Amplified Catalytic Detection of Nucleic Acids

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Abstract

Templated catalytic reactions have emerged as a powerful tool for the detection and diagnostics for DNA and RNA. Detection schemes that allow the amplification of the signal are of high interest in the search of polymerase chain reaction free nucleic acid diagnostics. The basis of templated catalytic reactions is the catalytic activity of template which can trigger numerous reaction for the generation of signalling molecules. Because of the catalytic activity of the template, a single template can give multiple turnovers. However, there are three important points to consider in the design of reactive probes: the reactivity of the functional groups, affinity for the template and the readout system. Recently, fluorogenic reactions have been exploited for the detection of nucleic acids. Fluorogenic reactions are particularly promising because they simplify and shorten the reaction protocol. The different reactions exploited for the generation of a fluorogenic signal can be classified into two classes. The first class is based on the activation of a fluorescein whereas in the second class a quencher is cleaved with accompanied fluorescence enhancement. Both classes offer the possibility for the detection of nucleic acid molecules in living cells. Additionally, the research of template reactions opens a door to position specific drug release.

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1. Introduction

A fundamental biochemical feature that is common to all cellular organism is the use of nucleic acid sequences for the storage of genetic information, called DNA and RNA.[1] Along with the discovery that nucleic acid sequences – mostly RNA – control critical cellular functions and are unique for each cellular organism, the development of methods for the detection and identification of nucleic acid sequences has gained an increase interest. To date, the detection of specific DNA and RNA sequences is used to explore genes and genomes in organisms, in forensic applications and most importantly in the healthcare for the identification of infectious organisms and diagnosis and/or prognoses of diseases, like cancer. To combat these diseases effectively, accurate detection in an early stage of the disease is of great importance. In combination with the focus of medicine on the genetic cause of diseases, which is linked to the development of more personalized medicine, this leads to the demand of ultrasensitive sequence specific nucleic acid detection. In modern-day research, Polymerase Chain Reaction (PCR) is the standard method for the detection of nucleic acid molecules.[2] However, PCR has some significant drawbacks and researches are continuously looking for new methods. Templated catalytic chemical reactions have turned out to be promising candidates for ultrasensitive and selective detection of nucleic acid sequences. Particularly appealing are fluorescent approaches that significantly shorten and simplify the detection scheme because of the lack of several purification steps.[3] Additionally, templated fluorescent reactions have the potential to be used in intact cells.

1.1. *Background and Perspectives*

The standard method for the detection of DNA and RNA – nucleic acid molecules – in present day biology, diagnostics and genomics is based on the enzymatic amplification of nucleic acid molecules by a method called Polymerase Chain Reaction (PCR).[2] PCR represent the ultimate limit of nucleic acid sensitivity. In PCR single or multiple copies of a specific nucleic acid sequence are amplified several orders of magnitude before being indentified and isolated afterwards.[2] However, PCR has some significant drawbacks.

Enzymatic methods like PCR require optimized conditions for the enzymes to achieve highly specific and selective amplification. As a result it is a complex and costly method. Besides, PCR is highly sensitive to contamination causing amplification of spurious sequences. To avoid the amplification of spurious sequences, various reagents can be added or the catalytic property of the enzyme has to be improved making PCR even more complex. If multiple targets need to be detected, multiplexing, PCR can not be used. That is the detection of multiple distinct target sequences in a single assay is not possible. Finally, because PCR is an enzymatic methods it can not be used in intact ells because it is very difficult to get the enzymes through the membrane.[4] Moreover, PCR only amplifies a target sequence which then still needs to be detected. This also limits the use in intact cells. At this point it is worth saying that chemical reactions triggered by the hybridisation of oligonucleotides to target nucleic acid sequences can take place inside cells as there are multiple potential methods for getting oligonucleotides into the desired cell.[5-7] Note, that not each chemical reaction is biocompatible.

Full Sanger sequencing is a nucleic acid detection method overcoming some of the short-comings of PCR. It is a technique which allows every nucleotide to be indentified one by one.

However, full Sanger sequencing is also an enzymatic method. Note, that although full Sanger sequencing is an enzymatic method its efficiency is increasing and dropping in cost.[8]

Because of the previously mentioned drawbacks of enzymatic methods, scientist are continuously developing new non-enzymatic methods for selective and sensitive nucleic acid detection. A promising candidate is the use of Molecular Beacons (MB) in which upon binding of a oligonucleotide probes to the target nucleic acid sequence, the fluorescence of an internally quenched fluorophore is restored.[9] Although, MB are based on oligonucleotide probes and can thus profit from the technique to introduce it into cells,[5-7] it only yields one signal per target which can give problems for detection if only a small number of target molecules are present, or if there is a high background signal. For MB to be useful in nucleic acid detection, a method for the amplification of the signal has to be applied. Templated chemical reactions however, could in principle reach multiple turnover per target molecule and thus amplifying the signal in situ without the need of an additional amplification methods.

Based on the brief previous discussion, templated chemical reactions belong to the most promising candidates for sensing of nucleic acid sequences. In templated reactions the signal is amplified without the need of an additional step. The use of a chemical reaction for obtaining a signal offers also the possibility to design a reaction that is orthogonal to other processes occurring in the sample obtaining a potentially clear on-off signal ratio.[10-11] The aim of this paper is to discuss recent advanced in the development of catalytic reactions that are triggered by the presence of a target nucleic acid sequence. First, PCR is briefly discussed followed by a discussion of why fluorescent reactions triggered by the hybridisation of short DNA strands – oligonucleotides – offer advantages and solve problems observed with other methods.

2. Basic Principles of Nucleic Acid Template Reactions

In the previous section it was briefly discussed why templated chemical reactions are promising candidates in comparison with PCR and MB. It was however not said why nucleic acid templated reactions are highly selective. In this section the basic principles and important considerations of nucleic acid templated reactions will be discussed.

2.1. *Basic Principle*

Template reactions are performed frequently in synthetic chemistry and nucleic acid chemistry to perform chemical reactions that are not efficient in the absence of a so-called template. A template is a particle (molecule or atom) which binds non-covalently to reactants. Upon binding the reactive groups are aligned which results in an increased effective molarity (Figure 1). This increase in the effective molarity results in acceleration of template reactions and allows selective reactions to be performed at low reactant concentrations compared with conventional synthesis, the reaction is catalyzed. In nucleic acid templated reactions the template is represented by the target nucleic acid sequence, DNA or RNA. In principle the probe(s) could be represented by any molecule that can bind to a nucleic acid target. However, in this study the probes are short synthetic oligonucleotides strands which bind with a high predictable affinity and high selective recognition to nucleic acid sequences.[10-11]

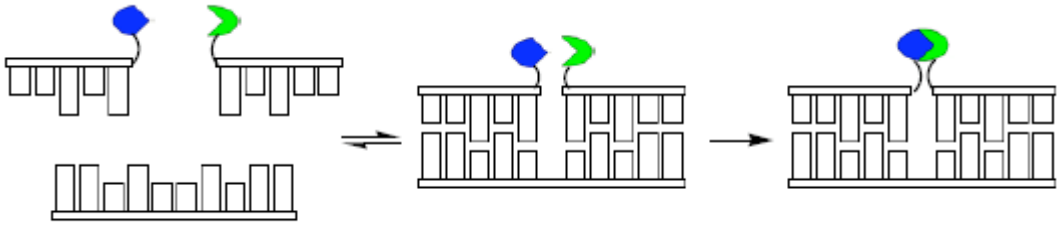

Figure 1: schematic representation of a Nucleic Acid Templated Reaction. the short synthetic oligonucleotides recognizes a unique nucleic acid sequence and binds to it via Watson-Crick base paring – hybridisation. Upon hybridisation the functional groups will react.

