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Achieving Turnover in DNA-Templated Reactions

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Introduction

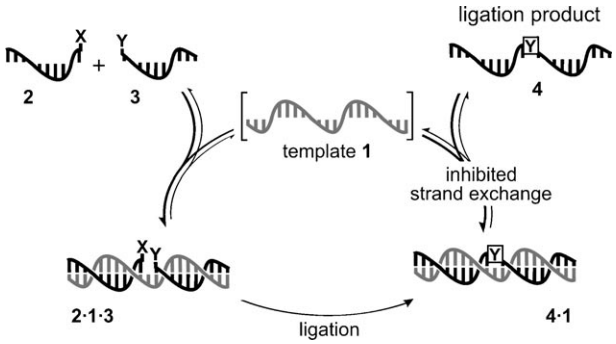
Templated reactions have frequently been applied in nucleic acid chemistry as well as in synthetic chemistry to facilitate conversions that proceed less efficiently when performed in the absence of the template. Templates bind the reactants and align reactive groups. The increase of the effective molarity results in accelerations of the templated reactions. Template-controlled reactions can mimic features of catalysed reactions. Turnover is one important hallmark of catalysis. Efficient catalysts can facilitate turnover by binding transition states with higher affinity than reactants or product molecules. This mode of action has rarely been put into effect in template-controlled reactions, and this probably is one reason why stoichiometric amounts of templates are usually needed to drive templated reactions to completion. The majority of templates have been designed for binding of starting materials.

The programmable self-recognition properties make nucleic acids particularly well adapted to templated synthesis.[1] Key processes of life, such as replication, transcription and translation, exemplify the importance of nucleic acid templates. The pioneer studies in DNA-templated synthesis were performed to unravel the molecular mechanisms of evolution.[2] Recently, the focus has shifted to the exploration of applications of DNA-controlled reactions. For example, programmed assembly of DNA-based nanomaterials has been explored,[3,4] the construction of amplifiable small-molecule libraries is under development,[5] and nucleic acid-templated reactions are used as a diagnostic means of detecting the presence of the nucleic acid targets[6–13] and to release drugs.[14] In the majority of cases one molecule of DNA template is used to promote the formation of one product molecule. However, some applications might benefit if the DNA templates were catalytically active. Significant efforts have been invested to develop reaction systems that facilitate the detection of low-abundance DNA or RNA by means of amplified signalling.[15–19] In yet another fascinating scenario, catalytic amounts of RNA could trigger the release of drug molecules, which would occur only in cells with altered (pathogenic) gene-expression programs.[14,20–22]

The design of a reaction system that is amenable to catalysis by unstructured, linear DNA or RNA is a challenge. Even though high-rate accelerations may be harnessed, turnover of reactants frequently is impeded by the high affinity of reaction products for the DNA or RNA template. Thus, the major problem is product inhibition, and it requires detailed insights into reaction mechanisms and the imagination of chemists to solve this problem. Enzymes can avoid product inhibition by offering binding clefts that have the highest affinity for the transition state or reaction intermediates. Analogous, nucleotide-based recognition patterns can be provided by folded structures of optimized DNAzymes or ribozymes. By contrast, the stabilization of transition states or reaction intermediates can less readily be implemented when unstructured nucleic acids are used as templates. Here opportunities in reaction design (rather than in template design) have to be explored in order to confer catalytic activity on unfolded templates. This will also suit the demands of applications in amplified DNA/RNA detection or DNA/RNA-triggered drug release systems, which call for a general applicability to any template sequence. This review was written with the aim of presenting the current state of templated reactions catalysed by DNA and RNA. It is important to note that the emphasis lies entirely on the development of chemical reactions that are triggered by DNA hybridization with unstructured templates rather than on the use of folded DNAzymes. For additional information on DNA-templated reactions the reader is guided to recent review articles.[1,23,24]

DNA-Catalysed Ligation of Nucleic Acids

In DNA-controlled ligation reactions two oligonucleotides or analogues thereof are armed with two mutually reactive groups, which mostly are affixed to the 3′- and 5′-ends. Without special adjustments, templates rarely exhibit catalytic turnover in chemical ligation reactions.[15,23,25,26] Although technically the nucleic acid template **1** might act as a catalyst for ligation of probes **2** and **3**, the catalysis suffers from strong product inhibition due to the high affinity of the ligation product **4** to the DNA template **1** (Scheme 1). As a result, stoichiometric



Scheme 1. Catalytic cycle of nucleic acid templated ligation reactions.

