Detecting RNA and DNA with Templated Chemical Reactions

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1. Introduction

Sensing nucleic acid sequences is critical in modern-day biology and medicine, and is likely to continue to increase in importance over the next 10−20 years. The current uses for identifying DNA and RNA sequences are many and include identifying individuals in forensic applications, sequencing of genes and genomes for basic biology and applied medicine, identification of microorganisms in food and environmental samples, identification of infectious organisms in human patients, diagnosis of cancer and drug resistance, and genetic prognosis of disease progress and response to drug treatment. In the future, there will be increasing focus in medicine on the underlying genetic causes of disease, and this will invoke greater demand to detect and identify RNA and DNA sequences. In addition, sequences will need to be detected at multiple points—in solution after amplification, in cells and biological fluids, and even directly in the living body. As a result, it is likely that multiple chemical and biological solutions for detecting and identifying RNAs and DNAs will be needed.

The aim of this review is to summarize recent advances in the development of chemical reactions that are triggered by hybridization of an oligonucleotide—a synthetic short strand of DNA—to a target RNA or DNA strand and outline how these reactions are being used, or could be used, in detecting and identifying such targets in a biological sample

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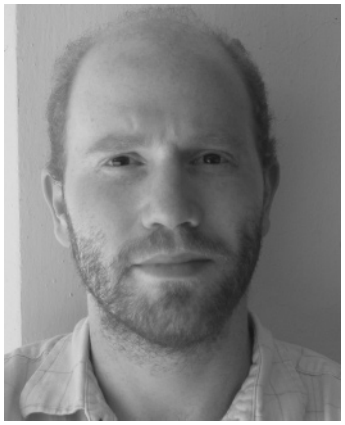

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Eric Kool received his Ph.D. degree from Columbia University in 1988 and was a postdoctoral fellow at the California Institute of Technology before taking a position as Assistant Professor at the University of Rochester in 1990. In 1999 he moved to Stanford University, where he is Professor of Chemistry. His research interests include the study of DNA replication and repair, development of templated chemistry for imaging cellular RNAs, design of new genetic systems, and discovery of new DNA-based fluorophores and sensors. His work has been recognized by the ACS Pfizer Award in Enzyme Chemistry and the ACS Cope Scholar Award.

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or cell. First, we will briefly discuss the basic principles of DNA and RNA molecular recognition that underlie this research. We will then outline the historical development of such templated reactions and then proceed to delineate the current state of the art. Finally, we will predict some future areas of special promise and mention some exciting new technologies that are under development in this field.

A number of related topics will not be covered in this review because of our present focus on chemical reactions. Our emphasis is entirely on covalent chemistries that are triggered or enhanced by DNA hybridization, and we will not explicitly cover noncovalent changes, such as DNA-triggered conformation changes. It is worth noting that this omits from the present discussion some other current and promising methods for nucleic acid detection, such as the conformation-changing "molecular beacons" first described by Tyagi and Kramer.[1] The reader is directed to recent reviews on this topic for more information.[2−4] Another example is the malachite green aptamers reported recently by Dieckmann[5] and Kolpashchikov.[6] Also not explicitly part of this review are enzymatic reactions that require DNA hybridization; important examples include ligase detection reaction,[7] ligase chain reaction,[8] polymerase extension,[9] and invader assays.[10] Once again, the reader can find those topics reviewed in the cited literature. A related strategy involves development of DNA and RNA enzymes (ribozymes and deoxyribozymes) for RNA or DNA detection; if interested, the reader can find more information on the development of these elsewhere.[11,12] Finally, we will not cover simple photophysical probes, such as fluorescently labeled oligonucleotide probes that rely simply on binding and do not involve covalent chemical changes.[13,14]

As the previous paragraph points out, DNA-templated reactions are not the first developed approach for sensing RNA or DNA sequences, nor are they the only promising approach. Before we go into the details of the currently known DNA-templated reactions, we will first briefly point out why chemically templated reactions offer some advantages over other current nucleic acid detection strategies and may solve some problems that other methods might not.

2. DNA and RNA Sensing: Possibilities for Chemical Reporting

The most widely used present-day strategies for detecting and identifying DNAs and RNAs in modern biology and medicine do not make use of chemically templated reactions. For example, between the cell and final analysis, polymerase chain reaction (PCR) is by far the most common intermediate step in identifying RNAs and DNAs, and there already exist several methods for real-time sequence identification during PCR, such as SYBR green,[15] invader assays,[10] and molecular beacons.[2] Moreover, full Sanger sequencing, in which every nucleotide is identified in order (rather than just identifying local nucleotides), is growing in efficiency and dropping in cost.[16] Given these advances, why then are an increasing number of laboratories doing research on new chemical strategies for sensing DNA and RNA?

There are multiple answers to this question, and some examples will serve to point out limitations in the current methods and reasons why templated chemical reactions offer some special promise. For example, enzymes that are employed in nucleic acid detection can have high activity and high specificity and may yield amplification of signal. However, enzymes cannot generally be used in intact cells because it would be difficult to deliver them there. Moreover, most enzymes cannot work with both DNA and RNA; for example, using ligases to detect RNA is markedly slower and less efficient than is the case with DNA.[17] There are no polymerases that reliably make RNA from RNA using primers. Deoxyribozymes have recently been examined for use in detecting cellular RNAs;[18] however, the signal-to-noise ratio has been low, possibly because of the preference for higher-than-cellular levels of magnesium, relatively slow turnover, and low selectivity against mismatches. DNA-templated chemistries, by contrast, may take place inside cells (depending on the chemistry involved), and within limits there are a number of established methods for introducing oligonucleotides into cells.[19−21] Moreover, many DNA-templated chemistries might work as well on RNA templates as on DNA templates.

Conformation-changing approaches, especially molecular beacons and variants, are among the most promising methods for sensing RNA sequences inside cells.[4,22,23] However, they can suffer from significant problems with background signals; for example, unintended binding to any protein in the cell may cause the conformation change that yields signal.[24] Moreover, MB approaches typically yield one signal per target RNA, and there are as yet no strategies for amplifying this signal in cells, which is a serious issue since many if not most RNAs in the cell exist in low numbers. Some of the templated chemical approaches, in contrast, have already demonstrated turnover, yielding amplified quantities of signal per equivalent of target DNA or RNA. Moreover, the chemical reaction offers the possibility of lower background signal as it may be more orthogonal to the reactivity of biological molecules and thus suffer from less interference.

Finally, purely photophysical approaches, such as FRET or excimer signals that might arise from side-by-side binding of two probes, have been demonstrated in solution in multiple labs.[25−27] However, the signal-to-noise ratios have to date been quite low due mostly to incomplete photophysical change. For example, FRET often occurs with only partial energy transfer, thus making it difficult to distinguish unbound probes from bound probes and other stray signals. Chemical reactions offer the possibility of a result much closer to an ideal digital (on-or-off) signal. They might occur in much higher yields and in some cases with much greater orthogonality than fluorescence signal changes alone might offer.

3. Basic Principles of Nucleic Acid Recognition for Templated Reactions

The first step in sensing of RNA or DNA in templated chemical reactions is for the probe to bind the template to induce the desired chemical reaction. In principle, the probe could be a small molecule that is induced to react by binding DNA, as is the case with the enediyne class of drugs.[28] However, virtually all probes under study at present are built from synthetic oligonucleotide (or analog) strands in order to make use of the predictable high affinity and highly selective recognition properties of DNA. Thus, modern probe designs have two features in common: first, the oligonucleotide part that allows recognition and binding of a particular sequence and, second, the reactive part that will undergo chemistry when binding occurs. DNA synthesis chemistry has advanced sufficiently over the last two decades to allow for simple and efficient automated construction of the first part. Second, DNA conjugation chemistry developments[29]

Table 1. Key Design Parameters for Designing DNA-Templated Reactions for Sequence Sensing

sensing environment	target analyte	reaction conditions	application
solution (pure DNA)	DNA or RNA	pH	PCR
solid support	single or double stranded	temperature	SNP typing
in situ−fixed cells	secondary and tertiary structures	ionic strength	cellular/organismal imaging
in situ−live cells			sub-nanomolar sensing

have made it (in most cases) straightforward to add a great variety of reactive groups to DNA.

There are a few points worth making about the molecular recognition design features that must be kept in mind when designing a new DNA-templated chemical reaction (Table 1). The first determining factor is the type of environment in which the sensing is to be carried out: in solution with pure DNA, on solid supports, in situ with fixed cells, or in intact cells. It is certainly possible (and useful) to sense pure DNA sequences that are derived from natural mixtures; this is the situation when PCR amplification is used to generate DNA from a small sample of RNA or DNA taken from a cellular sample. The only drawback in design for sensing pure DNA is that several powerful techniques to determine specific sequences and mutations in solution or on solid supports already exist. Perhaps the greater value added for future DNA-templated reactions will be for applications in direct sensing of cellular RNAs or DNAs, where the current state of the art is in much earlier stages. Sensing would be useful with RNAs or DNAs extracted from cells if it could succeed without the PCR or RT-PCR amplification step, which requires substantial additional reagents, time, and cost. In addition, templated reactions might be applied directly in the cells, removing even the need for the nucleic acid extraction step. However, if one is to design probes for cellular targets, one must take into account formidable issues such as intracellular delivery, cellular localization, stability against enzymatic degradation of probes, and background signals from the complex intracellular chemistry.

