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Amplification by Nucleic Acid-Templated Reactions

Julia Michaelis[†a], Alexander Roloff[†a] and Oliver Seitz*[a]

[†]these authors contributed equally to this review

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Nucleic acid-templated reactions enable the design of conditional reaction systems, in which bond formation occurs only when a particular DNA or RNA molecule is present. Such reaction systems are currently being explored for applications in DNA/RNA diagnosis, drug screening and as a means to design gene expression specific therapy. However, biological nucleic acid templates usually have low abundance. Therefore, either the targeted nucleic acid template has to be multiplied by means of an amplification step or the template itself has to act as a catalyst which amplifies product formation. This critical review highlights the recent advancements in nucleic acid-templated reactions that proceed with turnover in template and thereby provide a means for amplification. Improvements in reaction engineering and the development of new chemistries have pushed the limits from 10^1 to 10^2−10^3 turnovers. This includes reaction systems that lead to the ligation of oligonucleotides or to the interconversion of appended functional groups beyond ligation as well as templated chemistries that enable the activation of catalysts for subsequent triggering of reactions between non-nucleotidic substrates. Present limitations and future opportunities will be discussed.

Introduction

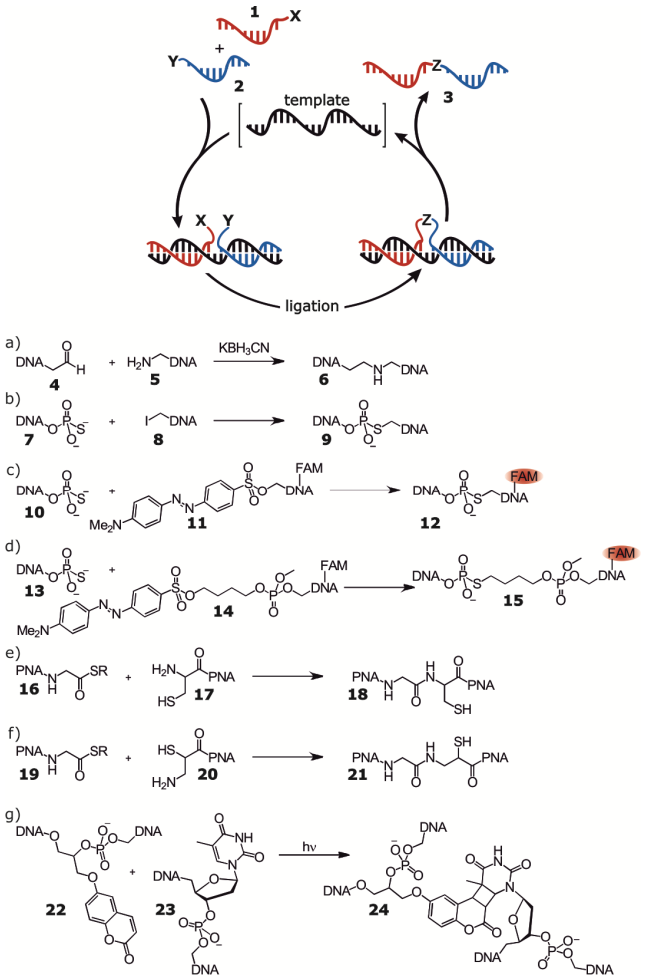
Replication, transcription and translation involve nucleic acid molecules acting as templates of biochemical reactions. The fundamental importance of these processes has inspired chemists to investigate the molecular basis of evolution. The pioneering investigations of self-replicating reaction systems[1–10] revealed that the proximity enhancement induced upon adjacent alignment of preactivated (oligo)nucleotides on the template enables dramatic increases of reaction rates. The current research on nucleic acid-templated chemistry extends beyond origin-of-life studies. A multitude of sequence-programmed reactions is explored with an aim to translate nucleic acid sequences into functions relevant to applications. As yet, nucleic acid templated reactions have predominantly been used in DNA/RNA diagnosis, where product formation detects or images the presence of the targeted nucleic acid template.[11–15] The extension of the repertoire of DNA-templated reactions has enabled the design of evolvable small molecule libraries that are envisioned to facilitate the drug screening process.[16–19] RNA-controlled reactions are explored as potential tools for a molecular therapy which engages itself upon exposure to a specific disease-related gene expression status.[20–24] According to a fascinating vista, templated reactions on nanosized DNA-architectures may once allow the replication of complex functional systems.[25–27]

Recurring problems in addressing biologically originated nucleic acid templates are the relatively low quantities which are available by common extraction methods or which might be present within cells. Therefore, either the target nucleic acid has to be multiplied by means of an enzymatic amplification step or the template itself has to act as a catalyst which amplifies product formation. Nucleic acid templates are evolutionary optimized self-recognition modules which can confer dramatic increases in reaction rate *via* proximity enhancement, but they typically are rather modest catalysts because both the starting materials and the reaction products usually bind with high affinity to the template. Hence, nucleic acid-templated reactions are severely affected by product inhibition which limits turnover in template. This *critical review* focuses on the current state of nucleic acid-templated reactions that provide turnover in template and, thereby, enable product amplification. Innovations and limitations will be discussed in the context of envisaged applications. Within the last 5 years since the publication of a similar review,[12] important advancements have been made in reaction engineering as well as in the development of new biocompatible reactions. Given their timeliness, emphasis will be placed upon the most recent achievements in ligation chemistry, transfer chemistry and Staudinger chemistry. In addition, templated chemistries that involve helper catalysts will be discussed.

Nucleic acid-templated ligation reactions

Nucleic acid-templated ligation reactions merge two reactive oligonucleotide probes **1** and **2** into a longer product oligonucleotide **3** (Scheme 1). The ligation product usually has a higher affinity for the template than the oligonucleotides before reaction. As a result, ligation reactions usually suffer strongly from product inhibition. On the other hand, ligation chemistries are more versatile and often easier to implement in a templated format than non-ligation based interconversions. Taking into account the progress in the rapidly emerging field of biocompatible conjugation methods,[28–31] it is of increasing interest to establish strategies that allow for catalytic conversions in nucleic acid-controlled ligations.

Lynn, Kool and Seitz were amongst the first who reported non-replicative DNA-promoted ligation reactions that proceeded with significant turnover in template under isothermal conditions.



Scheme 1 Nucleic acid-templated chemical ligation with turnover in template (X, Y: reactive groups, Z: ligation junction). Different ligation types such as a) reductive amination,[32,33] b) phosphorothioate-iodide[35-38] or c), d) -dabsyl substitution,[39-43] e), f) native chemical ligation[47-50] or g) coumarin-thymidine photoligation[51] have been shown to lead to the formation of multiple ligation products per template. (FAM: fluorescein dye, R: $CH_2C_6H_5$, $CH_2CH_2CONHGly\text{-}NH_2$, $CH_2CH_2SO_3Na$)