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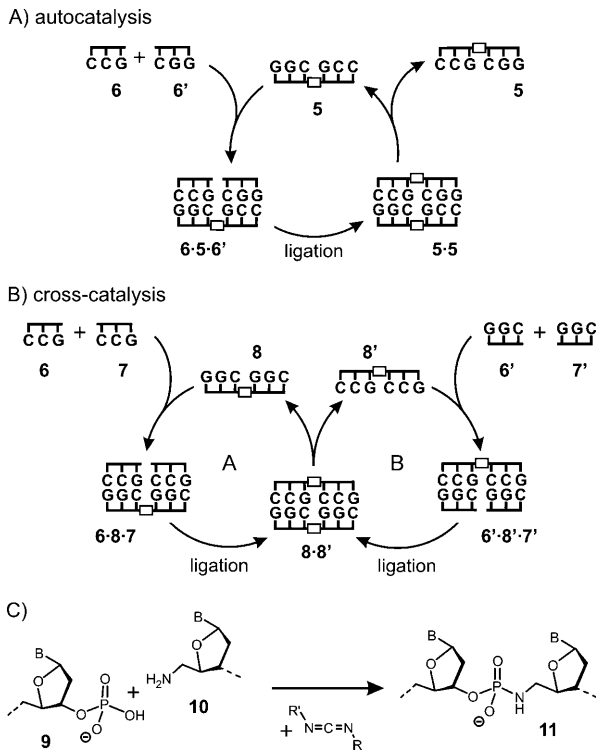
amounts of the template are required to achieve quantitative conversion.

In nucleic acid diagnostics the catalytic formation of the ligation product is desired when the DNA or RNA to be detected is present at low concentration. The high number of cooperatively formed Watson–Crick base pairs poses a problem in the design of catalytic ligations. The challenge is to decrease the DNA affinity of the ligation product without compromising the affinity of the starting compounds for the template or the ligation rate.[17]

Ligation Reactions with Self-Replication

Self-replicating systems have been designed and studied to derive a better understanding of the scope and limitations of self-organisation processes[27] that are believed to be relevant to the origin of life on Earth.[2,28] Self-replicating systems can exhibit growth characteristics that lead to amplification of the template concentration through autocatalysis. However, one requirement, is that the formed product molecules are self-complementary and serve as templates. Autocatalysis in self-replication of DNA has been first demonstrated by von Kiedrowski[28] and Orgel.[29] In a more recent experiment, four common trimeric precursors **6**, **6′**, **7** and **7′** were allowed to react to furnish either self-complementary (**5**) or complementary (**8** and **8′**) templates (Scheme 2).[30] These reactions were followed in the presence of one of the hexadeoxynucleotide templates. It was found that self-complementary hexamers **5** (Scheme 2A) were formed faster than non-self-complementary hexamers **8** or **8′** (Scheme 2B) owing to self-replication by the formation of phosphoroamidate linkages in **11** (Scheme 2C). However, the simultaneous incubation of all four trimeric precursors allowed both autocatalysis and cross-catalysis to occur, and the hexamers **5**, **8** and **8′** were formed at similar rates. Previous kinetic studies had indicated that autocatalysis suffered from product inhibition, which does not allow for exponential amplification of template concentration.[31] In a procedure that allows exponential replication (SPREAD, surface-promoted replication and exponential amplification of DNA analogues) the catalytic activity of the template was increased by surface immobilisation of the newly formed template.[32]

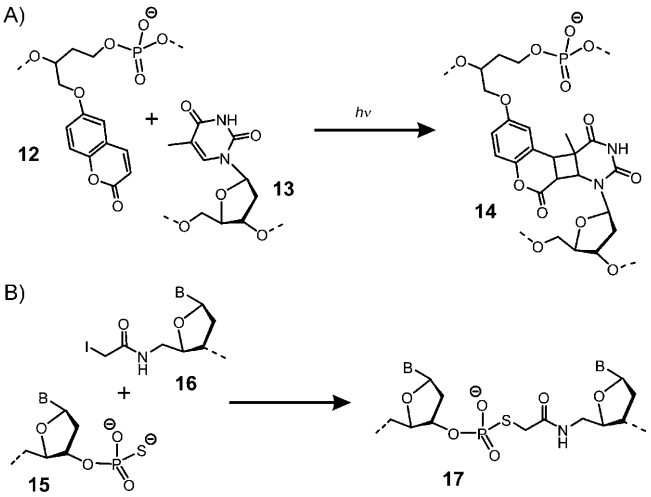


Scheme 2. A) Autocatalytic and B) cross-catalytic self-replication; C) carbodiimide mediated phosphoroamidate formation.[30,31]

Ligation Reactions in which Catalysis is Promoted by Thermal Cycling

The dissociation of product–template complexes is required to obtain turnovers. Dissociation can be enforced by thermal denaturation. A subsequent annealing process at low temperature is needed to continue the ligation process. This approach was taken by Albagli et al. who reported a photocycloaddition reaction between coumarin derivative **12** and thymidine **13** to covalently join two oligonucleotide probes in **14** (Scheme 3A).[26] Sequence-specific annealing was used to bring the coumarin group into the vicinity of a thymine base. Selective photochemical cross-linking provided a three-arm junction. It was demonstrated that a single-stranded DNA template was able to generate amplified amounts of cross-linked product by continuously repeating the three-step cycle of hybridisation, irradiation and denaturation. After ten cycles, the concentration of product exceeded the concentration of template by a factor of 5. Insertion of a second set of ligation probes that was complementary to the first probe set bypassed product inhibition and turned the amplification process into a self-replicating system.

