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Review

Design and Applications of Split G-Quadruplex DNAzymes for Construction of Gated Biosensor

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Abstract

Split G-quadruplex DNAzymes offer unique opportunities for building gated biosensors with a wide range of applications. Splitting G4 DNAzymes involves separating guanine tracts in the G-quadruplex DNA sequence into two non-functional sequences that reconstitute into a functional G-quadruplex with peroxidase activity upon hybridisation of the aptamer probe region within the split system with the target molecule. Several studies have demonstrated the reassembly of split G4 DNAzymes and their applications in the detection of various analytes. This approach offers unique opportunities for modular biosensor construction, target-dependent activation, lack of requirement for labelling, amplification-free high sensitivity, and specificity over traditional G4 sensing. In this review, we explore the strategies of splitting G-quadruplex and their applications in biomedical diagnosis, environmental sensing, food safety monitoring, cell detection, and the integration of the technology with nanomaterials for enhanced stability and sensitivity. We considered the classical intermolecular split strategies that utilise binary probes and intramolecular split systems, which integrate the spacer DNA that allow for single probes as the model G4 sequence. Finally, we explore the current challenges required to develop split G-quadruplex DNAzymes into tools for routine practical applications.

Keywords: split G-quadruplex; biosensor; DNAzyme; DNA aptamer

1. Introduction

Biosensors are analytical devices that detect analytes using biorecognition elements and convert the signal into a measurable electrical output using a transducer and signal processor. The biorecognition element could be an enzyme, antibodies, DNA/RNA, whole cells/microbes or molecular imprinted polymer [1]. The sensing mechanism could involve direct binding, enzymatic reaction, signal amplification, competitive assay, and sandwich assay. Several commercial biosensors, such as glucose sensors, pregnancy test strips, and biosensors for food safety and environment monitoring have been developed. These have shown important features such as portability, selectivity, sensitivity, speed, and reusability. There are several emerging trends in biosensor development, such as lab-on-a-chip and microfluidics, wearable biosensors, nano biosensors, AI, and wireless communication, which integrate biosensors and DNAzyme–aptamer-based biosensors. These can be

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classified into electrical, optical, piezoelectric, and thermal biosensors based on the way they transduce their signal.

Traditionally, protein enzymes such as alkaline phosphatase, catalase, β-galactosidase, glucose oxidase, urease and horseradish peroxidase are commonly used as signal generating units due to their ability to drive the oxidation or hydrolysis of a substrate-induced electron transfer or colour generation that is proportional to the concentration of the target analyte [2]. The conjugation of these enzymes to antibodies (recognition elements) makes this process more expensive. The advent of aptamers has led to the discovery of several oligonucleotide probes as more viable alternatives to the traditional method due to their unique advantages, such as specificity, sensitivity, cost effectiveness (no need for expensive equipment), simplicity (reduced number of analytical steps), and chemical and thermal stability over antibody-based sensing [3,4]. Most recent systems have employed the turn-on approach to reduce false positives by ensuring catalytic activity is activated only upon target-induced reassembly [5,6]. The colorimetric method is often employed due to its simplicity and naked-eye visualisation [5,7,8]. However, fluorescence [9,10] and electrochemical [11,12] readouts improve quantitation. Meanwhile, dual-modal [13,14] and nanoparticle-based [15,16] strategies enhance robustness.

G-quadruplex DNAzymes are four-stranded catalytic DNA formed when G-quartet structures are stacked on one another. These structures arise from guanine rich single stranded oligonucleotides joined by Hoogsten hydrogen bonding in the presence of univalent metal ion ions [17,18]. When these structures are complexed with hemin (iron III protoporphyrin), they display peroxidase activity (Figure 1) [19]. G-quadruplex DNAzymes can utilise hydrogen peroxide to oxidise a wide range of substrates that can generate appropriate signals in the presence of substrates such as 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonicacid), tetra methyl benzidine, guaiacol, o-phenylenediamine, luminol, etc. This has made them useful in biosensor development and in other biotechnological applications.

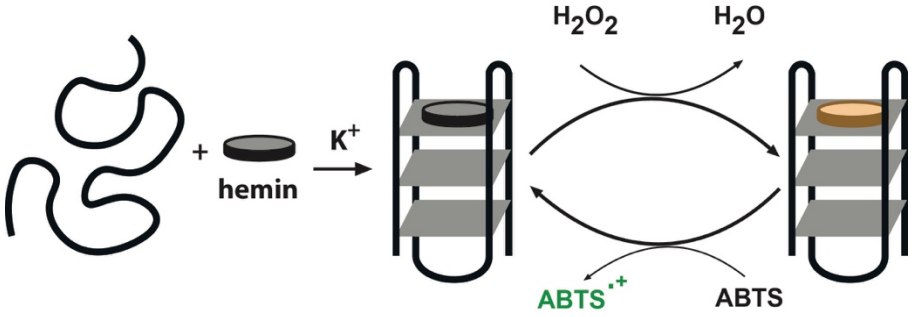

Figure 1. Formation of G-quadruplex-hemin DNAzyme and H_2O_2-activated oxidation of ABTS. Guanine-rich DNA oligonucleotides fold to form G-quartet interactions (grey planes) which bind to hemin cofactor (grey discs) in the presence of univalent metal ion (K^+) to form a catalytic complex. The resulting G-quadruplex DNAzyme activates H_2O_2 to oxidise a reductant donor substrate (ABTS). The formation of the reaction product ($ABTS^{\bullet}$) can be measured spectrophotometrically by monitoring absorbance at 415 nm.

Gating is the process of modulating the formation of a G-quadruplex structure and by extension the catalytic activity of the DNAzyme. Gating can be achieved structurally or functionally. In structural gating, the gate is closed by keeping the G4 forming sequence in an inactive conformation by splitting the guanine tracts into halves. The gate only opens when the target analyte hybridises with the aptamer sequence of the probe, this brings the split halves into proximity and corrects the spatial orientation needed that needs to be reassembled into a G-quadruplex structure with peroxidase activity (Figure 2).

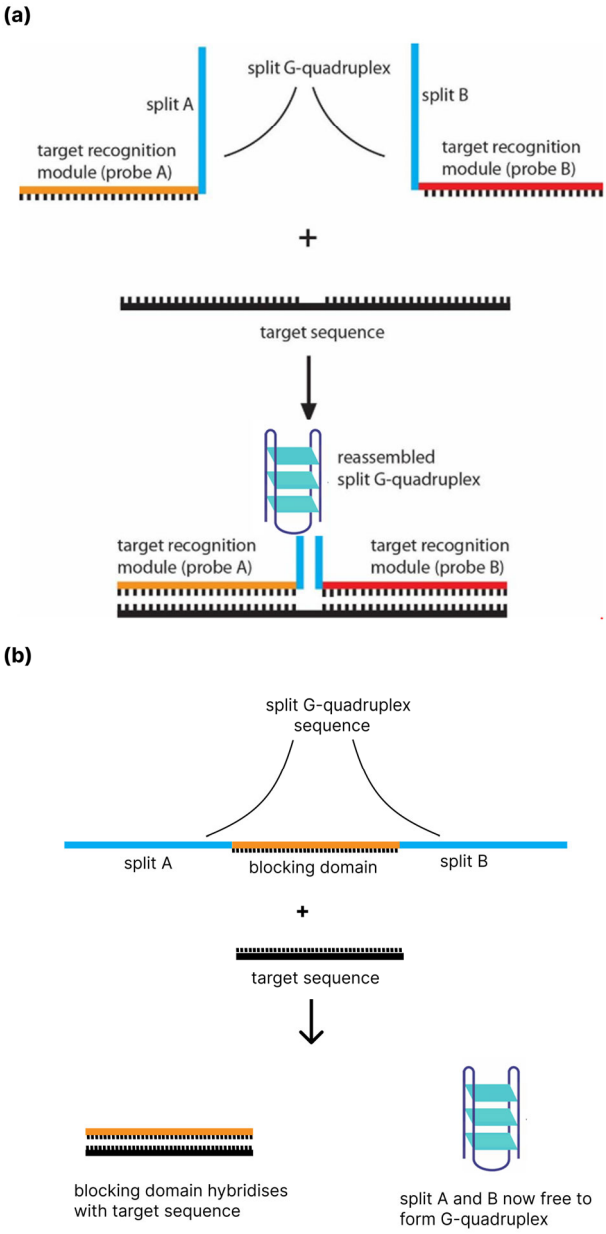

Figure 2. Design of G-quadruplex DNAzyme gating. (**a**) Structural gating. The system comprises two target recognition modules (probe A and probe B) each attached to one part of the split G-quadruplex DNA sequence. Hybridisation of probe arms A and B to their complementary target DNA brings the split parts into proximity to assemble the functional G-quadruplex DNAzyme. (**b**) Functional gating. The system consists of a single probe with a spacer or blocking sequence complementary to the target placed within the G4 DNAzyme sequence to prevent it from forming a functional G4 DNAzyme. The blocker is removed in the presence of a target; thus, a functional G4 DNAzyme that serves as a signal can be formed.

