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Nucleic acid detection using G-quadruplex amplification methodologies



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ABSTRACT

In the last decade, there has been an explosion in the use of G-quadruplex labels to detect various analytes, including DNA/RNA, proteins, metals and other metabolites. In this review, we focus on strategies for the detection of nucleic acids, using G-quadruplexes as detection labels or as enzyme labels that amplify detection signals. Methods to detect other analytes are briefly mentioned. We highlight various strategies, including split G-quadruplex, hemin–G-quadruplex conjugates, molecular beacon G-quadruplex or inhibited G-quadruplex probes. The tandem use of G-quadruplex labels with various DNA-modifying enzymes, such as polymerases (used for rolling circle amplification), exonucleases and endonucleases, is also discussed. Some of the detection modalities that are discussed in this review include fluorescence, colorimetric, chemiluminescence, and electrochemical methods.

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1. Introduction

Before the advent of modern genetic analysis, diagnosis of diseases was a cumbersome process that sometimes involved lengthy culturing of pathogens and analysis of species-specific markers. This could take days to complete, during which disease progression also worsened. Consequently, mortality rate did not only depend on the availability of appropriate drugs to treat a disease but also on the accuracy and speed of diagnosis. The detection of DNA or RNA is now used not only to identify disease states but also to identify mutated genes that could predispose one to certain diseases. Classic examples are BRCA1 or BRCA2 genes, which when mutated are known to drastically increase a woman's risk of breast cancer [1]. In the last few decades several workers have developed sensitive detection of DNA or RNA using either organic or nanoparticle fluorescent [2–4], electrochemical [5–7], colorimetric (usually via horseradish peroxidase or alkaline phosphatase) [8–11] tags or even personal glucose meters [12]. Protein-based nucleic acid detection methods, such as those that use peroxidases, require refrigeration for reagent storage and are not ideal for resource-poor areas or field detection.

G-quadruplexes have found increasing use in supramolecular applications [13] and in the last decade these fascinating structures have also been used for molecular diagnostics [14–17]. Following the seminal discovery by Sen [18] that DNA G-quadruplexes can enhance hemin peroxidation [19], there has been an explosion of the use of G-quadruplexes for the detection of DNA, RNA and other

molecules, including proteins, small molecules as well as metals. The advantage of using DNAzymes for diagnostics is that they can be stored as lyophilized powders at ambient temperatures, without significant degradation and can be reconstituted into active enzymes by simply dissolving in a buffer containing monovalent cations, such as potassium, sodium or ammonium [20].

In this review, we will discuss the detection methodologies that use G-quadruplexes. Assays that utilize enzymatic and non-enzymatic signal amplification strategies will be highlighted.

2. G-quadruplex-based amplification methodologies without protein enzymes

2.1. Split G-quadruplex detection

Nucleic acid containing four or higher tracts of guanines can form peroxidase mimicking DNAzymes. Several groups, including Kolpashchikov, Willner, Wang and Sintim, have designed a system whereby the G-quadruplex is split into two probes that lacked peroxidase activity but upon template-assisted formation of G-quadruplex from the split fragments, peroxidation activity is restored (Fig. 1) [21–24]. Two types of split G-quadruplexes, symmetric [21–23,25,26] and asymmetric [24,27,28], have been described. Split G-quadruplex probes (Figs. 1–4) have equivalent guanine composition whereas asymmetric G-quadruplex probes (Figs. 1, 5 and 6) have nonequivalent guanine contents.

2.1.1. Symmetric split G-quadruplex probes

The symmetric G-quadruplex probe consists of two regions, an analyte binding arm and a G-quadruplex forming region. The two

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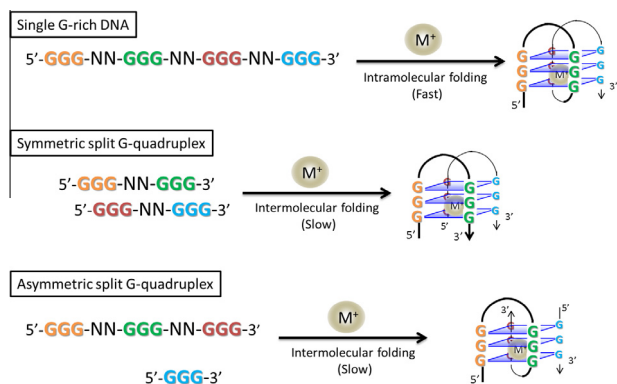


Fig. 1. Representation of symmetric and asymmetric split G-quadruplexes. NN represents two bases other than guanosine.

probes bind to a target nucleic acid in a juxtaposed manner with the analyte binding arm. This binding event puts the two G-rich regions of the probes in close proximity to facilitate G-quadruplex formation (i.e. maximization of effective concentration). Kolpashchikov and co-workers described a symmetric G-quadruplex probe, which could detect the tau protein encoding gene [21]. They were able to detect 1 μM of the DNA target via colorimetric means, using DAB (3,3'-diaminobenzidine tetrahydrochloride) as the reductant (Fig. 2 and Table 1) [21].

Willner and co-workers also reported nucleic acid detection using a symmetric split G-quadruplex probe but instead of DAB, the Willner group used luminol as a reductant for a luminescent readout (Fig. 3) [22]. Additionally, in contrast to Kolpashchikov's turn-on strategy, Willner's approach uses a chemiluminescence turn-off probe to detect the target DNA. In the absence of target DNA, the two split G-quadruplex probes associate with each other to form a peroxidase mimicking G-quadruplex. However, when the two probes bind to a DNA analyte, the G-rich regions are too far from each other to reconstitute into a G-quadruplex and therefore the chemiluminescent signal is reduced. A detection limit of 600 nM was obtained with this system, which is still impressive considering that it is a turn-off assay, which are known to be less sensitive than turn-on approaches.

Ma and co-workers used a symmetric split G-quadruplex probe to detect gene deletion (Fig. 4) [25]. In their study, they detected gene deletion in the CCR5 gene (CCR5- Δ 32) by designing a split G-quadruplex with recognition sequences, which would bind the sequences that flank the deletion target. For an intact gene, the

two split G-quadruplex probes would be too far apart to reconstitute into a G-quadruplex. However, when the region between the two binding sites of the two probes gets deleted, the two G-rich probes are now brought into close proximity for effective reconstitution into a G-quadruplex. The formation of the G-quadruplex was detected via the binding of cyclometallated iridium(III) complex, $[\text{Ir}(\text{ppy})_2(\text{biq})]^+$, resulting in a luminescent readout (Fig. 4).

2.1.2. Asymmetric split G-quadruplex probes

Two challenges that plague typical G-quadruplex probes as nucleic acid detection labels are: (1) background noise due to the association of the split probes in the absence of target and (2) inefficient reconstitution of an enzymatically proficient G-quadruplex from the split fragments. Efforts to address the above issues have resulted in the development of asymmetric split G-quadruplex probes (Figs. 5 and 6) [24,27]. Zhou and co-workers have used an asymmetric split G-quadruplex, and ABTS as the peroxidase substrate, to detect fragments of HIV genes, HIV-1 (NL4-3) or HIV-1 (HXB2), at a concentration of 5 nM [27]. They also demonstrated that the split asymmetric G-quadruplex probes could discriminate between sequences with only a single base difference (SNP detection).

2.1.3. Asymmetric split G-quadruplex probes with duplex regions

Soon after Zhou's report of asymmetric split G-quadruplex probes, the Sintim group reported that appending a duplex overhang to the G-quadruplex region of an asymmetric split G-quadruplex probes facilitated G-quadruplex formation and hence improved the signal-to-noise ratio (S/N) of detection via split G-quadruplexes (Fig. 6) [24]. By incorporating a complementary region each probe (labeled A and B in Fig. 6) that formed a duplex, the S/N was increased by fivefold. The kinetics of the reaction was also greatly enhanced leading to both a quick and sensitive detection of DNA.