Another recent approach, which included thermal cycling, was reported by Abe and Ito.[33] According to a ligation method from Letsinger,[34] 3′-phosphorothioate **15** was allowed



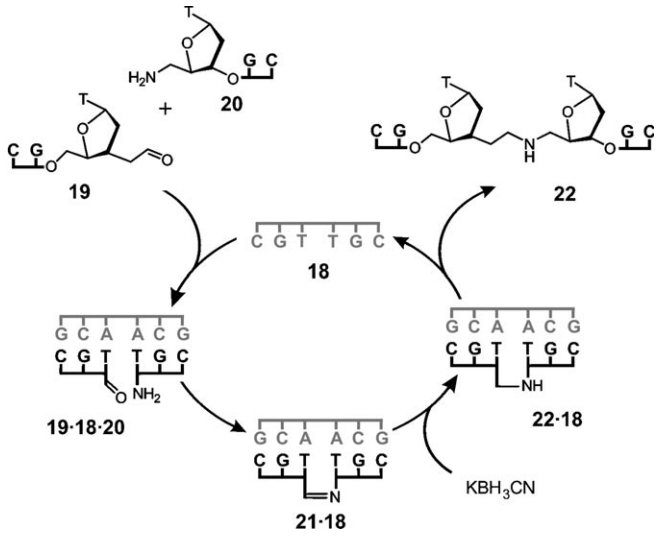
Scheme 3. A) [2+2] Photocycloaddition of coumarin derivative **12** and thymidine **13**;[26] B) phosphorothioate formation.[33]

to react with 5′-iodoacetylated oligonucleotide **16** (Scheme 3 B). The reaction was carried out in a thermal cycler instrument designed for polymerase chain reactions (PCR) and monitored by means of a gel-based analysis of radioactively labelled phosphorothioates **17**. After ligation, the reaction mixture was heated to 60 °C to promote the dissociation of the product–template complex. Up to 60 turnovers were obtained within 100–120 min when RNA was used as a template.

Thermal cycling might provide a technical solution to the problem of product inhibition; however, one possible drawback is that the high temperatures that are required to drive denaturation increase the rates of nontemplated reactions. Thus, background reactions might reduce the dynamic signalling range. Furthermore, it remains to be shown whether chemical ligation methods that require thermal cycling for signal amplification provide advantages to PCR-based analyses.

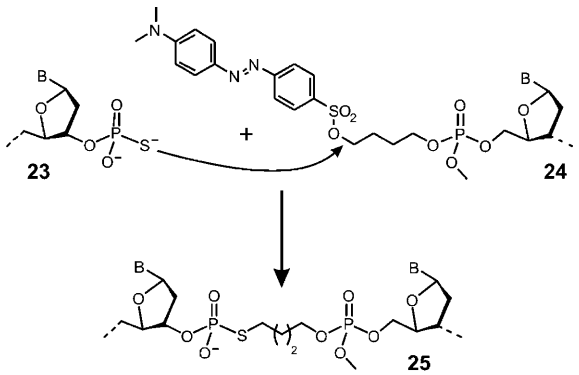
Ligation Reactions with Catalysis under Isothermal Conditions

The first reported approach in which a template-catalysed ligation reaction proceeded at isothermal conditions was introduced by Lynn in the 1990s.[25,35] In the catalytic ligation, an aldehyde **19** was coupled with an amine **20** in the presence of template **18** via reductive amination (Scheme 4). The reaction proceeds via a two-step mechanism in which the imine **21·18** is formed first, followed by reduction with KBH_3CN to yield the corresponding secondary amine-linked DNA-hexamer **22·18**. Interestingly, the product duplex **22·18** was estimated to be 10^6-fold less stable than the imine duplex **21·18**. This was explained by the increased flexibility of the ethylamine in product **22**.[25] More importantly, the product duplex **22·18** proved to be 30-fold less stable than the complex of the reactant probes with the template **19·18·20**. Thus, there was no product inhibition and the method afforded 50 turnovers in ligation.[36] There are no reports of the DNA-catalysed reductive ligation of larger oligonucleotides, but one can assume that the destabilisation by reduction might become less significant as the length of the oligonucleotide probes increases.