In functional gating, the G-rich region of the split halves is blocked by a complementary sequence that forms a Watson–Crick duplex structure, thereby preventing the split halves from forming a functional unit. The gate only becomes open in the presence of target analytes that bind to the blocking domain, thereby freeing G4 forming regions. Watson–Crick hybridization is often used for a nucleic acid (DNA/RNA) target based on a complementary sequence between the target and the recognition arm of the sensing

element [7,20], while aptamer recognition is often used for the detection of non-nucleic acid analytes/target based on multipoint interactions with its tertiary structure (Figure 3).

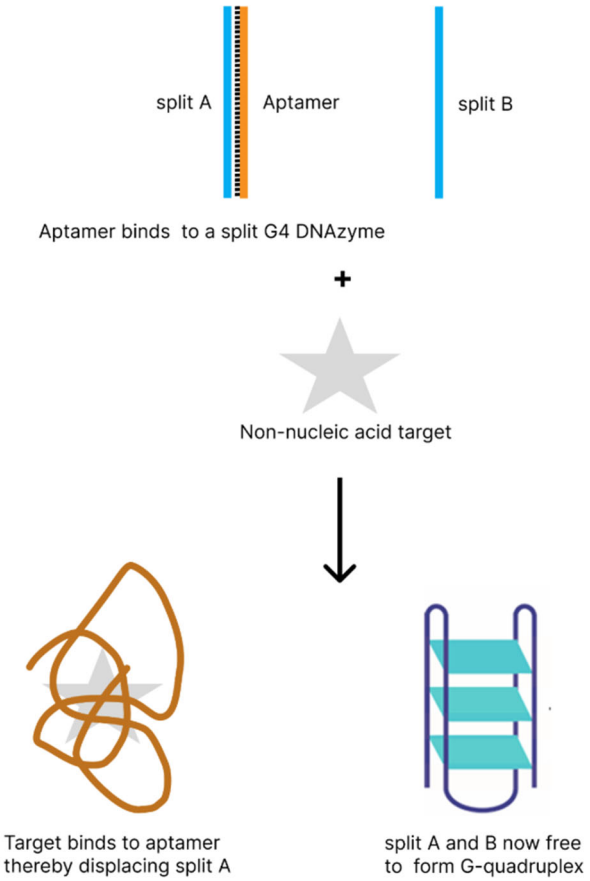

Figure 3. Principle of application of split G-quadruplex DNAzyme for the detection of non-nucleic acid targets such as protein and other small molecules. The target possesses a stronger binding affinity for the aptamer than the split, hence the split is dissociated and reassemble with the other half to form a G-quadruplex structure. This binding is due to multipoint interactions and represents a non-Watson–Crick interaction between the aptamer and the target.

Despite the availability of large amounts of data regarding high selectivity of split G4 DNAzyme based detection method, they are yet to reach commercial success due to background noise affecting the sensitivity of the system. Hence, it is imperative to understand the factors that interplay to attain high signal to background noise and to improve the sensitivity of the split G4 DNAzyme system. In this review, we aim to provide an overview of approaches for splitting G4 DNAzymes and highlight some applications in which the technology has been used. We discuss the strategies of splitting G-quadruplex and their applications in biomedical diagnosis, environmental sensing, food safety monitoring, logic gate system, and cell detection. We also explored the challenges associated with the reconstitution of an effective split system and designed approaches to address these issues.

2. Designing a Split G-Quadruplex DNAzyme

G-quadruplex (G4) formation requires four tracts of at least three consecutive guanines (G-tracts) to form stacked G-quartets. The guanines in a G-quartet are held together by Hoogsteen hydrogen, while the G-quartets are stacked coaxially to form a stable planar G-quadruplex through π–π interactions between the aromatic rings of the guanine bases. G4 stability relies on continuous π–π stacking between planar G-quartets, which are the

primary stabilizing force of the folded structure. (Figure 1). In non-split (intact) G4, the π–π stacking between G-quartets is stabilized by the sugar–phosphate backbone, which holds the guanines in a precise orientation. Splitting disrupts the continuity of stacked G-quartets (inter-quartet stacking).

Splitting G4 DNAzyme probe involves fragmenting guanine tracts in the oligonucleotide sequence flanked by target recognition sequence. Splitting helps to overcome the complex challenges associated with the use of intact G4 DNAzyme in biosensing. Splitting offers the following:

i. a simple detection system without the need for a label commonly used in the non-split/intact G4 i.e., no fluorophores/enzyme conjugation requirement;
ii. improved specificity and reduced background noise i.e., fragments/splits are inactive and only become activated after target-induced reassembly;
iii. modular and programmable design i.e., easy fusion with aptamer, nanomaterials;
iv. controllable activation;
v. separation of target binding domain (specificity) from signal production (catalytic function).

The split components are not functional parts and can only reconstitute into a G-quadruplex functional unit with peroxidase mimetic activity that generates a signal upon hybridisation of the aptamer probe region within the split system with the target molecule (Figure 2). Template-dependent hybridisation processes are often employed in a split system. Hybridisation of the target analyte to the aptamer recognition (sensing) site due to complementary binding brings the separated split G4 DNAzyme parts into proximity and induces their reconstitution [21]. Split components can also be kept apart using toehold sequences containing seven or less of the complementary Watson–Crick base pairs that block some regions of the split halves. However, in the presence of target molecules, the toehold block is relieved, and two split probes would reassemble into a G-quadruplex structure [22]. Split systems exhibit better selectivity, sensitivity and higher signal to background noise ratio than traditional G4 because of the resultant synergistic effect of the conformational restriction of the binary probes that can be activated only in the presence of a target analyte [23].

Split G4 DNAzyme can be classified as intermolecular and intramolecular. It is regarded as an intermolecular split if the full G-quadruplex-forming sequence is divided into two probes/fragments. If the split is caused by spacer/blocker introduced within the full sequence, resulting in only one probe, this is termed as an intramolecular split [24]. Intermolecular split G4 DNAzyme can be further classified into classical and non-classical based on whether the splitting occurs in the loop or within the G-tracts (Figure 4). The split point for classical intermolecular G4 DNAzyme is often in the loop region of the G4 sequence to give either 6:6, 3:9 or 9:3 (3:3, 1:3 or 3:1 of triads of G tracts) of 12 guanine bases. Classical intermolecular split G4 DNAzymes can be classified into symmetric and asymmetric designs. If the split G4 is designed to have the same number of GGG tracts in both probes, it is termed a symmetrical split (2:2 triads of guanine tracts). In asymmetrical splits, the two parts have an unequal number of GGG tracts (3:1 or 1:3 triads of guanine tracts) in both fragments (Figure 4).