2.2. Hemin covalently attached to G-quadruplex

2.2.1. Peroxidation by G-quadruplexes attached to hemin

Since Sen's discovery that G-quadruplexes can bind hemin (leading to an active peroxidase-mimicking DNAzyme) [19], it has been a matter of debate as to the role played by the topology of G-quadruplexes in the actual peroxidation step [24,37–40]. That is, is topology of the DNAzyme important for binding of hemin or the actual oxidation of peroxidase substrates or both? A few groups have demonstrated that parallel G-quadruplexes, but not anti-parallel G-quadruplexes, are good peroxidase

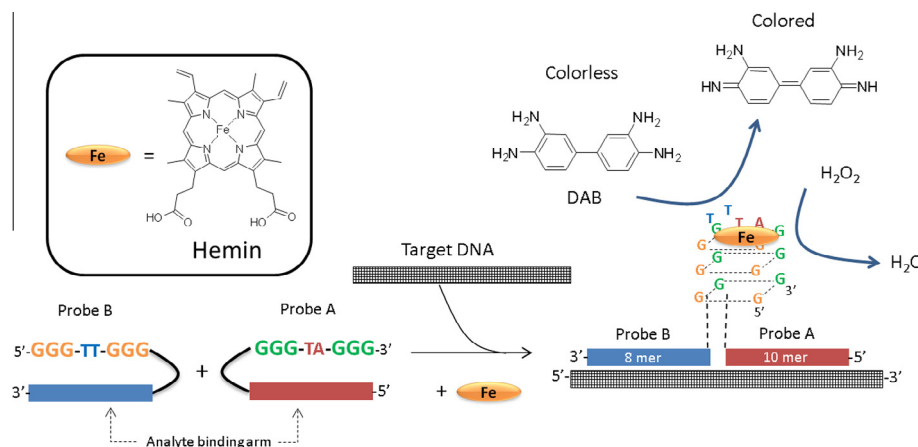


Fig. 2. Kolpashchikov's "turn-on" probe, which uses symmetric split G-quadruplex and DAB. (Adapted from Ref. [21] with permission. Copyright 2008, American Chemical Society.)

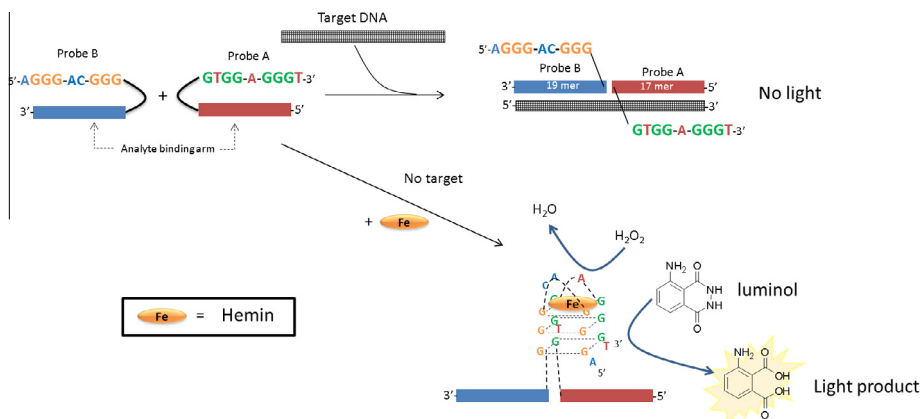


Fig. 3. Willner's symmetric split G-quadruplex probe. When target is present, no chemiluminescence is observed. (Adapted from Ref. [22] with permission. Copyright 2004, John Wiley and Sons.)

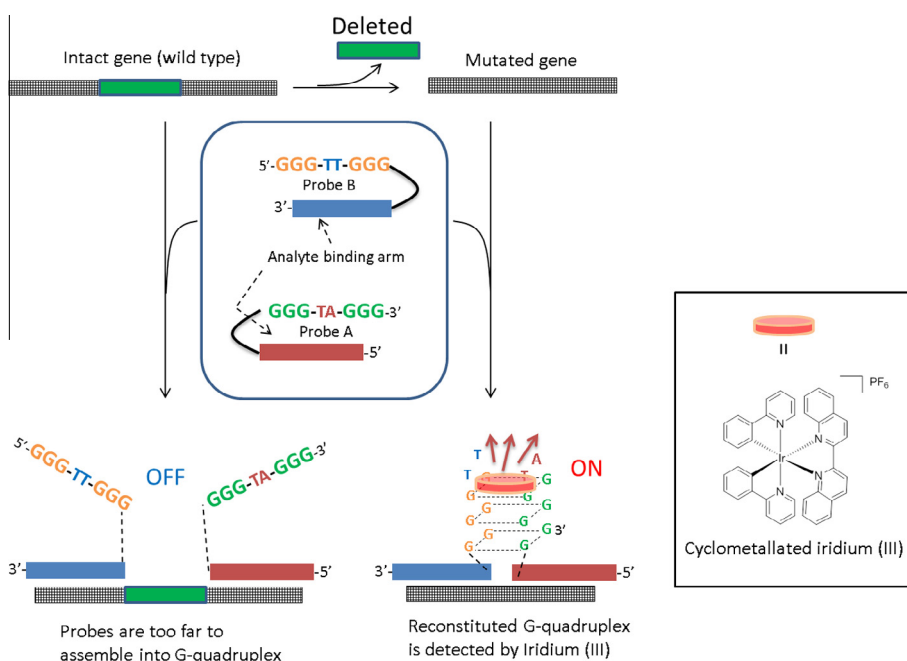


Fig. 4. Ma's DNA deletion detection system [25].

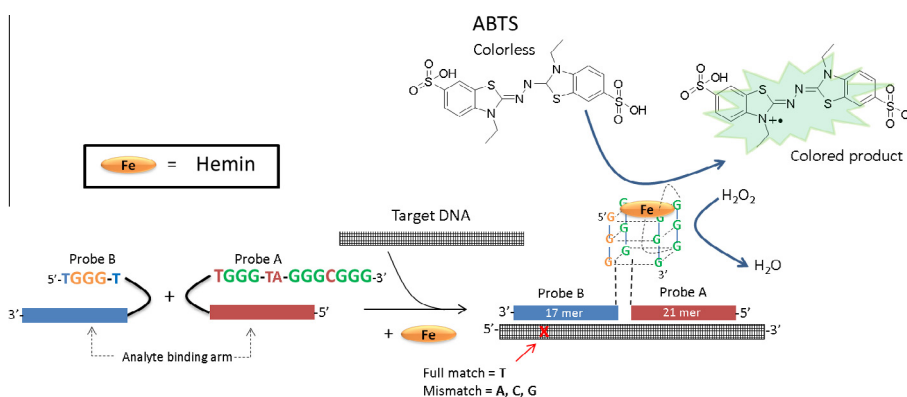


Fig. 5. Asymmetric split probe by Zhou et al. showing selectivity between mismatched targets and natural target sequences [27]. (Adapted from Ref. [27] with permission. Copyright 2008, American Chemical Society.)

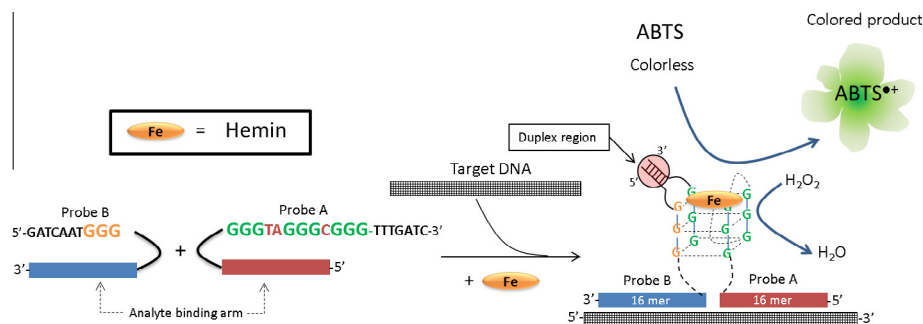


Fig. 6. Asymmetric split G-quadruplex probe, with duplex-forming regions, developed by Sintim and co-workers [24]. (Adapted from Ref. [24] with permission. Copyright 2009, American Chemical Society.)