2.1.1. *Selectivity of Nucleic Acid Template reactions*

With the discovery of DNA molecules in 1953 by James Watson and Francis Crick it turned out that DNA is build from four different building blocks and is responsible for the storage of genetic information.[1] In many cellular organisms and viruses, RNA is also used as genetic material. Additionally, RNA performs many critical cellular functions. Both DNA and RNA are linear polymers, called nucleic acid molecules, with a fixed backbone from which protrude variable substituents. The backbone is built of repeating sugar-phosphate units. In DNA the sugars are molecules of deoxyribose from which the name DNA is derived (Deoxyribonucleic acid), whereas in RNA (Ribonucleic acid) the sugar is referred to as riboses. In both DNA and RNA the sugars are labelled with one of four possible bases. In DNA each of the deoxyribose is linked with one of the four possible bases; Adenine, Cytosine, Guanine and Thymine (Figure 2). In RNA Thymine is replaced by Uracil. Thus, each monomer

of DNA or RNA consists of a sugar-phosphate unit with one of the four possible bases attached to the sugar. Each monomer is called a nucleotide, therefore DNA and RNA are also referred as nucleic acid molecules. The bases can be arranged in any order along the DNA and RNA strand. This variable sequence of bases acts as an very efficient way of storing genetic information, as each distinct sequence can represent a different message.[1]

Most DNA strands are accompanied by a second strand (Figure 3). The structure that Watson and Crick proposed is composed of two intertwined DNA-strands where the bases are located on the inside and the sugar-phosphate backbone on the outside– this the famous double helix structure (Figure 3). The helical structure is a result of the fact that each sugar points in the same direction. The most important observation deduced from this double helix is the formation of specific base pairs which are held together by hydrogen bonds (Figure 2). The base paring only occurs between specific bases: Adenine (A) pairs with Thymine (T) and Guanine (G) binds with Cytosine (C). It can be imagined that because of this paring, only complementary strands can form a double helix structure In other words, the sequence along one strand completely determines the sequence along the other strand. It is this specific base pairing which makes nucleic acid template reactions highly selective. In nucleic acid templated reactions the oligonucleotide probes can only bind with a strand that is complementary to each own sequence. With this in mind, the oligonucleotide probes will bind with a high affinity to its target sequences. Key-processes in cellular organism depend on the self-recognition of nucleic acids such as replication, transcription and translation.[1]

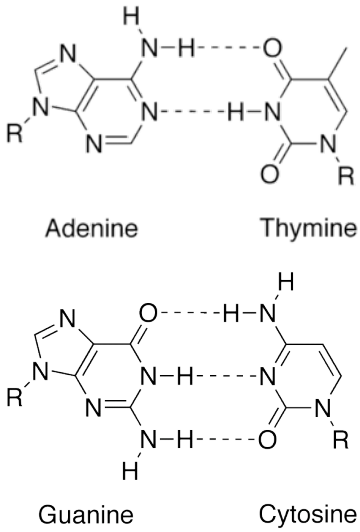

Figure 2: the specific base pairing between the four distinct bases: Adenine, Thymine, Guanine and Cytosine.

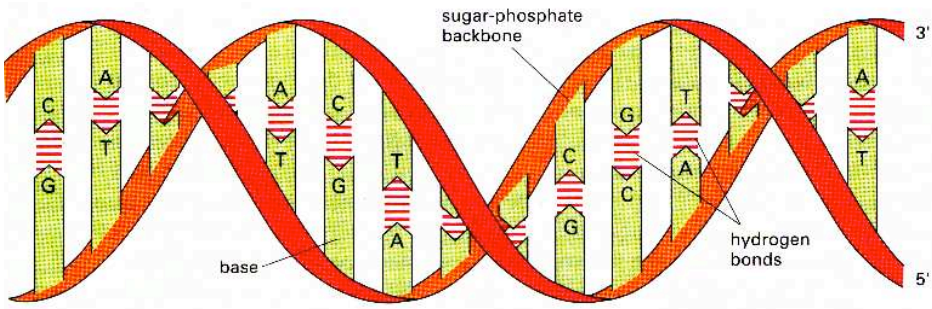

Figure 3: The double-helical structure of DNA proposed by Watson and Crick. The sugar-phosphate backbone of the two chains is shown in red and the bases are highlighted with their respective abbreviation. The strands are antiparallel, running in opposite directions with respect to the axis of the double helix structure.

2.1.2. *Parameters in Nucleic Acid Detection*

Table 1. Parameters that are of importance in nucleic acid templated reactions for the detection of nucleic acids[11]

Target	*Reaction conditions*	*Environment*
Double stranded	Temperature	Solution
Single stranded	PH	Solid Support
Geometric structure	Ionic Strength	Cellular environment

For the detection of specific nucleic acid sequences, the target nucleic acid sequence is used as the template and triggers a chemical reaction. Because of the inherent nature of nucleic acid molecules, this approach is highly selective and product is only formed if the target sequence is present. For the templated approach to be of use in nucleic acid detection a few factors have to be considered.

The first important factor that has to be considered is whether the target analyte is either a DNA or RNA strand. Additionally, it is important if the target strands are double or single stranded. RNA-strands have the potential to bind more strongly to oligonucleotide probes than DNA strands and therefore product inhibition can form a problem in the detection of RNA.[1] Secondly, DNA strands are mostly accompanied by a complementary strand. If the target strand is already involved in Watson-Crick base paring it is more difficult to use it in nucleic acid templated reactions. The recognition step of the oligonucleotides is inhibited.[11] To solve this problem, synthetic oligonucleotide probes with a stronger affinity to the target strand can be used, that is the free energy of binding with the probes is larger. Another procedure which can be used to overcome the previous mentioned problem is partial denaturation of the target strand before one adds the oligonucleotide probes.[1] As can be seen in figure 2, the specific base pairing occurs because of hydrogen bonding. Increasing the temperature and hence the energy of the strands will disrupt the hydrogen bonding and the strands will cleave from each other.[1,11] Note however, that a denaturation step prior to detection is time-consuming. This article will focus mainly on single stranded nucleic acid sequences. In this case, however, still the occurrence of stems and loops in a single strand, in which strong base pairing occurs, has to be considered – the geometric structure of the target analyte has an influence on the detection.[1,12-13]