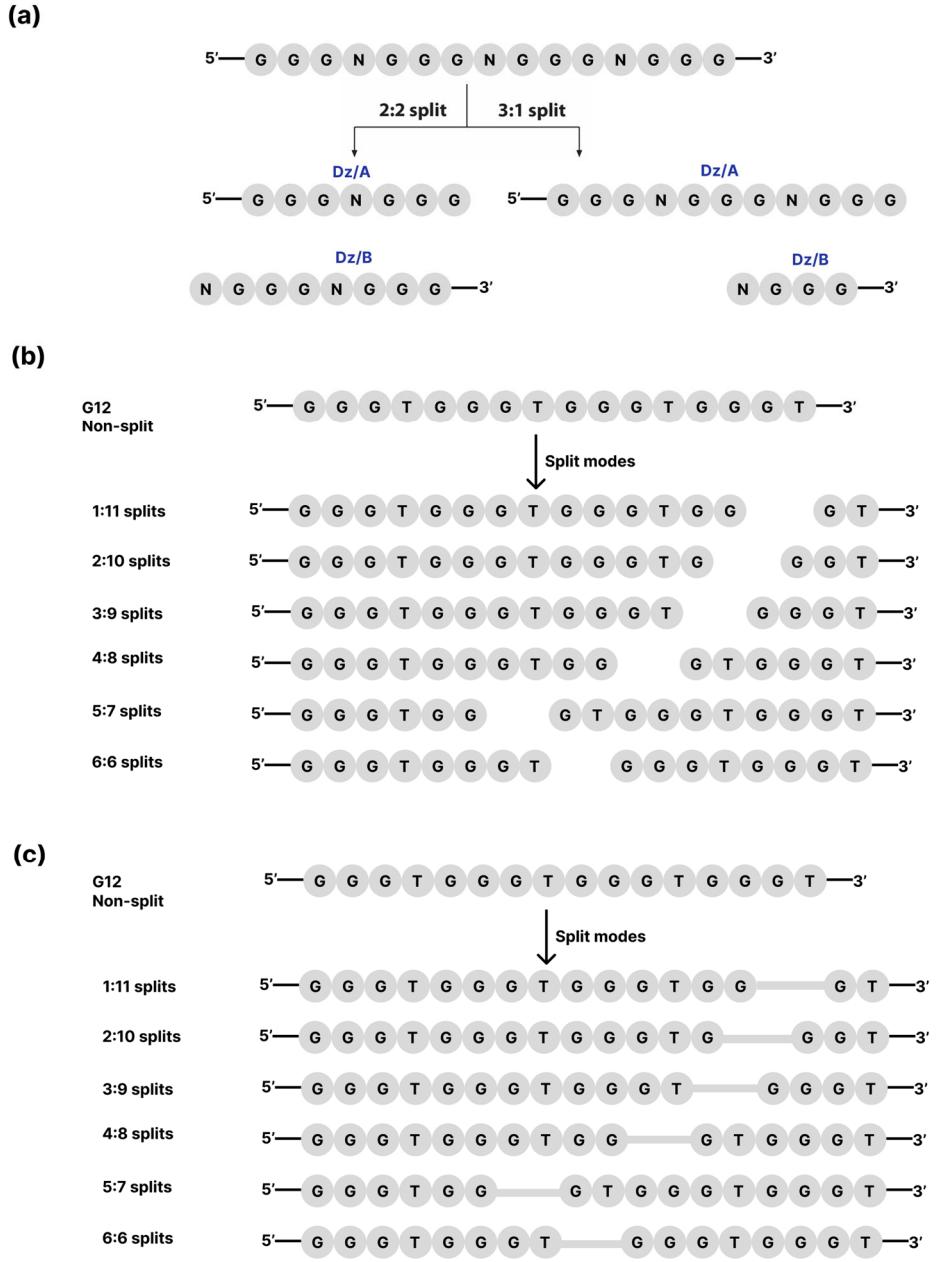

Figure 4. Various split modes of G-quadruplex DNAzyme (**a**). Intermolecular classical split involves splitting outside of the G-tracts, 2:2 mode represents symmetrical splits, while 3:1 mode is the asymmetrical split. N represents other nucleobases other than guanine (**b**). The intermolecular non-classical split involves splitting within the G-tracts (**c**). The intramolecular split utilises a single probe instead of a binary probe, with the split achieved by spacer sequence (—) corresponding to TTCTTT repeat.

Several factors decide which type of split is suitable for designing sensing devices. Deng et al. [7] and Ida et al. [23] reported better selectivity for the asymmetrical split. They have reported that the symmetrical split generates background noise (signal) in the absence of a target analyte because it is prone to self-reassembling to form a dimeric G-quadruplex. There are several reports on the better performance of asymmetrical splits in terms of lower background noise when compared with the symmetrical split [25]. Ren et al. [26] have reported that the symmetrical split of c-myc displayed better selectivity than in the

asymmetrical split mode. However, both studies agree regarding the better sensitivity of the asymmetrical split mode.

Although there are still unresolved controversies regarding which split pattern is preferable for the classical intermolecular split G4 DNAzyme, Osalaye et al. [27] have reported better performance for a 2:2 splits model for PS5.M, though this model was found to be undesirable in C-myc, Bcl2 and C-kit sequences due to higher risk of self-dimerisation or G4 formation, especially at high concentration and even in the absence of target analyte. The performance differences between specific split G-quadruplex architecture are due to how the split position influences G-tract continuity, loop geometry, π–π stacking, ion coordination, and the structural pre-organization required for efficient folding and function (e.g., hemin binding in G4-DNAzymes).

Hence, it is safe to say that the most efficient splits model is dependent on the type of DNAzyme sequence used as partition between the G-tracts that prevent exposure of the terminal tetrad in the split, loop and flank composition around the G-tracts, which could also influence reassembly and hemin access to the 3′ end of the splits. Moreso, optimum performance is also dependent on the target analyte. Zhu et al. [20] have reported better sensitivity and signal-to-noise ratio for non-classical splits of G4 DNAzyme (splits within guanine bases core) (Figure 4b) than for classical intermolecular splits of G4 DNAzyme (splits within the loop region of the G-tracts). These studies suggest that the preferred splitting symmetry is dependent on the sequence of the G4 used, the flanking aptamer sequence, type of metal ion, buffer salts, and other factors.

Non-classical intermolecular splits partition G-tracts within a G-tract core i.e., between G2–G3 in a GGGTGGGTGGGTGGGT sequence to give 2:10 splits or between G4-G5 to give 4:8 splits (Figure 4b). This severely destabilizes the core (reduces their thermodynamics), suppresses spontaneous folding and would require the de novo synthesis of the G-quartet before it can undergo restacking. This is in contrast to classical splits between G-tract boundaries, i.e., between A or T in a GGGATTGGG sequence, which are well tolerated, especially those within the linkers and loops, because the integrity of the G-quartets is preserved and because their reassembly simply entails stacking the pre-organised quartets rather than reconstructing them de novo.

Zhu et al. [20] explored six different split modes for non-classical intermolecular splits and concluded that the 4:8 split mode yielded the highest signal-to-background ratio in fluorescence assays. This split mode was found to minimize background fluorescence due to the specific interactions of the G-rich segments, which enhances the sensitivity of the assay. In their work, the G4 sequence was split in the G-region instead of the loop. In this approach, the G4 sequence was split using individual guanines into 1:11, 2:10, 3:9, 4:8, 5:7, and 6:6 rather than splits based on G triads (Figure 4c). Their approach led to effective splitting, as confirmed by circular dichroism characterisation, a lower background noise and higher fluorescent signal when compared with the traditional/classical splitting patterns using G triads reported by Deng et al. [7] and Nakayama and Sintim [25].

Zhu et al. [20] revealed that G7, G8 and G9 have lower fluorescence, showing weak binding to protoporphyrin IX under different experimental conditions in their split form. In addition, it was found that better results are obtained when splitting is from the 3′ end due to the low background noise of G8. Moreso, splitting affects the cation's binding environment by altering the local electrostatic landscape required for potassium and/or sodium/ammonium ion(s) coordination between the g-quartet. While loop-based splits maintain intact cation-binding channels within each fragment, splits within the G-tracts do not, thereby making it difficult for fragments to reassociate through cation binding and Hoogsteen hydrogen bonding. The closeness of the split position within a G-tract to the coordination site could allow water molecules to infiltrate into the core and hydrate the

cation, consequently weakening the electrostatic attraction that holds the G-quartet together.

Because flanking the target recognition site adjacent to the split G4 could also affect the success of the split symmetry, it is noteworthy that this splitting symmetry, recommended by Zhu et al. [20], is based on only one model target. Hence, further work would be required in testing several other recognition sites, G4 sequences and targets to check if this conclusion is universal.

The sensitivity of an assembled G-quadruplex is influenced by various parameters, such as the thermodynamic stabilities of the sequence and the distinct architectural features of the split G4, which consequently alters topology, hemin binding, stability and catalytic efficiency of the split G4 DNAzyme upon reconstitution. It is important to understand and verify the role of individual nucleobases using structural (CD, NMR), computational and systematic mutagenesis studies with single base substitution/swap and determining how they affect the performance of the split system. Analyte recognition sequence flanking of the split G4 sequence and reaction conditions should be undertaken to carefully design a successful split system that does not perturb the stability and affinity of the binding sites.

Fragmentation at an unfavourable position might prevent the split components from reconstitution into a functional three-dimensional unit [28]. Secondary structure prediction (i.e., mfold and NUPACK) could be used to model folding of the flanking regions and target binding, with this process being able to be validated by experimental procedures such UV–vis analysis, CD, native PAGE, SPR, ITC and FRET. Reaction conditions, such **as** cation screening (K^+, Na^+, NH_4^+, mixed cations i.e., NH_4^+ substituted for Na^+), pH and buffer studies, hemin titration/binding kinetics and readout methods, could be optimised and standardised.