A second important factor that must be considered is whether the target analyte will be DNA or RNA and whether it is double stranded, single stranded, or a combination of both (e.g., a folded structure). Targets that are already involved in Watson−Crick pairing are more difficult to recognize than single-stranded targets. If partially or completely involved in paired structure, the current options are to partially denature the targets prior to recognition or use chemically modified probes that have free energy of binding sufficient to afford thermodynamically favorable displacement of the target secondary structure with pairing by the probe.[30] In intact cells, DNA is double stranded and RNA is overtly single stranded, although it is folded into stems and loops that undergo substantial and often extensive pairing.[31,32] Because of its partial single-stranded structure, RNA has become an attractive target for intracellular probes. However, a given RNA molecule often has an unknown or incompletely known structure, and it takes a combination of calculation and trial and error to find a site that is accessible in practice.[33,34] In addition, duplexes containing RNA backbones take on an A-form helix, which has a different twist and shape than the B-helix that the majority of DNA duplexes adopt.[35] In principle, this structural difference could affect the success of DNA-templated chemistries, depending on the geometric requirements of the specific reaction.

A third factor in the design of nucleic acid-templated reactive probes is the physical conditions in solution, in particular pH, ionic strength, and temperature. DNA recognition is particularly sensitive to the latter two factors. Full stabilization of natural DNA or RNA duplexes typically requires as much as 1 M Na^+ or K^+ or 5 mM of a divalent cation such as Mg^{2+}.[36] DNA duplexes (especially short ones) show strong sensitivity to temperature, and high temperatures will cause probes to cooperatively dissociate, or "melt", from their targets. In DNA-templated chemistries the reactions must be compatible with conditions that allow molecular recognition to occur with the target DNA or RNA.

Beyond this, there is the issue of the length of the oligonucleotide probes that are employed in DNA-templated chemistry. Longer probes bind more tightly than short ones, and typically it takes at least 6−7 nucleotides to allow a probe to bind substantially at room temperature.[37] Probes around 18 nucleotides or longer are required to specify one target site in complex genomic settings (such as the human genome of 3 gigabases), as short contiguous sequences may occur many times in such a large sequence space. There are some disadvantages of longer probes, however. First, sequence specificity is inversely proportional to probe length, so longer probes are less accurate for small sequence differences. The smallest target sequence differences one would want to distinguish in biological samples are single nucleotide polymorphisms (SNPs), which are generally quite difficult to distinguish with probes >15 nucleotides in length.[38] A second disadvantage of longer probes is higher cost and lower yield in their preparation.

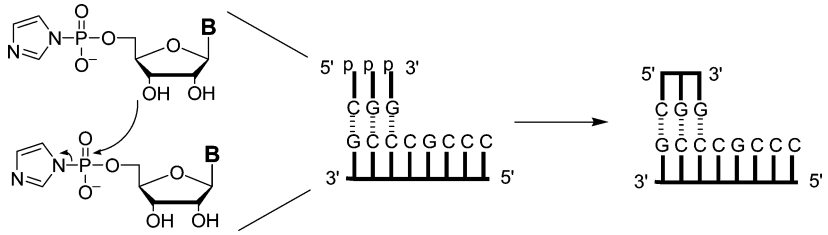
A final consideration in the design of DNA-templated sensors is the application in which they are to be used. For example, if they are to be used during PCR, they must be resistant to heating and cooling cycles and must yield a signal rapidly during these cycles. If they are to distinguish single nucleotide differences, they must be highly selective. If they are to be used in sensing tiny quantities of RNA or DNA, they need to be highly sensitive and offer excellent signal-to-background ratios. If they are to be used in intact cells, they may benefit from some resistance to enzymatic degradation and their reaction must not require any reagents or catalysts that cannot be delivered into the cell.

4. Reagent-Mediated DNA-Templated Chemistry

4.1. Ligation of Mono- and Oligonucleotides

Among the earliest examples of DNA-templated reactions were the nucleophilic bond-forming reactions described by Orgel.[39] He reported extensive studies (too many to fully enumerate here) of the ability of activated mononucleotides to dimerize, trimerize, and further oligomerize in the presence of a complementary DNA template (Scheme 1). His goal was the study of the early origins of life on Earth (prebiotic chemistry).[40] Before proteins existed there would have been a need for chemical mechanisms that join nucleotides together in specific sequences. Orgel prepared imidazolide-activated 5′-monophosphate derivatives of nucleosides and measured rates and yields for formation of oligomers over a period of days at millimolar concentrations of monomers.[41]

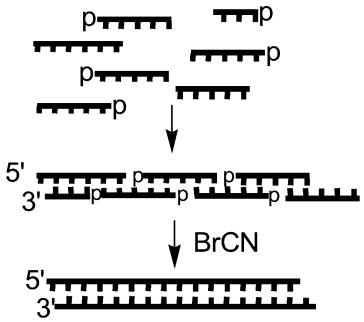
Scheme 1. Trimerization of Imidazole-Activated Mononucleosides[40]



Pyrimidines were not successfully oligomerized by this approach, but purines (primarily guanine nucleotides) were to a significant degree. Later, Switzer showed that modified DNA bases promoted such oligomerizations.[42]

Another important early example of DNA-templated nucleophilic reactions was the ligations reported by Shabarova of Moscow State University. Shabarova found that synthetic oligonucleotides, in the presence of a complementary strand of DNA, could bind side-by-side and react to form a phosphodiester bond, mediated by cyanogen bromide in the presence of divalent metal ions (Scheme 2).[43] This

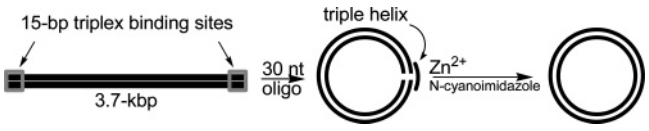
Scheme 2. Cyanogen Bromide-Mediated Formation of Phosphodiester Bonds[43]



reaction could proceed in high yields and short times (typically only a few minutes) and was used in one instance to assemble a biologically active 183-nt transfer RNA gene.[44] Shabarova studied the reactions of both 5′-phosphoryl and 3′-phosphoryl oligonucleotides in the presence of DNA templates and produced both linear and circular oligonucleotides by this approach.[45] This reaction was not pursued as a strategy for sequence detection but rather viewed as a preparative method.

Subsequent studies carried out at Caltech made use of related chemical approaches, again for preparative purposes. Dervan demonstrated the use of *N*-cyanoimidazole and Zn^{2+} in ligations converting linearized double-stranded plasmid DNA, several thousand base pairs in length, to circular form (Scheme 3). This required both strands to react. In one

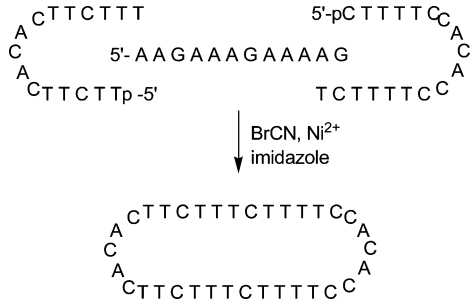
Scheme 3. Conversion of Double-Stranded Plasmid DNA to Circular Form Using Templated Triple Helix and *N*-Cyanoimidazole/Zn^{2+} [47]



example, a sticky-ended linear DNA created by restriction enzyme cleavage was closed in a self-templated reaction.[46] In a second experiment, opposite ends of a blunt-ended plasmid DNA were joined by templating the reaction using a third triplex-forming oligonucleotide that brought the ends into proximity.[47]

Kool also developed DNA-templated reactions for closing circular DNAs; however, these were small circular single-stranded oligonucleotides ca. 30−70 nucleotides in length. In this case the chemistry used 3′- or 5′-phosphorylated DNAs combined with BrCN/imidazole/Ni^{2+} as a condensing reagent (Scheme 4).[48,49] The circular DNAs were designed

Scheme 4. Templated Formation of Circular DNA from Linear Segments[48,49]



to bind to complementary RNAs and DNAs, and templates contained the same sequence that the circles were eventually intended to bind. Later studies by Turnbull further optimized these cyclizations and also addressed the converse situation, in which circular DNAs templated the BrCN-mediated ligation of linear oligonucleotides.[50,51] Circular "dumbbell" oligonucleotides, which are DNA duplexes with closed ends, have been chemically closed in self-templated reactions pursued by several laboratories.[45,52,53]

Templated ligations of oligonucleotides—distinct from the monomer nucleotides of Orgel—have also played important roles in studies of prebiotic chemistry scenarios. It was realized that if an oligonucleotide could template the ligation of two-half-length oligomers, this was halfway to autocatalytic self-replication. Von Kiedrowski carried out a series of studies in which short oligomers (commonly 3−5 nucleotides in length) were ligated by the complement, and then the ligated product could template the ligation to form the original strand sequence.[54−56] The ligation chemistry involved the reaction of 3′-amino-substituted oligomers with 5′-phosphoryl oligomers to form phosphoramides in the presence of the carbodiimide condensing agent EDC. Later studies carried out these templated reactions on surfaces and made use of denaturation cycles to promote turnover of the templates.[57] These ligations were not studied as possible reactions for detection of DNA and RNA but were rather explored mainly for the study of the emergence of autocatalysis on Earth.