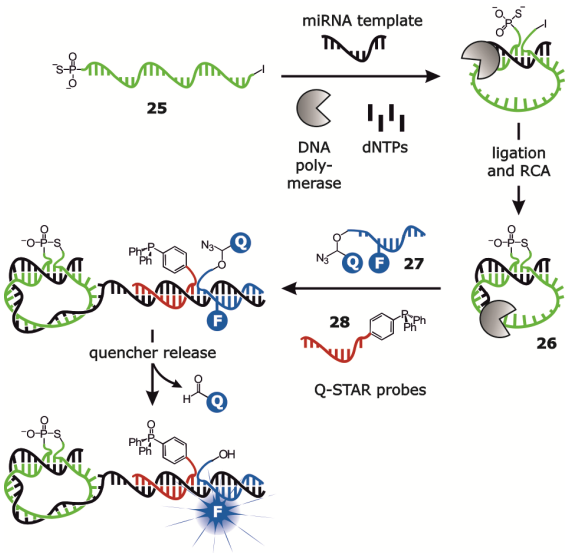
Lynn's templated reductive amination between aldehyde- and amino-modified oligonucleotide trimers **4** and **5** provided approx. 50 product molecules (**6**) per template (Scheme 1a).[32–34] No attempts were reported about catalytic reactions with larger oligonucleotides. Kool and co-workers developed the templated 'autoligation', i.e. a ligation that is not dependent on chemical additives. For example, the DNA-templated nucleophilic substitution reaction between 3'-phosphorothioate- and 5'-iodothymidine-terminated DNA-based probes **7** and **8** afforded 40 product molecules (**9**) per template at low nanomolar template concentration (Scheme 1b).[35–38] In an advanced version of this approach, the iodine moiety was replaced by a sulfonate leaving group (Scheme 1c).[39] This allowed the design of so-called quencher assisted ligation (QUAL) reactions, in which the displacement of a sulfonate-linked quencher group in **11** (dabsyl) by the phosphorthioate in **10** restores fluorescent emission of a fluorophore. The formation of the fluorescent ligation product **12** can be monitored in real-time. The method was applied in the detection of ribosomal RNA (rRNA)[40,41] and messenger RNA (mRNA)[42] within live cells. The incorporation of flexible ligation junctions opposite to unpaired template bases (see product **15** in Scheme 1d) increased the catalytic efficiency to 92 turnovers in template.[43]

Our group explored the use of peptide nucleic acid (PNA)-based ligation reactions. PNA is a non-ionic DNA analogue that binds complementary nucleic acids with very high affinity and sequence specificity.[44,45] The peptidic nature of the PNA backbone facilitated the application of powerful ligation chemistries developed for protein synthesis. For example, the native chemical ligation (NCL) is a highly chemoselective reaction which relies on the reaction of a thioester with a vicinal aminothiol structure usually provided by an *N*-terminal cysteine residue.[46] The nucleic acid-templated NCL involved a PNA-glycylthioester **16** and a cysteinyl-PNA-conjugate **17** (Scheme 1e).[47–49] The templated PNA ligation affording product **18** showed high sequence specificity and enabled the detection of single base mutations on PCR-DNA targets. Up to 226 (as opposed to 51) ligation product molecules were formed per DNA template when cysteine was replaced by the β-amino acid isocysteine (*i*Cys) in **20** (Scheme 1f).[50] This increase in turnover was attributed to the rather low template affinity of ligation product **21** that has a Gly-*i*Cys junction opposite to an unpaired template base.

The aforementioned strategies achieved product amplification by promoting the dissociation of the product-template duplex *via* enhanced flexibility at the ligation junctions. Thermal cycling offers an alternative means to promote dissociation of product-template complexes. Albagli[51] developed a photocycloaddition reaction between a coumarin-functionalized DNA **22** and a thymidine in the second probe **23** (Scheme 1g). Formation of a three arm junction during template hybridization prevented cross-linking of the coumarin probe with the template strand and enabled the specific generation of product **24** when irradiated with UV-light. The use of a second probe set complementary to the first one turned the system self-replicating, yielding ~30-fold amplification after 21 cycles consisting of subsequent hybridization, irradiation (reaction) and denaturation steps. Abe and Ito[52] showed that Kool's phosphorothioate-iodide substitution furnished up to 60 product molecules per RNA-template after 100 thermal cycles.

Although turnover numbers of > 200 are impressive, there are drawbacks. Usually, vast excess of probes over template and long incubation times of up to 24 h are required to obtain high turnover.[38,43,50] Under these conditions, the speed of the off-template reactions such as hydrolysis or template-independent ligation can be significantly higher than the speed of templated synthesis, and with decreases in template concentration it is becoming increasingly difficult to detect product signals above background. The work of Albagli is a notable exception. The exponential amplification method allowed to trigger the reaction with 200 pM template.[51]

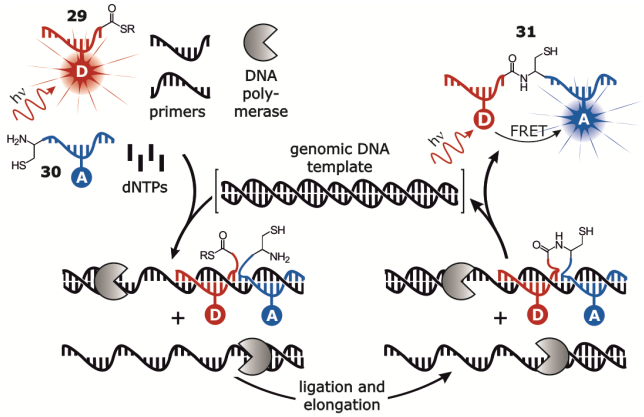
To enable ligation reactions at subnanomolar template Kool and co-workers introduced a chemoenzymatic double-amplification system.[53] In a first step, a miRNA template functioned as a splint for the intramolecular ligation (cyclization) of a linear DNA probe **25** *via* sulfur-halide substitution (Scheme 2). This finds similarity in the concept of padlock probes, which require a ligase to execute cyclization.[54] The cyclized DNA **26** served as a



Scheme 2 A miRNA template initiates the cyclization of a terminally iodinated DNA phosphorthioate (**25**), which templates a target primed rolling circle amplification (RCA). The RCA products are detected by means of a fluorogenic Q-STAR reaction (see also Scheme 13) between **27** and **28**.[53] (F: fluorophor; Q: quencher).

template for rolling circle amplification (RCA) using ϕ29 DNA polymerase. The polymerase is primed by the miRNA itself (target-primed), which prevents RCA to be initiated in the absence of the template. Long, single stranded DNA repeats were produced and used as templates for the second ligation step. Q-STAR probes (**27** + **28**, *vide infra* for a detailed explanation) capable of turnover in template annealed adjacently on multiple binding sites of the RCA product and promoted a fluorescent signal upon reaction. It was estimated that RCA yields 188-fold template amplification, whereas an additional 15-fold gain in signal was achieved through turnover of the Q-STAR probes. The detection limit of this system was 200 pM, pointing to the benefit in sensitivity through enzymatic template amplification. Single base specificity was demonstrated. The use of a miRNA carrying a point mutation at the ligation site reduced the signal increase by a factor of 5. The hallmarks of this reaction system are negligible background, fluorescence-based real-time read-out and isothermal reaction conditions (30 °C). Splint ligation and RCA can be carried out simultaneously. It proved advantageous to perform the signal generating reaction afterwards. The long incubation time (24 h) is a drawback.