Lv et al. [24] have reported an **intramolecular split G4 DNAzyme (intra SG),** which is quite different from the intermolecular split G4 previously discussed. Intra SG has short non-guanine DNA spacers inserted into intact G4 strand. This consequently divides the G4 sequence into two modules (Figure 4c). This design simplifies split G4 DNAzyme binary probes into a single probe. In this model, the G4 sequence is divided into two modules and linked using DNA spacer bases. This offers the opportunity of simplifying split G4 DNAzyme binary probes into a single probe. In this study, eleven types of intra SG split modes (1:11, 2:10, 3:9, 4:8, 5:7, 6:6, 7:5, 8:4, 9:3, 10:2 and 11:1) were generated from T30695 sequence (GGGTGGGTGGGTGGGT) rather than the split types 1:3, 2:2 and 3:1 or 1:11, 2:10, 3:9, 4:8, 5:7, 6:6, 7:5 usually obtained in the classical intermolecular SG. In addition, intra SG from 8:4 inter G4 was tested, as it was the best performing split mode previously reported by Zhu et al. [20]. Spacer DNA of varying lengths corresponding to 0, 2, 5, 10, 20, 30, and 50 consecutive thymine bases were inserted at the split site of the T30695 parent sequence. Circular dichroism was used to determine the G4 structure of the various split [24,29,30] modes, and constructs with spacer T = 5, 10, 20, 30 and 50 displaying positive peaks at 264 nm, showing that they form the functional parallel G4 structures capable of binding NMM porphyrin. The authors reported that splits with space T20, T30 and T50 gave the highest fluorescence. The study also found that, in consistency with the superior performance of inter SG modes 4:8 and 5:7 over 3:9 and 6:6 inter SG, intra SG 2:10, 4:8 and 5:7 displayed superior sensing performance.

Space length within the intramolecular splits G4 could significantly affects folding kinetics and reassembly by controlling effective concentration, conformation and orientation of guanine tracts. A short spacer (2–5 nt) may sterically hinder reassembly as there would not be flexibility to allow the G-tracts to rotate into the right orientation. Spacers of 10–20 nt often yield the fastest reassembly and highest activity due to their ability to maintain effective molarity between complementary fragments, which facilitates the bringing

of G-tracts into close proximity, as well as facilitating cation coordination and π–π stacking. The intramolecular split not only helps to reduce background noise when compared with intermolecular splits but also adds additional advantages in terms of allosteric control and higher target specificity. However, intermolecular splitting offers simplicity in design and synthesis [24,29,30].

Nakayama and Sintim [25] describe the features of the probe architecture and reaction needed for the reassembling of the split DNAzyme into the G-quadruplex structure. They proposed the following: i. Replacement of the loop that connects the G3-tract in the G4 structure with a stem loop motif, which will endow the split probes with the regions of limited complementarity needed for effective reconstitution; ii. The design of the DNA motifs adjacent to the G4 structure can influence the stability and peroxidase activity of the reconstituted split system; iii. The type of monovalent cation that is in excess can greatly affect the turnover of G4 DNAzyme. Decomposition of the G4Dz is reported to be faster in sodium and potassium ion but slower in ammonium ions; iv. The DNA spacers connecting the split G4 region and the aptamer recognition sequence can have impact on the success of the split strategy.

Connelly et al. [31] have reported that the primary and/or secondary structure of the target could significantly affect the performance of the probe. They also reported unintended interactions between the target and its recognition sites, especially if the target is rich in cytosine. This and many other factors should be considered when designing split G4 DNAzyme. Typically, optimisation studies are required to characterise the various factors that could affect the performance of the split G4 DNAzyme before designing for specific applications.

3. Applications of Split G-Quadruplex DNAzyme in Biosensors Development

Split G4 DNAzyme has gained applications in medicine, environmental sensing, food safety monitoring and cell detection. In this section, we briefly review the principles behind selected applications and highlight examples of systems in which they have been used.

3.1. Nucleic Acid Detection

DNA detection is of great significance due to its applications in molecular diagnostics, forensic and human genetics. Polymerase chain reaction (PCR) is used as the standard method for nucleic acid detection. However, this method requires the use of sophisticated equipment and signal detection using fluorescent probes or DNA-binding dyes. The utilisation of expensive and bulky equipment hinders its application in point of care (POC) treatment, especially in resource-limited settings. **Single nucleotide polymorphism** (SNP) is the most common type of genetic variation in the human genome. Large-scale analysis of SNP is often carried out for genetic risk assessment within a population upon detection of mutation. This process is laborious and time consuming; hence, an inexpensive, simple and fast diagnostic strategy is desirable.

Kolpashchikov et al. [32] were the first to report the splitting of G4 DNAzyme and this has since been applied to the fluorescent detection of single nucleotide polymorphism (SNP) in Haplotype H1c carrying SNP rs242557, which substitutes G to A at microtubule-associated protein tau, which in turn is commonly associated with Alzheimer's disease. They designed a symmetrical split G4 DNAzyme that utilises a probe aptamer that can form a duplex in the presence of the target sequence, thereby making the split components reassemble. The analyte/target binding arms were added to each half of the split G4Dz and were tailor made to be complementary to the rs242557-G sequence target. This

aptamer is highly sensitive to single nucleotide substitution and would not hybridise to the target sequence containing SNP. A fluorescent signal is only generated in the presence of the full complementary target sequence.

Deng et al. [7] were the first to utilise an asymmetrical (unequal) 3:1 split mode for the colorimetric detection of SNP. Probe split A contained **three GGG** repeats (9 G's) and its 5′ end matches the 3′ end of the target DNA, while probe B contained **one GGG** repeat (3 G's) and its 3′ end matched with the 5′ end of the target sequence. The addition of target DNA to the mixture of the split probes A and B causes the target recognition site in the probe to hybridise with target DNA to form a double stranded DNA. This process brings the two split components into proximity to form a G4 structure capable of exhibiting peroxidase activity. They were able to detect mutation with a single mismatched nucleotide and demonstrated a detection limit in the nanomolar range.

Ren et al. [26] have reported a split G4 fluorescent method without the need for H_2O_2, hemin, and ABTS for the detection of **target DNA in biological fluids**. Their method was found to be able to discriminate mismatch perfectly using a N-methylmesoporphyrin IX fluorescent probe for the turn-on (enhanced fluorescence) approach and tetrakis (diisopropylguanidino)-zinc-phthalocyanine for the turn-off (diminished fluorescence) approach. The design detected target DNA with a limit within a nanomolar range.

Nakatsuka et al. [33] reported split G4 DNAzyme DNA nano-tweezers (NTs) for the colorimetric detection of **norovirus** mRNA using ABTS as the chromogenic substrate, with a detection limit of 4 nM (Table 1). The split DNAzyme-NT changes from an open to a closed conformation upon binding the target, thereby shortening the distance between the separated split domains and making them able to reconstitute to form a G-quadruplex structure with peroxidase activity. It was proposed that linker length ($T > 0$) is essential for the system to be able to detect NV-RNA, as DNA-NT with $T = 0$ would not be able adjust the distance between the split DNAzyme needed to restore G4 formation/peroxidase activity. They also reported that it is important for the tweezer to be in the open state to effectively separate the split G4 DNAzyme. Wu et al. [34] also described a let-7a miRNA detection that can discriminate single base difference among the miRNA family using split G4 DNAzyme. They obtained a linear range of 10 fM–10 nM and a detection of limit of 7.4 fM.

Guo et al. [35] developed a label-free electrochemical sensor based on split G4 DNAzyme coupled with a DNA nano-tweezer (DNT) for the detection of nucleic acid. In the presence of the target, the two hairpin structures in the DNT hybridise, making the opened DNT close and thereby facilitating the reconstitution of the split G4 DNAzyme. The hairpin structures were designed to prevent a false positive signal and improve the sensitivity of the system. In their approach, the guanine-rich sequence was positioned on the outside rather than the inside to improve the signal and to reduce background noise. This method achieved a detection limit of 22 aM and a linear range of 1 fM to 1 nM for the detection of circulating tumour DNA. Zhu et al. [36] developed a nanopore multiplex method of detecting the nucleic acid of SARS-CoV2 and various serotypes of influenza A simultaneously using glass nanopores (14 nm), five identical asymmetrical split (1:3) G4 DNAzyme probes, and DNA carriers with multiple binding locations to differentiate signals from different targets.

Table 1. Biosensing applications of G-quadruplex DNAzymes for the detection of nucleic acids, proteins, and diagnosis of diseases.