Table 1

Summary of split G-quadruplex probes discussed in the text.

| Style | Detection limit | Substrate | Output signal | Target | Figure | References |
|---------------------------|--|---------------|-------------------|---|---------|------------|
| Symmetric split | 1 μ M | DAB | Colorimetric | Tau protein coding DNA | Fig. 2 | [21] |
| Symmetric split | 600 nM | Luminol | Luminescent | 5'-TTGAGCATGCGCATTATCTGAGCCAGTACCGAATCG-3' | Fig. 3 | [22] |
| Asymmetric split | 5 nM | ABTS | Colorimetric | HIV-1 (NL4-3) or HIV-1 (HXB2) | Fig. 5 | [27] |
| Asymmetric split + duplex | 800 pM | ABTS | Colorimetric | 5'-ATCTAGAGTCCACCAAGCTTCAGATTAGAAAAG-3' | Fig. 6 | [24] |
| MB | 200 nM | ABTS | Colorimetric | 5'-AATGGCAGCAATTCACCAGTACTACAGTTAAGGC-3' | Fig. 10 | [29] |
| MB | – | Luminol | CRET luminescence | 5'-TCGAATAAGCACTGAGGT-3' | Fig. 11 | [30] |
| MB | 5.4 nM | TMB | Colorimetric | 5'-TAGAACTATCAGATGTGCT-3' | Fig. 12 | [31] |
| MB-symmetric split | 1 pM | ABTS | Colorimetric | 44-mer segment of <i>ureC</i> gene sequences in <i>Ureaplasma Urealyticum</i> | Fig. 13 | [32] |
| TB-switch | 33 pM | Rhodamine 110 | Fluorescence | 5'-AATGGCAGCAATTCACCAGTACTACAGTTAAGGC-3' | Fig. 14 | [33] |
| TB-switch | 20 nM | – | Clotting time | Severe acute respiratory syndrome (SARS) coronavirus gene | Fig. 15 | [34] |
| MB | 1 pM | – | Electrochemical | 5'-TCGAATAAGCACTGAGGT-3' | Fig. 16 | [35] |
| Hybridization | 2 nM | Luminol | Electrochemical | 5'-AGCGTAGGATAGATACGGTTCGGCG-3' | Fig. 17 | [36] |
| RCA | 10 pM | ABTS, Luminol | Colorimetric | M13 phage target DNA | Fig. 18 | [56] |
| Endonuclease | 0.02 fM | – | Electrochemical | 5'-CAGAAAGACTCTCGTTCITT-3' | Fig. 19 | [60] |
| Exonuclease | 36 pM | NMM | Fluorescence | 5'-CTGTTGTGACTCGTCGCAATAAC-3' | Fig. 20 | [61] |
| Mg ²⁺ DNzyme | 1 fM | ABTS | Colorimetric | 5'-GTGTTGTAGTAGTGTAGTACAACIT-3' | Fig. 21 | [65] |
| MB | 500 Cells | ABTS | Colorimetric | Telomerase in HeLa cells | Fig. 22 | [29] |
| MB | 43,000 Selective than Na ⁺ ^a | FAM/TAMRA | Fluorescence | Potassium (K ⁺) | Fig. 23 | [68] |
| Endonuclease | 1.5 pM | ABTS | Colorimetric | Thrombin | Fig. 24 | [76] |
| Gold surface | 5.5 nM | Oligreen | Fluorescence | Adenosine | Fig. 25 | [77] |

MB = molecular beacon strategy, Th-switch = thrombin switching by aptamer strategy, Mg²⁺ DNzyme = Mg²⁺ dependent DNzyme cleavage reaction.

^a Selectivity of K⁺ (not limit of detection).

enzymes. In 2011, the Sintim group demonstrated that anti-parallel G-quadruplexes are equally as good peroxidase enzymes as parallel G-quadruplexes when the hemin cofactor was covalently linked to the DNzyme (Fig. 7) [41]. Thus, it was concluded that topology is important for hemin binding and not necessarily for catalytic activity. When hemin was covalently attached to single stranded DNA (ssDNA) that could not form G-quadruplex, peroxidation enhancement was minimal, implying that the G-tetrad floor was a prerequisite for nucleic acid peroxidation enhancement of hemin. Based on the observation that the covalent linkage of hemin to G-quadruplexes overcomes any limitation that is imposed by the G-quadruplex topology, attempts were made by the Sintim group to detect nucleic acids using probes that are covalently linked to hemin (see Section 2.2.2).

2.2.2. Hemin-G-quadruplex conjugates for DNA detection

One of the limitations of the detection of DNA/RNA using split G-quadruplex probes is that complete reconstitution of the G-quadruplex peroxidase is not always assured. Sintim

and Nakayama therefore wondered if they could detect nucleic acids using two probes, whereby one probe contained a pre-formed G-quadruplex and the other, a covalently linked hemin cofactor (Fig. 8). The rationale behind this design is that the probe that contains G-quadruplex but no hemin would be unable to catalyze peroxidation because hemin is required for the oxidation whereas the other probe that is covalently linked to hemin but does not contain a G-quadruplex would also have low peroxidation efficiency because the G-quadruplex motif is a necessity for nucleic acid enhanced peroxidation. In line with this expectation, in the presence of target analyte the G-quadruplex/hemin–DNA conjugate probe combination gave a higher peroxidation than both probes in the absence of target (Fig. 9). However, the background from the hemin–DNA conjugate probe alone was too high to allow for a sensitive detection of DNA. It appears that although non-G-quadruplex DNAs are not good peroxidases, when covalently attached to hemin, some peroxidation can occur, albeit not as effectively as when G-quadruplex motif is present.

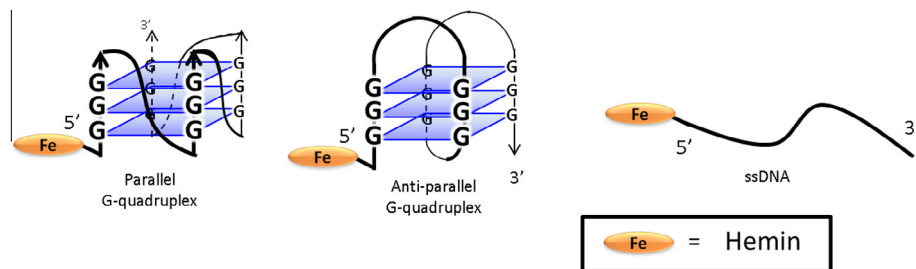


Fig. 7. Different design for Hemin covalently attached probe [41a].

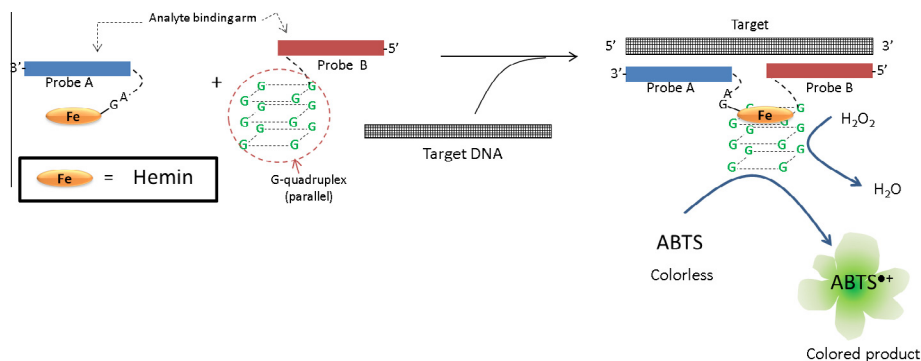


Fig. 8. A new design of a covalently linked hemin-probe [41b] target: 5'-CTAGCCACGAAGTACGCTTCAGATTAGAA-3', Probe A: 5'-(hemin)-GAGTAGTTCGTGGCCTAG-3', Probe B: 5'-TTCTAATCTGAAGC-GGGTAGGGCGGTTGGG-3'.

2.3. G-quadruplex based molecular beacons

2.3.1. Molecular beacon G-quadruplex probes

Several groups have described the detection of nucleic acids using a G-quadruplex molecular beacon strategy [29,31,42,43]. Willner described a strategy whereby some of the guanines needed for the formation of G-quadruplex were involved in the stem formation of a molecular beacon. Binding to a target opened the molecular beacon stem, and the guanines became available to engage in G-quadruplex formation (Fig. 10) [29]. The formed G-quadruplex could then catalyze the oxidation of ABTS to give a colorimetric signal. Using this strategy 200 nM of DNA target could be detected.

In a different rendition of MB G-quadruplex detection of nucleic acids, the Willner group attached a CdSe/ZnS quantum dot (QD) to the 3'-end of a MB G-quadruplex [30]. Upon DNA target binding to

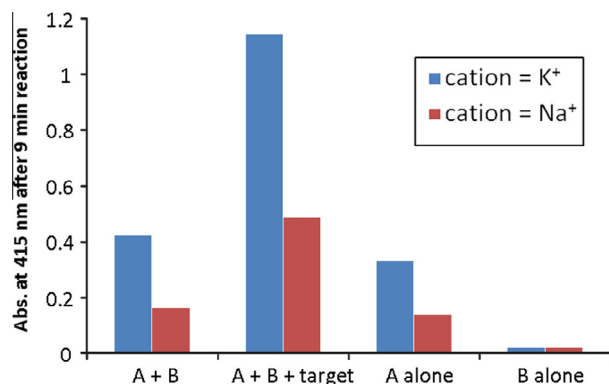


Fig. 9. Signals obtained from hemin-G-quadruplex conjugate probes (see Fig. 8 for the strategy). Conditions: [Probe A] = 300 nM, [Probe B] = 1 μ M, [target] = 1 μ M, [ABTS] = 5 mM, [H₂O₂] = 2 mM, buffer = 50 mM Tris-HCl (pH 7.9) containing 50 mM KCl or 200 mM NaCl.

this probe, the MB opens to form a G-quadruplex. The group then used the reformed G-quadruplex to oxidize luminol. The 420 nm light emitted from the oxidized luminol (chemiluminescence) excited the QD, which then emitted 620 nm light (Fig. 11). The overall process is termed a chemiluminescence resonance energy transfer (CRET). The emission signal from quantum dots can be readily tuned by changing the size of the quantum dot so this approach has the potential to be used for multiplexing by simply using QDs with different sizes.