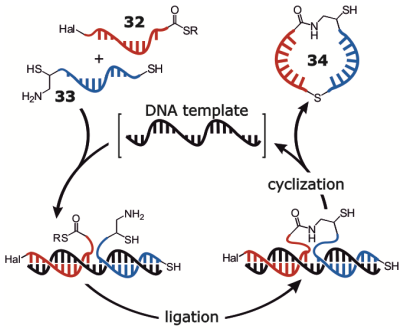
Kool's method relied on a linear enzymatic amplification reaction (RCA). To achieve exponential amplification of the template, we interfaced a fluorogenic DNA-templated native chemical PNA ligation with polymerase chain reaction (PCR) (Scheme 3).[55] PCR comprises thermal cycles of denaturation (95 °C, 10 sec), annealing (50 °C, 30 sec) and extension (72 °C, 20 sec). The process poses a challenge to ligation chemistry because on the one hand the reactive probes must remain stable during the denaturation step while on the other hand only little time is available to accomplish templated ligation during the annealing phase. We settled on templated native chemical ligation chemistry between reactive PNA-conjugates **29** and **30**. We showed that the use of the less reactive β-aminoacid (β-alanyl) rather than α-aminoacid (glycyl) thioesters reduced template-



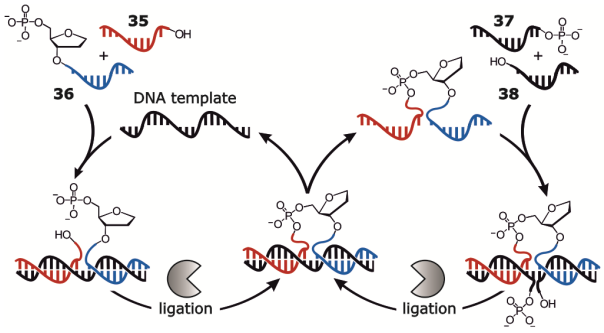
Scheme 3 An amplicon templates the fluorogenic native chemical ligation of donor and acceptor fluorophore labeled thioester- (**29**) and cysteinyl-PNA (**30**) probes during its amplification *via* PCR.[55,56] (D: fluorescence donor; A: fluorescence acceptor).

independent background and improved the resistance towards hydrolysis. To enable very fast signaling rates within the given sequence context, the ligation was designed to proceed through seamlessly base-paired template-probe complexes.[56] The human BRaf-gene served as template in an asymmetric PCR, which yields an excess of single stranded PCR product. The FRET signal from ligation product **31** formed along PCR was detected in this one-pot procedure within a few hours, starting from as little as 2.5 aM template concentration. No signal was generated in absence of template or in presence of single base mismatched templates. These features attest to the sensitivity and sequence specificity of the in-PCR ligation. Moreover, native chemical PNA ligation is amongst the few methods that allow the use of double stranded DNA templates. Interfacing chemoselective nucleic acid-templated chemistry with PCR may provide a general solution to the problem of product inhibition in DNA/RNA-templated ligation reactions. However, the requirement for a thermocycling device as well as the rather elaborated reactivity tuning is disadvantageous.

The RCA- and PCR-based methods aimed at *bypassing* product inhibition in templated chemistry by employing enzymatic amplification steps. This resulted in significantly improved sensitivities, enabling the detection of picomolar to attomolar concentrations with single base specificity. However, this gain of sensitivity was not achieved by improved turnover of the underlying chemical reactions, but rather by simply increasing the amount of template available that can in turn trigger the ligation step. Recently, we introduced a non-enzymatic approach to *reduce* product inhibition. A ligation-cyclization reaction sequence (termed 'cycligation', see Scheme 4) can generate cyclic ligation products (**34**) which exhibit significantly reduced template affinities.[57] During 'cycligation', the perturbation of the product-template duplex is rather global compared to the earlier examples[32,33,43,50] which led to localized perturbations around the flexible ligation junctions. The 'cycligation' reaction involved the bis-electrophiles **32** and bis-nucleophiles **33** which undergo a templated native chemical ligation step followed by an intramolecular sulfur-halide substitution. The yields achieved upon 'cycligation' on substoichiometric DNA-templates were 2–3-fold higher than yields obtained in ligation-only reactions, provided that dissociation of the initially formed product–



Scheme 4 The DNA-templated ligation of haloacetylated PNA-thioesters **32** with thiolated isocysteinyl-PNAs **33** is followed by cyclization of the ligation products. The cyclic products **34** have low template affinity which promotes turnover in template.[57] (R: CH_2CH_2CONH-Gly-NH_2)



Scheme 5 The DNA-templated enzymatic ligation of two DNA probes (**35** + **36**) yields products with abasic sites reducing their template affinity. A second set of complementary probes (**37** + **38**) implements an additional amplification cycle that enables DNA replication in a cross catalysis format.[58,59]

template duplex was enabled by thermal cycling (or when dissociation was favored in the early phase of reactions at low product concentration). However, the reported sequence of ligation and cyclization reactions failed to provide advantages at low nanomolar template loads. This was attributed to the template affinity of the cyclized ligation product. Though cyclization destabilized the product by $\Delta T_M = -11$ °C this was not sufficient to overcome the T_M-increase conferred upon PNA ligation ($\Delta T_M = 19$ °C). It remains to be shown whether product cyclization in combination with other templated chemistries will lead to reaction products with lower template affinities than the reactants; an ultimate goal on the road to highly catalytic nucleic acid-directed chemistry.

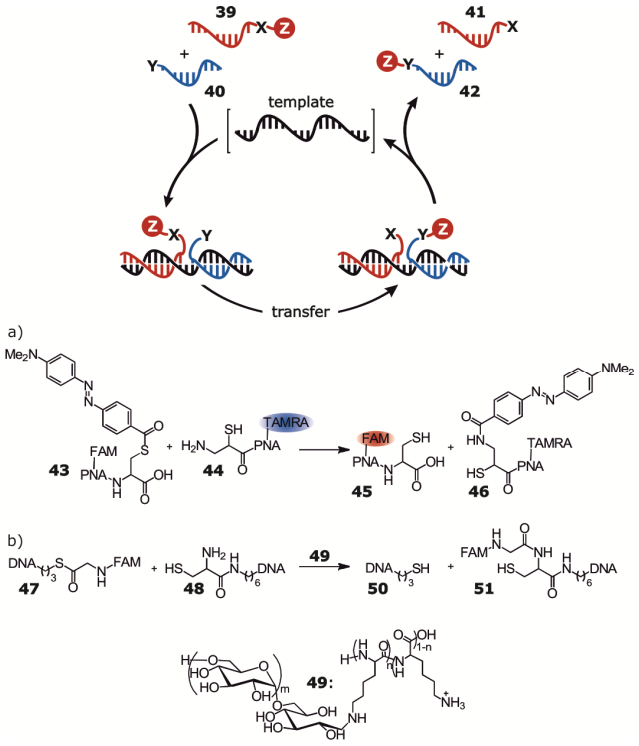
An approach that is based on templated enzymatic ligation was introduced by Gibbs-Davis.[58] A T4-ligase mediated ligation reaction between 3'-OH and 5'-phosphate modified DNA probes delivered up to 18 product molecules per template. It was key to incorporate an abasic site at the ligation junction. The resulting destabilization of the product-template duplex facilitated dissociation and, thereby, provided access to fresh reactants. The reaction had low background, because the ligase requires the reacting oligonucleotides arranged in a ternary complex. Low nanomolar template concentrations sufficed to trigger ligation. The sensitivity was improved by implementing a second amplification cycle. In a cross catalysis format, two complementary sets of probes (**35** + **36** and **37** + **38**) were utilized in a self-replicating system (Scheme 5). Here the abasic site-containing product of the **35** + **36** ligation templated the ligation of the unmodified DNA probes **37** + **38**. Up to 32 product molecules per template could be obtained within 20 h, yet at the expense of a non-negligible background rate (the ligase recognizes the complex comprised of all 4 probes and initiates a pseudo-blunt-end ligation). An improvement in turnover efficiency was achieved by using very high ligase concentrations.[59] Under these 'high-enzyme' conditions and in the presence of 14 nM template (0.01 equiv), about 100 ligation reactions per template occurred within only 40 min, accompanied by complete consumption of the reactants. The reaction in absence of template lagged behind the target-directed process but also reached completion after 80 min. By decreasing the concentration of the reactants in a first reaction and using higher concentrated probes and fresh ligase in a subsequent reaction, more than 2 million-fold amplification was observed. This facilitated the detection of 140 fM template.