Target Analyte	Method	Linear Range	LOD	Signal	Reference
Norovirus mRNA	Dimer G4	-	10 nM	Colorimetric	[37]
	S. Split G4-DNT	-	4 nM	Colorimetric	[33]
miRNA	G4	0.1–1 μM	4.5 nM	Colorimetric	[38]
	G4	1 pM to 100 nM	1 fM	Colorimetric	[10]
	G4 MB	1 pM to 5 nM	1 pM	Colorimetric	[39]
	A. Split G4	10 fM–10 nM	7.4 fM	Colorimetric	[40]
	S. Split G4	2–10 nM	0.43 nM	Colorimetric	[30]
	I. Split G4	0–500 fM	10.36 fM	Fluorescent	[14]
	S. Split G4	10 fM–10 nM	2.43 fM	Colorimetric	[41]
Tumour DNA	S. Split G4-DNT	1 fM to 1 nM	22 aM	Electrochemical	[35]
p53 DNA	G4 + A	0.25 fM–2.5 nM	25 fM	Colorimetric	[42]
	S. Split G4	5 pM to 5 nM	3.8 pM	Colorimetric	[32]
Hepatitis-B virus	G4	10 pM–10 nM	0.5 pM	Electrochemical	[11]
	S. Split G4	0 to 200 nM	5 nM	Fluorescent	[26]
	G4 + NP	0.3–1000 pg/mL	0.19 pg/mL	Electrochemical	[21]
Chlamydia trachomatis	S. Split G4	100 nM to 1μM	100 nM	Electrochemical	[12]
Staphylococcus aureus	S. Split G4	50 to 10^6 CFU	23 CFU/mL	Colorimetric	[6]
Salmonella	S. Split G4	5.2×10^1–5.2×10^6 CFU	4.33 CFU/mL	Colorimetric	[43]
Helicobacter pylori	S. Split G4	0–10^5 CFU	0.105 CFU/mL	Colorimetric	[44]
Cronobacter sakazakii	Split G4	2–1200 CFU/mL	1.2 CFU/mL	Colorimetric	[38]
Dengue virus	N. Split G4	3.125 nM–6.4 μM	5.1 nM	Colorimetric	[23]
Thrombin	Split G4 + NP	0.5 to 20 nM	0.5 nM	Colorimetric	[45]
T4 polynucleotide kinase	G4	-	0.2 U/mL	Fluorescent	[46]
T4 DNA ligase	S. Split G4	0–2.5 U/mL	0.012 U/mL	Colorimetric	[47]
Carcinoembroyonic antigen	G4	1 ng/mL to 30 ng/mL	0.961 ng	Fluorescent	[48]
	S. Split G4	1 to 50 ng	1 ng/mL	Colorimetric and Fluorescent	[49]

G4 = G-quadruplex; MB = molecular beacon; CFU = colony-forming unit; DNT = DNA nano-tweezer; S. split = symmetrical split; A. split = asymmetrical split, N. split = non-classical intermolecular split (splitting with the G-tract); I. split = intramolecular split (G-quadruplex-forming ability is initially blocked or partitioned within a larger structure—not as a two-part intermolecular split.); C = colorimetric; F = fluorescent; E = electrochemical; L = luminescent.

Micro RNAs (miRNAs) are small non-coding sequences, they play an important role in post translational modification gene changes; hence they are significant biomarkers of many disease conditions because of their unique properties, exceptional specificity and remarkable stability in blood and body fluids. Asadollahi et al. [14,30] designed a split G4 DNAzyme system for the turn-off colorimetric detection of miRNA-21 and miRNA-155, commonly used in the early diagnosis of breast cancer with a linear range of 2–10 nM and detection limits of 0.43, 0.54 and 0.63 nM in buffer, blood and urine samples, respectively. In this design, the matching length must be short to ensure the system is sensitive to low target concentration. The authors utilised a competitive sequence (blocker) to identify

SNP when the matching length is not short. Their method was able to detect a signal when the mutation formed only 5% of the total target DNA. Ma et al. [14] have reported split G4 DNAzyme for the fluorescent detection of miRNA-21 and from there for the detection of acute kidney injury, with a linear range of 0–500 fM and a limit of detection of 10.36 fM (Table 1).

3.2. Diagnostic Tools

Ren et al. [26] described a symmetrical split c-myc promoter (a G4Dz) for the detection of the **hepatitis-B virus** (HBV). Their design made the region-flanking split G4 to be complementary to the target HBV target. The split probes could not assemble into the functional G4 in the absence of the target. Once the target hybridises to the complementary regions on the split probes, it brings the probe fragments into proximity, allowing them to reassemble and form a G-quadruplex (G4) structure. They utilised N-methyl mesoporphyrin IX as a fluorescent probe based on its ability to bind G4 and release fluorescence proportional to the HBV target concentration.

Swine fever virus (SFV) is a highly contagious disease that affects pigs and wild boar, leading to economic losses. A virus neutralisation test is often use for detecting this disease. However, this method is laborious and time-consuming, rendering it unsuitable for testing a large number of samples. In addition, the method does not differentiate between the field and vaccine strain of this disease. Lu et al. [50] reported a combination of split G4 DNAzyme and an amplification technique for the colorimetric detection of SFV. The probe contains an aptamer for the SFV target recognition RNA sequence, which makes the split halves reassemble once the 3′ end of the target hybridises with the 5′ end of the probe, in turn allowing for the formation of the G-quadruplex structure, which, upon binding to the hemin, catalyses the colorimetric oxidation of ABTS using H_2O_2. Their system was found to be 100 times better than RT-PCR and has a detection limit of 10 copies/mL of viral RNA.

Lee et al. [12] designed a novel split G4 DNAzyme electrochemical sensor capable of exhibiting peroxidase activity in the presence of the target DNA of sexually transmitted disease (STD) pathogens due to the precipitation of 4-chloronapthol on the electrode surface. This method offers a miniature, portable, user friendly, fast, and less expensive alternative. They utilised two split DNA probes each containing the G4 DNAzyme sequence and a complementary DNA target sequence. The first split has a G4 DNAzyme sequence at the 3′ end while the second split has a G4 DNAzyme sequence at the 5′ end. After the target binding, the split probes form a G-quadruplex structure that binds hemin and utilises H_2O_2 to precipitate 4-chloronapthol on the electrode surface. The electron transfer resistance or increase in impedance signal due to this precipitation was used to identify and quantify the concentration of the target DNA. A Nyquist plot for the electrode with and without samples and a Faradaic impedance plot of the sample with the target DNA-induced precipitation were used to determine the feasibility of this system. They used the system to detect pathogenic STDs such as *Trichomonas vaginalis*, *Herpes simplex* virus and *Chlamydia trachomatis* from infected patients, with a linear relationship in the range of 100 nM to 1μM.

Ida et al. [23] reported an asymmetrical split G4Dz capable of distinguishing various serotypes of **dengue virus** and those of **zika virus** and **yellow fever virus,** despite very close sequence similarities. They used a 4:8 split mode for serotype I, while a 10:2 split mode was used for serotypes II–IV. The target-binding arm was joined to each of the split G4 DNAzyme sequences. The split parts are brought together when the target dengue virus DNA hybridises with the aptamer target recognition site in the probes, thus allowing the formation of G4, which, when complexed with hemin, oxidises ABTS to produce

a proportional colour signal. The obtained detection limits of 8.8 nM, 4.9 nM, 9.3 nM and 5.1 nM are for serotypes I, II, III, and IV, respectively.

Exosomal surface proteins are commonly found in patients with breast cancer and they have clinical importance in diagnosis. Common detection techniques for breast cancer require complex, expensive and laborious procedures. Cheng et al. [13] reported asymmetrical split G4 DNAzyme for the electrochemical and colorimetric detection of breast cancer exosomes. They utilised aptamers not bound by exosomes to induce G4 formation by complementary binding. Their system could distinguish between nonmetastatic, metastatic breast cancer and healthy individuals. They obtained a detection range of 1.0×10^3 to 1.0×10^7 particles/μL for colorimetric detection and 1.0×10^3 to 1.0×10^8 particles/μL for electrochemical detection.

A carcinoembryonic antigen (CEA) is a type of cell surface glycoprotein used as a biomarker for tumours. Shahbazi et al. [49] have reported a split G4 DNAzyme and aptamer probe for the detection of CEA in saliva. Complementary CEA aptamer sequences are situated at the flanks of both of the split G4 DNAzyme halves. In the absence of the target, the aptamer sequences hybridise to form a duplex structure that keeps the split G4 fragments apart, thereby preventing them from forming functional G4 structures. When present, the target CEA causes the split fragments to hybridise with the aptamer recognition sequence and reconstitute the active G4 DNAzyme structure. Their method detected CEA with a range from 1 to 50 ng/mL and a limit of detection of 1 ng/mL (Table 1).

Zhang et al. [18] integrated split G-quadruplex structures with CRISPR-Cas systems, enabling a signal-on colorimetric detection method for pathogens like the monkeypox virus and respiratory syncytial virus. They report that this approach allows for the detection of as little as one copy of target nucleic acid per test, achieving sensitivity akin to PCR results in clinical samples.