Related work was reported by Nicolaou and Orgel, who also were interested in prebiotic chemistry and self-replication. Nicolaou reported *N*-cyanoimidizole-mediated ligations in a three-stranded DNA context and observed kinetics that

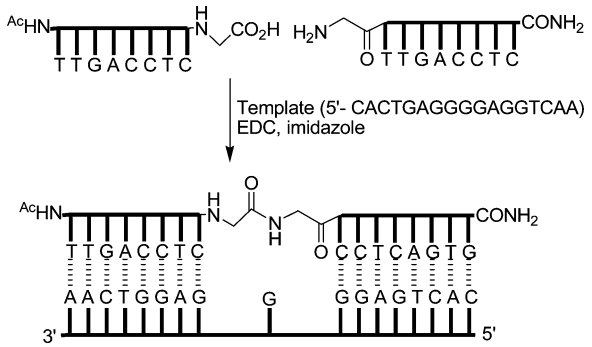
were characteristic of autocatalytic self-replicating systems.[58] Orgel reported chemical ligations of DNAs on PNA and hexose nucleic acid (HNA) templates.[59,60]

Lynn carried out related studies with different chemistry and made an important observation regarding the issue of turnover in DNA-templated chemistry. As with von Kiedrowski, Lynn's goal was the study of autocatalysis with respect to prebiotic chemistry. Lynn initially demonstrated ligations of 3′-amine-substituted oligonucleotides with 5′-aldehyde-derivatized ones to form imines (again, templated by a longer complementary DNA).[61] A notable difference in this chemistry in comparison with the aforementioned strategies is that the reaction is a self-ligation, requiring no added reagents beyond the synthetic DNAs themselves. Self-ligations will be discussed further below; they are especially significant because the lack of a need for reagents makes such reactions potentially viable in the cellular context.

A later variation on this reaction involved addition of a reducing agent ($NaBH_3CN$), which converted the imines to amines and permanently linked the ligated oligomers.[62−65] It was observed that this reduction destabilized the binding of the ligated product as compared to the imine-ligated product. Lynn observed that this destabilization promoted the turnover of the ligating species on the template, again leading to a viable catalytic system.[62]

Orgel et al. reported the template-directed ligation of DNA and PNA to form chimeras on DNA and PNA templates using 1-(3-(dimethylamino)propyl)-3-ethyl-carbodiimide hydrochloride (EDC) as the activating agent.[59] Seitz reported a PNA−PNA ligation on DNA templates similarly using carbodiimide coupling reagents (Scheme 5).[66,67] Excellent

Scheme 5. Seitz's Templated PNA−PNA Ligation[66,67]



mismatch selectivity was obtained when short PNA probes were used (7−8mers), and an abasic site was generated between the two ligating strands.[66] This was achieved by adding a glycine residue to the terminus of one PNA probe. In competition assays using matched and singly mismatched probes, only the ligation products formed by binding to fully complementary templates were observed by MALDI-TOF mass spectrometry.[67]

4.2. Metal-mediated Chemistry

4.2.1. Templated Redox Chemistry

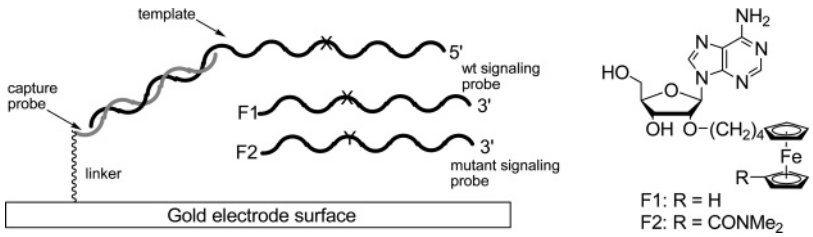
An important class of chemical reactions that can be promoted by the presence and nature of a complementary DNA or RNA strand is redox chemistry. After the notion of facilitated charge transfer through DNA was introduced by Barton,[68,69] a large number of laboratories have investigated the mechanism by which electrons or holes move through the double helix.[70−73] Although DNA is not a highly conductive molecular wire, it has been clearly demonstrated that holes can be transported through DNA over surprising distances.[74] When a hole is injected into a double helix, it can migrate through several turns of the helix and the end result is often the oxidative reaction of a guanine base, which is the most readily oxidized of the natural DNA bases. Alternatively, if there is another route or acceptor of the migrating charge, one can, in principle, use this redox chemistry to detect the presence of a double helix.

A few research groups have explored the utility of DNA-promoted charge transfer in the detection of complementary sequences of DNA. Prominent among these have been the laboratories of Meade and of Barton. Meade demonstrated that DNA hybridization-based capture of target DNAs on metal surfaces can be used to direct further hybridization of a redox-active oligonucleotide probe. Meade cleverly used two different ferrocenyl tags (having different redox potentials) on two separate probes to distinguish between single base differences in the target DNA (Scheme 6).[75] Signals were detected by alternating current voltammetry. Although the localization of the ferrocenyl reporter probes close to the metal surface requires hybridization to a complementary strand, in this case the charge transfer does not necessary occur through the DNA itself.

In a different approach, Barton and co-workers focused on facilitated and in some cases long-range charge transfer through the stacked bases of a DNA double helix. Experiments have shown convincingly that an intact double helix is required for charge transport,[76] thus establishing this redox chemistry as being templated by formation of the double helix. Among the most interesting observations in this work is that small perturbations to the intact stacking of even a single base pair that lies between the charge donor and acceptor can lead to a great diminishment of charge-transport yield. For example, base mismatches strongly inhibit hole migration beyond them to a nearby guanine residue.[77,78]

Barton and co-workers made use of this observation by developing a redox-based reporting system for detection of complementary DNAs and discrimination of single nucleotide differences in a target strand (Figure 1).[79] As with the Meade system, the charge is detected through a metal (gold) electrode and a DNA oligonucleotide is attached to the metal surface. However, in the Barton system, the charge migrates through the stacked DNA bases and the degree of charge

Scheme 6. Electrochemical Detection of SNPs Based on Ferrocenyl Tags with Different Redox Potentials[75]



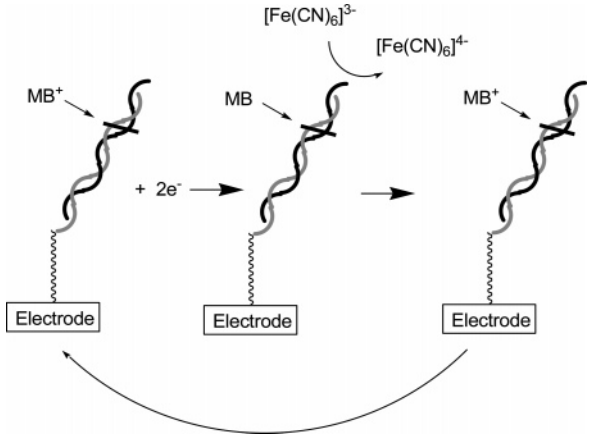


Figure 1. Detection of DNA on electrode surface. Methylene blue (MB^+) intercalates double-stranded DNA but does not interact when there is a mismatch at the intercalation site. Reduction of MB^+ is triggered by electrons from the surface of the electrode. Reduced MB can reduce $[Fe(CN)]^{3-}$, regenerating MB^+.[79]

migration varies quite significantly when one of the bases is mismatched. Methylene blue is used as an intercalating charge donor, and when the capture DNA probe on the surface binds a complementary strand, methylene blue can then bind by an intercalative mechanism. Inducing a potential difference on the gold surface causes redox in the methylene blue, and the charge is detected by the gold electrode.[79] Significantly, the charge transfer can be amplified by recycling the methylene blue via addition of ferricyanide to the solution. Most importantly, the signals vary strongly in the presence of a mismatch, and reading the signal does not depend on carefully balancing the binding of matched and mismatched probes. A recent report by the Kelley group demonstrates a new method for attaching DNA to electrodes for methylene blue reduction, which might provide additional utility in the development of DNA-redox processes.[80]

Thorp also made use of oxidation of nucleobases in electronic detection of DNA sequences.[81] This also was carried out on gold surfaces, and it was reported that quantities of target DNAs as small as attomoles could be successfully detected.

Such approaches are appealing because they could be potentially integrated into electronics manufacturing processes and might be useful in typing nucleic acids after extraction from cells and amplification by PCR. However, it is not clear whether templated redox chemistries could be easily applied to yield signals with intracellular targets, whether fixed or intact. It is also not reported whether RNAs could be directly sensed by such an approach, as the charge transport of duplexes containing one or two RNA strands is as yet largely unexplored.