Nucleic acid-templated reactive group interconversions

Interconversions of chemical moieties between two oligonucleotide probes represent one answer to the problem of product inhibition found in ligation reactions. These reactions do not change the length of the involved reactive oligonucleotides. Therefore, molecules after reaction have similar template affinities than molecules before reaction, enabling significantly increased turnover in template.

Nucleic acid-templated transfer reactions

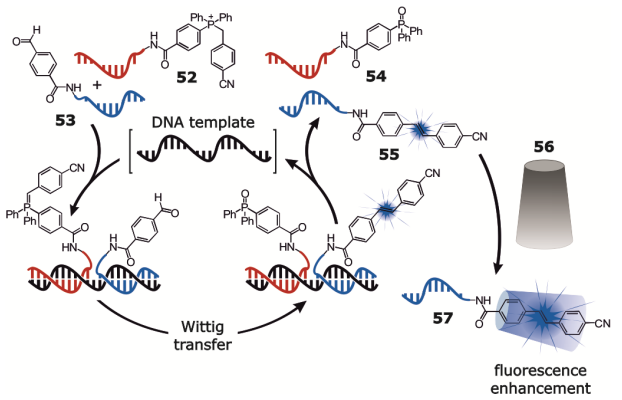
Nucleic acid-templated transfer reactions involve the relocation of groups from one donating oligonucleotide conjugate **39** to another accepting oligonucleotide conjugate **40**, yielding the two product oligonucleotides **41** and **42** (Scheme 6). Grossmann and Seitz demonstrated acyl transfer reactions which proceed according to a NCL-type mechanism.[60] A reporter group such as a dabcyl quencher was attached to a fluorescein-labeled PNA-probe **43** *via* a thioester linkage (Scheme 6a). Adjacent annealing of a rhodamine-labeled isocysteinyl-PNA conjugate **44** triggered the dabcyl transfer which was accompanied by increases of fluorescein emission in product oligonucleotide **45** and decreases of rhodamine emission in conjugate **46**. The reaction system afforded 402 product molecules per template at 10 pM target concentration after a reaction time of 24 h, required that a high excess of reactants (10^5-fold) was used to drive turnover. The background remained low (3.4%) and the reaction proceeded with single base mismatch specificity. The method was applicable to other reporter groups. For example, a RNA-promoted reaction was used to transfer a biotin reporter to an acceptor that was subsequently immobilized to the surface of microtiter well plates.[61] The immobilized biotin triggered a second enzymatic amplification step, which included a chromogenic reaction mediated by streptavidin-conjugated horseradish peroxidase. This facilitated the detection of 500 amol RNA target. Subsequent studies included the transfer of pyrene and reaction monitoring by excimer formation.[62]



Scheme 6 Nucleic acid-templated acyl transfer reactions with turnover in template (X, Y: reactive groups, Z: transferred moiety). a) Transfer of a quencher group between PNA probes following an NCL mechanism.[60] b) Fluorophore transfer between DNA probes in which strand exchange is accelerated by a polycationic copolymer **49**.[65] (TAMRA: carboxytetramethylrhodamine)

Recently, template-promoted acyl transfer reaction were adapted to DNA-based reaction systems.[63,64] Our study was aiming for the elimination of a central bottleneck of NA-templated chemistries; i.e. the exchange of the template-bound product by incoming reactants.[65] The negatively charged DNA-backbone offers the possibility to accelerate strand exchange reactions through interactions with polycationic additives.[66] A comb-type copolymer comprised of a polylysine backbone with dextran side chains (**49**) was added to a reaction system that involved a DNA-templated fluorophore transfer reaction between conjugates **47** and **48** (Scheme 6b). After 24 h reaction time 29 product molecules of **51** had been formed per template, which could be detected at 500 pM concentration. Contrarily, no turnover was observed in the absence of the copolymer. Only recently the DNA-triggered transfer reaction was used to immobilize a Cy5 analogue on the surface of a highly emissive quantum dot.[67] The reaction was accompanied by a 35-fold increase of a FRET signal at stoichiometric template loads.

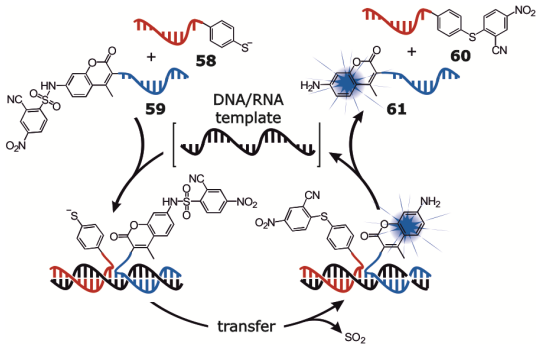
A method for consecutive signal amplification was reported by Chen *et al.*[68] A Wittig reaction was designed to proceed *via* the transfer of a benzylidene unit from the DNA-phosphonium conjugate **52** to a DNA-linked benzaldehyde **53** (Scheme 7). The fluorescent stilbene **55** was formed with turnover in template. Encapsulation of the stilbene by a suitable receptor molecule (**56**) such as α-cyclodextrin enhanced stilbene emission by closing non-emissive decay channels in the inclusion complex **57**. The reaction system afforded a 100-fold gain in fluorescence after 24 h reaction on 0.1 equiv template. The detection limit of this



Scheme 7 Consecutive signal amplification: A DNA-catalyzed Wittig reaction leads to the formation of fluorescent stilbenes. Fluorescence enhancement is achieved *via* encapsulation with α-cyclodextrin.[68]

method was found to be 40 pM, which corresponded to 0.0002 equiv of template and a turnover number of 84 after 24 h. A thermocycling protocol allowed the detection of double-stranded DNA. Very low background, tolerance to high temperatures and very strong gain in fluorescence are the hallmarks of this reaction, yet the rather long reaction times and the need for basic pH (8.5) are disadvantageous. The use of more reactive phosphonium ylides may provide opportunities for improvements.

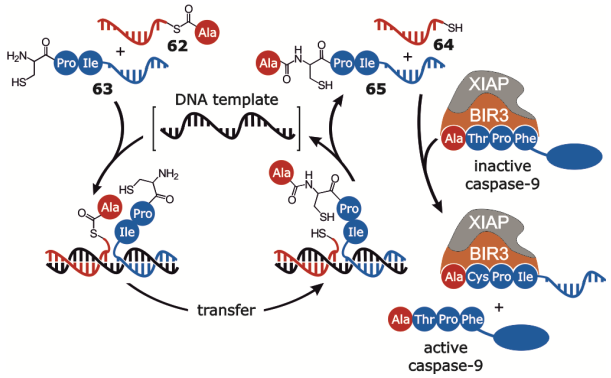
Very recently, Abe and Ito introduced a nucleophilic aromatic substitution which enabled an extremely rapid quencher transfer.[69] The reaction system was comprised of a thiolated oligonucleotide **58** and a nitroarylsulfonamido coumarin derivative **59** (Scheme 8). The template triggered the attack of the



