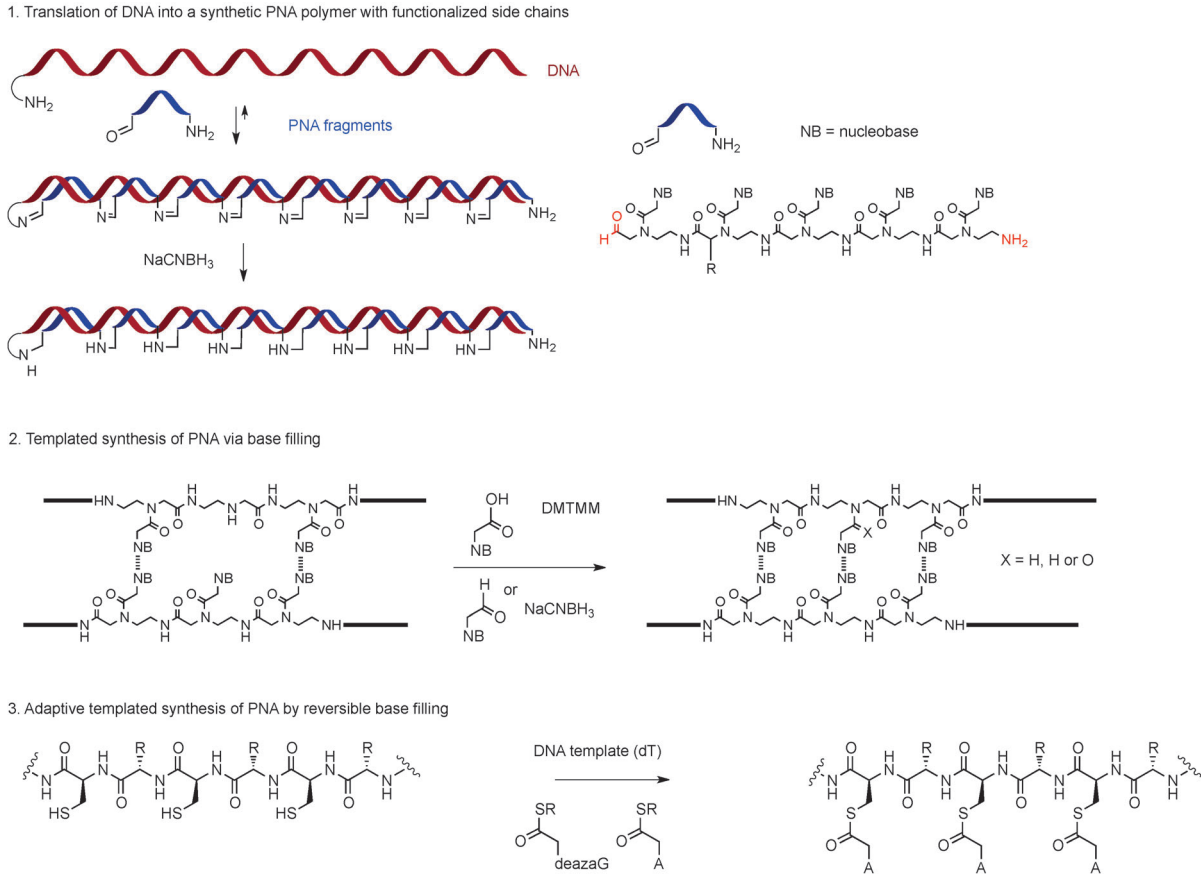

Figure 18. Translation of DNA and PNA into synthetic polymers.

backbone was acylated with adenine through trans-thioesterification in the presence of a polyT with 89% yield and more than eightfold selectivity for the adenine nucleobase. Furthermore, the tPNA of a given sequence adapted to a novel sequence when challenged with the complementary DNA template. The tPNA combines the base-pairing interaction of oligonucleic acids with the side-chain functionalities of peptides and proteins. However, the ability to cycle through multiple rounds of replication without compromising the sequence integrity of tPNA oligomers was not demonstrated.