4.2.2. Metal Complex Formation and Metal-Catalyzed Hydrolysis

DNA-templated assembly of metal complexes for detection of sequences is a relatively new field. Recognition of DNA sequences followed by site-specific cleavage has long been considered the realm of restriction endonucleases,[82,83] but an increased understanding of ribozymes and DNAzymes over the past decade[84] has opened the door for development of new methodologies for nucleic acid sequence recognition and template hydrolysis.

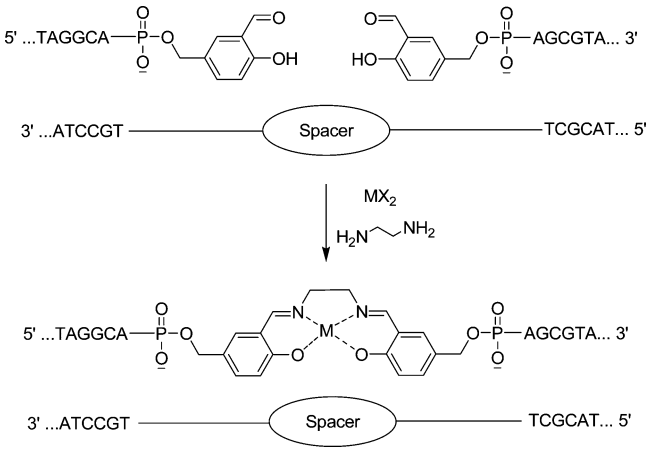
Hydrolytic cleavage of DNA is challenging due to the stability of the phosphate−ester bond, and while RNA cleavage is made simpler by the 2′-hydroxyl which can serve as an internal nucleophile, development of sequence-specific cleavage methods remains difficult. The are a number of reasons why specific sequence recognition followed by template hydrolysis is desired, most notably catalytic biosensing, in vivo degradation of mRNA for gene inactivation, and in vivo destruction of viral, oncogenic, or mutant mRNAs.[85]

There are many examples of DNA being used as a scaffold for assembly of metal ions; several groups, such as Schultz,[86,87] Tor,[88] and Shionoya,[89] have reported examples of high-affinity metal-mediated base pairs, but these examples rely on modified bases in both strands, making them inapplicable to biological detection strategies. Strategies for detection instead require that probe strand(s) that allow for metal complex assembly can bind to a natural DNA or RNA template. Such methods for template-directed metal-catalyzed cleavage of DNA tend to utilize two different approaches: redox metals such as iron, copper, and manganese and associated oxidants and reductants or hard Lewis acids such as calcium, magnesium, zinc, and lanthanides.[90−92] Nature's method for doing this, ribozymes, is a subject that will not be discussed in detail here, but the reader is directed to reviews for more information.[84,93−95] Rather, we shall consider several examples of ribozyme functional mimics where the position and coordination environment of the catalytic cation is controlled through chemically synthesized linkers.[96]

Many of first examples of templated hydrolysis aimed to mimic the activity of DNases by using redox-active metal complexes. For example, Dervan provided an early example of template-mediated metal-catalyzed chemistry by attaching an EDTA moiety to a thymidine residue in a 19-mer oligonucleotide and showing that when this probe bound to a 167-mer template in the presence of Fe(II), O_2, and DTT, the template strand was cleaved over a range of 16 nucleotides near the probe binding site.[97] A variety of other DNA-tethered Fe(II)-binding complexes have also been shown to lead to site-specific cleavage of DNA, including iron−bleomycin,[98] iron−porphyrins,[99] iron−2,6-pyridinedixarboxylate,[100] iron−2,2-dipicolylamine,[101] and iron−bipyridine.[102] Other metals such as manganese, copper, and the lanthanides have also been applied to this strategy. Meunier and co-workers reported a manganese porphyrin complex linked to a DNA strand that gave very efficiently template cleavage.[103,104] Chen and Sigman demonstrated that 1,10-phenanthroline−Cu(I)-linked DNA, in the presence of H_2O_2, could cleave DNA or RNA templates within a few bases of the metal-binding site.[105−107] This scheme was elaborated to improve activity by development of better chelators[108] and through internal conjugation of the phenanthroline moiety.[109]

Another well-explored functional ribozyme mimic involves linking a terpyridyl group to DNA.[110−113] These substrates can cleave complementary RNA templates in the presence of divalent metal ions, typically Cu(II), albeit with relatively poor yield and long reaction times.[110,111] This method was improved by switching to ligands derived from 2,9-dimethylphenanthroline (neocuproine), which increased catalytic activity 30-fold over the terpyridyl complexes due to suppression of hydroxide-bridged dimers and greater activation of coordinated water by Cu(II) neocuproine.[114] A related ribozyme mimic that, like natural ribozymes, used Mg(II) as the metal catalyst was also reported but proceeded with only moderate yields.[115] It is worth noting that while many

Scheme 7. Template-Mediated Formation of Metal−Salen Complexes[123]



of these aforementioned examples were efficient in strand cleavage, the site of hydrolysis was always over a range over several bases near the metal-binding site; none offered recognition for cleavage at a particular base.

A method for base-specific cleavage that employs acridine-modified oligonucleotides and lanthanide ions was developed by Komiyama.[116,117] Lanthanide ions and their complexes had previously been shown to nonspecifically cleave RNA.[118,119] Following previous work that employed lanthanide chelates for the templated cleavage RNA,[92,120,121] Komiyama showed that DNA bearing an acridine modification would lead to specific cleavage of a complementary RNA strand at the position 5′ or 3′ to the acridine, depending on the lanthanide used.[116] The specificity of the reaction is mediated by the intercalation of the acridine, resulting in the unpaired opposing base being flipped out from the duplexes, thereby placing the RNA backbone confirmation at the scissile phosphodiester linkages. The site-selective scission is then mediated by acid catalysis by the protonated acridine residue and lanthanide ion.

Recent work by the Sheppard group has impressively demonstrated the assembly of metal complexes, namely, metallosalen-conjugates, using DNA templates.[122] Sheppard designed DNA−metallosalen building blocks by attaching salicylaldehyde moieties to the 3′ or 5′ end of oligonucleotides. The conjugate is assembled in the presence of ethylenediamine and divalent metal when two strands bind to a template strand with a spacer region between them (Scheme 7).[123] Two nucleotide residues provided the optimal spacer length to maximize complex formation. Using Ni(II) and piperidine, these metallosalen−DNA complexes were able to effectively cleave the DNA template within 1−2 bases of the spacer residues.[124,125]

In addition to metal-mediated cleavage of the phosphate bond, there are a few notable examples where metals are used for the template-promoted cleavage of substrates synthetically attached to oligodeoxynucleotide probes. Perhaps the most promising such approach was reported by Kraemer et al., who used a DNA template as a catalyst for a metal-cleavable linker.[126−128] Using PNA oligomers, they designed two probes, one of which contains a Cu(II) complex and the other possessing a carboxylate linker that can be hydrolyzed by the metal. When the two probes bind adjacently to a DNA template, the substrates are brought in close proximity and the linker is cleaved, releasing the carboxylate (Scheme 8).[126−128] Because there is no product inhibition, turnover is possible and was observed with turnover numbers up to 35.[127] A single mismatch reduced the initial cleavage rate up to 100-fold.[128] Most significantly, this method may be compatible with biological conditions because the Cu(II) ion is tightly bound to the substrate PNA, although no data have been reported on this yet.

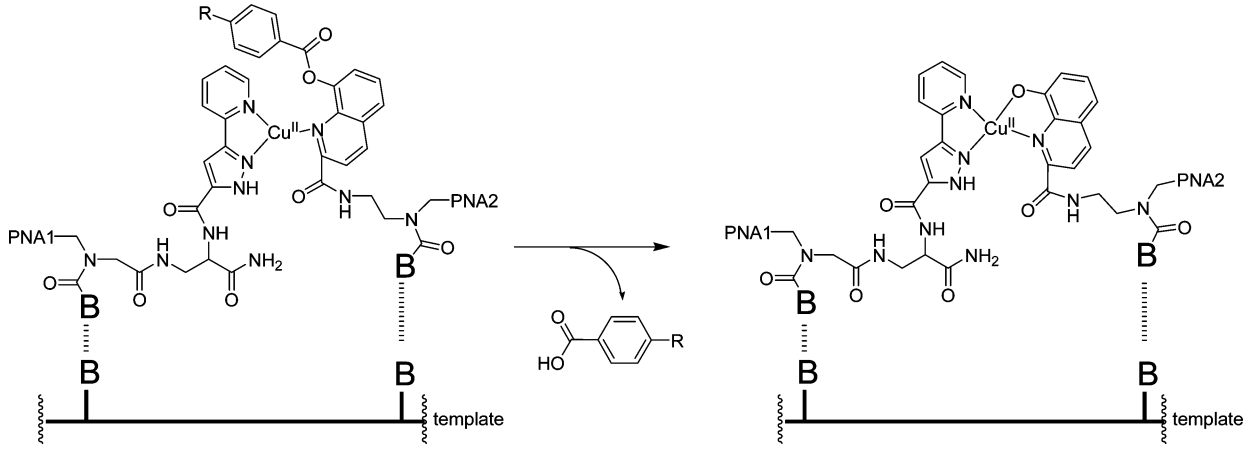
5. Reagent-Free DNA Templated Reactions

In designing DNA-templated reactions for sensing sequences, it is highly desirable to develop methodologies that do not require any reagents aside from the oligonucleotide probes themselves as reagent-free reactions are often more rapid and selective than those requiring additional catalysts. Furthermore, addition of reagents may make a methodology incompatible with detection in vivo as it may be difficult or impossible to deliver the necessary reagents into cells and even if they were to enter cells they may be toxic.