Wang and co-workers have described an interesting “split” molecular beacon G-quadruplex probe that can be used to detect DNA or thrombin (a dual probe, Fig. 12) [31]. This probe has two stem-loop structures (or molecular beacons), one of which (MB-I) is complementary to the target DNA. When there is no target DNA, and both molecular beacons are intact, the probe can form a complete G-quadruplex, resulting in an active DNAzyme peroxidase. However, in the presence of target DNA, MB-I unfolds resulting in the unfolding of the G-quadruplex as well. The turn-off probe gives rise to a limit of detection of 5.4 nM. The other stem-loop (MB-II) contains an aptamer for thrombin. Thus in the presence of thrombin, MB-II unfolds to bind to thrombin (which is also a G-quadruplex structure). The central peroxidase-mimicking G-quadruplex melts during the thrombin binding event, and hence peroxidation is also inhibited in the presence of thrombin.

Others have also described various themes of G-quadruplex molecular beacons. Park and co-workers were able to detect a 44-mer segment of the *ureC* gene from *Ureaplasma urealyticum* using a G-quadruplex molecular beacon with a detection limit of 1 pM (Fig. 13) [32]. The molecular beacon probe had a symmetric split G-quadruplex at the 3'- and 5'-termini. When blocker DNA is bound, the two halves of the G-quadruplex could associate to form an active DNAzyme leading to a colorimetric signal by oxidation of ABTS. However, when the blocker DNA, associates with the MB the split G-quadruplex is broken and no signal is observed. To create a turn on probe the authors used the blocker DNA in a two-step amplification process, in which the blocker DNA would lose

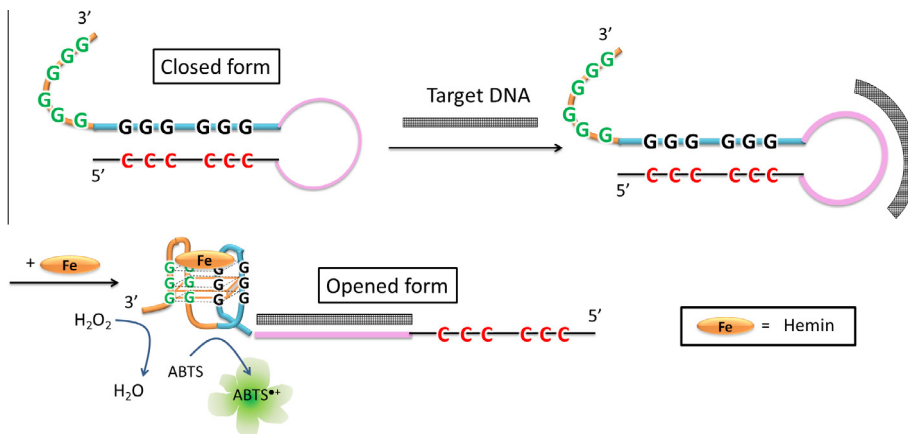


Fig. 10. G-quadruplex-based molecular beacon [29]. (Adapted from Ref. [29] with permission. Copyright 2004, American Chemical Society.)

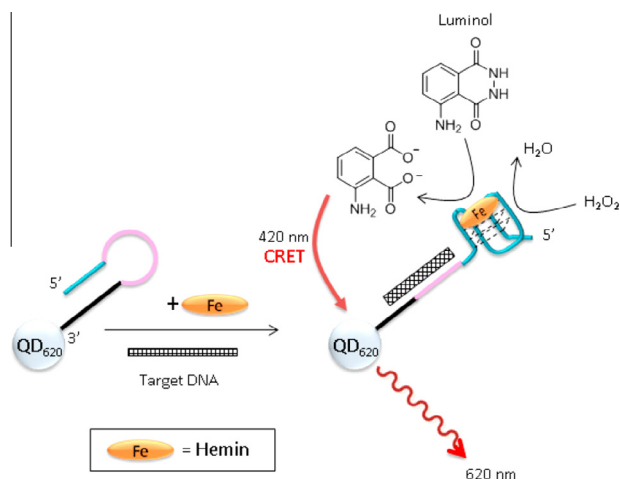


Fig. 11. CdSe/ZnS quantum dots (QDs) covalently linked to G-quadruplex molecular beacon. (Adapted from Ref. [30] with permission. Copyright 2011, American Chemical Society.)

affinity for the MB and bind the DNA analyte of interest resulting in the molecular beacon refolding and forming an active G-quadruplex peroxidase. Once the target DNA and the blocker DNA are bound (step 1), a primer associates (step 2) and DNA polymerase extends the primer (step 3) resulting in the target DNA falling off (step 4). The target DNA may then associate with another blocker DNA resulting in the reformation of another active G-quadruplex-MB peroxidase thereby creating a turn on probe for this system.

Willner and co-workers have described an interesting strategy to detect nucleic acids with G-quadruplex probes but unlike the aforementioned strategies that utilized the enzymatic properties of G-quadruplexes, this time the G-quadruplex was used as an inhibitor of the enzymatic activity of thrombin (Fig. 14) [33]. For this strategy, thrombin was covalently linked to a thrombin aptamer, which is a G-quadruplex, via an intervening sequence that could bind to a DNA analyte. Upon binding to the target DNA, the G-quadruplex aptamer was released from thrombin, resulting in an active thrombin, which could then cleave a non-fluorescent rhodamine 110-conjugated peptide, into a fluorescent rhodamine (see Fig. 14).

Ikebukuro and co-workers also used thrombin and thrombin-binding aptamer G-quadruplex to detect DNA. However in their strategy, the target DNA binding site was located in the G-quadruplex loop sequence. This method gave a detection limit of 20 nM (Fig. 15) [34].

2.4. G-quadruplex-based detection via electrochemical readouts

The detection of analytes via electrochemical means is attractive for two principal reasons: (a) such methods have the potential to be miniaturized on electrochemical chips and (b) electrical signals can be amplified. In the aforementioned peroxidation reactions catalyzed by G-quadruplex-hemin complexes, hydrogen peroxide oxidizes hemin into a high valent oxo species, which accepts electrons from reductants, such as ABTS. In the absence of an organic peroxidase substrate, but presence of an electrode, such as gold electrode, the electrons needed for the reduction of H_2O_2 can be provided by the electrode to complete the cycle (Fig. 16) [35].

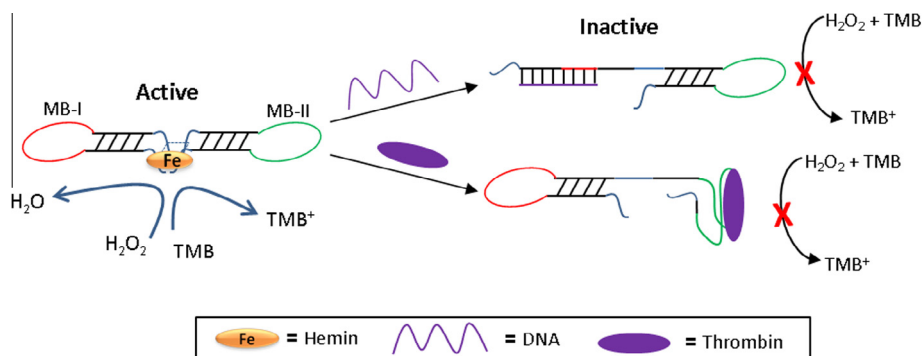


Fig. 12. Wang's combined split G-quadruplex and molecular beacon probe. (Adapted from Ref. [31] with permission Copyright 2011, American Chemical Society.)