A second remark concerns the physical condition in which the templated catalytic reactions are carried out. The most important physical condition that has to be considered is the temperature. At higher temperatures, strands with single base mismatching can also hybridize with the probes and function as template for the chemical reaction. On the other hand, at lower temperatures, the probes can hybridize with the target strand but subsequently do not cleave and the templated reaction becomes inhibited. As a result, an optimum temperature has to be find.[1,11] Secondly, the acid and ionic strength of the reaction mixture is of importance as these can influence the hybridisation of the probes to the target strand and the subsequent triggered chemical reaction.[1,11] Also the environment in which the templated chemical reaction is performed must be kept in mind. For example, a cellular environment is completely different than sensing that is carried out on a solid support which can be washed several times before the identification of the DNA or RNA.[1,11]

Finally, the length of the synthetic oligonucleotides is of influence on the templated chemical reaction. The synthetic oligonucleotides strand must be long enough such that sufficiently strong binding occurs. A stronger binding between the probes and target allows for a longer time scale in which the probes can bind to the same target and subsequently allow the chemical reaction to occur.[14] Additionally, the probe must have an number of oligonucleotides that is at least long enough such that it only binds to a specific target, as short oligonucleotide sequences can occur more frequently in DNA or RNA strands. Probes consisting of at least 18 nucleotides are necessary to acquire specific recognition. However, the probe must not be too long as a longer probes binds more strongly to the target and are less sequence specific, that is the longer the probe the more base mismatches can occur.[6] Also, longer probes are more costly in preparation.[15]

2.2. *The Triggered Chemical Reactions*

Although high-rate accelerations may be obtained for the chemical reaction in comparison with the reaction in the absence of the template, turnover of the reactants is often impeded because the reaction may give products with a higher affinity for the template. Product inhibition is the most significant problem in nucleic acid template reactions. It was mentioned in the previous section that the oligonucleotide probe must not be to long but long enough that it can only bind to specific regions of a target strand. Nevertheless, a high turnover number is needed for a sufficient signal amplification. Here opportunities lie to design a system in which product inhibition is circumvented.[10-11] Because the ultimate goal is to design a general system for the detection of nucleic acids in which each random sequence could in principle be detected, reaction design rather than template design has to be explored. Different reaction types have been explored in recent years including ligation, cleavage, hydrolysis and transfer reactions as well as organometal mediated reactions. In the remainder of this paper these different reaction types will be explored with the focus on those reactions in which a fluorescent group is formed or activated.

3. Fluorogenic Template Chemical Reactions

In the previous section the basic principles of nucleic acid templated reactions have been discussed, and it was argued that nucleic acid templated reactions are highly selective and sensitive. Several chemical reactions have been explored in nucleic acid templated reactions with the purpose of ultrasensitive nucleic acid detection. Fluorogenic reactions have already been proven to be a excellent tool in biodetection schemes. Fluorogenic systems are for example extensively used for the labelling of proteins and visualisation of biologically processes.[19-22] As a result, fluorogenic reactions have also been investigated in the search of ultrasensitive nucleic acid detection. Besides fluorogenic reactions are particularly appealing because these can significantly shorten and simplify the detection protocol by obviating several purification steps including the extraction of DNA or RNA strands out of the cells.[23-26] It was also mentioned in previous sections that it is highly desirable to have a reaction where the template can reach a turnover number to provide a sufficient fluorescent signal with single nucleotide mismatch resolution. To date, fluorescent reaction can be classified into two types of reactive probes. First, non-fluorescent probes recognize a target and upon hybridisation a reaction is triggered which results in a the activation of a fluorescent product. The second type of reactive probe relies on the cleavage of a quencher moiety upon hybridisation of the probes and subsequent enhancement of the fluorescence.

3.1. *Activation of a Fluorescein*

The first class of fluorescent templated reactions to be explored are those reaction types in which a fluorescein is activated upon hybridisation of the probes. Although there are many fluorescent functional groups in chemistry which can be activated by a chemical reaction, not all chemical reactions are suited for templated reactions. In view of nucleic acid detection two types of chemical reactions are extensively used. The first reaction type to

consider is the Staudinger reaction which offers some significant advantages over other reaction types. The second reaction which will briefly be considered are metal mediated reactions.

3.1.1. *Staudinger Reaction*

An important chemical reaction used for the detection of nucleic acids by fluorescent means is the Staudinger reaction. In a Staudinger reaction an azide reacts with a phosphine to produce an aza-ylide intermediate. spontaneous hydrolisation yields an amine and the oxidized form of the phosphorus group.[16] Bertozzi had shown in 2003 that the Staudinger reaction could be used to activate a fluorophore as a result of the phosphorus oxidation.[17] In 2004 Taylor and co-workers presented a concept in which the Staudinger reaction was used to switch on a fluorescent signal. Here, an ortho-acylated fluorescein-probe was able to react with an azide that was connected to a second probe.[18] Whenever the probes bind the template on adjacent positions, the acyl moiety is transferred to the azide yielding an amide and a fluorophore (Figure 4). In other words the azide is reduced by the triphenylphosphate moiety generating a fluorescent signal. A 188-fold increase in the rate of the reaction in the presence of the DNA template is observed. additionally, the reaction showed a mismatch discrimination with a 30-fold increase in initial rate for a fully matched versus mismatched template. However, the reaction has not been described with substoichiometric amounts of the template.

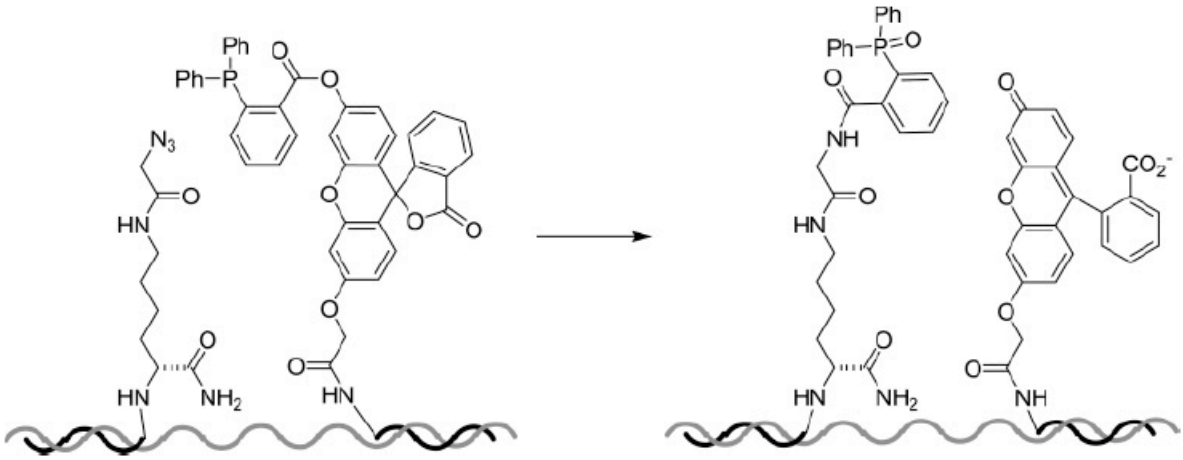

Figure 4:Activation of a Fluorophore by a templated Staudinger reaction.