Scheme 4. DNA-catalysed reductive amination.[36]

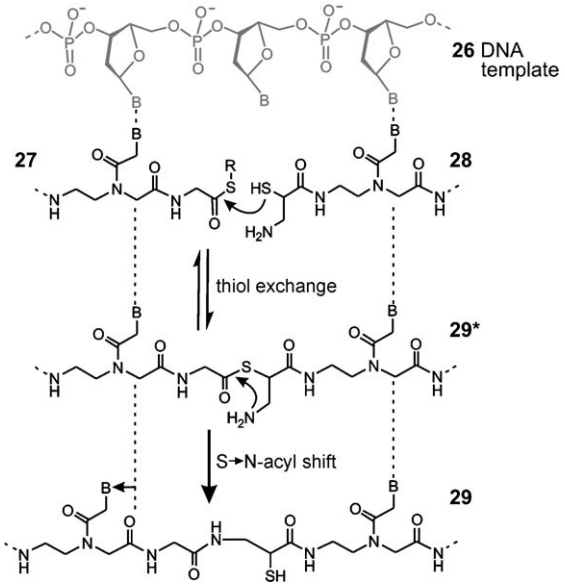
Kool and co-workers[6] reported a simple, reagent-free method for the ligation of oligonucleotides. An autoligation was mediated by nucleophilic displacement of a 5′-iodide with a 3′-phosphorthioate group.[37] The introduction of fluorescently labelled ligation probes facilitated readout of product formation and marked an important step towards applications in live cells.[7–10,15,38–40] An elegant concept relied on so-called quenched autoligation probes.[8,38–41] A suitable pair of ligation probes consists of a Cy5-labelled 3′-phosphorothioate probe and an electrophilic FAM-labelled probe in which the dabsyl quencher serves as leaving group. The quencher is lost upon ligation. Thus, the progress of ligation reactions can be monitored by measurements of fluorescence emission.[40] Interestingly, the introduction of fluorophores influenced the sequence specificity of the reaction as the differences in ligation rates on matched versus single-mismatched templates were reduced from 180-fold for unlabelled ligation probes to 35-fold for labelled probes.[6,7] To promote turnover, a flexible linker was inserted at the ligation site (Scheme 5).[15] The dabsyl group was



Scheme 5. Phosphorosthioate formation with a dabsyl leaving group at the end of a flexible hydrocarbon linker.[15]

installed at the end of a hydrocarbon linker in **24** such that the ligated product **25** contained a several-atom-long flexible linker that interrupted the Watson–Crick base pairing and destabilised the product–template duplex.[42,43] This approach yielded 92 turnovers at 10^4-fold excess of ligation probes after 24 h reaction time.[15]

We recently suggested a potentially generic concept to reduce product inhibition in template-catalysed ligation reactions.[17] The design implements the central feature of Lynn's two-step ligation reaction in which the ligation product that is formed in the first step should bind the template with high affinity, whereas the second reaction step should result in the reduction of the stability of product–template duplexes. The native chemical ligation of peptide–nucleic acid (PNA) probes **27** and **28** proceeds via a ligation–rearrangement sequence wherein the rearrangement step alters the chain length of the backbone (Scheme 6). Ligation of a PNA-glycine thioester **27** with an *iso*-cystein-PNA **28** forms the thioester intermediate



Scheme 6. The native chemical ligation proceeds via a reaction sequence wherein the rearrangement form **29*** to **29** alters the length of the backbone.[17]

29*. A subsequent S→N acyl shift leads to an extension of the main-chain. The accompanying increase of flexibility destabilises the product–template duplex.[11,17]

The ligation occurred opposite to an unpaired nucleobase. This arrangement on the one hand enhanced the sequence fidelity to a match/mismatch selectivity of 10^3, and on the other reduced the affinity of the ligation product **29** for the template **26**.[12] At 10^4-fold excess of ligation probes **27** and **28**, 226 turnovers were obtained after 24 h.[17] To enable real-time measurement of the ligation reaction a FRET-based read-out was developed that is specific for product formation. The labelled ligation probes furnished very high sequence fidelity, and the signalling of mismatched templates was below the limit of detection.[17,44]