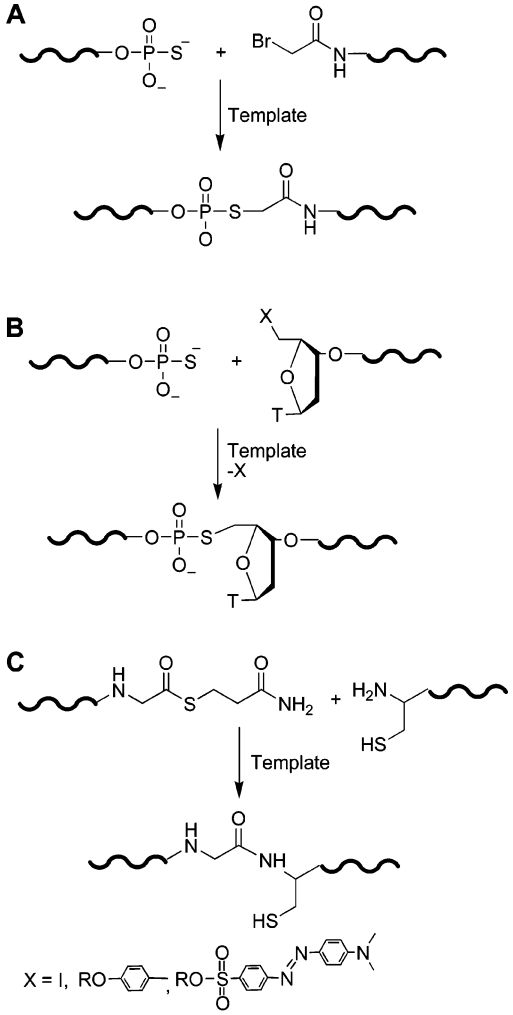
5.1. Self-Ligations

DNA-templated self-ligations are bond-forming reactions that require no added reagents to proceed. In such cases, the oligonucleotides being joined are modified during DNA synthesis to contain reactive ends. The most important early examples of self-ligations (termed "autoligations") were from the laboratory of Letsinger,[129−134] who demonstrated the reaction of oligonucleotides containing electrophilic 5′-bromoacetyl groups with adjacent oligonucleotides containing a 3′-phosphorothioate group (Scheme 9A). This reaction was noteworthy for its rapid rate, and high yields were

Scheme 8. Template-Mediated Release of Synthetically Attached Substrates Using Cu(II)[126]



Scheme 9. Self-Ligation Reactions[a]



[a] (A) Reaction of an oligonucleotide containing a 5′-bromoacetyl group with an adjacent oligonucleotide containing a 3′-phosphorothioate group.[130] (B) Reaction of an oligonucleotide containing a 5′-iodo,[37] tosyl,[132] or dabsyl[139] group with an adjacent oligonucleotide containing a 3′-phosphorothioate group. (C) Native chemical ligation strategy using two adjacent PNA strands.[145,146]

observed in as little as a few minutes. Preparation of the electrophilic species was complicated by the fact that it reacted with water at an appreciable rate, thereby inactivating it.

This work by Gryaznov and Letsinger was especially significant because of the realization that it might be used as a means of detecting a complementary strand of DNA. The authors quantified yields by gel electrophoresis and compared yields using a complementary strand with yields when a single mismatched base was present in the template. In one case, yields were higher by about 20-fold when a mismatch was not present, suggesting significant discrimination was possible even for detection of the smallest mutations.[131] The authors did not further develop this reaction for sensing nucleic acids: in particular, signaling mechanisms that would report on the reaction were not studied.

Letsinger later developed a different electrophilic group, the 5′-tosyl modification, which also led to autoligations with 3′-phosphorothioate oligonucleotides in the presence of a complementary DNA (Scheme 9B). This was used as a "covalent lock" in the preparation of short cyclic oligonucleotides.[135] The reactivity of the tosyl end group required the use of rapid-deprotection chemistry on oligonucleotides containing it.

Xu and Kool explored the reactivity of a different electrophile, namely, the 5′-iodide (Scheme 9B). This leaving group was developed in an attempt to avoid the rapid degradation of the electrophilic group (which was observed with tosylate) during both the deprotection step of synthesis and storage and use. The iodide proved to give high yields in quantitative ligations,[37] although the rate for reaction with phosphorothioate was slower than with tosylate. The iodide-mediated ligations typically proceeded over 6−24 h under typical laboratory conditions. Early iodide-modified DNAs were prepared by incorporating a 5′-iodo-thymidine phosphoramidite derivative (now commercially available) at the terminus of oligonucleotides during DNA synthesis. This placed some sequence restrictions on templated reactions with iodides. However, later synthetic studies led to the development of a method for placing a 5′-iodo group on the terminus of any oligonucleotide (replacing the 5′-hydroxyl) by carrying out an iodination reaction with the detritylated oligomer while it remains on the solid support.[136] Typical phosphorothioate−iodide self-ligations involved short- to medium-length probes of 6−20 nucleotides in length. Discrimination of DNA sequences in solution was possible to the level of single nucleotide differences; optimum selectivity was observed with the polymorphic nucleotide being placed at the center of a short reactive probe. This inhibited binding of mismatched probes, thus preventing their ligation with the other partner. Ligation efficiencies were quantitated with all possible mismatches in one context; selectivities were as low as 10-fold and as high as 1000-fold against a single mismatch and rivaled the enzyme T4 DNA ligase in selectivity.[137]

Recent studies out of Georgia Tech by Hud have described the same phosphorothioate−iodide ligations involving very short DNA oligomers, including trimers.[138] Such short oligomers would not typically react in the earlier studies because they would not bind sufficiently well to the templating DNA. However, Hud demonstrated that addition of an intercalator to the reaction allowed very short oligomers to self-ligate in solution with high efficiency. The end goal of this research was to observe whether such reactions could be used to assemble longer oligomers from these small reactive blocks, thus leading eventually, it is hoped, to self-replication schemes.

A limitation of the phosphorothioate−iodide and phosphorothioate−tosylate self-ligations for detection of nucleic acid sequences is that the reactions do not inherently generate an easily detectable signal. In the early studies it was necessary to isolate and characterize the ligated oligonucleotide product (by gel electrophoresis or HPLC) to observe whether it had reacted, thus confirming whether the target was present.[37,135,138] One strategy developed to overcome this was the use of a donor fluorophore on one probe and an acceptor on the other, leading to a fluorescence resonance energy transfer (FRET) signal during templated ligation.[137]

More recently, quenched autoligation was developed as a molecular strategy to yield a fluorescent signal directly as a result of covalent reaction. The initial report of this strategy entailed conversion of a standard quencher (dimethylaminoazobenzenesulfonyl or "dabsyl") into a leaving group (Scheme 9B).[139] Since arenesulfonyl groups are commonly active as efficient leaving groups (as in the case of tosyl, described above), Sando and Kool realized that functionalization of the 5′-hydroxyl of DNA with dabsyl might make use of this group as both a leaving group and a quencher.

When a fluorophore (initially fluorescein) was conjugated to such an oligonucleotide, it was nearly nonfluorescent; however, such dabsyl-activated probes were shown to react efficiently with phosphorothioate probes in the presence of a complementary template to yield ligated product. The reaction released the dabsylate quencher, thus causing the probe to "light up". The advantage of the dabsylate probes as opposed to iodide FRET probes[137] was their simplicity; only one fluorophore was needed to yield a signal, allowing the possibility for several colors to be used simultaneously. Four-color applications with single-nucleotide selectivity were described.[140] One limitation of this approach, however, was that the dabsyl group could only be added as a thymidine phosphoramidite derivative. This placed sequence limitations on the target, requiring that an adenine be near the desired polymorphic site.

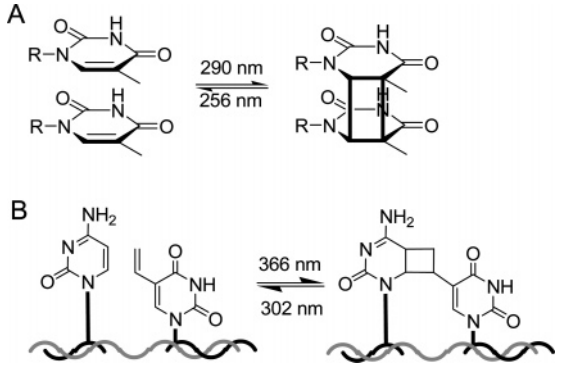
Development of the dabsyl-quenched probes enabled the first cellular detection of RNAs using templated chemistry (see section 6).[141] Additional alterations of the design of such probes later allowed detection of RNAs in living bacteria[142] and detection of RNAs in intact human cells.[143] This is discussed in more detail below.