The results of Taylor and co-workers show that the Staudinger reaction is particularly well adapted to templated synthesis and could be used to activate a fluorescein. Therefore, researches have been utilizing the Staudinger reaction extensively in the view of nucleic acid detection. Pianowski and Winssinger have used the Staudinger reaction to unmask a 7-azidocoumarin fluorophore.[27] Also here a detection scheme with single nucleotide resolution and signal amplification by virtue of the template turnover is observed. It was found to show a 10-fold difference in fluorescence intensity for the perfect matched template relative to single nucleotide mismatched template using catalytic amounts of template. The reaction showed a conversion of 50% within 15 minutes and concentrations down to 1 nM could be detected.

From the obtained results it could be concluded that the Staudinger reaction has particularly good kinetics which is desired for fast detection. Additionally, the Staudinger reaction can be used for signal amplification by virtue of template turnover. Another important remark is that the Staudinger reaction does not require additional reagents and is orthogonal to biological processes meaning that it has the potential to be used in intact cells.

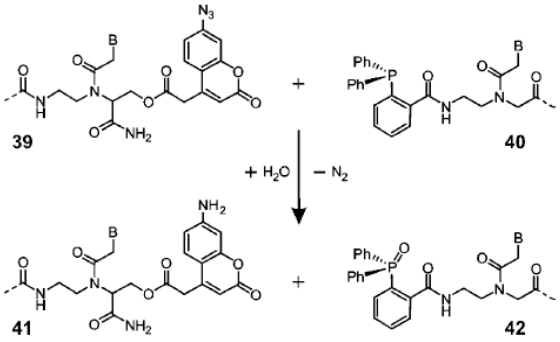

Figure 5: Staudinger reaction between an azidocoumarin (39) and a phosphin-modified PNA probe (40).[27]

Although the templated Staudinger reaction has certain advantages over conventional techniques like PCR, it still contains some points of consideration. To date, known examples that include the Staudinger reaction for the activation of a fluorescein are limited to azide based fluoresceins. These examples include 7-azidocoumarin and azidesubstituted rhodamines. However, certain drawbacks limit the scope of these structures. First, the phosphine moiety is particularly prone to oxidation and secondly the ester is easily hydrolyzed. It is very likely that these degradation reactions cause the often observed low catalytic turnover of templated Staudinger reactions. Besides, this instability hinders the use of the Staudinger reaction in intact cells.[28]

3.1.2. *Metal Mediated Activation of a Fluorescein*

Metal complexes can provide useful functionalities for the activation of a fluorescein upon binding of the probes to the template or target strand. Besides, most organometallic complexes are characterised by unique chemical reactivities and also utilize metal-ligand interactions that are inert to physiological conditions. As a result, organometal complexes may provide stable, bioorthogonal reactants that are capable of performing reactions in living cells. Franzini et *al*. has explored the use of nucleic acid templated reaction between an organomercury-oligonucleotide probe and a masked fluorophore linked to the second probe (Figure 5).[29] The organomercury- probe is composed of Phenylmercury group linked to an oligonucleotide strand. The masked fluorophore is the Rhodamine B phenylthiosemicarbazide. When the two probes bind on adjacent position on the template, the presence of the mercury functional group mediates the occurrence of a cyclization reaction of the thiosemicarbazide functional group generating an oxadiazole. Upon formation of the oxadiazole the highly fluorescent rhodamine spirolactam ring is unmasked. The reaction mediated by the presence of a mercury functional group is completely biostable and therefore future studies for in cell use is needed. It was found that after 110 min of reaction time, the fluorescence in the presence of the complementary template was 11.3 times the fluorescence of background signal.

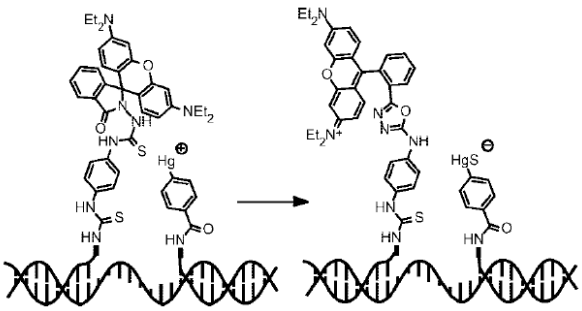

Figure 5: Chemistry of DNA-Templated Activation of rhodium Fluorescence by Phenylmercury-DNA Conjugates

Unfortunately, Franzini did not show the results for single nucleotide mismatching and substoichiometric amounts of template. However, the fact that metal mediated reaction is bioorthogonal and biostable is promising.

Other metal mediated or catalysed templated reactions have been reported.[30] However, these systems do not produce a fluorescent product. Consequently, the detection assay relies on other techniques, which often requires multiply steps or advanced techniques.

Recently, a completely different approach for generating strong fluorescence upon hybridisation of the probe molecules to the target strand, and the subsequent chemical reaction, has been proposed by Herrmann and co-workers.[31] Herein, a nucleic acid (DNA) templated generation of a potent fluorophore by the Pd-catalyzed displacement of iodine from a Boron dipyrromethane (BODIPY) core, a Heck-reaction, is reported. BODIPY chromophores exhibit high fluorescence quantum yields, and in addition, their absorption and fluorescence spectra can be easily changed by chemical modifications of the pyrrole rings. Additionally, BODIPY derivatives have been shown to exhibit heavy-atom quenching effects. Upon removal of the Iodine by utilizing the Heck-reaction the fluorescence is restored. They observed a 20-fold increase in fluorescence intensity for fully matched versus single base-mismatched templates where 90% of the fluorescence maximum was reached after 10 min. Complete saturation was achieved within 20 min where the detection limit was found to be 10 pM.

The obtained detection limit by Herrmann and co-workers is exceptionally low and promising for future detection schemes of nucleic acids. However, because of the fact that a Pd-metal is needed for the reaction to occur is difficult to be used in intact cells. Nevertheless, the method offers the possibility for fast detection of nucleic acid molecules that are present in low amounts.

3.2. *Fluorescent enhancement*

The second class of probes used for fluorescent detection of nucleic acids is the enhancement of the fluorescence through transfer or cleavage of a quencher moiety. In this view two general approaches exist. The first approach to consider is a reaction type in which a reporter group is transferred from one probe molecule to the an other probe molecule, the so-called transfer reactions. The second type used for the enhancement of a fluorescence signal widely employed is the removal or cleavage of quencher moiety from a oligonucleotide probe.