Scheme 8 Rapid DNA-catalyzed quencher transfer *via* aromatic nucleophilic substitution that yields a fluorescent coumarin (**61**).[69]

thiolate which displaced the coumarin-aminosulfone at the *2*-cyano-*4*-nitrobenzene core. The accompanying extrusion of SO_2 led to the formation of the fluorescent *7*-aminocoumarin in **61**. In presence of stoichiometric template, the reaction yielded 67% product within only 30 sec, at negligible background even after 30 min. Turnover was demonstrated by using 50 nM reactants and as little as 500 fM template (10^{-5} equiv). Under these conditions, up to 1500 product molecules per template had been formed within 15 h at 22 °C. Especially noteworthy is the > 2-fold gain of fluorescence at 50 pM template (0.001 equiv) after only 60 min. A detailed steady-state kinetic analysis showed that the rate of the templated reaction is within the range of the dissociation step of the oligonucleotide conjugates. The

significance of this finding is apparent. To accomplish fast turnover in template it is necessary to perform reactions at fast dissociation rates of the product-template complex. However, this will have little effect when the templated reaction is rate limiting. Therefore, reactions that proceed as fast as or even faster than the rate of product-template dissociation are particularly suited to provide high turnover rates. The templated nucleophilic aromatic substitution was used for the detection of rRNA in living *E. coli*. Envisaging applications beyond nucleic acid diagnosis, an NCL-based aminoacyl transfer reaction was used to explore options for a gene-expression specific molecular therapy system *via* syntheses of proapoptotic peptides.[24,70] As depicted in Scheme 9,

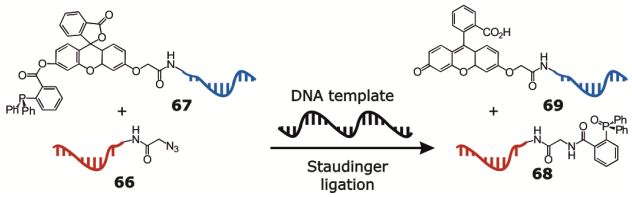


Scheme 9 A DNA-triggered alanyl transfer generates a proapoptotic peptide **65** which activates a caspase-9.[24,70]

a DNA template triggered the transfer of an alanine residue from a thioester-modified PNA conjugate **62** to an acceptor peptidyl-PNA conjugate **63**. The alanine-transfer led to the formation of a proapoptotic peptide motif in conjugate **65** which was intended to bind the BIR3 domain of the X-linked Inhibitor of Apoptosis Protein (XIAP) known to be overexpressed in several tumors. This, in turn, allowed release of activated caspase-9 from the antiapoptotic XIAP-caspase-9 complex. The reaction provided 85% yield after 1 h in presence of stoichiometric template. Substoichiometric template loads (0.1 equiv) generated 24% product within the same time showing the potential for turnover. When the reaction was conducted in presence of matched template in cell lysate spiked with BIR3, up to 27% of caspase-9 (45% of caspase-3) activity was restored. This gain of enzyme activity did not occur when the reaction was attempted on single base mismatched template. This study proved the feasibility of an artificial translation system and encourages further investigations of RNA-triggered peptide synthesis at a cellular level.

Nucleic acid-templated nitrogen release reactions employing azide-quenched fluorophores

Various nucleic acid templated chemistries rely on Staudinger reactions. The Staudinger reduction involves a reaction between a phosphine and an azide which leads to the formation of phosphinoxide, nitrogen and an amine. In the Staudinger ligation, an *o*-diphenylphosphinobenzoic ester reacts with an azide to furnish an *o*-diphenylphosphinoxidobenzoic acid amide.[28,71] The first example of a DNA-templated Staudinger reaction was introduced by Taylor and co-workers.[72] The reaction system comprised an azido-PNA **66** and an *o*-diphenylphosphinbenzoate-quenched fluorescein linked to a second PNA in **67** (Scheme 10). The templated Staudinger ligation led to a transfer of the benzoate to the amine formed upon reduction of the azido group,



Scheme 10 DNA-templated fluorogenic Staudinger ligation between a phosphine-quenched fluorescein PNA **67** and an azide-bearing oligonucleotide **66**.[72]

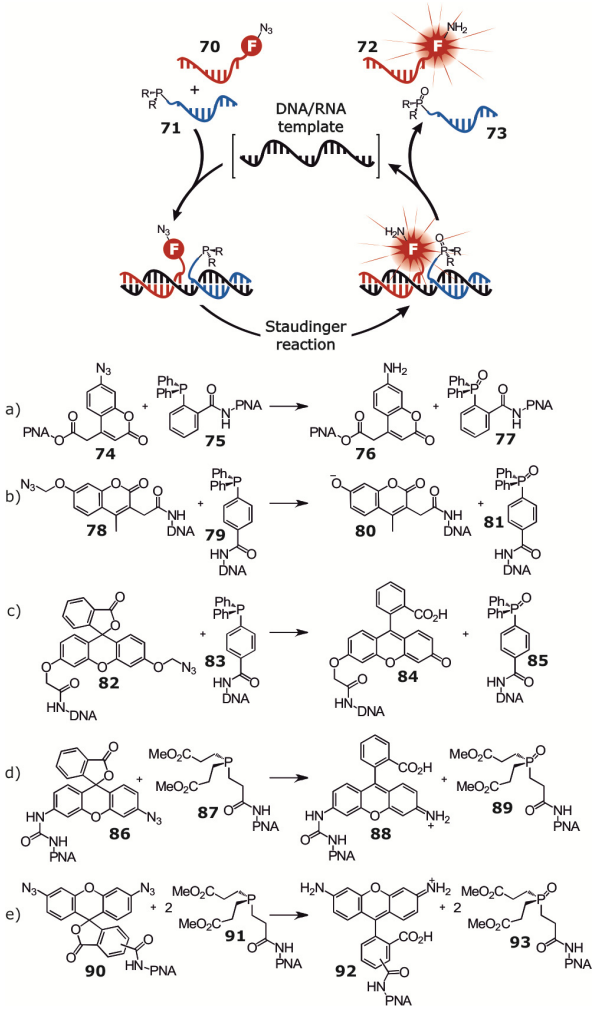
which was accompanied by an increase in fluorescein emission in conjugate **69**. The templated reaction was 188-fold faster than the conversion in absence of the template. The reaction reached a plateau at about 50% which was attributed to the presence of oxidized phosphine probes.

In 2007, Pianowski and Winssinger demonstrated a DNA-templated Staudinger reduction which employed a triphenylphosphine (TPP)-PNA conjugate **75** and a PNA-tethered azidocoumarin **74** (Scheme 11a).[73] The conversion yielded a highly fluorescent coumarin derivative **76** with 20 turnovers at 5 nM template concentration within only 30 min, required that formamide was included in the reaction buffer. Kool and co-workers described the DNA-templated activation of a 7-azidomethoxy-coumarin profluorophore.[74] As depicted in Scheme 11b, reduction of the azido group in DNA conjugate **78** triggers the release of nitrogen and formaldehyde, the latter being formed upon a fragmentation of a hemiaminal intermediate. The formation of **80** was accompanied by a 29-fold increase in fluorescence. The detection limit was found to be 2.5 nM at a turnover number of 20 after 30 min.

Furukawa *et al.* used the templated Staudinger reduction of the azidomethyl-caged fluorescein DNA probe **82** (Scheme 11c) to detect RNA within living cells.[75] The reaction of 50 nM reactive probes on 500 pM template led to 69% yield of fluorescent product **84** after 4 h. This translates into a turnover number of 50, if the yield of the background reaction (10%) is taken into account. To demonstrate the biocompatibility of the Staudinger reduction, the reactive probes were introduced into human leukemia cells aided by streptolysin O (SLO) mediated permeabilization. Fluorescence microscopic imaging and flow-cytometry was used to localize and quantify intracellular rRNA and β-actin-mRNA targets.