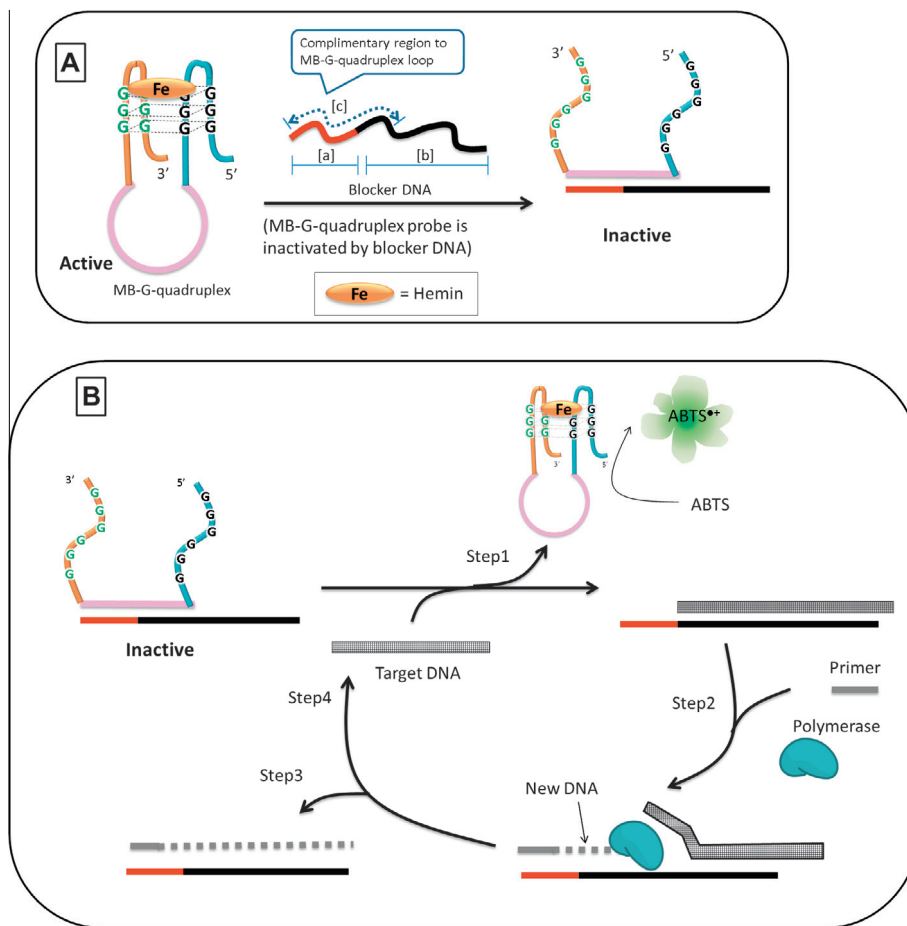


Fig. 13. Two step amplification by Park et al. [32]. (Adapted from Ref. [32] with permission. Copyright 2011, American Chemical Society.)

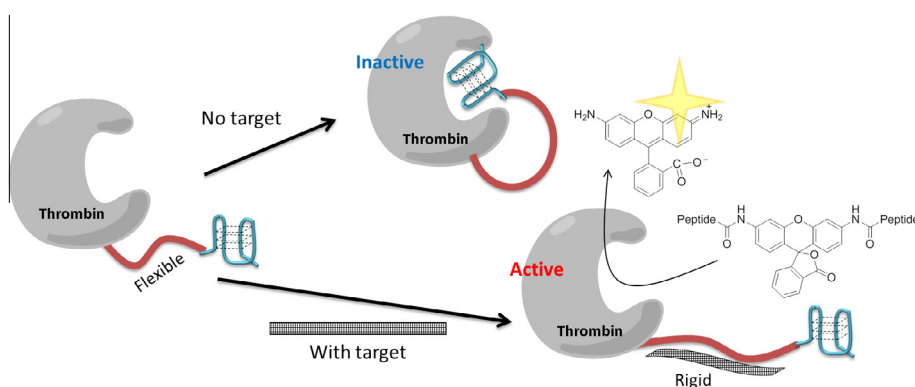


Fig. 14. Fluorescent DNA detection by thrombin–DNA conjugate [33]. (Adapted from Ref. [33] with permission. Copyright 2005, American Chemical Society.)

Willner and co-workers showed that upon binding of a target to a MB G-quadruplex probe that was attached to a gold electrode, a G-quadruplex forms. Upon the addition of hemin, this DNAzyme reduces H_2O_2 , which can be detected by the gold electrode resulting in a detection limit of 1 pM [35].

The Willner group has also described photoelectrochemical detection of analytes using G-quadruplex/hemin complexes [36]. For this approach, a quantum dot (QD) is sandwiched between an electrode and a detection probe, which binds to one half of a target DNA whereas the other half is bound by a probe containing a G-quadruplex. Thus the target brings the G-quadruplex, QD and the gold electrode in close proximity. The G-quadruplex/hemin

complex is used to oxidize luminol and the light produced is transferred to the QD (CRET). Upon excitation, the excited QD transfers electrons to the Au-electrode, which is then measured as an electrical signal (Fig. 17) [36].

3. Enzymatic signal amplification methods

Various G-quadruplex-based detection techniques use enzymes to enhance the signal for the detection event. Of these, polymerase chain reaction (PCR) amplification is probably the most utilized [44–46]. Enzymatic amplification techniques that have been used

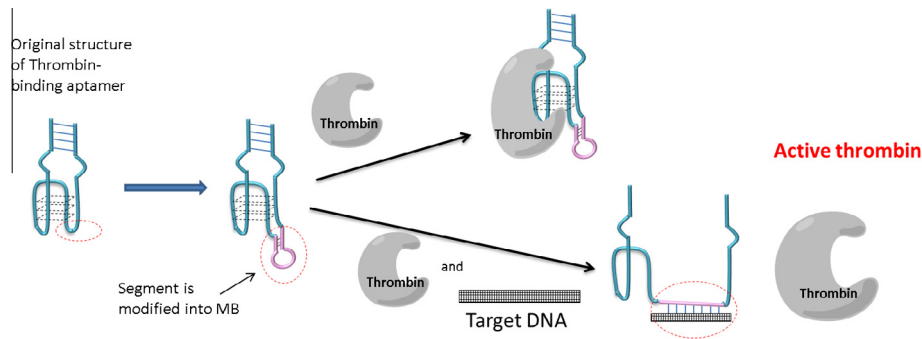


Fig. 15. Fluorescent DNA detection by thrombin-DNA conjugate with a modified thrombin aptamer [34]. (Adapted from Ref. [34] with permission. Copyright 2006, Elsevier.)

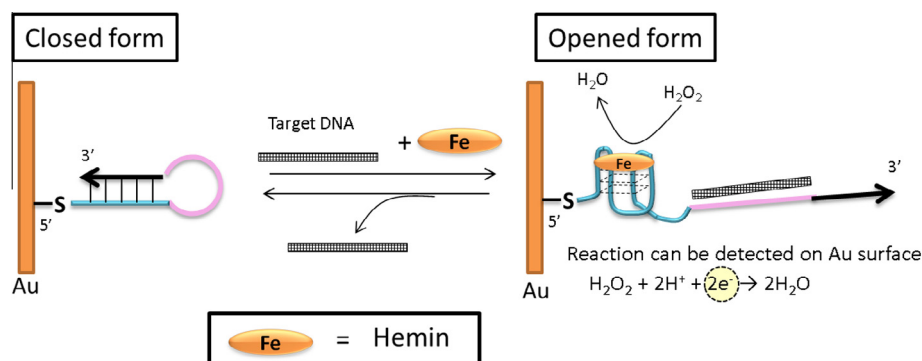


Fig. 16. DNA target detection on gold electrode using the bioelectrocatalytic properties of reduction of H_2O_2 [35]. (Adapted from Ref. [35] with permission. Copyright 2010, American Chemical Society.)