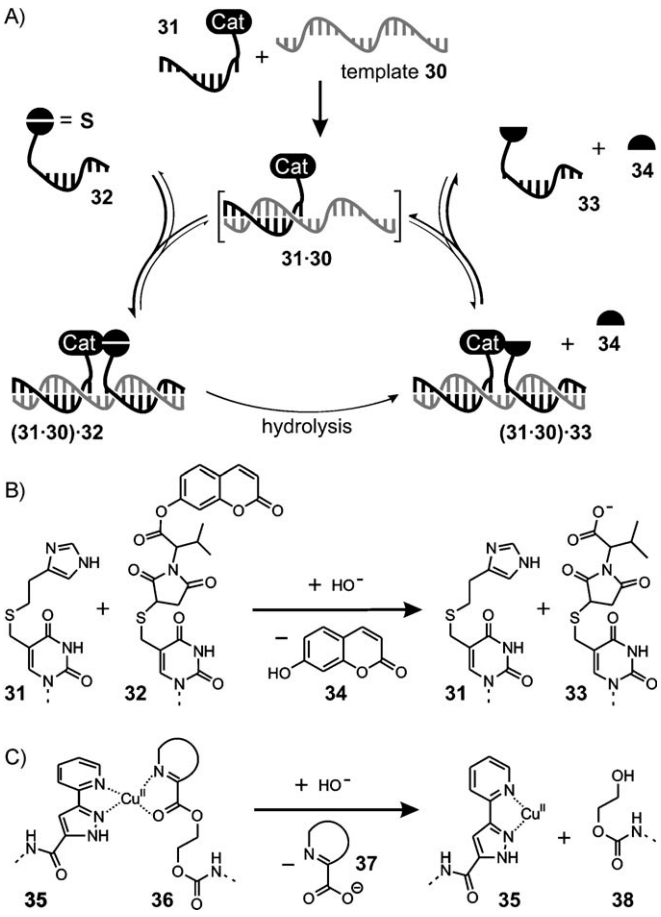
Despite the considerable efforts invested, the ligation products commonly bind to the template with higher affinity than the probes before ligation. The problem of product inhibition requires the use of short ligation probes, which should be applied at high concentration and in large excess to the template in order to drive displacement of the product from the product–template complex. However, these conditions typically increase the rate of background ligation. In these cases, signal amplification is achieved at the cost of increased signal background. For example, in the PNA-based ligation system (Scheme 6) the templated reaction had to be detected on 17% background from nontemplated reactions.

DNA-Catalysed Cleavage Reactions

Catalysis requires that the product–template complexes dissociate at least as readily as reactant–template complexes form. Ideally, templated reactions should be designed to avoid an increase in the number of paired nucleotides in the reaction product. Cleavage reactions fall under this category.

DNA-Catalysed Ester Hydrolysis

Ma and Taylor introduced DNA/RNA-catalysed hydrolysis reactions.[14,20–22] They proposed a concept for the design of a drug-release system triggered by disease-specific mRNA or DNA (Scheme 7 A). The potential for nucleic acid detection was also addressed. In this concept, one probe, **31** is equipped with a catalytic moiety (Cat) and the other probe **32** with a suitable substrate (S). Probe **31** and the template **30** form the catalytically active duplex **31·30** that is capable of binding substrate probe **32**. The cleavage of S furnishes product probe **33**, which has an unchanged affinity to the target **30**. In the initial approach DNA-based probes were employed and up to three turnovers were demonstrated.[14] The use of PNA-based reaction systems allowed for increases of the catalytic rate.[21,22] The hydrolysis of oligonucleotide coumarin ester **32** by adjacent hybridisation of an imidazole-containing oligonucleotide **31** enabled the release of fluorescent coumarin **34** (Scheme 7 B). Even at relatively low excess of substrate **32** the duplex of imidazole-conjugate and template **31·30** showed catalytic activity.[14]

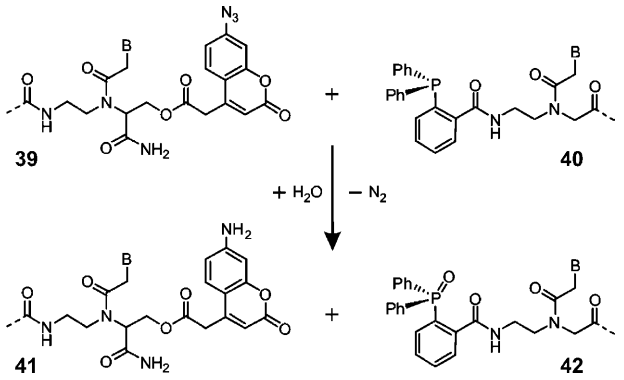


Scheme 7. A) Concept of the nucleic acid catalysed hydrolysis;[14] B) imidazole[22] and C) Cu^{II}-mediated[45] ester hydrolysis.

Mokhir and Krämer described a DNA-templated cleavage reaction in which a copper(II) complex acted as catalyst of ester hydrolysis (Scheme 7C).[13,16,45] A 3-(pyrid-2-yl)-pyrazole fragment **35** and the substrate picolinate ester **36** were attached to short sequences of PNA and brought into close proximity at a complementary DNA template. The functionalities served as ligands for a copper(II) complex, which accelerated the hydrolysis of the substrate ester to give **37** and **38** (Scheme 7C). This approach yielded ~10 turnovers,[16] and proved the ability of hydrolysis reactions to achieve signal amplification of one order of magnitude at relatively low substrate concentration and excess.