3.2.1. *Transfer Reactions*

Transfer reactions should be considered in view of templated reactions and is a promising reaction type since the number of paired oligonucleotides remains unchanged and product inhibition is unlikely, that is both the reactant and products will show similar affinity for the template with the result that the exchange may proceed quickly. Transfer reactions allow the modification of the probes along with the transfer of a reporter group. Grossmann et *al.* used a nucleic acid controlled reaction to transfer a fluorescence quencher (**Q**) from one probe to the other.[32-33] Upon transfer of the dabsyl quencher the emission of fluorescein (**F**) is turned on while the fluorescence of (**T**) is switched off (Figure 6). The transfer reaction

proceeded via a native chemical like fashion. The hybridisation of the probes to the template triggers a transthioesterfication followed by an irreversible S→N-acyl migration. As a result the reporter group, the quencher, is transferred. In the design of the template reaction Grossmann has indeed fashioned reactants and probes with similar affinity. The template reaction showed 402 turnovers at 10^4-fold excess of the probe after 24 h but more importantly a useful 7-fold fluorescence enhancement after 5h. A signal was obtained at relatively low probe concentrations (100 nM) where most other nucleic acid template reactions require higher probe concentrations (10µM). Additionally, the transfer reaction showed low background signal (3.4% non-templated transfer). These result could be obtained in the presence of 0.1 nM of template (target DNA). The method reported by Grossmann is highly selective but takes a relatively long 5 h. to obtain a useful fluorescence signal.

Note that although Grossmann used a quencher as the reported group, other reported groups could be envisioned resulting in different readout strategies. This is well illustrated by Grossmann in 2007 where an RNA-catalyzed transfer of a biotin allowed for the pre-amplification for an enzyme-based readout (Figure 7).[34] Here, the biotin reported group is transferred from one probe to a probe bearing a terminal His tag (His_6). The nucleic acid templated transfer reaction of biotin showed a 10^2-fold amplification, which is comparable with PCR. After the transfer one probe bears both a biotin label and a His tag. As His-tags are capable of forming strong interactions with nickel, it is possible to immobilize the probe on a surface of Nickel-coated well plates. After washing, the walls were incubated with the enzyme horse-radish peroxidase-streptavidin conjugate (HRP-SA). The HRP-SA binds only with the biotin reported group, and catalyses the oxidation of colourless tetramethylbenzidine to the coloured quinoid. Consequently, the oxidation reaction is only observed when the biotin is transferred via the template reaction to the probe bearing the His tag. By means of this double-amplification strategy a sensitivity was reached down to 500 attomol. Although this approach is highly sensitive and claims to be comparable with PCR it still contains some drawbacks. The method makes use of an enzyme-based readout resulting in the need

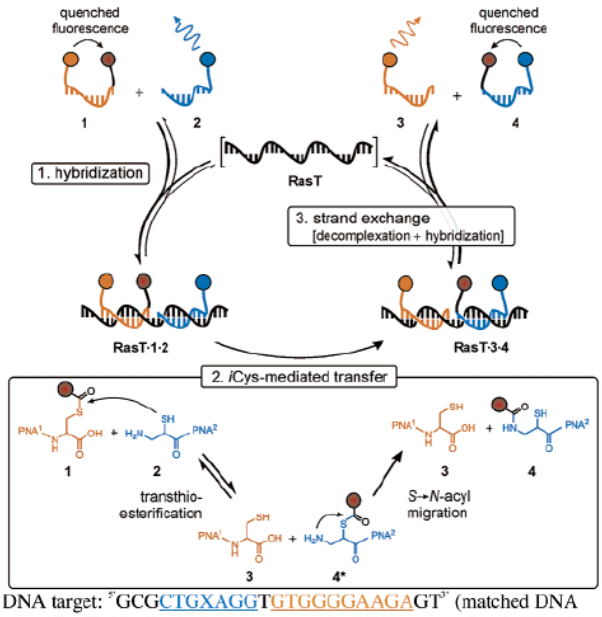

Figure 6: Transfer of a fluorescence quencher (Q) from a fluorescein (F) to a fluoroscein (T).

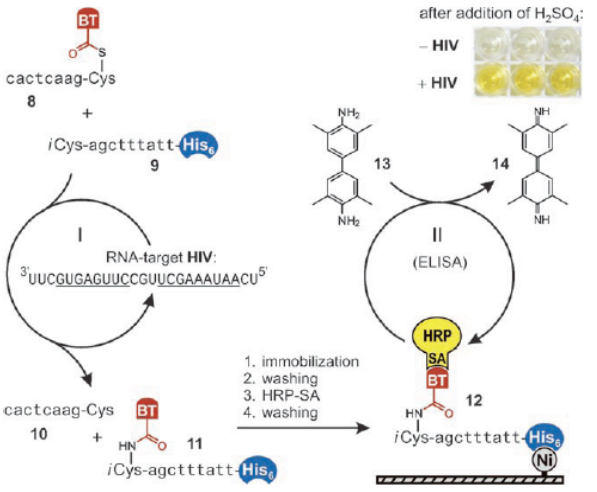

Figure 7: Double Signal Amplification using RNA-catalyzed biotin transfer reaction as preamplification and a modified ELISA as the final amplification.

of specific environments for the enzyme to be efficient and reach a high turnover number. Besides, the procedure requires several washing steps making it time-consuming and costly. Despite these drawbacks, nucleic acid template transfer reactions again show to be a potential method for ultrasensitive nucleic acid detection.

3.2.2. *Quenched Autoligation*

In nucleic acid controlled ligation reactions the oligonucleotide probes are labelled with mutually reactive groups and connect the two oligonucleotides upon hybridisation to the template. In principle the template can act as catalyst for the ligation product, however the templated ligation reactions suffer from strong inhibition of the products. This is due to the fact the products of ligation reactions often show a stronger affinity for the template – the product formed consists of coupled oligonucleotide probes and hence has a higher binding energy. To reach a sufficient conversion of the reactants stoichiometric amounts of template are needed. The challenge is to decrease the affinity of the ligation product without affecting the affinity of the probe molecules.[35]

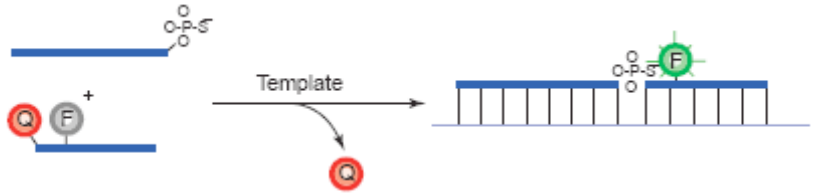

Figure 8: Chemistry of QUAL probes. After hybridization to template, thephosphorothioate displaces the quencher, leading to self-ligation of the two probe strands and unquenching of the fluorophore.