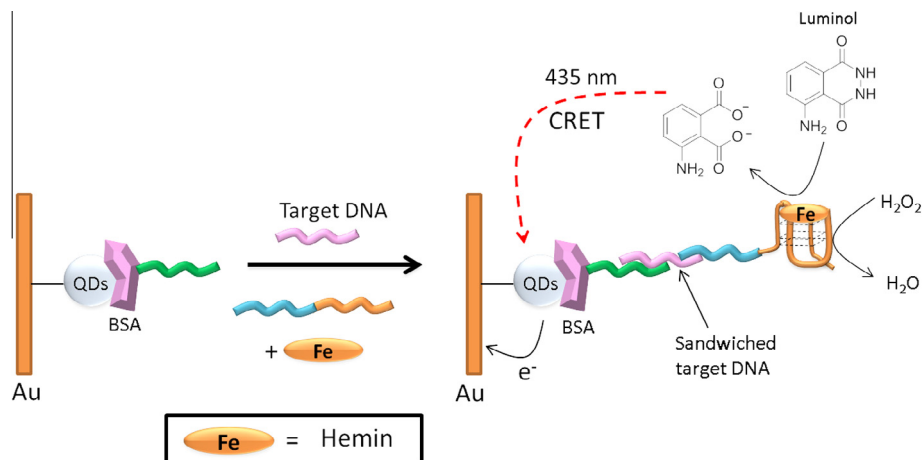


Fig. 17. DNA detection by photocurrent detection by CdS quantum dots (QDs) functionalized gold [36]. (Adapted from Ref. [36] with permission. Copyright 2012, American Chemical Society.)

to detect various analytes, including small molecules [47–50], metal ions [47,50,51] and proteins [48–52] have been described.

3.1. Rolling circle G-quadruplex formation amplification

Rolling circle amplification (RCA) has been utilized extensively in the amplification of circular genes and the sensing of nucleic acids [53]. RCA uses a relatively short, circular ssDNA, primer and DNA polymerase to form a linear chain of DNA that is complementary to the circular ssDNA template but significantly longer than the circular ssDNA template. RCA of circular DNA using RNA polymerase to make long RNA has also been described [54,55]. In a study done

by Willner and co-workers, a RCA machine was employed, which allowed the circular ssDNA template to recognize a target DNA sequence which then acts as a primer [56]. Once the primer is hybridized with the circular DNA, RCA was used to create a sequence of tethered G-quadruplexes [56]. Using a slightly modified strategy this method was able to detect 10 pM of the M13 phage target DNA (Fig. 18).

Another recent study has utilized RCA as a method for the detection of DNA methyltransferase [57]. Regarding the assembly of multiple G-quadruplex labels from one target, Willner and co-workers have also described an interesting detection of DNA via an enzyme-free autonomous assembly of G-quadruplex nanowires [58].

3.2. Nicking nuclease signal amplification

Originally applied to molecular beacon systems, nicking endonuclease signal amplification (NESA) is an efficient way to improve sensitivity in DNA detection [59]. NESA obviates the need for a 1:1 binding stoichiometry of DNA probe to target DNA. This allows amplification of signal from minimal amounts of target DNA. Chen and co-workers used an endonuclease amplification approach, in conjunction with a gold immobilized DNA to detect oligonucleotides (Fig. 19) [60]. A sequence containing a 3'-terminal thiol group, a G-rich sequence and a sensing sequence was immobilized on a gold electrode [60]. Upon hybridization of the target sequence to the immobilized DNA the endonuclease nicks the DNA thus cleaving it from the G-rich sequence still attached to the gold and the G-rich sequence was then allowed to fold into a G-quadruplex and the excess DNA could be washed away [60]. The immobilized G-quadruplex was then used to oxidize TMB (3,3',5,5'-tetramethylbenzidine), using a hemin/H₂O₂ system [60]. The peroxidation reaction could be followed by either a color change or an increase in current across the electrode [60]. This method allowed for the detection of DNA at a concentration as little as 0.02 fM [60].

Qu and co-workers used Exo III to accomplish an exonuclease amplification utilizing a G-quadruplex motif in place of the molecular beacon [61]. Upon release of the target another portion of probe DNA would be released, which was capable of folding into a G-quadruplex in the presence of monovalent cations. This

G-quadruplex would then bind NMM (N-methyl mesoporphyrin IX), a fluorescent heme analog, resulting in a fluorescent output. The target molecule could then bind another DNA probe and continue signal amplification. While not G-quadruplex peroxidation specifically, this method uses the heme binding capabilities of G-quadruplex structures (Fig. 20). Other reports that detect nucleic acids using a nicking endonuclease strategy have also been reported [62].

3.3. DNAzyme cascade amplification

DNAzyme and enzyme cascades allow for sensitive detection techniques. This method has been used to detect proteins as well as other target analytes [63,64]. A novel DNA detection using reconstituted DNAzyme in tandem with DNA peroxidases has been shown by the Willner's group (Fig. 21) [65]. In this work, they utilized a DNAzyme cascade to produce a folded G-quadruplex capable of a colorimetric output by the oxidation of ABTS. In this system, two probes hybridize with the DNA analyte forming a duplex. Each probe contains an overhang, which can form an active, Mg²⁺-dependent DNAzyme that can nick RNA. In addition, the overhangs contain a region, which hybridizes with a pseudo-circular DNA, which contains two G-rich regions capable of forming a G-quadruplex. Each of these G-rich regions is, however, blocked by a small DNA as to prevent G-quadruplex folding. After hybridization with the target DNA the active DNA machine assembles. In the presence of Mg²⁺, the DNAzyme then nicks the circular

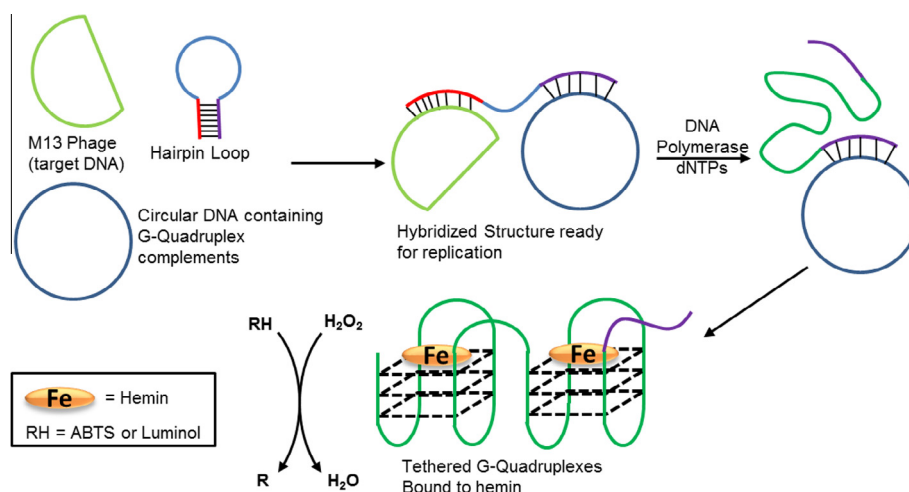


Fig. 18. Rolling circle amplification utilized by Willner and co-workers [56]. The rolling circle amplification only occurs when target is bound to the circular DNA. Once bound the DNA polymerase makes long tethered G-quadruplex structures, which can act as peroxidase mimics when bound to hemin.

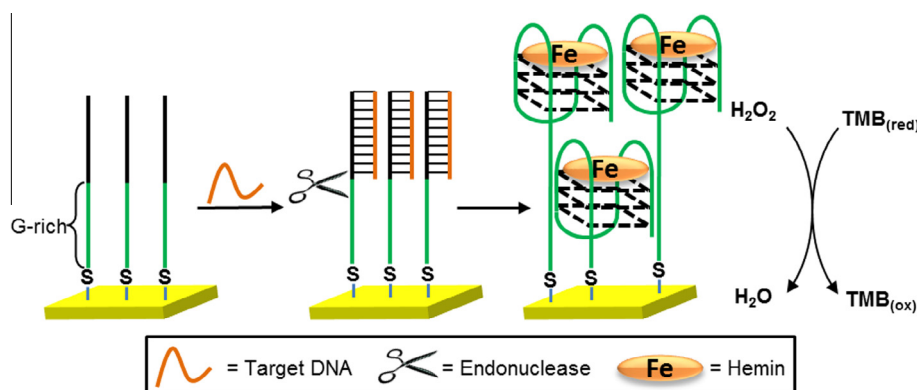


Fig. 19. Endonuclease amplified DNA detection using gold-immobilized DNA sensor [60].

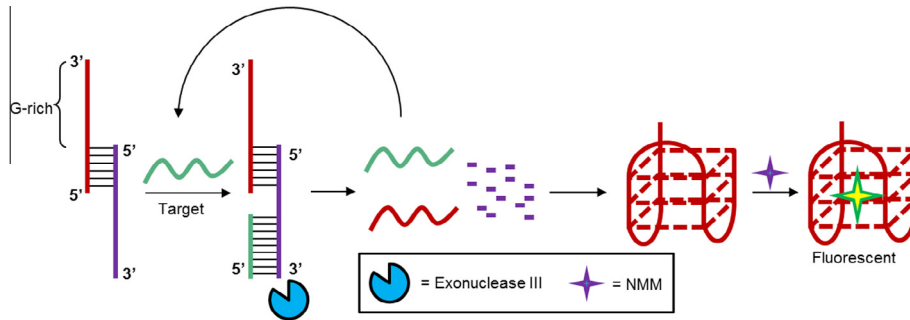


Fig. 20. Qu and co-workers method of DNA detection with exonuclease III amplification [61].