The Seitz group demonstrated that the "native chemical ligation" reaction (originally developed for peptides)[144] could be achieved between PNA strands using DNA template by adding a cysteine residue to the N-terminus of one of the ligating probes and including benzyl mercaptan in the reaction mixture to maintain a reducing environment (Scheme 9C).[145,146] This strategy removed the need for carbodiimide reagent but required addition of a reducing agent for ligation to proceed. However, the reaction could conceivably be compatible with reaction in cells given the reducing environment of the cytosol. Both of the PNA ligation strategies were successfully used to detect single-nucleotide polymorphisms in PCR reactions with analysis of the ligation products by mass spectrometry.[146]

5.2. Photoligations

A second class of reagent-free reactions is one that is triggered by light (assuming one does not consider light a "reagent" per se). The [2 + 2] photochemical cycloaddition reaction has particular significance in DNA chemistry as it has been known for decades that exposure to UV light can trigger formation of intrastrand cyclobutane−pyrimidine dimers (Scheme 10A).[147,148] In the earliest report of a DNA-templated photoligation, Hanawalt formed dimers, trimers, and tetramers of [d(pT)$_{10}$] by incubating it with [poly(dA)] and irradiating the strands with UV light >290 nm. He also demonstrated that subsequent irradiation at 254 nm reversed the reaction.[149] Products greater than tetramers were not observed because of the high rate of internal DNA damage, particularly internal TT dimers, and low overall reaction yield.

Scheme 10. Photoligations[a]



[a] (A) Intrastrand cyclobutane−pyrimidine dimer formation.[147,148] (B) Reversible photoligation using 5-vinyl pyrimidines.[150−152]

The concept of using pyrimidine dimers to photoligate DNA was greatly expanded upon by the Saito group, who developed a series of 5-vinyl pyrimidines and related modified nucleotide substrates for photoligation reactions (Scheme 10B).[150−156] These substrates were photoligated with long-wavelength UV light of 366 nm, which does not promote significant DNA damage, and gave reliable photoreversibility when the products were irradiated at 302 nm.[155,156] This reaction has proven useful for the templated synthesis of circular,[150,151] branched,[153] and catenated DNA.[151] The specificity of this reaction and its potential applicability to RNA templates has not yet been reported.

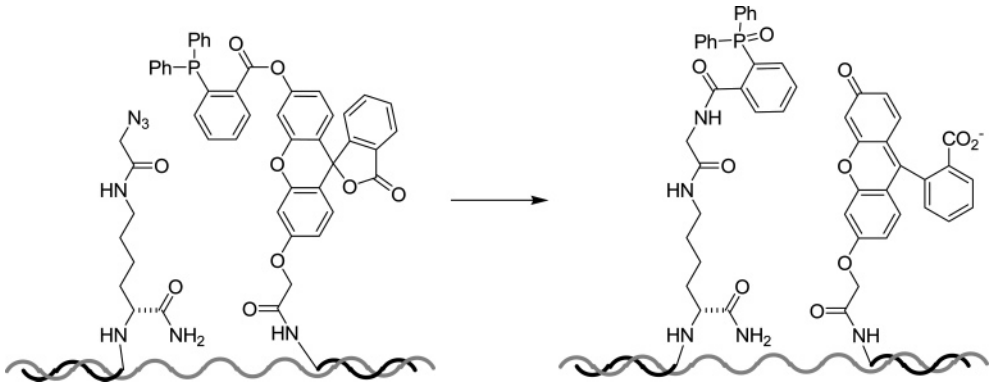
Other photoligation reactions showed significant selectivity for a fully matched template over one containing mismatches at the ligation site,[134,157,158] suggesting that photoligation approaches may also offer some template selectivity. Letsinger reported using stilbenedicarboxamide groups attached to the ends of two adjacently binding DNA probes to afford a photoligation reaction,[134] and Ihara demonstrated photoligation of anthracene-conjugated DNA through formation of anthracene cyclodimerization.[157] Taylor showed that photoligations employing 4-thiothymidine offered a high-yielding method for forming circular DNAs and sequence sensing.[158] Vlassov demonstrated the photocrosslinking of two oligonucleotides, one conjugated with a sensitizer and the other conjugated with a phenyl azide.[159,160] All of these reactions gave severalfold selectivity preference for matched templates versus templates containing one or two mismatches at the ligation site.

Given the simplicity of DNA-templated photoligation reactions, in particular the fact that the nucleotide substrates are relatively easy to make, the reactions are reliable and often high-yielding, and no reagents need to be added, it is somewhat surprising that there are not more reports in the literature. The critical problem at this point seems to be the lack of any sort of easily distinguished reporter for determining whether a reaction has taken place. Since mass spectrometry and radioactive PAGE are currently the only methods to determine the presence of photoligation products,[153] photoligation reactions are inadequate for many assays. Compatibility with cells may also be an issue, as exposing cells to UV light may trigger undesired chemical and biological responses.

5.3. Other Reagent-Free Reactions

The Taylor group demonstrated some interesting examples of DNA-templated chemistry for sequence detection and drug release. A reagent-free DNA-templated Staudinger reaction was used to trigger the turning on of a fluorescent signal (Scheme 10).[161] In the standard Staudinger reaction, a phosphine and an azide react to form an aza−ylide, which spontaneously hydrolyzes to yield an amine and the oxidized phosphorus species.[162] Bertozzi had previously shown that the Staudinger reaction could be used to activate a fluorophore as a result of the phosphorus oxidation.[163] In the DNA-templated version of this reaction, Taylor linked a nonfluorescent fluorescein ester containing a triphenylphosphine moiety to one PNA strand and an azide to another.[161] When

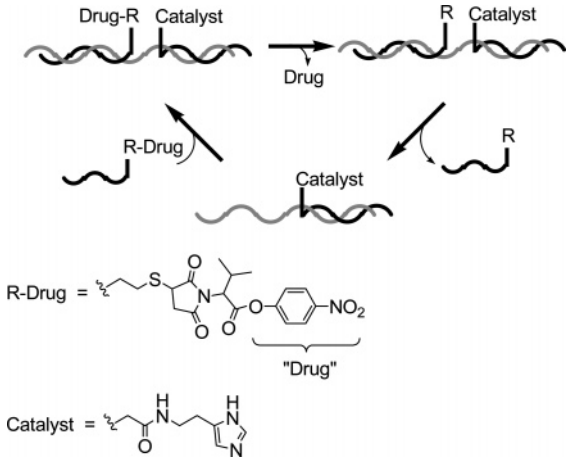
Scheme 11. Activation of a Fluorophore by a Templated Staudinger Ligation Reaction[161]



the strands adjacently bound to a template, the Staudinger reaction proceeded under physiological conditions, leading to cleavage of the fluorescein ester and activation of fluorescence (Scheme 11). The reaction also showed excellent mismatch sensitivity with a greater than 30-fold increase in initial rate for a fully matched versus mismatched template. Significant susceptibility of the phosphine to air oxidation was noted, however, which could limit the utility of the approach.

Another example from the Taylor lab uses two probes, one bearing a 5′-linked imidazole "catalyst" the other bearing a 3′-linked *p*-nitropheyl ester, to release a substrate upon adjacent binding of the two probes on a complementary template (Scheme 12).[164,165] Their system was intended to

Scheme 12. Taylor's Templated Release System for Release of a *p*-Nitrophenol.[164] Such a Strategy Could Potentially Release a Drug upon Sensing a Genetic Sequence



be a model for chemotherapeutic agents that would use the mRNA or DNA specific to the cancer to trigger release of a drug. The model system was shown to efficiently release *p*-nitrophenol in the presence of complementary template with high sensitivity to single nucleotide differences. Because there is no ligation of the strands, there is no product inhibition, and the reaction was demonstrated to occur catalytically. This approach was later applied using PNA probes[166] and profluorescent substrates[167] but has not as yet been shown to work in cells.