Only recently Saneyoshi *et al.* were able to improve the reaction's turnover with the same reactive probes (**82** + **83**) through the addition of the earlier mentioned comb-type polylysine/dextran copolymer **49** (compare Scheme 6b).[76] At a 1 µM polymer concentration a turnover number of 128 was detected after 1 h reaction time at 40 °C with a detection limit of 10 pM template.

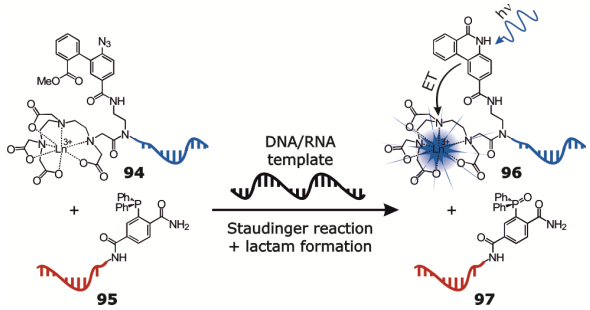
Winssinger and co-workers explored conjugates comprised of cell-permeable GPNA (guanidine-based peptide nucleic acid) oligomers[79] and an alkyl-based phosphine such as tris-(2-carboxyethyl)phosphine (TCEP) in **87** (Scheme 11d).[77] They



Scheme 11 Nucleic acid-templated Staudinger reactions with turnover in template employing azide-quenched fluorophores.[73–78] (F: fluorophore)

found that the reduction of the rhodamine-azide conjugate **86** by TCEP was much faster than by the previously used arylphosphines. The reaction system enabled the fluorometric detection of mRNA coding for *O*-*6*-methylguanine-DNA methyl transferase which is highly expressed in HEK cells. The use of the (bis-azido)-rhodamine GPNA probe **90** increased the signal-to-noise ratio and the sensitivity (Scheme 11e).[78] Fluorescence measurements showed 20% conversion into product oligonucleotide **92** at 2 nM (0.02 equiv) DNA template, with virtually no signal in absence of the target. This reaction allowed the visualization of miRNA in living cells. Most intriguingly, cell lines expressing high, moderate or low levels of miRNA-21 were distinguishable by means of the fluorogenic reaction. The authors furthermore showed that the reaction system enabled miRNA quantification in suspended cells.

Fluorescence detection in cell suspensions can be difficult due to autofluorescent background. Recently, Abe and Ito developed a Staudinger reaction-based method to address this issue (Scheme 12).[80] They used a TPP-DNA **95** that converted an azido-masked pro-antenna moiety of a second DNA conjugate **94** into the amine form. Subsequent lactam formation generated the active *6*(*5H*)-phenanthridinone antenna in **96**, which in turn could transfer energy upon excitation at 340 nm to an adjacently attached



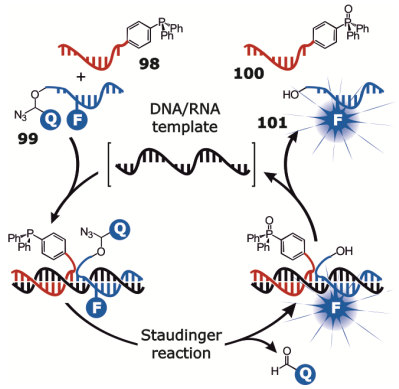
Scheme 12 A nucleic acid-templated luminogenic Staudinger reaction generates a phenanthridinone sensitizer. This enables energy transfer to the adjacently attached lanthanide, which results in a long lived fluoresence emission.[80]

lanthanide. The life-time of the resulting lanthanide luminescence is in the millisecond range, which allows cutting off any biologically originated short-lived autofluorescence by applying time-gated monitoring. The system achieved a 10-fold turnover in template after 4 h, when 2.5 nM (0.01 equiv) of the template was included. The reaction enabled detection of rRNA in suspended *E. coli* bacteria. Additionally, this technique facilitated the detection of DNA templates in presence of high concentrations of fetal bovine serum.

A potential drawback of the Staudinger reaction is the susceptibility of phosphine to oxidation. Although an excess of phosphine probe can be used to compensate for oxidized reactants, the latter will still compete for template binding, thereby limiting the achievable turnover rates. In addition to that, excess reactants lead to enhanced template independent background reactions. However, it should be noted that reactions within cells likely profit from the reducing environment.

Nucleic acid-templated nitrogen release reactions employing cleavable linkers

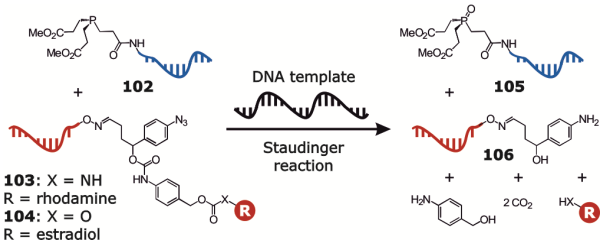
In earlier examples, Kool and co-workers used a nucleophilic substitution reaction in order to release a quencher from a fluorophore-labeled oligonucleotide probe (QUAL).[39–41,43,42] There, fluorescence turn-on was accompanied by ligation of the reactants. An alternative approach that also leads to fluorescence emission through quencher release but does not involve ligation of probes was reported by Franzini and Kool in 2009.[81] The Q-STAR (quenched Staudinger-triggered α-azido-ether release) concept is based on the Staudinger reaction of a 3'-TPP-labeled probe **98** with a second oligonucleotide **99** bearing a 5'-α-azido-ether (Scheme 13). A dabsyl group was tethered *via* this linker and used to quench an internal fluorescein. Adjacent hybridization triggered the reduction of the azido group. The formed hemiaminal releases a quencher imine which upon hydrolysis forms the quencher aldehyde. The reaction was fast and yielded 90% of product **101** after 32 min. A > 60-fold signal increase was obtained in less than 2 h on stoichiometric DNA template. Of note, the same fluorescent signal was obtained after 12 h when the reaction was performed on 2 nM (0.01 equiv) template, with less than 15% of the signal arising from background reaction. A set of FAM-labeled and FAM/TAMRA-labeled Q-STAR probes was utilized to distinguish two bacterial strains (*E. coli* and *S. enterica*) that differed by only one nucleotide in a certain region of their ribosomal RNA. The development of a Q-STAR probe that included a NIR label



Scheme 13 Fluorescence activation *via* nucleic acid-triggered quencher release through Staudinger reduction of an α-azido-ether (Q-STAR) by a phosphinylated oligonucleotide. [81] (F: fluorophor; Q: quencher)

(Quasar 670) and a cleavable black hole quencher (BHQ-2) facilitated cell sorting by dual-color flow cytometry.[82] In a subsequent study it was shown that higher signal gains (up to 370-fold) could be achieved when the fluorophore-bearing probe was equipped with two cleavable quenchers.[83] The Q-STAR concept was applied to double-stranded DNA templates, which contained purine rich segments.[84] Homopyrimidine Q-STAR probes were designed to form triple helices with the homopurine stretch *via* Hoogsteen base pairing. The reaction had to be performed at pH 5.6 in order to assure protonation of cytosine (C+) in the probe strand, which is necessary for stable Hoogsten interactions with G-C pairs within the template. Product formation was significantly slower (3 h) than on ssDNA templates (20 min) and no turnover activity was reported. This concept was further explored for the recognition of abasic sites within double stranded DNA.[85] The fluorescence labeled probe contained an imidazophenanthrene or a pyrene base surrogate that enabled base stacking with next base neighbors around the abasic site. The occurrence of an abasic site in the double-stranded template induced a 36-fold gain in fluorescence upon reaction. Interestingly, the reactive probes could also be applied to monitor enzymatic DNA repair.