DNA-Catalysed Staudinger Reaction

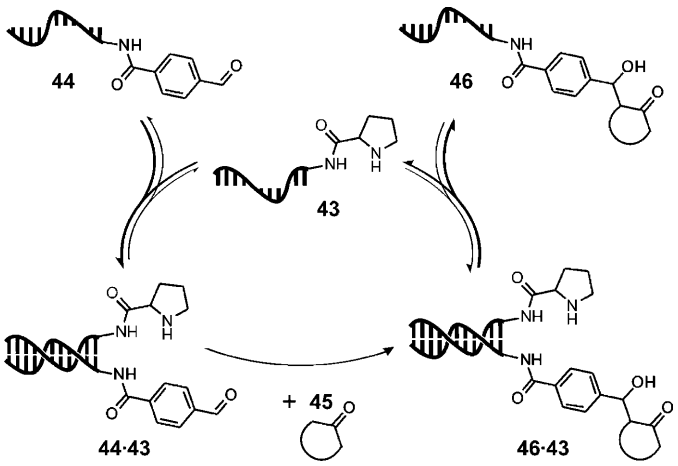
Pianowski and Winssinger reported a DNA-catalysed version of the Staudinger reaction.[19] They used a low-fluorescent azidocoumarin, **39** and a triphenylphosphine-modified PNA probe **40** (Scheme 8). Hybridisation with the DNA template triggered the reduction of the azide function in **39** to release nitrogen and yield a highly fluorescent coumarin derivative **41**. In this setup, both product probes **41** and **42** have to be replaced by the starting probes **39** and **40**. At 10^2-fold excess of probes **39** and **40**, 20 turnovers were obtained after only 30 min.



Scheme 8. Staudinger reaction between an azidocoumarin (**39**) and a phosphin-modified PNA probe (**40**).[19]

DNA-Catalysed C–C Bond Formation

Tang and Marx used proline-modified DNA **43** as catalyst in a proline-catalysed aldol reaction between a complementary aldehyde–DNA conjugate **44** and several non-tethered ketones **45** (Scheme 9).[46] The displacement of the product probe in duplex **46·43** by aldehyde-modified probe **44** was promoted by thermal cycling. After 50 cycles (25 °C for the aldol reaction and 80 °C for duplex denaturation) at 20-fold excess of probe **44**, eight turnovers were achieved. In principle, the adjacent hybridisation of two modified probes (catalyst and substrate) to an unmodified target should result in a system similar to the catalytic cycle depicted in Scheme 7A, and therefore expand the scope of this approach.

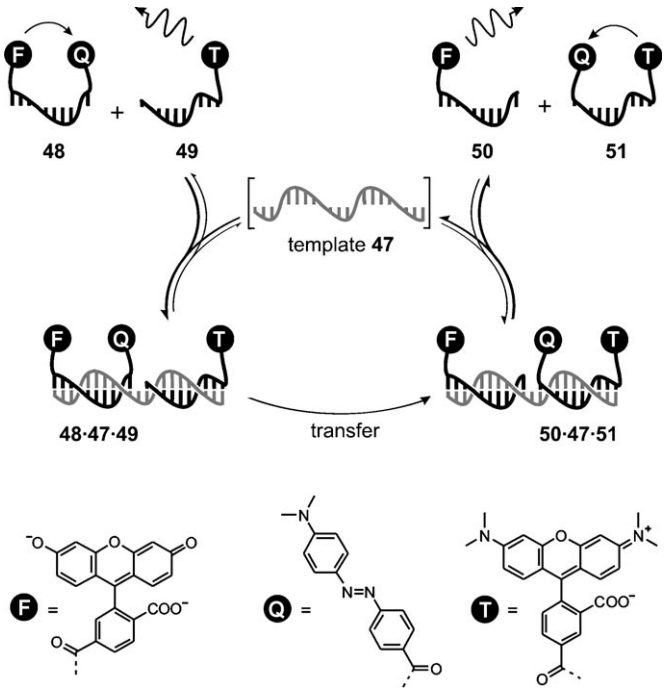


Scheme 9. DNA-catalysed aldol reaction.[46]

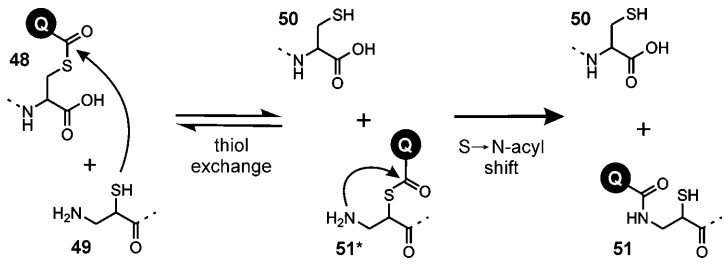
DNA-Catalysed Transfer Reactions

Transfer reactions should also be amenable to template catalysis since the number of paired nucleotides remains unchanged. In addition, transfer reactions allow the target-directed modification of detector probes with readily detectable reporter groups. Taylor and co-workers presented a concept in which the Staudinger ligation was used to activate fluorescein emission.[47] In this approach an O-acylated fluorescein–PNA conjugate was allowed to react with a second PNA probe that was equipped with an azide function. Upon hybridisation the acyl moiety was transferred to the azide-bearing probe to yield an amide bond and the fluorescing deacylated fluorescein–PNA conjugate. This elegant chemoselective reaction has not been described with substoichiometric amounts of template so far.