6. Applications

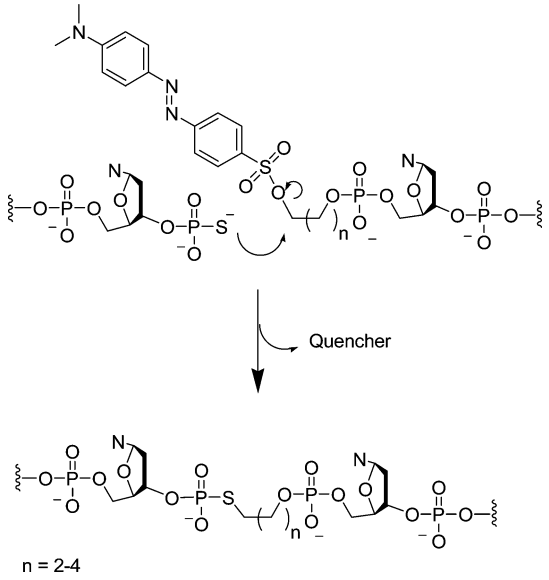
6.1. Detection of Cellular RNAs

As mentioned above, development of the dabsyl-quenched autoligation probes enabled the first cellular detection of RNAs using templated chemistry.[141] Green and red probes were developed for sensing of 16S ribosomal RNAs in *E. coli*. Formalin-fixed cells were used to ensure that probes could access the target without requirement for passing through the bacterial cell wall and without concern for degradation by cellular nuclease activities. It was reported that probes of either color could be used to successfully detect this RNA, which was imaged by fluorescence microscopy. Ribosomal RNA targets are of interest for a number of reasons: first, they are highly abundant, which avoids the issue of sensitivity which might cause difficulties in more rare RNAs; second, they are well mapped for accessibility to binding of oligonucleotides;[33,34] and third, ribosomal sequences are widely available in databases for many bacterial strains.[168,169] Indeed, rRNAs are the most widely used target for microbial diagnostic development,[170] and a few standard oligonucleotide probes are now available commercially.

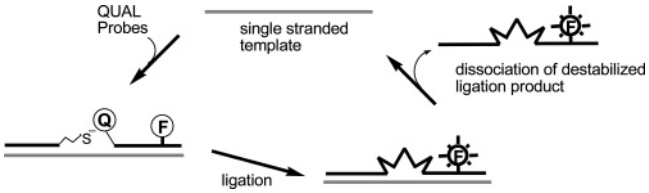
If simple fluorescence-tagged oligonucleotides have been used for identification of bacteria, then why are more complicated reactive probes needed? The answer is 2-fold: first, quenched self-ligating probes are much more highly sequence selective and can identify single nucleotide differences,[139] whereas standard longer nonreacting oligonucleotides typically need multiple nucleotide differences before two sequences can be distinguished.[38] Second, the quenched probes are simpler to use because they are merely added to the cells and imaged. By contrast, standard labeled probes do not change their signal on binding; this means that they must be carefully hybridized to the target and then carefully washed to remove unbound probes before signal can be detected. Finally, this requirement for washing also prevents the use of standard probes in intact cells, whereas reactive probes have been employed in live bacteria (see below).

A recent advancement in quenched autoligating probes has been the development of quenched linkers (Scheme 13).[171] These have been used as phosphoramidite derivatives for reaction with the 5′ terminus of the desired oligonucleotide electrophilic probe. The linker carries a dabsyl group at its remote end. Interestingly, the dabsylate group at the end of these 2−4 carbon linkers was still able to react with an adjacent phosphorothioate probe and in fact reacted more rapidly, producing useful yields in 2−6 h.[171] The linkers yield two other advantages: first, they can be added to any probe sequence, allowing the quenched autoligation to become universal in sequence, and, second, the ligated product was destabilized in its binding to the target as compared with the previous 5′-dabsylate-mediated ligations.[171] This promoted dissociation of the ligated probe pair from the target, which allowed other probe pairs to generate signals as well

Scheme 13. Universal Linker/Quencher Probes for Quenched Autoligation (QUAL) Chemistry[171]



Scheme 14. Catalytic Cycle of "Universal Linker" QUAL Probes[171]



(Scheme 14). This turnover generated 17- to 100-fold amplification of signal. In effect, the template became a catalyst for generation of many signals, which increased sensitivity of such probes measurably.

Quenched autoligation (QUAL) probes with the universal linker were shown to be useful in detecting multiple bacterial ribosomal RNA sequences.[142] Notably, they were shown to detect these RNAs even in intact bacterial cells in the presence of a small amount of sodium dodecyl sulfate to increase permeability. This allowed for appealing simplicity in bacterial identification: one could simply add the probes to the bacteria, wait 2−3 h, and then examine the result under a standard fluorescence microscope. It was shown that a two-color system could be used to detect single nucleotide differences in *E. coli*.[140] Later experiments showed that probes could distinguish between *E. coli*, *Salmonella*, and *Pseudomonas* strains simply by their color.[142] It was also shown that flow cytometry could be used conveniently for identification of such organisms.

Most recently it was shown that quenched autoligation probes (again using the universal linker) could successfully detect ribosomal RNAs in intact human cells.[143] For increased resistance to degradation, 2′-*O*-methyl RNA backbones were used for the probes. Probes were introduced into the cells by use of the pore-forming peptide streptolysin O (SLO).[19] In these most recent probes a further development was used to increase signal-to-noise ratios, namely, a quenched FRET strategy. In this approach the phosphorothioate probe contained a nonstandard Cy3 fluorescence acceptor coupled with the previous dabsyl-quenched fluorescein probes. Once ligation occurred, the fluorescein became unquenched and donated energy to the acceptor dye, yielding the signal. This had the useful effect of removing false signal from simple hydrolysis of the dabsyl probe. This advance made possible the first detection of messenger RNAs using templated reactive probes.[143] Several high-abundance housekeeping genes were detected by this approach. The signals from the RNAs could be seen by laser confocal microscopy (Figure 2) or flow cytometry.

Despite these recent advances, certain limitations still exist for such templated chemistries in sensing RNAs in cells. First is the accessibility through the cell wall; although SDS succeeded for some bacteria,[142] certain Gram-positive bacteria (such as *Mycobacterium tuberculosis*) cannot be penetrated by this approach (Silverman, A. Unpublished data). For human cells, delivering and keeping probes in the cytoplasm can be challenging.[172] Perhaps the greatest limitation still remaining is sensitivity. Many if not most RNAs exist in low numbers in the cell and cannot yet be detected by current probes.

If the technological goal is fluorescence detection of cellular rRNAs and mRNAs, then one must ask whether templated reactions can compete with other detection strategies under investigation. The most difficult challenge is the detection of native mRNAs in intact cells. To date there exist only a few reports of detection of mRNAs in intact nonengineered cells.[20−23,143] Most of those reports involve the use of molecular beacons (MBs), which are quenched probes that make use of a conformational change on binding the target to engender a fluorescence signal. To date, although there are now many examples of templated reactions that detect nucleic acids, only quenched autoligating probes have been employed in detection of cellular RNAs. Like MBs, these probes are also currently limited to detection of abundant mRNAs. Overall, it appears that this one class of templated reaction probes is at least competitive with MBs as a technology;[142] it remains to be seen whether other templated reactions could solve the ongoing problem of sensitivity and signal-to-noise.

6.2. Reaction Discovery Strategies

Development of new methodologies and reactions for templated detection of DNA and RNA remains crucial for the discovery of better methods for detection. The Liu lab pioneered DNA-templated synthesis and reaction discovery methodology that offers great potential in this regard.[173] By attaching substrates to complementary DNA strands, thereby increasing local concentrations of reactants, Liu and coworkers demonstrated that a large number of organic reactions can be achieved in a DNA-template-directed fashion. A rather wide reaction scope has been demonstrated,[174] including reagent-free linking thiols and amines with maleimides and haloacetals[175] and reagent-added reactions such as amide-bond formation, reductive amination, nitro-Aldol, nitro-Michael, dipolar cycloadditions, Heck couplings, Wittig olefination, and functional-group conversions of azides to amines, thiols, and carboxylic acids.[176−181] These reactions proceed with reasonable template selectivity as reactions are not observed on DNA templates containing three mismatches. Reactivity on RNA templates has not been reported.

In addition to the wide scope of reactions potentially available for sensing sequences, a number of structural architectures are available for DNA-templated reactions, notably end-of-helix, hairpin,[175] bulge, and internal[182] (Figure 3). Surprisingly, in each of these architectures reactivity was