3.3. Food Safety Monitoring

Food derived from genetically modified organisms (**GMO**s) are becoming common and acceptable in several countries. However, there remain concerns about ethical, environmental and biosafety issues. DNA analyses are used to ensure compliance with the allowable modified genes in food. Though polymerase chain reaction (PCR) is the gold standard for the detection of GMOs, it is laborious, time consuming, and requires expert personnel and use of expensive equipment. Hence, the development of a rapid, cheap and simple analytical method for detecting GMOs is highly desirable. Qiu et al. [9] have reported a split G4 DNAzyme for the detection of GMOs in food. The hybridisation of the GMO target with the recognition site on the probes allows the split G4 halves to be brought into proximity, in turn allowing their reconstitution into the G4 structure and the oxidisation of 2′,7′-dichlorodihydrofluorescein diacetate into a fluorescent signal upon complexation with hemin, using H_2O_2 as the oxidant. The study reported a linear detection range of 50 nM to 50 μM and a detection limit of 7 nM (Table 2).

Aflaxotin B1 (AFB1) is a mycotoxin that is a secondary metabolite of mould and poses a serious public health concern to humans and animals. Hence, a method for the rapid and sensitive detection of AFB1 is desirable for food safety and quality control. Seok et al. [51] have reported a split G4 DNAzyme and DNA aptamer for the turnoff colorimetric detection of target AFB1, the complementary binding of the aptamer to the target leads to the structural deformation of the aptamer–G4 DNAzyme complex and results in the splitting of the G4 DNAzyme into fragments, consequently leading to a reduction in peroxidase activity. This approach detected AFB1 target in the linear range of $0.1–1.0 \times 10^4$ ng/mL, with a detection limit of 0.1 ng/mL. This system is highly specific only for AFB1 and could distinguish it from other mycotoxins.

Traditional methods of detecting **antibiotics,** such as HPLC, GC-MS, capillary electrophoresis, ELISA, electrochemical methods, etc. are laborious, time consuming, and require the use of expensive equipment and trained personnel. Cui et al. {53] reported an asymmetrical split G4 DNAzyme integrated with signal amplification using dual nicking enzymes for the visual colorimetric detection of kanamycin. The split G4 DNAzyme fragments are blocked by being caged in hairpin structures, thereby preventing them from reassembling into G4 structures in the absence of the target antibiotic. In the presence of a target antibiotic, the restriction endonuclease Nt.AlwI cleaves the hairpin probe, thereby releasing the split halves and allowing them to form G4 structures. The peroxidase activity of the reassembled complex oxidises ABTS to generate a colour signal for the visual detection of antibiotics from food samples with the naked eye. The system is highly sensitive and specific for kanamycin, with a detection limit of 14.7 pM and linear range of 50 pM to 500 nM.

Zhang et al. [52] have reported a split G4 DNAzyme integrated with enzyme amplification for the label-free electrochemical detection of **kanamycin**. This approach is similar to the method used by Cui et al. [53], which blocks the split parts of the G4 sequence into different hairpin probes. They reported a linear range of 100 fM to 1 nm and a detection limit of 83 fM. Wu et al. [34] reported the turnoff visual detection of chloramphenicol in foods based on a split G4 DNAzyme and aptamer. The split probes both contain a G4 sequence and regions complementary to the chloramphenicol aptamer. When the target is absent, the aptamer sequence hybridises with the complementary regions of the split G4, causing the split G4 halves to reconstitute and form functional G4 structures capable of oxidising ABTS to generate green colour visible to the naked eye. On the contrary, in the presence of the target, it preferentially binds to the aptamer, thereby making the split G4 sequence drift apart, collapsing the G4 structure and quenching the colour signal. The system detected chloramphenicol with linear range of 10 to 200 nM and detection limit of 87.3 pM.

Table 2. Biosensing applications of G-quadruplex DNAzymes in food safety, environmental pollutant and drug detection.

Target Analyte	Method	Linear Range	LOD	Signal	References
GMOs	S. Split G4	50 nM to 50 μM	7 nM	Fluorescent	[9]
	S. Split G4	5 nM–90 nM	2 μM	Fluorescent	[54]
	S. Split G4	50 nM to 0.5 μM	5 nM.	Colorimetric	[55]
Aflaxotin B1	G4 + A	0.0032 to 50	0.0032 ng/mL	Colorimetric	[56]
	S. Split G4	0.1 ng–1 μg	0.1 ng/mL	Colorimetric	[51]
	Split G4	0.5–15 ng	0.02 ng/mL	Fluorescent	[57]
Kanamycin	G4	0.1–20 μM	59 nM	Fluorescent	[58]
	S. SplitG4	0.6 to 20.0 nM	0.33 nM	Fluorescent	[59]
	A. Split G4	50 pM–500 nM	14.7 pM	Colorimetric	[53]
	A. Split G4	100 fM to 1 nm	83 fM	Electrochemical	[52]
	S. Split G4	0–500 fM	10.36 fM	Colorimetric	[34]
	S. Split G4 + NP	0.1 pg to 10 ng	76 fg/mL	Colorimetric	[16]
Chloramphenicol	G4	1–10 ng/mL	0.518 ng/mL	Fluorescent	[60]
	G4 + A	10 to 0.1 ng	90 pg/mL	Colorimetric	[61]
	S. Split G4	10 to 200 nM	87.3 pM	Colorimetric	[34]
Glyphosate	G4	0.5–9 μM	0.13 μM	Fluorescent	[62]
	G4 + Ir	100 to 500 nM	26.4 nM	Luminescent	[63]
	I. Split G4	100 to 400 nM	1.37 nM	Colorimetric	[64]
Ocharatoxin A	G4	4–30 nM	4 nM	Colorimetric	[65]
	Split G4	0.1–2.5 ng/g	0.011 ng/g	Colorimetric and	[66]

				fluorescent	
Copper	G4	50 nM–50 μM	5.9 nM	Colorimetric	[67]
	G4	8 nM–2μM	3 nM	Fluorescent	[68]
	Split G4	8–200 nM	3.9 nM	Colorimetric	[69]
Mercury	G4	50–2500 nM	50 nM	Colorimetric	[70]
	A. Split G4	5–500 nM	19 nM	Colorimetric	[68]
	S. Split G4	1–500 pM	0.5 pM	Colorimetric	[5]
Cadmium	A. Split G4	10 pM to 1 μM	10 pM	Colorimetric	[71]
Bisphenol A	A. Split G4	20 pM to 2μM	5 nM	Luminescent	[72]
Coralyne	G4 + A	0.01–5 μM	5.8 nM	Fluorescent	[73]
	A. Split G4	0.06 to 10 μM	19 nM	Colorimetric	[74]
	A. Split G4	0.033 to 1.667 μM	16 nM	Colorimetric	[75]
P. hetrophylla	Split G4	1 nM to 30 μM	0.31 nM	Colorimetric	[76]
	S. Split G4 + NP	10 fM to 10 μM	1.87 fM	Fluorescent	[77]
Cocaine	G4 + Ru (II)	12–1300 nM	5 nM	Fluorescent	[78]
	G4 + NP	0.1 mM to 20 mM	50 nM	Colorimetric	[15]
	S. Split G4	30 to 300 nM	30 nM	Luminescent	[79]
	Split G4	50 pM to 25 nM	16 pM	Fluorescent	[80]

Ir = iridium(III) complex; GMOs = genetically modified organisms, DNT = DNA nano-tweezer; S. split = symmetrical split; A. split = asymmetrical split, N. split = non-classical intermolecular split (splitting with the G-tract); I. split = intramolecular split (G-quadruplex-forming ability is initially blocked or partitioned within a larger structure—not as a two-part intermolecular split.), C = colorimetric; F = fluorescent; E = electrochemical; L = luminescent.

Glyphosate is one of the most used and effective non-selective herbicides. However, its residues can be found in food, soil, and water, thereby causing toxic effects, including disruption of cycles, endocrine disorder, reduction in testosterone, increased death from Parkinson's disease, autism, tumorigenesis, and the hepatorenal effect [64]. Hence, it is essential to reliably monitor its residual levels in food and water. Current methods used for measuring the amount of glyphosate in food have several shortcomings, such as expensive equipment, long time analysis, stability of reagents, cost, and the need for trained personnel. Hence, a simple, fast and cost-effective method of detecting this herbicide is desirable. Mohammadi et al. [64] have reported a split G4 DNAzyme for the turnoff colorimetric detection of glyphosate based on the reconstitution of split G4 sequences that is dependent on the presence of glyphosate. The device has a linear detection range of 100 to 400 nM and limit of detection of 1.37 nM for glyphosate.