We recently showed that high catalytic turnover numbers can be obtained in a DNA-controlled aminolysis reaction. This reaction was used to transfer a reporter group from a donating to an accepting probe.[18] In one example the fluorescence quencher (Q) was transferred from the fluorescein (F) to the tetramethylrhodamin (T)-modified PNA probe (**48** and **49**, Scheme 10). The relocation switched on emission of F in **50**, while switching off emission of T in **51**. The transfer reaction proceeded via a native chemical ligation-like mechanism (Scheme 11). Reporter group Q in **48** is attached as thioester. Upon hybridisation, a thiol exchange with the *iso*-cysteine moiety in probe **49** leads to intermediate **51***. A subsequent S→N-acyl shift forms transfer product **51**. After 24 h the DNA-catalysed transfer furnished 402 turnovers at 10^4-fold excess and relatively low concentration (100 nM) of reactant probes. The reaction combines high catalytic activity of the target DNA with useful yields (69% and 25% yield after 24 h on 0.01 and 0.001 equiv template, respectively) and low background (3.4% non-templated transfer after 24 h). The modular setup allows the use of various types of reporter groups. Therefore, other readout strategies can be envisioned.[48]



Scheme 10. Transfer of a fluorescence quencher (Q) from a fluorescein (F) to a tetramethylrhodamin (T)-modified PNA probe, **48** and **49**, respectively (F: 6-carboxyfluorescein, Q: dabcyl, *T* = 5-carboxytetramethylrhodamin).[18]

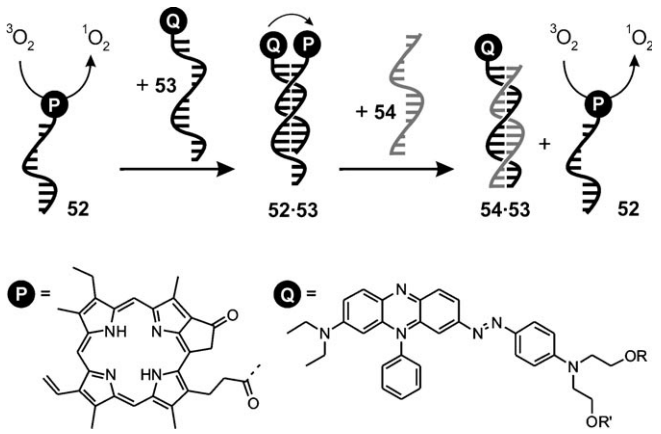


Scheme 11. Transfer of a reporter group (Q) by a native chemical ligation-like mechanism.[18]

Nontethered Reagents in DNA-Catalysed Reactions

The basic feature of most DNA/RNA-catalysed reactions is that nucleic acid hybridisation is used to increase the effective molarity of the reactants. These concepts require that at least one reactant be linked to an oligonucleotide. Alternative approaches involve nontethered reagents and DNA that acquires catalytically active moieties either by chemical modification or by noncovalent binding.

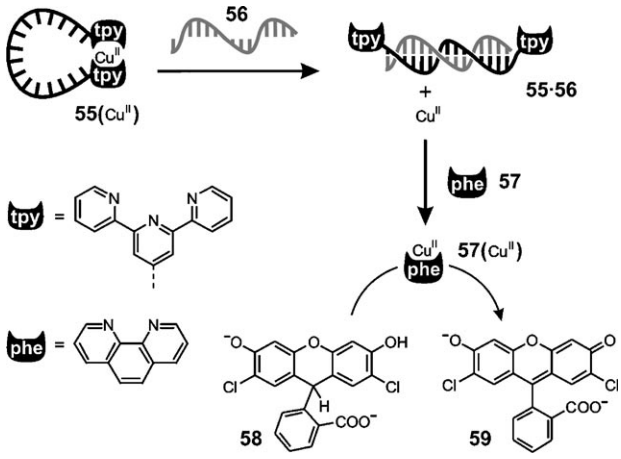
Ogilby and Gothelf presented a strategy that allows the off and on switching of the synthesis of singlet oxygen (Scheme 12).[49] The photosensitiser pyropheophorbide-*a* (P) was attached to a 15-mer DNA sequence **52**. Excitation of P initiates the conversion of $^3O_2$ to $^1O_2$. The excited state of P was quenched and hence the production of $^1O_2$ deceased when quencher-labelled complementary 21-mer **53** was present. To reactivate photosensitiser P unmodified DNA **54** was added. The release of catalytically active **52** proceeded because **54**



Scheme 12. Off and on switching of a singlet oxygen photosensitiser P (P: pyropheophorbide-*a*, Q: black hole quencher 3).[49]

was designed to be the full-length complement of quencher-labelled probe **53**. The authors envisioned DNA-triggered activation of $^1O_2$ production as a potential photodynamic therapy, in which pathogenic nucleic acid sequences could be used to trigger a $^1O_2$-induced cell death.