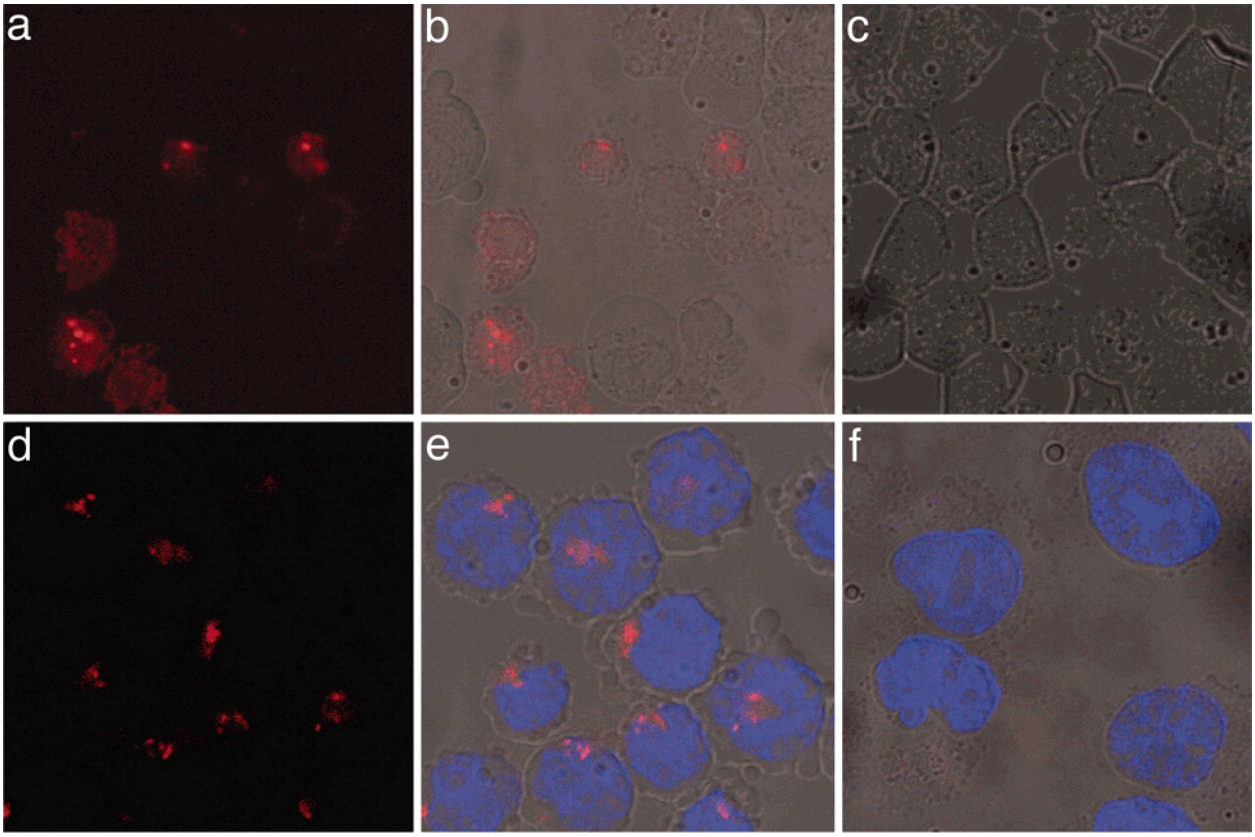


Figure 2. Imaging of 28S rRNA and β-actin mRNA in HL-60 cells by laser confocal microscopy: (a−c) 28S RNA probes and control; (d−f) β-actin probes and control. (a and d) Cy5 signals only. (b and e) Overlay with bright field image. (c and f) Overlay of signal from control (scrambled) probe with bright field image. FRET signals from Cy5 are shown in red; Hoechst 33342 stain (blue) was used as a reference, showing localization of nuclear DNA in the β-actin probes case. Reprinted with permission from ref 143. Copyright 2006 National Academy of Sciences.

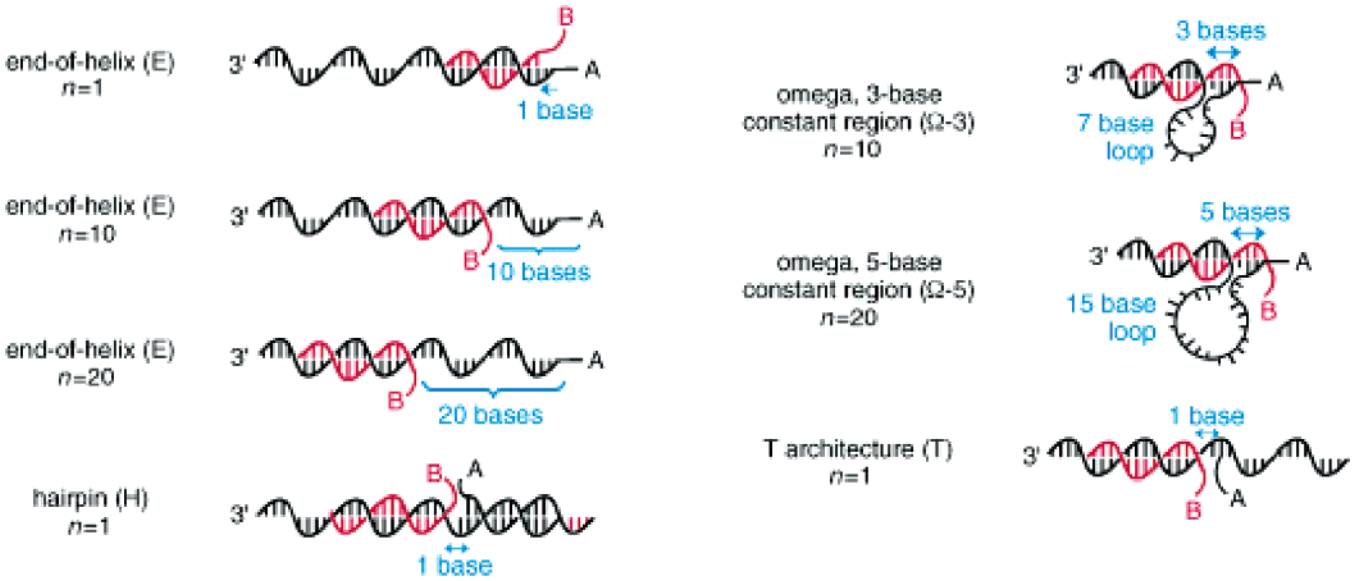


Figure 3. Structural architectures for DNA-templated reactions. Reprinted with permission from ref 182. Copyright 2003 Wiley-VCH Verlag GmbH & Co.

observed when the reagents were bound both side-by-side and up to 30 nucleotides apart.[175,182] The most likely explanation for the distance-independent rates of the reactions is that DNA hybridization, rather than the bond-forming reaction itself, is at least substantially rate determining. Thus, DNA-templating seems to sufficiently speed up these reactions relative to nontemplated reactions by increasing effective local concentrations of reactants from nanomolar to millimolar range,[175] enough that DNA templating can enhance reaction rates to the point that annealing becomes rate determining.[173,175,182] This methodology was applied to new reaction discovery by combining DNA-templated synthesis and in vitro selection using PCR on the reaction products and sequence detection using microarrays to simultaneously evaluate a series of substrates for bond-forming reactions.[183]

The DNA-templated reactions of Liu have been set up such that both probe and template strand are modified with reagents; thus, the scope of this method for using natural DNA or RNA to achieve templated reactivity has yet to be investigated. From the reactivity thus far described in the literature, it seems likely that such an architecture will be successful and might open the door for new detection methods based on organic reactions that were otherwise unavailable for nucleic acid sensing.

7. Conclusions and Future Prospects

In the past 10 years there has been a veritable explosion of research in DNA-templated reactions. As the above discussion points out, there now exist quite a number of DNA-templated chemistries available for detecting DNA or RNA in vitro, and indeed, the number of sequence detection methodologies not involving templated chemistry has also expanded. It would seem that given the large array of technologies for detecting and identifying nucleic acids, many of the in vitro applications in nucleic acid sequence detection that one could currently imagine might already be addressed well without further development of new templated reactions.

Why then does there remain a need for further study of DNA-templated reactions? The answer to this is multifold and depends on new applications that are not yet well addressed by current technologies. Prominent among these coming applications is the detection and imaging of nucleic acid sequences in living cells and ultimately in living organisms. These applications are currently in their embryonic stages with current probes and reactions,[184] and clearly more development is needed to make the approach generally workable. Most of the existing DNA-templated chemistries do not inherently generate signals, so new molecular strategies and reactions that involve signal generation are needed. There is also a serious problem of sensitivity; most of the templated reactions that do produce a signal produce only one signal per template. Thus, methods of amplifying signal strength or quantity would be valuable.[128,171] It would also be useful if methods could be made quantitative, so that not only sequence of the targets would become known but also their numbers as well. Finally, there still remains a serious problem of how to reliably deliver reactive probes through cell walls and into the desired cellular compartments.[19,172]

Of course, cellular applications are only one direction for future development of DNA-templated reactions. Other applications, some far removed from biomedicine, may well be developed. The architecture and assembly properties of DNA make it a promising building material for design of nanoscale structures and machines,[185−188] and templated chemistry may well be useful for building and operating such devices. In addition, DNAs encode and store information and can even be used in computation,[189,190] and it would seem to be only a matter of time until templated reactions find applications in those directions.

Finally, there remain many types of new chemistries to explore, many of which might lend themselves to DNA templating. For example, a broad class of reaction that has not yet been adopted for DNA-templated use is thermal cycloadditions. Many standard 4 + 2 cycloadditions can proceed well in water,[191−193] as can the 3 + 2 cycloadditions involving alkynes and azides.[194] Many more aqueous reaction classes remain possibilities for exploration. Some of these investigations may be carried out with specific applications in mind, but some may be deserving of effort even without a specific applied goal. It is likely that some such explorations will not only lead to efficient new reactions but also inspire previously unforeseen applications.

8. Acknowledgments

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9. Note Added after ASAP Publication

This review was posted ASAP on August 30, 2006, with part of Scheme 9 missing. The scheme was corrected, and the review was reposted on August 31, 2006.

10. Reference

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