Ocharatoxin A (OTA) is a recalcitrant and carcinogenic mycotoxin commonly found in cereals and cereal-based products. Hence, it is a serious public health concern. Traditional methods of detecting OTA have shortcomings, such as complex pre-treatment steps, need for expensive equipment, the requirement for highly trained personnel, and long detection time. Lin et al. [66] developed a magnetic microfluidic device coupled with a symmetrical split G4 DNAzyme for the turnoff fluorescent and colorimetric detection of OTA in wheat. The OTA aptamer is fixed to a magnetic bead and allows for the reconstitution of the split G4Dz halves through complementary binding to form catalytically active G4 structures capable of oxidising amplex red (ADHP) using H_2O_2 to generate red fluorescence. However, in the presence of the OTA target, the aptamer preferentially binds it, thereby allowing the split G4 DNAzyme parts to uncouple and lose the G4 structure, and consequently leading to loss of fluorescence and colour. The magnetic bead–aptamer conjugate is also beneficial because it helps to separate the probes from the mixed system, thereby suppressing the background noise due to the presence of hemin in the solution. The system achieved a linear range of 0.1–2.5 μg/kg and a detection limit of 0.011 μg/kg.

3.4. Detection of Environmental Pollutants

Li et al. [69] have reported a label-free split G4 DNAzyme and a Cu^{2+}-specific DNA-cleaving DNAzyme for the detection of **copper ion** (Cu^{2+}) and **mercury ion** (Hg^{2+}). In this design, the guanine rich sequence is linked to the 3′ end of the double helix stem of the stem–loop structure and the 5′ end is extended by the complementary sequence of the G4. The G4 sequence was partly caged in the intramolecular duplex to form a split G4 DNAzyme which is unable to form a G-quadruplex structure. Cleavage of DNA by the Cu^{2+}-specific DNAzyme at specific sites decreased the stability of the duplex stem. This brings the split region into proximity, resulting in the formation of G4 DNAzyme with peroxidase activity capable of oxidising ABTS to generate a colour signal proportional to the concentration of Cu^{2+}. The system was reported to have a linear range of 8–200 nM and a detection limit of 3.9 nM. They also exploited the ability of the system to bind Hg^{2+} to stabilise T-T base mismatch and therefore detect Hg^{2+}, with a linear range of 10–100 nM and detection limit of 4.8 nM. This system can efficiently keep the background signal very low comparable to the catalytic activity of hemin alone because the intramolecular double helix stem structure blocks the formation of G4 by the split DNAzyme in the absence of Cu^{2+}. The relative short stem of the duplex structure employed by this system also makes it easy for the split G4 DNAzyme to reconstitute in the presence of Cu^{2+}.

Zhou et al. [71] have reported a 3:1 asymmetrical split G4 DNAzyme capable of detecting **cadmium ions** without the use of labelling, washing, or enzyme. In this system, the authors designed hairpin structures that form a three-way G-quadruplex junction. The presence of Cd^{2+} makes the probe aptamer initiate branch migration of the hairpins, leading to the formation of the unstable complex. This triggers the hybridisation of additional branched junctions that assemble the split G4 DNAzyme fragments to form G-quadruplex structures with peroxidase activity with a linear range of 10 pM to 1 μM and detection limit of 10 pM.

Bisphenol A (BPA) is a persistent toxic environmental pollutant that disrupts the endocrine system. Efforts aimed at designing a reliable detection device for BPA have been hampered by a lack of stable bioreceptor and signal generator. Xu et al. [72] have reported turnoff chemiluminescence aptamer split G4 DNAzyme for the detection of bisphenol A. The BPA target recognition sequence is incorporated into the one GGG tract of the split G4 DNAzyme sequence (probe A) and the other split fragment (three GGG tracts) into the complementary sequence (probe B) to act as bait. In the absence of BPA, probes A and B can partially conjugate through their complementary parts. This enables the split parts of G4 DNAzyme to form a G-quadruplex structure that could produce a chemiluminescence signal upon binding to hemin by using luminol and H_2O_2 as substrates. However, when BPA is present, probes A and B form a duplex structure that keeps the split halves of the G4 DNAzyme far apart and unable to reconstitute the G-quadruplex structure, consequently leading to a reduced signal. The level of reduced signal is directly proportional to the concentrations of BPA. This system has a linear range of 20 pM to 2μM and detection limit of 5 nM.

Coralyne (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo- [4,5-i]phenanthridium) is a planar alkaloid that has been reported to possess anti-leukemic activity. It is a poly deoxyadenosine (poly dA)-binding drug that intercalates DNA and induces topoisomerase I-mediated DNA cleavage. DNA sequences containing several poly dA may affect chromatin structure due to nucleosome exclusion, thereby assisting in the entry of transcription proteins. Moreso, poly (dA) sequences may serve as DNA disease biomarkers due to their sensitivity to nucleases. Only a few strategies have been developed for the detection of coralynes and to study their interaction with poly dA, and most of those suffer their own shortcomings. Hou et al. [74] have reported a label-free and colorimetric turn-on detection of coralyne based on a split G4 DNAzyme and adenine rich probe, obtaining a

detection limit of 32 nM. Kan et al. [75] also reported a split G4 DNAzyme capable of detecting coralyne. They designed a hairpin probe (HP) containing three GGG tracts of split DNAzyme and an adenine-rich region; the second probe (P1) contains the remains of the split halves of one GGG tract and another region rich in adenine. When a coralyne target is present, it hybridises with the adenine-rich region on the hairpin probe and the second probe, thereby making the split G4 sequence come into proximity to form a functional G4 DNAzyme with peroxidase activity capable of oxidising ABTS and thus giving a colour signal. In the absence of coralyne, the split G4Dz remains separated by the HP and P1 probes and is able to neither form a G4 structure nor generate a colour signal. The system selectively distinguished coralyne from other DNA intercalating agents, such as methylene blue and rhodamine, as well as other drugs like berberine and jatrorrhizine which are similar in structure to coralyne. They obtained a linear range of 0.033 to 1.667 μM and a detection limit of 16 nM.

Pseudostellaria hetrophylla **(PH)** is a commoly used plant in Chinese alternative medicine for treating various lungs and spleen diseases, hyperirritability, night sweats, etc. Due to other counterfeit species that are commonly sold, it is now often characterised to identify the marker compound within it. However, environmnetal factors and processing conditions can affect its chemical composition. Hence, genetic analysis is often used as a reliable means of identifying the plant species. Internal transcribed spacers (ITRs) of 18S, 5.8S and 26S nuclear ribosomal DNA (nrDNA) are commonly used for phylogenetic analysis as genetic markers for identifying plant species. However, this technique is expensive and there is variation in its sensitivity and reliability. Zheng et al. [76] have reported a label-free split G4 DNAzyme (1:1:1:1 GGG repeats mode) integrated with a molecular beacon for the detection of PH nrDNA ITR sequence. The one GGG repeat is tethered to the two ends of the reporter, while the aptamer recognition site is located in the loop region. In the absence of the target, the split G4 DNAzyme combines and forms a G4 structure that produces a colour signal. When the target DNA is present, it hybridises with the loop of the molecular beacon, causing the stem to open and form duplex NDA preventing the split G4 DNAzyme from reassembling and thereby turning off the colour signal. The authors report a linear range of 1 nM to 30 μM and a detection limit of 0.31 nM.

Luo et al. [81] have reported a multimodule split G4Dz for the detection of cocaine. In a design similar to those described above, two split parts of G4 DNAzyme were flanked with complementary cocaine-binding aptamer sequences. In the absence of cocaine, the split parts are kept apart through duplex formation by the aptamer in each fragment. When the target is present, it hybridises with the aptamer, thereby making the split halves reassemble to form a G4 DNAzyme structure and generate a colour signal proportional to the cocaine target concentration. They obtained a linear range of 0 to 100 μM and detection limit of 10 μM.

4. Integration of Split G-Quadruplex DNAzyme with Nanomaterials for Enhanced Stability and Sensitivity

Zhu et al. [45] have reported an optical turnoff for **thrombin** detection based on a combination of a split G4 DNAzyme and magnetic nanoparticles. Binding of the thrombin analyte to its aptamer recognition domain on the probe leads to a decrease in the flexibility of the probe, making the split G4 parts unable to associate into a functional G4 structure. In the absence of thrombin, the probe is flexible and the split G4 DNAzymes are in proximity to form a G4 structure capable of binding hemin and generating a colour signal when ABTS and H_2O_2 are provided. The magnetic nanomaterial is used to coat the DNA, helping to separate the interfering substances from the analyte and leading to improved

detection of thrombin from human plasma. The authors report a linear detection range of 0.5 to 20 nM and a detection limit of 0.5 nM.