Winssinger and co-workers introduced a self-immolative linker which allowed the release of functional molecules such as rhodamine or estradiol by a nucleic acid-controlled Staudinger reduction (Scheme 14).[86] The reaction system involved a GPNA-tethered *p*-azidobenzyloxycarbonyl unit, which was attached to a *p*-aminobenzyloxycarbonyl-linked amine (in **103**) or alcohol (in **104**). The adjacent alignment of a TCEP-GPNA probe **102** triggered the Staudinger reduction, which initiated a fragmentation reaction resulting in the release of the functional

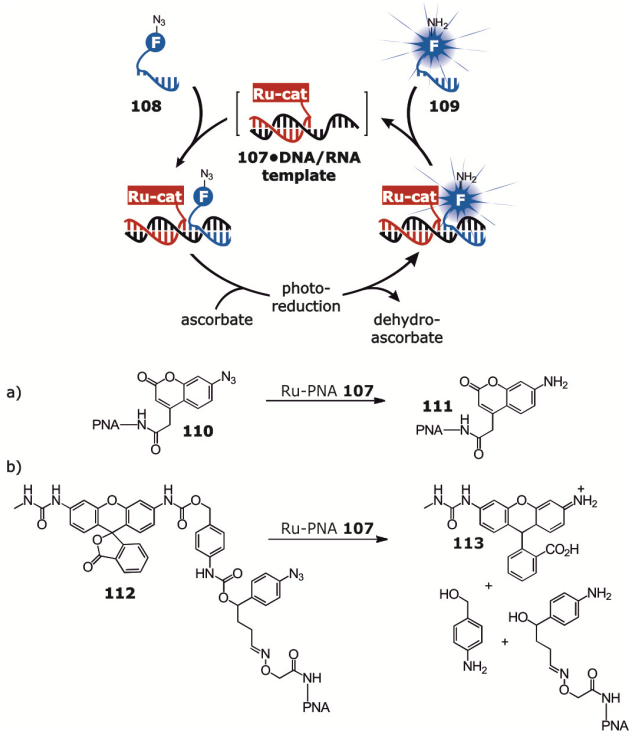


Scheme 14 The DNA-catalyzed release of functional molecules such as rhodamine or estradiol is induced by the Staudinger reduction of a self-immolative linker.[86]

molecule along with *p*-aminobenzyl alcohol and CO_2. Estradiol was liberated in 85% yield after 30 min in presence of 0.5 equiv (100 nM) template. This method showcased an approach towards the design of gene expression-specific prodrugs.

Ruthenium-promoted nucleic acid-templated nitrogen release reactions

The Staudinger reduction requires stoichiometric amounts of phosphine conjugates which usually are applied in excess to compensate for losses caused by oxidation. Recently, Winssinger and co-workers introduced a nucleic acid-templated version of a photocatalyzed azide reduction (Scheme 15)[87] which was discovered by DNA-encoded reaction screening in the labs of Liu.[88] The ruthenium complex $[Ru(bpy)_2phen]^{2+}$ in GPNA conjugate **107** was used as a replacement of the phosphine conjugates in templated Staudinger reduction. When excited by 455 nm light, the adjacent annealing of both the Ru-GPNA **107**



Scheme 15 A nucleic acid-triggered fluorogenic azide reduction is photocatalyzed by a PNA-tethered ruthenium complex (**107**).[87,89]

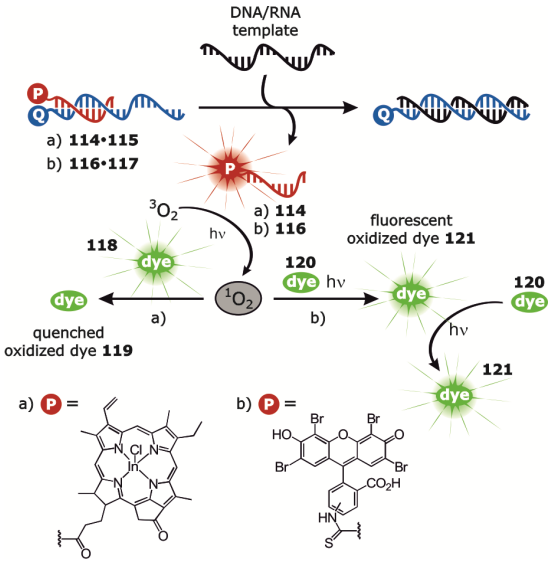
and the azido-PNA (**110** or **112**) triggered the reaction. At a 10 nM load of catalyst and template (corresponding to 0.02 equiv with respect to the azido-probe) the azido-coumarin profluorophore **110** (Scheme 15a) was found to yield 30% of highly fluorescent product **111** within 3 h. This corresponds to 10 turnovers when the 10% yield of background is considered. In this study, ascorbate was employed as reductant to reform the metal complex after light-induced oxidation by the azido-GPNA. However, a subsequent study showed that ascorbate was redundant when the reaction was performed within the reducing environment provided by live cells.[89] A Ru-catalyzed rhodamine-release reaction (Scheme 15b) enabled the detection of miRNA in zebra fish. In this study, γ-ser-PNA[90] was used as this derivative provided a better distribution within zebra fish than GPNA. The

reaction provided nearly 10% yield of rhodamine **113** after 24 h in the presence of 5 pM DNA-template ($2x10^{-5}$ equiv corresponding to the profluorogenic substrate), which was 3.6-fold higher than the background and calculates to an impressive turnover number of > 4000.

Incorporation of catalysts into nucleic acid-controlled reactions

The incorporation of catalysts into nucleic acid-controlled reactions can be used to trigger the conversion of freely diffusing reactants in solution. Because the products of the reaction are no longer connected with nucleic acids, turnover in template is no longer dependent on the dissociation of the involved nucleic acid duplexes.

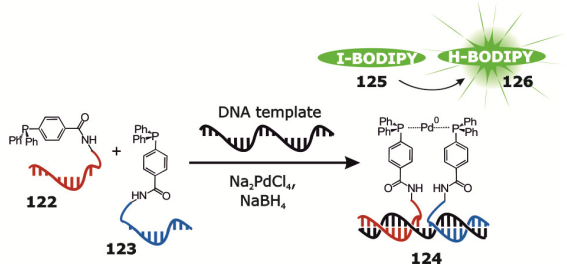
An early example was introduced by Krämer and co-workers who released copper(II) ions from a pincer-like oligonucleotide upon target hybridization. The released Cu(II) subsequently assembled with phenanthroline into an active Cu(II) redox-catalyst that converted a non-fluorescent substrate into a fluorescent dye.[91] Mokhir, Ogilby and Gothelf have reported on a nucleic acid-templated reaction that led to the generation of cytotoxic singlet oxygen from nontoxic triplet oxygen.[22,23] A DNA-conjugated pyropheophorbide-*a* photosensitizer **114** (or an indium(III)-complex thereof) is quenched upon hybridization with a complementary strand **115** equipped with a black hole quencher (Scheme 16a). Strand displacement by the target nucleic acid