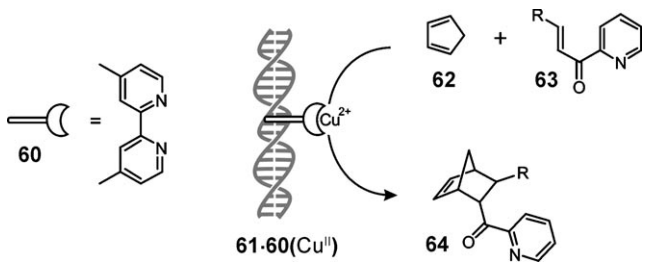
Krämer and co-workers reported the target-induced allosteric release of copper(II) ions which served as cofactor for a redox catalyst that is capable of catalysing the formation of a fluorescent product. (Scheme 13).[50] In the initial probe **55**(Cu^{II}) the copper ion was complexed by two terpyridine (tpy) fragments that were attached to both termini of a 20-mer DNA segment **55**. The addition of target DNA **56** induced the formation of a rigid duplex structure in **55·56**, thereby disrupting the intramolecular chelation, and releasing Cu^{II}. This allowed the formation of complex **57**(Cu^{II}), which was capable of catalysing the oxidation of up to 20 nonfluorescent molecules **58** to fluorescent 2′,7′-dichlorofluorescein (**59**). The authors explained that the signal amplification was limited by the low catalytic turnover rate (2 h^{-2}) in the final oxidation step



Scheme 13. DNA-trigged release of Cu^{II} ions and their subsequent detection by a catalytic oxidation reaction (typ: terpyridine, phe: 1,10-phenanthroline).[50]

and suggested the use of more-active catalysts such as apoenzymes in combination with their metal ion cofactors.

Roelfes and Feringa employed double-stranded DNA as chiral ligand in copper-catalysed enantioselective Diels–Alder reactions (Scheme 14).[51–54] Small aromatic compounds served



Scheme 14. Enantioselective DNA-based Diels–Alder reaction.[51]

to anchor metal complexes to DNA. For example, DNA-intercalators were linked to metal-binding groups via spacers.[52] Bidentate ligands that included both the metal binding site and the intercalator, such as in **60** enabled a closer contact of the metal centre with the enantiodiscriminating double helix. As a result, high enantioselectivities were obtained in the Cu^{II}-catalysed Diels–Alder reaction of cyclopentadiene (**62**) with azachalcones **63** in water.[51] Catalyst **61·60**(Cu^{II}) was also used in enantioselective Cu^{II}-catalysed Michael addition reactions.[54]

Summary and Outlook

The DNA- and RNA-triggered catalytic formation of product molecules has been investigated with the aim to improve the sensitivity of DNA and RNA detection, to explore gene-targeted activation of prodrugs and as an opportunity to exploit helical chirality in asymmetric catalysis. Mostly the unique pairing properties of complementary nucleic acid strands are used to align reactive groups of modified oligonucleotides; this allows the acceleration of reactions at very low reactant concentrations. Particularly, high rate accelerations have been achieved in DNA-controlled ligation reactions. However, the product inhibition causes problems. The highest turnover numbers in ligation reactions have been obtained in systems that exhibit high flexibility at the ligation site or involve a flexibility-inducing step. Nevertheless, it appears that the destabilisation conferred by flexible junctions is not sufficient to prevent inhibition of the template by larger ligation products. It might thus be required to design ligation systems that undergo more global changes of conformations subsequent to ligation. DNA-catalysed cleavage and transfer reactions offer a partial remedy to the problem of product inhibition. The number of paired nucleotides in each of the reaction components recognised by the template remains constant. Thus these reactions can be performed under conditions in which exchange of oligonucleotide-based reactants and products is fast. Under these conditions, and amplified product signals can be detected even at a low excess of reactants. Transfer reactions offer the fascinating opportunity to put conjugation chemistry under the control of the nucleic acid templates. It is thus imaginable to use DNA-catalysed transfer reactions as a means to modify probes in homogenous and heterogeneous formats; this might provide unique opportunities for the design of coupled catalytic circles. Nontethered reagents hold much potential in DNA-catalysed reactions as the turnover numbers are, in principle, no longer governed by the dissociation rates of nucleic acid duplexes. Regardless of the method used, some DNA-catalysed reactions have reached a state of art that allows applications in realistic scenarios, for example, in the PCR-free detection of DNA and RNA.

Keywords: catalysis · DNA · nucleic acid detection · oligonucleotides · signal amplification

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