6. Summary and Outlook

Over the past decade, a variety of oligonucleotide-templated reactions have been reported. This growing toolbox has enabled the development of powerful applications to translate nucleic acid instructions into diverse collections of small molecules, synthetic oligomers, and functional nanomaterial or to uncage fluorophore or bioactive molecules. In the latter case, it has been shown that cellular nucleic acids (rRNA, mRNA, or miRNA) can be used to template reactions in live or intact cells. Furthermore, some reactions now have been demonstrated to reliably provide information about secondary structures such as G-quadruplexes, hairpin loops or Lariat junctions. The past five years have been marked by the development of several new systems that take advantage of templated transformations rather than templated ligations and that greatly aid template turnover. The amplification associated with template turnover has been particularly useful for nucleic acid sensing. In parallel, two systems that respond reversibly to nucleic acid templates have also been reported and offer new possibilities for dynamic responses. Furthermore, templated reactions also have been used to access nucleic linkages that are simply not possible with the enzymatic toolbox of molecular biology. These novel junctions may open new frontiers in the growing field of DNA-based nanotechnology. Although all of these reactions are designed to respond to a nucleic acid template, several reactions now have been reported that require a secondary trigger in the form of oxidation or light. These reactions also have been used to design networks with unique behaviors in response to changing nucleic acid cues. Undoubtedly, these developments will inspire novel applications. Although nucleic acids provide an attractive platform to engineer such reactions, it is clear that the high effective concentration achieved through nucleic acid-templated supramolecular assemblies will be implemented in other supramolecular systems.

7. Addendum (May 21, 2013)

Since the submission of this Review, the following important contributions have been published:

1. The templated reduction of azidorhodamine (Figure 15.1) was used to detect pre-mRNA spicing in vitro.[147]
2. In an extension of azide-based immolative linkers discussed in Section 4.7 (Figure 15.2), Shibata et al. have succesfuly used a nucleic acid tempalted reaction to unmasks the function of a molecule that induce protein expression.[148]
3. The templated reduction of azide-based immolative linker using photocatalysis with ruthenium conjugates (Figures 11.10 and 15.2) has been achieved in live cells and shown to provide very turnover (>4000), thus providing a detection down to 5 pM of template.[149]
4. Comb-type polylysine–polydextran copolymers were shown to increase turnover in templated NCL fluorophore transfer reactions (Figure 12).[150]
5. A $^1O_2$-sensitizer (Figure 11.12) was used to cleave a linker in a templated fashion, leading to the release of fluorescent dye.[151]
6. Templated NCL fluorophore transfer (Figure 12) was shown to be possible with dsDNA obtained from PCR.[152]
7. Enzyme-free translation of DNA into diverse synthetic polymers of up to 26 kDa was achieved.[153]
8. Templated reduction of azidorhodamine (Figure 15.1) has been achieved using homo-DNA.[154]

8. Abbreviations

BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene
Bpy	2,2-bipyridine
CuAAC	copper-catalyzed azide–alkyne cycloaddition
Dabsyl	dimethylaminoazobenzenesulfonamide
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
dsDNA	double-stranded DNA
DTS	DNA-templated synthesis
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
FRET	Förster resonance energy transfer
LED	light-emitting diode
PNA	peptide nucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NCL	native chemical ligation
NHS	N-hydroxysuccinimide
NPPOC	2-(2-nitrophenyl)propyloxycarbonyl
PAGE	polyacrylamide gel electrophoresis
Phen	phenanthroline
SLO	streptolysin O
SNP	single nucleotide polymorphism
ssDNA	single-stranded DNA
TAMRA	tetramethylrhodamine

We thank our collaborators who have contributed to our work in the area of programmed assemblies and templated reactions as well as the funding agencies (HFSP, ERC, FRC, IUF). K.G. gratefully acknowledges a fellowship from Boehringer Ingelheim Fonds. This review was not intended to be comprehensive but rather to highlight some recent work, and we apologize for arbitrary omissions.

Received: October 20, 2012
Published online: June 12, 2013

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