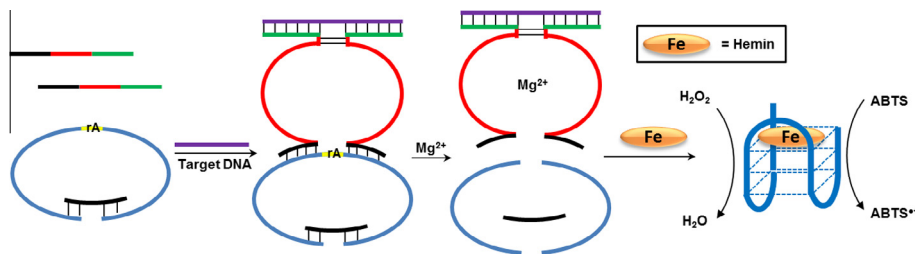


Fig. 21. DNAzyme cascade presented by Willner and co-workers [65].

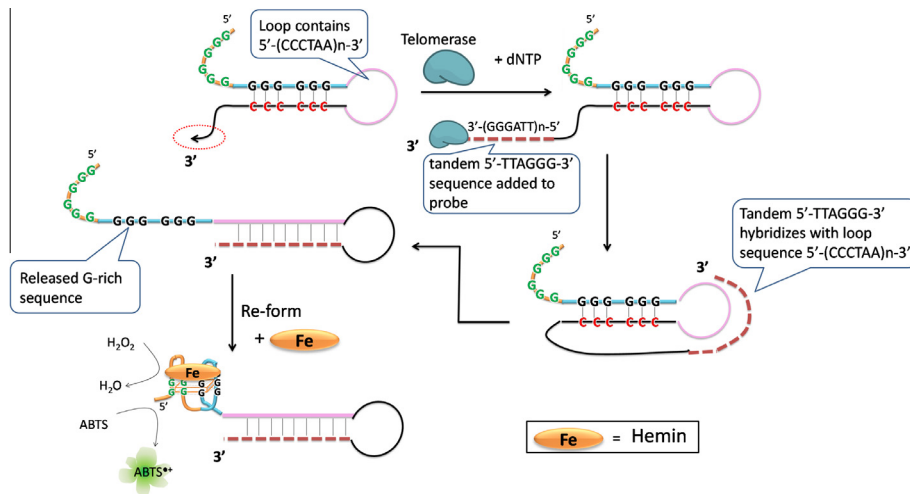


Fig. 22. Telomerase activity detection by MB-G-quadruplex probe [29]. (Adapted from Ref. [29] with permission. Copyright 2004, American Chemical Society.)

DNA next to an inserted rA nucleobase. This results in the release of the G-rich regions and as a result these sequences fold and carry out the oxidation of ABTS in the presence of hemin.

Others have used DNAzyme/enzyme cascades as well [66,67]. Tang and co-workers used DNAzyme/enzyme cascade for an amplified detection of microRNAs [67]. Tang's method also included a DNA-based nicking enzyme, which allowed the initiation of a complex enzyme cascade resulting in a detection method capable of detecting as little as 1 fM of target RNA.

4. Telomerase assays

Telomerase, which maintain telomere integrity, are important enzymes that are implicated in cancers so there are interests to develop sensitive assays to monitor telomerase activity. Because the telomere can form G-quadruplexes, others have developed various

G-quadruplex peroxidation-based assays to quantify telomerase function [29,68,69] (see Fig. 22 for a representative example). Willner used a G-quadruplex-MB probe to detect the activity of telomerase [29].

5. Detection of other analytes, apart from nucleic acids

The main focus of this review is the amplified detection of nucleic acids using G-quadruplexes. However G-quadruplexes have also been used to detect a variety of analytes, such as metals (K^+ , see Fig. 23, [70], Cu^{2+} [71], Ag^+ [72], Hg^{2+} [73], Pb^{2+} [74], Tb^{3+} [75]), proteins [76,77], and other metabolites [78], such as ATP [30].

Liang and co-workers detected thrombin with a limit of detection of 1.5 pM (Fig. 24) [78]. In this method the authors used three probes: (1) a G-quadruplex probe; (2) a blocker DNA and (3) a MB.

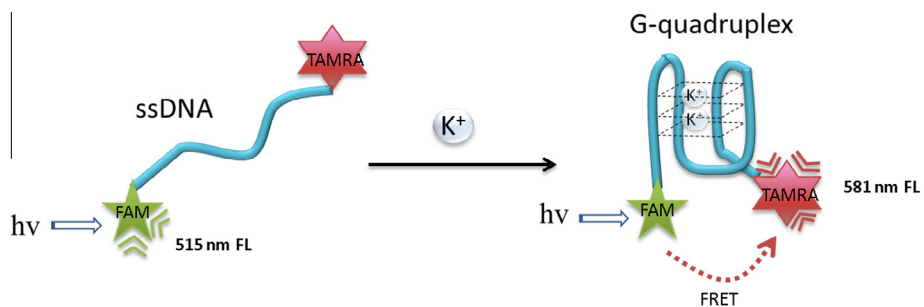


Fig. 23. Potassium detection by Takenaka and co-worker using FRET strategy. (Adapted from Ref. [70] with permission. Copyright 2002, American Chemical Society.)

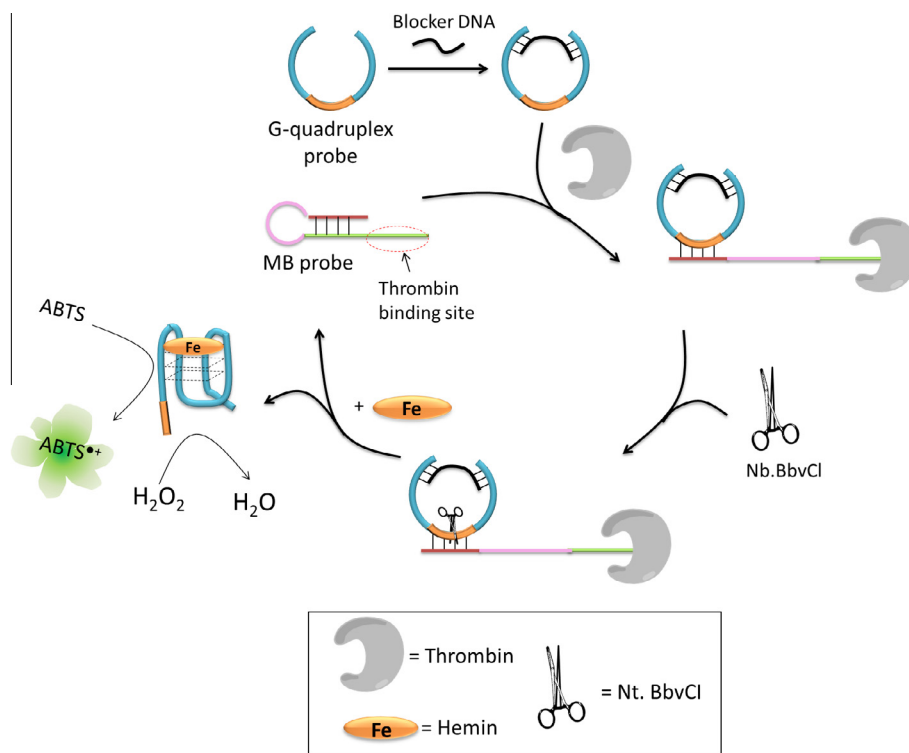


Fig. 24. Detection method utilizing released G-quadruplex DNAzyme after Nt.BbvCI cleavage reaction [78]. (Adapted from Ref. [78] with permission. Copyright 2013, American Chemical Society.)