Scheme 16 The activation of an oligonucleotide-bound photosensitizer (**P**) is achieved by the template-induced separation from a quencher oligonucleotide. **P** catalyzes the production of singlet oxygen that a) leads to the decomposition of a fluorescent dye [23] or b) triggers the generation of a fluorescent dye which promotes its own formation autocatalytically.[92]

separated the photosensitizer in **114** from the quencher in **115**. Irradiation with red light triggered the formation of $^1O_2$, which was detected by the decrease of fluorescence intensity caused by decomposition of a $^1O_2$-sensitive *1,3*-diphenylisobenzofurane dye **118**.[23] The authors reported > 4000 turnover numbers when 300 nM probe and target were allowed to react for 24 h in presence of vast excess of dye (3 mM, 10^4-fold). This impressive result nicely demonstrates that turnover can be achieved even at stoichiometric template loads. The reaction system was shown to proceed in live cells where endogeneous rRNAs were used to trigger the reaction. This approach is the first example of a template-mediated synthesis of a cytotoxic species from a non-toxic precursor in live cells. Disadvantageous is that product formation was signalled by the decomposition of a dye resulting in a negative signal as the reaction proceeds. Only one year later, Dutta and Mokhir reported a reaction system that provided a positive signal.[92] An eosin-modified oligonucleotide **116** was used for the photocatalytic generation of $^1O_2$, which triggered the oxidative conversion of dichlorofluorescin **120** to the fluorescent dichlorofluorescein **121** (Scheme 16b). Interestingly, this product can act as a photosensitizer, which enables amplification of the product signal by autocatalysis. In more recent approaches, the quenched fluorophore was tethered to the oligonucleotide *via* $^1O_2$-sensitive linkers such as *1,2*-dithioethylene or *9,10*-dialkoxyanthracene bridges.[93,94] In one example, the templated photochemical reaction led to the release of 15 nM fluorescein triggered by 0.04 equiv DNA (2 nM), which indicates modest turnover in template.

Building on DNA-templated Heck reactions,[95] Herrmann and co-workers introduced a conceptually new method for the amplified, fluorimetric detection of nucleic acids.[96] In this approach, the catalytically active palladium species was generated *in situ* as a response to the presence of the target nucleic acid (Scheme 17).



Scheme 17 The DNA-templated arrangement of oligonucleotide-bound phosphine ligands (**122** + **123**) generates a palladium complex that catalyzes the fluorogenic deiodination of a heavy atom-quenched BODIPY dye (**125**).[96]

Adjacent hybridization of two phosphinylated oligonucleotide probes (**122** + **123**) presumably facilitated the formation of a Pd-bisphosphine complex such as **124** (from Na_2PdCl_4 in the solution). The Pd-containing complex catalyzed the reaction between the iodo-quenched BODIPY dye **125** and $NaBH_4$, which led to deiodination and, therebye, strong enhancements of fluorescence in compound **126**. At 1 µM probe and 1 nM target (0.001 equiv), 90% of the maximal fluorescence intensity was obtained within only 4 min reaction time. The authors reported an impressive 10 fM detection limit. Under optimal dye/template ratios, each template triggered the conversion of 1000 dye molecules. The reaction was performed at pH 5 in order to prevent complexation of Pd by DNA nucleobases. The templated dehalogenation reaction also proceeded in cell lysate but was hindered in the presence of bovine serum albumin (BSA) due to the containing cysteine residues that poison the Pd catalyst.

Conclusion and Outlook

Nucleic acid-triggered reactions that proceed under turnover

conditions enable signal amplification. Turnover is the central requirement when the template has low abundance. The most frequently used methods can be categorized into systems that lead to 1) ligation of oligonucleotides, 2) interconversions of functional groups between oligonucleotides and 3) activation of catalysts in response to the presence of a certain nucleic acid template.

In DNA-templated chemistry, ligation reactions were studied first. Although the functional groups needed are readily implemented, product inhibition is a serious issue that limits turnover. The use of flexible ligation junctions[32,33,43,50] and ligation-cyclization[57] conferred significant improvements in turnover, yet, the formed products still have higher affinity for the template than the starting materials. To bypass this problem, chemoselective chemistries have been combined with enzymatic template amplification, which resulted in largely decreased limits of detection.[53,55] High turnover was achieved when ligation reactions were linked to chemical self replication.[58,59]

Significant efforts have been invested into the development of chemistries that proceed without ligation. Because the number of paired nucleotides within reactive oligonucleotide probes does not change, the reaction can be performed under conditions of dynamic strand exchange. One approach includes the nucleic acid-directed transfer of moieties from one probe to a second probe. Transfer reactions employing native chemical ligation-type,[24,60–62,65] Wittig-type[68] and nucleophilic aromatic substitution-derived[69] chemistries have been used to transfer quenchers, fluorophores and amino acids. Many of these reactions provide 10^2-fold gains of signal and proceed with 10^2-10^3 turnovers in template. This is an attractive feature which may prove useful not only for the purpose of nucleic acid sensing but also for the development of reaction systems that provide drug-like molecules only in diseased cells.[24]

Staudinger-type chemistry has proven extremely versatile. The phosphine-triggered release of N_2 from azide-quenched fluorophores[74,75,77,78,80,76] or cleavable linkers[81–86] led to the template-dependent activation of highly emissive fluorophores. This chemistry has been developed to a degree of maturity that enabled the detection of rRNA,[81] mRNA[77] and miRNA[78] within living cells. The recent discovery of ruthenium-promoted photocatalytic azide reductions provides new opportunities.[87] The Ru-conjugates can be applied in substoichiometric amounts and their long term stability towards oxygen facilitates turnover.[89] Furthermore, the ability to trigger the reaction in a spatially and temporarily defined manner is of interest for subcellular imaging.

The last few years have seen the development of a new type of templated chemistry. Rather than triggering reactions between oligonucleotide-based reactants, the template can be used to activate or assemble a catalyst that triggers the reaction between non-nucleotidic substrates. For example, strand exchange has been used to remove quenchers from oligonucleotide-linked photosensitizers which - *via* production of singlet oxygen – induce the release of fluorophores from quenched precursors.[23] In another embodiment, a template-assembled Pd-catalyst allowed the conversion of many fluorogenic reactant molecules as a consequence of a single hybridization event.[96]

The contributions discussed in this review showcase the significant advancements in nucleic acid-catalyzed chemistry. The developments of the last few years have pushed the limits from 10^1 to 10^2-10^3 turnovers. The recent contributions have shown that substantial sensitivity improvements can be achieved by optimizing the kinetics of the templated reactions,[69] by accelerating strand exchange,[65,76] or by reducing the number of substrates that need to be cycled.[89] Further improvements would be conceivable for reactions which generate products with template affinities below reactant probes. This could accelerate dissociation of the product-template complex without detriment to the rates of association of the starting materials. If such an approach would be combined with a chemistry that provides high rates of the templated reaction together with negligible off-template conversion, sensitivities were only limited by the molecular recognition of the template by the reactants. An alternative way to achieve a high degree of amplification is to decouple the signal generating step from the template recognition event.[23,96] Contrary to nucleic acid-templated chemical reactions, where the template increases the *effective molarities* of the reactants by adjacent alignment, the nucleic acid-controlled formation of catalysts from inactive precursors leads to an increase in the catalyst *concentration*. This fundamentally different approach makes it principally possible to use highly concentrated reactants. This can facilitate read-out. Reports about DNA-based organometallic catalysis are encouraging.[97–101]

Regardless of the approach pursued, the quest for sensitive nucleic acid templated chemistries will continue, because chemists are intrigued by the future applications in DNA/RNA diagnosis, DNA/RNA-guided therapy as well as DNA nanotechnology.

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Notes and references

[a] *Institut für Chemie der Humboldt-Universität zu Berlin, Brook-Taylor-Strasse 2, 12489-Berlin (Germany). Fax: (+49) 30-2093-7266; Tel: (+49) 30-2093-7446; E-mail: oliver.seitz@chemie.hu-berlin.de.*

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