Although product inhibition forms a major problem in ligation reactions for the detection of nucleic acids, there is method for the detection of nucleic acids based on ligation reactions called Quenched Autoligation (QUAL). The signal of QUAL probes is a result from a chemical reaction between two probes binding on adjacent sides of the template or target. One of the probes is labelled with an internal fluorophore and a quencher attached by an electrophilic sulfonic acid linkage, whereas the other probe is labelled with a nucleophilic phosphorothioate (Figure 8).[36-37] Whenever the probes bind on adjacent positions of the template, the phosphorothioate is in a position to replace the quencher on the other probe via a nucleophilic displacement reaction. In this reaction the probes will become ligated with the accompanied generation of a fluorescent signal. However, this process results only in one ligation product per target molecule because of product inhibition. Therefore stoichiometric amounts of the template is needed. To solve this problem Abe et *al.* introduced a modification to the just described QUAL method.[38] By attaching the Quencher to a short 'universal linker' that forms a bulge upon ligation, the dissociation of the ligation product from the target increased significantly and, in addition, the reaction rate showed a 4-fold increase. Using the universal linker they observed 100 signals per target strand. The QUAL approach allowed also for the distinction of closely related sequences by applying a two-colour approach. Herein, two probes complementary to distinct targets are labelled with either a red or green fluorescein and the accompanied quencher, respectively. Consequently, a green or red signal is observed. Because QUAL can be used to distinguish two distinct strands insitu, it is widely used for the distinction of closely related bacteria.

In 2009 Kool and co-workers proposed a probe design for templated fluorescence activation that combines the strong fluorescence enhancement and generality of quencher release probes such as QUAL, together with the kinetic advantages and bioorthogonality of the Staudinger reaction.[39] The approach, called Q-STAR, is based on the release of a dabsyl quencher that is initially attached to the probe via a α-azidoether (Figure 9). Reduction of the azide by triphenylphosphine triggers the cleavage of the α-azidoether linker and the accompanied release of the dabsyl quencher. Upon release of the quencher a strong enhancement of the fluorescence is observed. The reaction showed 90% conversion after 32 min and a 61-fold increase of the fluorescence was observed after 115 min. With this method Kool and co-workers could detect target sequences down to 2nM.

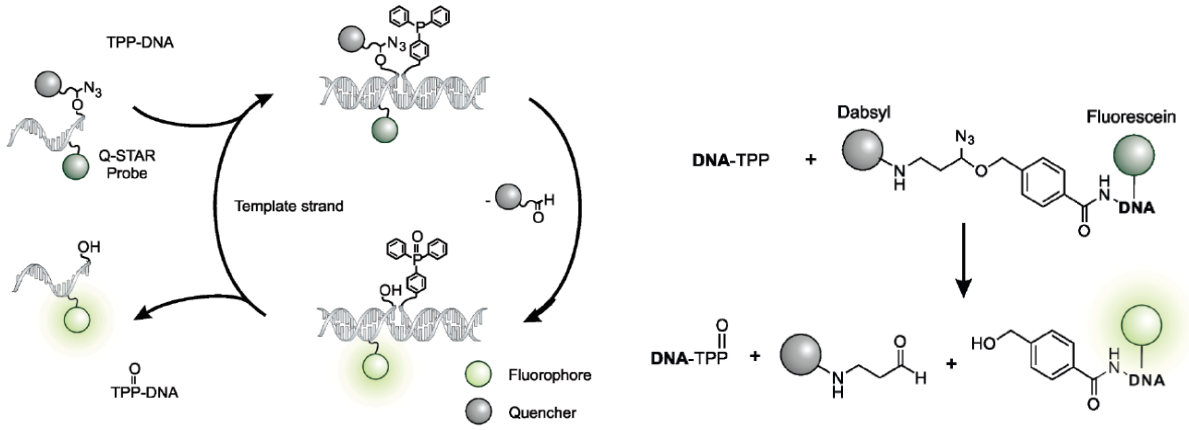

Figure 9: Detection of nucleic acids by templated fluorescence activation of Q-STAR probes. Left) A schematic representation of Q-STAR. The probe bearing the fluorophore and quencher (Q-STAR probe) bind together with the probe bearing the triphenylphosphine (TPP-Probe) to a common target strand (the template). Proximity-induced reduction of the Q-STAR azide functionality results in cleavage of the R-azidoether linker and release of the quencher, yielding a fluorescence turn-on signal. Subsequent probe exchange on the template allows for multiple turnovers; Right) Molecular structures of reactants, including (top) the quencher-conjugated release linker and (Right) the products after reduction, cleavage, and dissociation from the template.[39]

3.3. *Nonethered Reagents in Nucleic Acid Template Reactions*

The basic feature of nucleic acid template reactions is the increase of the effective molarity of the reactants. An alternative approach is based on the release of catalytic moieties, either by chemical modification or by noncovalent binding upon hybridisation of the probes. In 2006 Kramer and co-workers presented a method based on a target induced

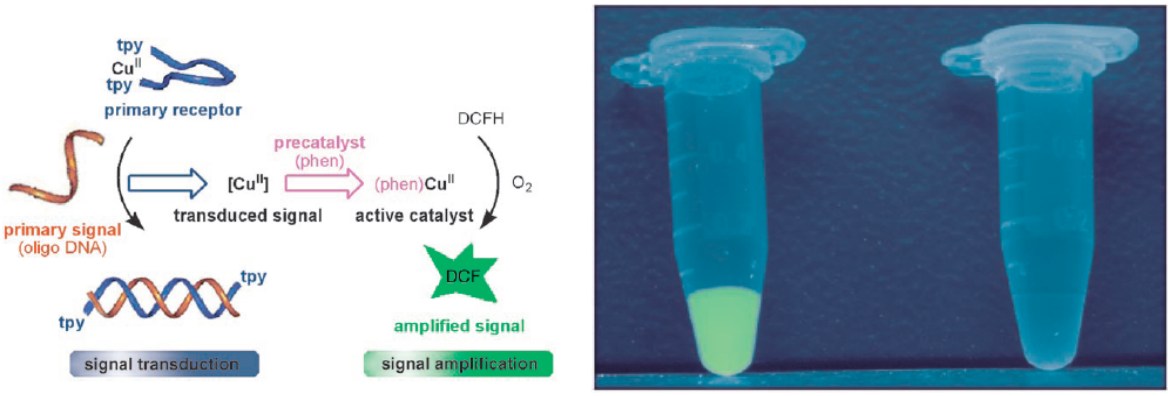

Figure 10: Left) Detection of DNA by allosteric signal transduction and catalytic amplification with formation of the fluorescent dye; Right) Reaction solution containing only the probe (left) and the probe plus the target strand (Right).[40]

allosteric release of Cu(II) ions.[40] The Cupper ions served as a co-catalyst for the formation of a fluorescent product (Figure 10). Upon binding of the probe to the target sequence, the Cu(II) ion is released from its initial situation where it was chelated by two terpyridine fragments attached to both ends of the oligonucleotide. After hybridisation the Cu(II) ion forms a complex with the phenanthroline, which is capable of catalysing the oxidation of the non-fluorescent molecule 2',7'-dichlorodihydrofluorescein (DCFH) to the fluorescent product 2',7'-dichlorofluorescein (DCF). Formation of the product was observed by following the increase in the relative fluorescence. It was observed that rate of oxidation increases dramatically in the presence of template. The activity is about two thirds of that of a template-free reaction mixture. The described method is sensitive to single base matches and target sequences were detectable down to 5 nM with a turnover number equal to 20. A slight disadvantage of this approach is that takes 12 hours to obtain a good signal.