Wang et al. [16] used a similar approach to integrate split G4 DNAzyme technology with gold nanoparticles for the colorimetric detection of kanamycin. The split halves of the G4 DNAzyme were designed into two hairpins that serve as gold nanoparticle-modified substrates for a Mg^{2+}-dependent DNAzyme (MDz). Once the kanamycin target binds to the aptamer recognition site, the release of MDz brings the split G4Dz into proximity, thereby allowing their reconstitution into a G4 structure in the presence of a potassium ion. The generation of the G4 structure induces the crosslinking of the gold nanoparticles (Au-NP), leading to an aggregation-based colour signal based on the strong extinction coefficient and distance-dependent absorbance properties of the Au-NP, which can be monitored at 520 nm. In addition, the MDz also aids in signal amplification, leading to increased sensitivity of the system. This is because the split MDz can reassemble into another MDz with the inner catalytic core, thereby initiating a second catalytic round that can trigger the reassembling of more of the split G4Dz, leading to an amplified signal response. The authors obtained a linear range of 0.1 pg/mL to 10 ng/mL and a detection limit of 76 fg/mL.

Zheng et al. [76] integrated a split G4 DNAzyme with graphene oxide nanoparticle for the aunthentication of ***Pseudostellaria hetrophylla*** based on nrDNA ITR sequences. In the presence of the target DNA, the probe leaves the graphene oxide surface, and its aptamer recognition sequences hybridises with the target to form Watson–Crick duplex structures, thereby allowing the split G4 DNAzyme parts to reassemble to a G4 structure that reacts with quinaldine red to produce fluorescence signal. The device was reported to have a linear range of 10 nM to 2 μM and a detection limit of 7.8 nM of the target nrDNA ITR. The graphene oxide aids in capturing the probe and consequently reducing the background signal. Similarly, Zheng and Hu [77] combined a split G4 DNAzyme with a magnetite graphene oxide (Fe_3O_4/GO) nanoparticle. When the target DNA is present, the aptamer recognition site on the probe hybridises with it. This brings the split G4 DNAzyme halves into proximity to form a G4 structure that binds hemin and utilises H_2O_2 to catalyse the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate, with a concomitant release of fluorescent signal. The magnetic graphene oxide aids in reducing the background signal by absorbing the excess hemin and probes, and the system has a linear range of 10 fM to 10 μM and a detection limit of 1.87 fM of the target nrDNA ITR. The integration of the Fe_3O_4/GO nanoparticle offers a several-fold improvement in sensitivity to this system, with a detection limit of 1.87 fM as against the 0.31 nM previously reported without nanoparticles.

Nanomaterials help to concentrate and align the split G4 DNAzyme fragment for effective stimulated target reassembly through spatial confinement, leading to a low detection limit and faster response time. Magnetic nanoparticles (MNPs) have a large surface area to volume ratio and magnetic properties that aid it to separate the analyte from interference substances in a complex sample such as human serum [45,77]. It also stabilises the active G4 DNAzyme through conjugates formed by these structures. These also amplify detection signals through their unique physical properties; for instance, gold nanoparticle aggregation-induced absorbance change can serve as an excellent signal transduction [16]. Graphene oxide (GO) provides a **large surface area,** with abundant oxygen-containing functional groups that strongly adsorb hemin and the split DNAzye probe in their inactive state through hydrophobic interaction. Consequently, they can remove undesirable noise that may be generated in the absence of the target. This prevents non-specific self-assembly and promotes the concentration of the DNA probes. Moreso, GO nanoparticles are water soluble, thereby enhancing their suitability in assay. The ability of graphene oxide nanoparticles to distinguish between various DNA structures

further enhances their applications in biosensor development [76,77]. Iron (III) oxide nanoparticles incorporated into a graphene oxide-based split system offers the separation and concentration of the formed G4 DNAzyme for enhanced substrate oxidation. This separation also helps to prevent background signals that might be caused by substrate oxidation due to the intrinsic catalytic activities of the Fe_3O_4/GO nanoparticles [77].

5. Practical Points in the Biosensor Applications of Split G4 DNAzymes: Sensitivity, Selectivity, Readouts, and Response Time

In this section, we provide a summary of the salient points relating to the practical use of a split G4 DNAzyme in various biosensor applications. The points summarised below are based mainly on the information provided in Tables 1 and 2.

Sensitivity: Typical performance for a split G4 DNAzyme system ranges with a sensitivity (LOD) that lies within the fM–nM range; this is generally sufficient/acceptable to assay clinical samples, such as with miRNA detection and with food and environmental pollutant screening.

Selectivity: Selectivity for some nucleic acid targets is highly remarkable; this system can discriminate between single-base difference and different strains of diseases. However, matrix intolerance due to aptamer cross-reactivity and G4 refolding in complex matrices such as whole blood and urine samples could limit specificity in real-world applications.

Readouts: The colorimetric method is often employed due to its simplicity and naked-eye visualisation [5,7,30,33,34]. However, fluorescence [14,26,46] and electrochemical [12,21,35,52] readouts improve quantitation. While dual-modal [49,66] strategies enhance robustness. Colorimetric methods offer naked-eye visualisation without the need for complex equipment. This yes/no effect based on colour generation is good for real-life point of care application, especially in settings where there is limited access to equipment.

Time: Most published articles reviewed here did not include the response time for each DNAzyme system. However, G4 systems are fast enough for real-life applications with response times ranging between 10 and 20 min. This is a reasonable wait time for point-of-care applications, food safety screening, and on-site environmental testing.

6. Challenges to the Use of Split G-Quadruplex DNAzymes in Biosensing

The considerations and examples above show that splita G-quadruplex DNAzymes offer exciting possibilities for biosensor design. However, several current limitations still hinder their broader application and development into translation for field use and other real-world applications. The following are a summary of the key challenges.

Limited catalytic efficiency: The peroxidase-mimicking activity of G-quadruplex/hemin DNAzymes is significantly lower than that of natural enzymes like horseradish peroxidase (HRP). This reduced activity can compromise sensitivity, especially in low-abundance target detection scenarios [37,82]. Several approaches have been developed to improve the catalytic activities of GQ-DNAzymes, including multimerization [37,83–85], addition of additives [8,86–88], introduction of terminal poly A and poly C bases [82,89–93], crosslinking of hemin to DNA sequence [94–97], etc.

Ion-dependent folding and stability: G-quadruplex formation is highly dependent on monovalent cations (e.g., K^+, Na^+, NH_4^+), which stabilize the structure. Variability in ion concentration can affect folding topology and catalytic performance, complicating sensor reproducibility [85,98–100]. Additional studies will be required to determine the unique blend of monovalent cations for the optimal activity of split systems and the application they are designed for.

Weak π–π stacking and incomplete assembly: The application of systems with split configurations are often limited by inefficient reassembly due to weak interactions between fragments. This can lead to incomplete G-quadruplex formation and reduced catalytic output [82].

Background noise and signal leakage: In some designs, spontaneous reassembly of split strands or non-specific interactions can generate false-positive signals, reducing assay specificity [101]. This remains one of the challenges that limits the use of split systems in designing diagnostic biosensing, especially when analytes are present at low concentrations. Sequence analyses and simulations can help predict split systems with minimal background activities. Generally, to achieve low target-free activity, neither fragment should retain a tract configuration capable of forming a catalytically competent G-quadruplex alone. For example, fragments that preserve ≥3 aligned G-tracts or that readily dimerize should be avoided. In addition, splitting within loop regions or introducing tract-weakening substitutions (G to A/T; GGG to GG), non-G spacers/basic linkers, or hairpin clamps that sequester G-tracts can suppress leakage.

Structural complexity and optimisation challenges: Designing split G-quadruplexes that fold correctly and interact predictably with targets requires precise sequence engineering. Multimeric or higher-order structures may enhance activity but add complexity and unpredictability [83]. Advances in DNA structure prediction and modelling tools along with simulations may provide additional insights into addressing these complexities.

Limited multiplexing and real-time capabilities: Although split G-quadruplexes can be adapted for multiplex detection in diagnostic biosensing, signal overlap and cross-reactivity remain concerns. In addition, real-time monitoring is limited by the need for external reagents (e.g., hemin, H_2O_2) and slow reaction kinetics [36].

Lack of standardisation: There is no universally accepted protocol for split G-quadruplex biosensor design, leading to variability across studies and difficulty in comparing performance metrics.

7. Conclusions

Split G-quadruplex DNAzymes represent a versatile and powerful platform for the design of diagnostic biosensors. Their conditional assembly, catalytic activity, and compatibility with diverse detection modalities make them suitable for a wide range of applications, from pathogen detection to environmental monitoring. Continued research into their structural optimization and integration into novel biosensing architectures will further expand their utility in molecular diagnostics.

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