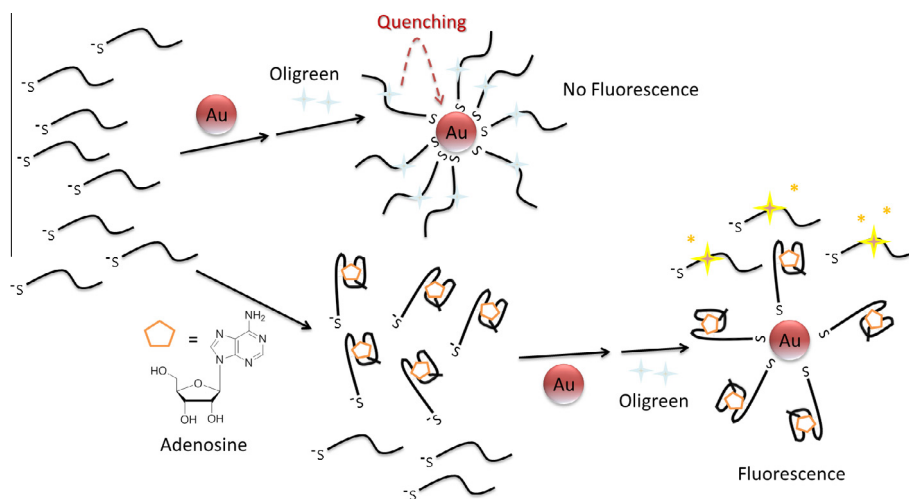


Fig. 25. Adenosine detection by G-quadruplex forming adenosine aptamer [79].

The G-quadruplex could associate with the blocker DNA to form a pseudo-circular DNA. In turn this complex could associate with the loop sequence of the MB, which would then associate with thrombin via a thrombin aptamer. After complete association, the Nt. BbvCI nicking enzyme cleaves the pseudo-circular DNA resulting in the release of two equivalents of active DNAzyme, which may then oxidize ABTS in the presence of hemin and H₂O₂.

Chang and co-workers investigated the use of a thiol-modified adenosine aptamer attached to a gold nanoparticle (Fig. 25) to detect adenosine in solution using oligo green fluorescent dye. Upon addition of adenosine, the adenosine aptamer binds adenosine and converts to a G-quadruplex. This G-quadruplex is more sterically demanding than the ssDNA aptamer alone. As a consequence, a larger concentration of ssDNA remains unassociated with the gold surface, which oligo green can then bind and in turn fluoresce.

If however, adenosine is absent the majority of the ssDNA aptamer associates with the gold surface. Although oligo green still binds this ssDNA its fluorescence is quenched by the gold nanoparticle resulting in no signal. This system allows for a 5.5 nM, turn on detection of adenosine [79].

6. Summary of substrates used for G-quadruplex based peroxidation

G-quadruplex peroxidases can oxidize a variety of substrates giving rise to several different colorimetric, fluorescent and luminescent signals. Table 2 summarizes the various substrates that have been used in various G-quadruplex-based analyte detection assays. Fig. 26, shows the structures of peroxidation substrates that have been used, as well as the structures of the oxidized products.

Table 2
List of peroxidation substrates that can be used in G-quadruplex peroxidation reactions.

| Abbreviation | Name | Detection method | pH of reaction | Absorbance of product (nm) | Emission of product (nm) | Color of product | References |
|----------------------|--|------------------|--|----------------------------|--------------------------|------------------|------------|
| DAB | 3,3'-Diaminobenzidine tetrahydrochloride | Colorimetric | 7.4 | 500 | – | Brown | [21] |
| ABTS | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid | Colorimetric | 7.9 | 415 | – | Green | [27] |
| TMB | 3,3',5,5'-Tetramethylbenzidine | Colorimetric | 7.5 Conc. H ₂ SO ₄ | 650 450 | – | Blue Yellow | [60] – |
| OPD | o-Phenylenediamine | Colorimetric | 5.0 | 450 | – | Yellow–orange | [80] |
| 4-CN | Chloronaphthol | Colorimetric | 7.2 | 400 | – | Blue–purSSple | [81] |
| Tyramine | 4-Hydroxyphenethylamine | Fluorescence | 8.5 | 320 | 410 | – | [82,83] |
| p-Cresol | 4-Hydroxytoluene | Fluorescence | 8.5 | 320 | 410 | – | [82] |
| Amplex red | 10-Acetyl-3,7-dihydroxyphenoxazine | Fluorescence | 8.5 | 570 | 588 | – | [82] |
| Tyrosol | 2-(4-Hydroxyphenyl)ethanol | Fluorescence | 8.5 | 320 | 410 | – | [82] |
| H ₂ DCFDA | 2',7'-Dichlorodihydrofluorescein diacetate | Fluorescence | 8.5 | 504 | 525 | – | [82] |
| Luminol | 5-Amino-2,3-dihydro-1,4-phthalazinedione | Luminescence | 9.0 | 460 | – | – | [22] |

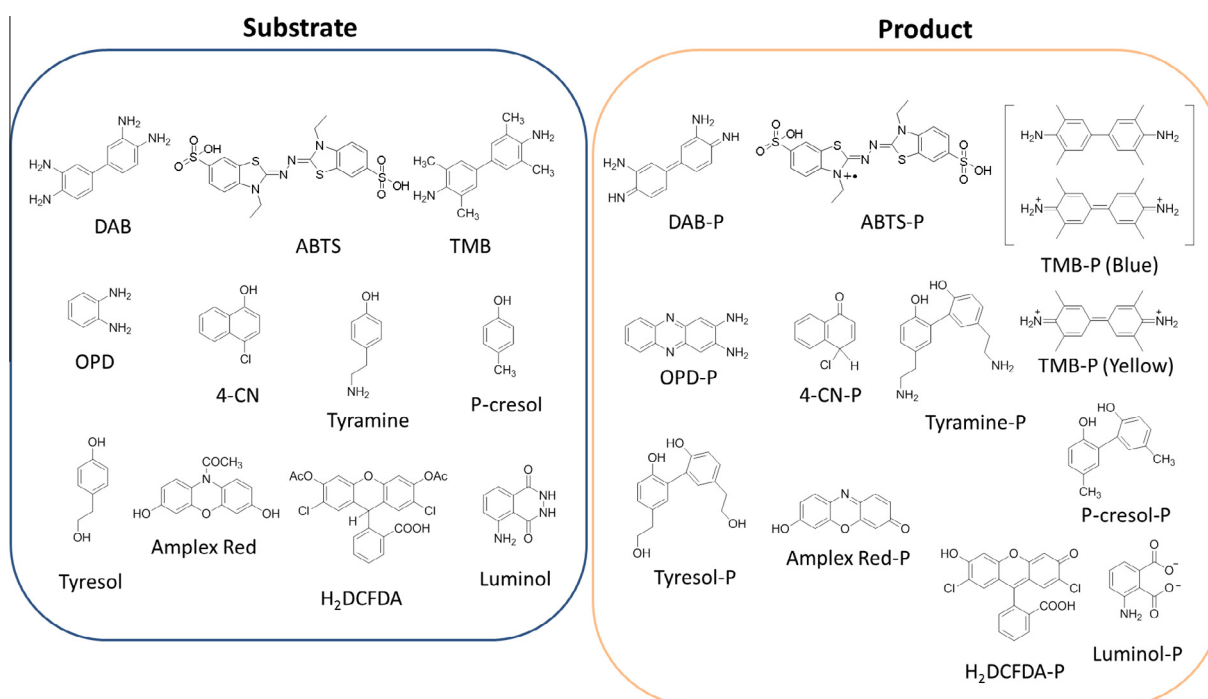


Fig. 26. Structure of reductants/peroxidation substrates used in the DNA peroxidation reactions and their oxidized products. P means product so for example tyramine-P means the product from tyramine oxidation.

7. Conclusion

Due to the importance of the G-quadruplex structure in cancer biology, there has been an enormous interest in studying G-quadruplexes as well as design small molecules that could be used to stabilize the G-quadruplex structure, with the hope that these molecules could become anti-cancer agents [84–111]. In the last decade or so, it has been shown that there is more to G-quadruplexes than just cancer studies and that they can also be used for sensitive detection of nucleic acids and other analytes. Despite the enormous progress that has been made in G-quadruplex-based detection protocols, there is still room for improvement. G-quadruplexes enhance hemin peroxidation but hemin on its own has some residual peroxidation activity so future studies that could help suppress the peroxidation of hemin, when not complexed to G-quadruplexes, could enhance the sensitivity of G-quadruplex-based peroxidations. Additionally the development of assay conditions [112–114] or G-quadruplex modifications [115] that enhance catalysis should allow sensitive detection of nucleic acids and other analytes. Recently a few reports have also shown that nucleotides as well as simple guanine based molecules can also catalyze peroxidation reactions [116–119]. The incorporation of these nucleotide-based peroxidations into methodologies that detect other analytes could be forthcoming.

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