4. Discussion

4.1. *Nucleic Acid Detection in Living Cells*

In the previous sections different reaction types that are used in templated catalytic reactions to produce a fluorescent signal are discussed. It can be concluded that nucleic acid templated reactions promising candidates for the detection of nucleic acid molecules for diagnostic with detection limits down to 1 pM. The most important reaction type that is exploited is the Staudinger reaction because of its bioorthogonality and reagent free character. The bioorthogonality and reagent free reaction makes it particularly useful for use in intact cells, in which procedure steps such as DNA or RNA extraction are obviated. Although this is true, limited research has been exploited on the use of template catalytic reaction in intact cells despite the number of methods that are known for the introduction of oligonucleotide probes into cells. to date only a restricted amount of research is performed on amplified catalytic reactions inside a cell.

For nucleic template reactions to be a well-adapted technique for usage in cells,[5-7] a few points have to be taken into account. First, the oligonucleotide probes have a tendency to aggregate in physiological conditions which gives erratic results. Secondly, the intrinsic fluorescence of cellular organelles must not overlap which otherwise leads to high background fluorescence that severely compromises the sensitivity.[11] Pianowski et *al.* proposed a detection scheme that was particularly well adapted for in cell use (Figure 11).[41] Herein, the use of two cell-permeable Gaunidine-based cell-permeable peptide nucleic acid (GNPA) probes, in which one probe is labelled with a aziderhodamine fluorescein and the other with trialkylphosphine, enabled the detection of cellular mRNA in

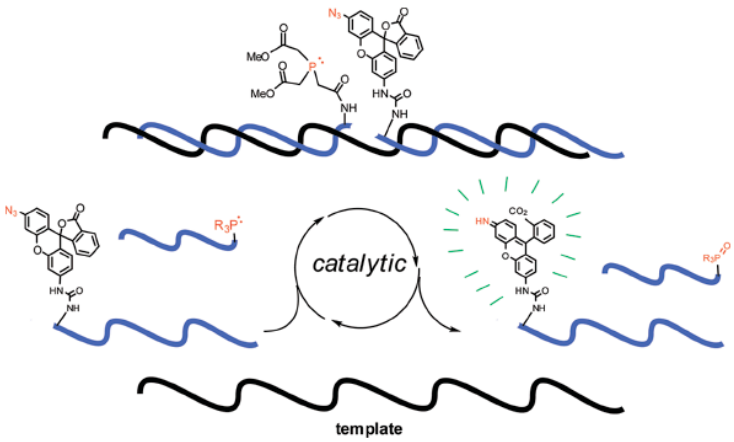

Figure 11: Schematic representation of the templated unmasking of azidorhodamine by Staudinger reduction.

intact cells. In the initial situation the rhodamine fluorophore is azide-quenched. Upon hybridisation of the probes the rhodamine fluorophore is activated by a templated Staudinger reduction. It was observed that the presence of a template let to a 30-fold increase of the fluorescence intensity. The rate of template reaction yields a 80% conversion in 10 min using 1 equivalent of template. The use of the rhodamine fluorophore enabled to visualize the detection of mRNA in intact cells (Figure 12) because the fluorescence excitation/emission falls in a different region of the electromagnetic spectrum than the excitation/emission of the cellular organelles. Satisfactory signal to noise ratios were obtained with the use of 250nM of probe with a significant decrease in the reaction rate for probes bearing a single base mismatch. Pianowski found that the signal increased significantly between 15 and 30 minutes of incubation time for the probes into the cells with only a modest increase for longer incubation times. Consequently, the described procedure allowed for a relatively fast nucleic acid detection scheme by application of a acid templated Staudinger reaction.

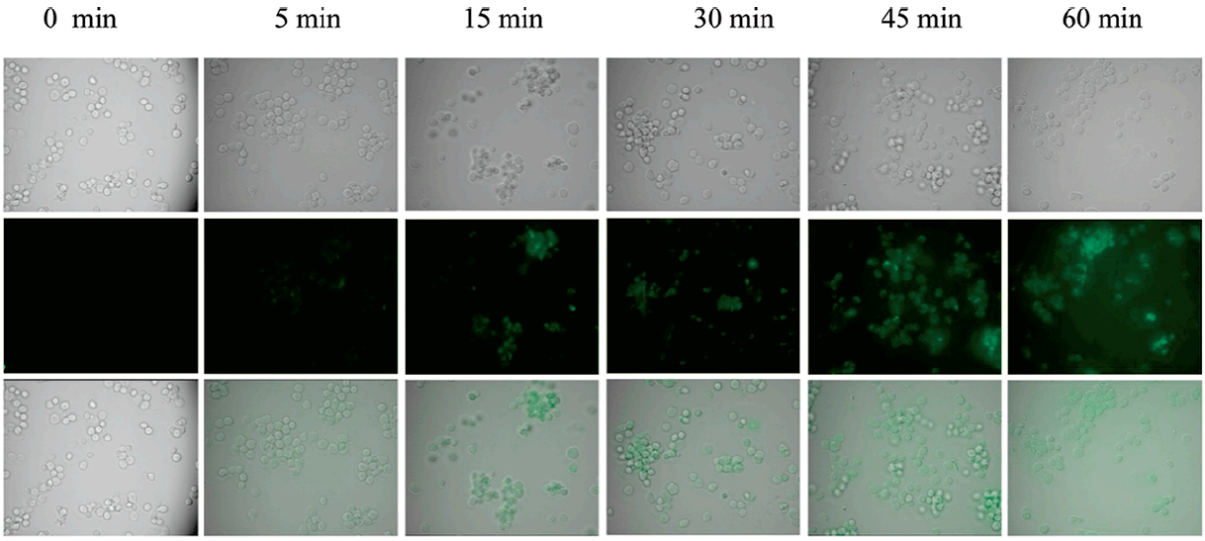

Figure 12: (top) White-light, (middle) fluorescence, and (bottom) composite images of cells incubated with the oligonucleotide labelled with a aziderhodamine fluorescein for 0, 5, 15, 30, 45, and 60 min and then treated with oligonucleotide labelled with the trialkylphosphine.

Although, the Pianowski describes one of the first experiments for nucleic acid detection using catalytic systems in intact cells one has to be critical and further investigations must be performed. In the experiments, the template was a strand of mRNA that was present in excess inside the cells. Therefore, a further look has to be done on the sensitivity of this approach when used in intact cells and if indeed the nucleic acid template approach can be used to detect DNA or RNA fragments only present in small amounts. The low sensitivity of this technique is probably due to the fact the oligonucleotide probes aggregate in the cellular environment.

4.2. *Sequence Specific Fluorogenic Detection of Double Stranded DNA*

DNA is most often present in the double helix structure. The obtained results for sequence specific fluorogenic detection of nucleic acids sequences is mostly performed on single stranded DNA or RNA. Double stranded DNA (dsDNA) has received very limiting

attention as a template for the of chemical reactions in order to amplify a signal. The examples that exist for the detection dsDNA include association of polyamides in the grooves of the helical structure of DNA or oligonucleotide probes binding in a triple-helix formation. However these methods show little or no fluorescence enhancement upon binding of the probes. Recently, Kool and co-workers described a template chemical reaction for the detection of dsDNA.[42] The method is based on a previously described method (Q-STAR) in which a quencher is released via a Staudinger reaction (Figure 13). To summarize, a fluorescein labelled probe contains a dabsyl quencher with at the same terminus an α-azidoether. A second probe carries a phosphine group. The template chemistry allows the reactive groups to react because of increased effective molarity, resulting in an azide reduction and rapid linker hydrolysis. As a result the quencher is released and enhanced fluorescence is observed. The probes were designed such that they will bind side by side in a parallel manner by a pyrimidine motif triple helix formation. In the pyrimidine motif triple helix formation a thymine recognizes an A-T base pair and cytosine recognizes a G-C base pair forming so-called Hoogsteen base paring interactions. To obtain complementary paring at physiological conditions the cytosine was replaced by pseudoisocytosines. Kool and co-workers observed that the reaction is slower than its analogue on single stranded sequences. They related this observation to the fact that with dsDNA the reactive groups are not optimal aligned due to sterical hindrance of dsDNA.

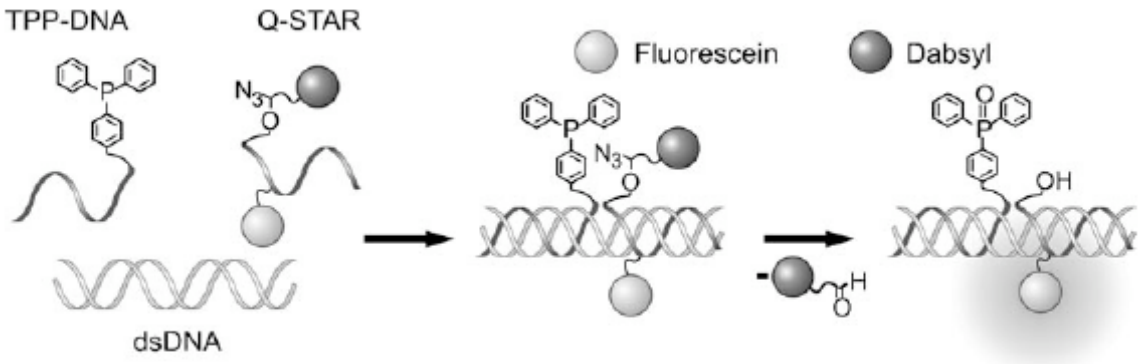

Figure 13: Strategy for the detection of dsDNA by template-mediated fluorescence activation of Q-STAR probes.

The fact that no denaturation of the DNA strand is necessary to detect a specific sequence contributes to a highly simplified method, as special equipment to separate nucleic acid strands is no longer needed. To conclude, the described probe has the potential to detect small amount of DNA, although further studies are needed as only ideal dsDNA is exploited by Kool and co-workers. If however, the problems steric hindrance and detection limit could be overcome denaturation steps to obtain single stranded DNA is no longer needed.

5. Future Prospects

It was mentioned in the introduction that the cancer disease can be detected using nucleic acid template reactions because cancer is characterised by a specific nucleotide sequence in the DNA strand of cells. Instead of a template chemical reactions which triggers the formation of a fluorescent group, the target strand could trigger a reaction in which a drug is released to combat the disease. If this could be realized, than the drug needed to

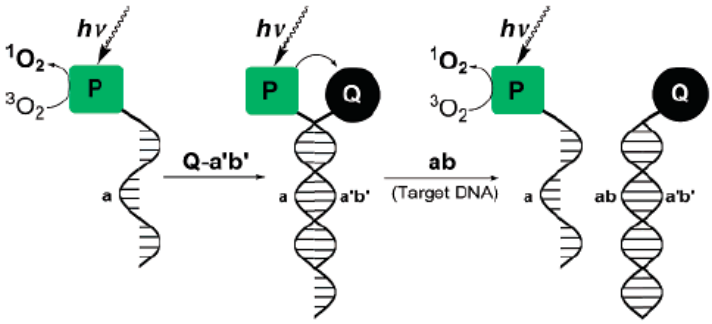

Figure 18: Schematic representation of the templated catalytic production of singlet oxygen.

combat a disease is only released if the DNA or RNA strand characteristic for the disease is present, that is the drug is only released in cancer cells. For cancer this would mean that only cancer cells will be killed whereas healthy cancer-free cells are unaffected. This is in contrast to present day cancer drugs where the drug affects both the cancer as cancer-free cells. A good example of a template reaction for the detection of a target strand and the accompanied particle-induced cell death is that of Ogilby and Gothelf.[41] Herein, a strategy is presented that allows for the control of the synthesis of singlet oxygen (Figure 14). The photosensitiser pyropheophorbide-α (**P**) was linked to a oligonucleotide probe. Upon excitation of the photosensitisere **P** the conversion of oxygen from its triplet state ($^3O_2$) to its singlet state ($^1O_2$). Adding a oligonucleotide probe labelled with a quencher the conversion of $^3O_2$ is $^1O_2$ suppressed. To activate the photosensitiser again, the oligonucleotide probe labelled with the quencher has to be cleaved. This can be achieved for example a by the presence of a complementary target strand. This method proves that it is possible for nucleic acid catalyzed reactions to be used in drug delivery systems.

Another point of interest is the fact that the above described methods for nucleic acid detection only gives quantitative results. It would be interesting if in the future the not only the presence of a particular sequence is known but also the amount. Secondly, the methods that exist to introduce oligonucleotides in cells is limited. Not each oligonucleotide probe can be introduced efficiently. Therefore further studies are needed.

6. Conclusion

As the above discussion points out there exist quite a number of different reaction types for the detection and visualisation of nuclei acid sequences, for both in reaction mixtures as in intact cells. The most promising reaction type for the use in intact cells is the Staudinger reaction because of its bioorthogonality and reagent free character. Although, many fluoresceins are explored to visualize nucleic acid strands further study is necessary in terms of sensitivity. To date, the templated reaction giving the best results in terms of sensitivity still relies on enzymatic methods. The non-enzymatic methods can detect down the picomolar regime. Additionally, only strands that are present in excess inside the cell can be visualized. Here, the technique for introducing oligonucleotide probes in cells needs to be improved. Besides, it is found that only RNA or single stranded DNA can be easily visualized using templated chemistry. To best of my knowledge only one article describes to the detection of double stranded DNA, where only ideal strands can be detected.

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