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Research Article

Enzyme-free, Colorimetric DNA Detection System Powered by Autocatalytic Assembly Circuits and G-quadruplex DNAzymes

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ABSTRACT

Objectives: To develop an enzyme-free colorimetric DNA detection system combining autocatalytic assembly circuits with G-quadruplex DNAzymes for highly sensitive and visual DNA identification.

Methods: The platform utilized toehold-mediated strand displacement (TMSD) integrated with autocatalytic hairpin circuits and a G-quadruplex DNAzyme. Buffer conditions, hemin concentration, temperature, and reaction time were optimized. Spectrophotometric detection at 416 nm was employed, and NUPACK software was used for oligonucleotide design validation.

Results: The optimized system exhibited a detection limit as low as 31 pM. Clear electrophoretic band patterns confirmed correct structure formation. The system showed high reproducibility, sensitivity, and a visible signal without enzymatic components.

Conclusion: This detection method is simple, cost-effective, and highly sensitive, with potential for point-of-care diagnostics, food safety testing, and environmental monitoring. Its enzyme-free nature, label-free operation, and modular design make it adaptable to other nucleic acid targets.

Keywords: DNA Nanotechnology, DNAzyme, TMSD, Colorimetric Technique

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Introduction

The rapid advancement of DNA nanotechnology has placed it at the forefront of new biosensing platforms, offering a unique opportunity to harness the inherent properties of nucleic acids. Toehold-mediated strand displacement (TMSD) is a process in which a single-stranded nucleic acid binds to a short exposed "toehold" region on a double-stranded complex, initiating strand displacement through branch migration. This fundamental mechanism in dynamic DNA nanotechnology enables the construction of precisely programmable molecular interactions. These systems have the potential to create autonomous, self-supporting platforms with enhanced signal amplification and target detection (1, 2).

The integration of TMSD with the principles of autocatalytic assembly has emerged as a particularly promising approach in DNA diagnostics. By exploiting the exponential amplification properties of autocatalytic cycles, researchers have developed highly sensitive nucleic acid detection systems (3, 4). These autocatalytic circuits show significant potential for point-of-care applications.

One of the most impressive applications of autocatalytic assembly circuits in DNA diagnostics is the use of catalytic DNA structures (CDAs), engineered DNA molecules that, through the formation of specific three-dimensional structures, can catalyze chemical reactions like enzymes. Specifically, G-quadruplex DNA structures—four-stranded configurations formed from guanine-rich nucleic acid sequences—consist of guanine tetrads linked by Hoogsteen hydrogen bonds and stabilized by cations. These structures can adopt various molecular arrangements and are employed in biological detection systems. These enzyme-free DNA-based catalysts generate visible color changes through peroxidase-like activity using chromogenic substrates.

By further coupling this technology with TMSD-based networks, researchers aim to create a new class of enzyme-free colorimetric DNA detection systems. The resulting systems demonstrate improved performance in complex samples (5, 6). In this study, we present a novel colorimetric DNA detection platform that integrates TMSD, autocatalytic circuits, and G-quadruplex DNAzymes to achieve ultra-sensitive detection (picomolar range), enzyme-free operation, and instrument-free visual readout. The system is designed to be cost-effective, accessible, and adaptable—promising for future multiplexing or integration with portable detection formats.

Methods

Electrophoresis Buffer Systems

A 1× TBE buffer with pH maintained between 7 and 8 served as the primary electrophoresis medium, providing optimal conditions for DNA fragment separation. The buffer preparation involved combining 10.8 g of Tris with 5.5 g of boric acid and 4 mL of 0.5 M EDTA in 100 mL of deionized water. For agarose gel preparation, a 3% concentration was used.

Physiological Buffer Systems

A 10 mM phosphate-buffered saline (PBS) solution with pH between 7 and 8 was prepared. This pH range is close to physiological conditions and helps maintain the stability of DNA and RNA structures. The preparation involved dissolving 1.56 g of NaH_2PO_4 (Biobasic) and 1.42 g of Na_2HPO_4 in 100 mL of deionized water. The buffer formulation was completed by incorporating 11.68 g of NaCl into the solution.

The study also employed a 50 mM HEPES 2× buffer system (pH 7.4) to maintain a stable physiological pH optimal for enzyme activity and cell viability during the experiments. It was prepared by combining 0.595 g of HEPES powder with 0.585 g of NaCl and 0.555 g of KCl in 50 mL of deionized water. Then, 1 mL of 2% DMSO and 46.73 µL of Triton X-100 were added to the buffer.

Detection System Reagents

For colorimetric detection, a 10 µM hemin solution was meticulously prepared from hemin powder using a sequential dilution approach. Initially, 0.001 g of hemin powder was dissolved in 200 µL of DMSO to ensure complete solubilization of this hydrophobic compound. Subsequently, 1 µL of this concentrated solution was diluted in 99 µL of deionized water to obtain a 100 µM intermediate solution. The final 10 µM working solution was achieved by further diluting 10 µL of the 100 µM solution in 90 µL of deionized water.

The colorimetric detection system utilized a 1 mM ABTS solution prepared in a citrate-phosphate buffer with a precisely controlled pH of 4.2, as this pH optimizes the stability of the ABTS radical and enhances the reactivity of antioxidants for more accurate and sensitive measurement. The buffer preparation involved dissolving 2.83 g of sodium phosphate dibasic and 1.92 g of citric acid anhydrous separately in 100 mL of deionized water. These solutions were then combined by mixing 2.06 mL of the sodium phosphate solution with 2.94 mL of the citric acid solution and diluting to a final volume of 10 mL with deionized water. The ABTS working solution was prepared by combining 1 mL of this citrate-phosphate buffer with 0.548 mg of ABTS powder and 1 µL of hydrogen peroxide (30% w/w). This solution was stored in darkness at 4°C to prevent photooxidation of the ABTS substrate.

Oligonucleotide Design and Computational Analysis

The development of the DNA detection system using an autocatalytic assembly circuit required sophisticated oligonucleotide design combining theoretical principles

Table 1. Sequences, lengths, and domains of the designed oligonucleotides designed in this study.

Name of the Sequence	Domain	Sequence (5'-3')	Length (nt)
H1	e, c, d, m, h, j, d*, c*	ACT GTC TTG GGT TTG GGT ACT GTC TGT GCA TCT CAG TAC CCA AAC CCA A	**65**
H2	c, d, l, k, o, f, k*, l*, d*, n	TTG GGT TTG GGT ACT GAG ATG CAC TCC CAA GAC AGT GTG CAT CTC AGT ACC CAA GAC AGT	**79**
H3	f, n, p1, P2, K2, e, c, q, r, v, q*, c*, e*, k2*	GAC AGT ACC CAA GCC GAG ATG CAC ACT GTC TTG GGT GAC AGT ACC CAA ACC CAA GAC AGT GTG	**83**
Hp	a, r, b, f, a*, w	CTA ATC GTG ATA GGG GT GAC AGT ACC CAA ACC CAA GAC AGTA CCC CTA TCA CGA TTA GCA TTA A	**85**
Input Strand	w*, a*	TTA ATG CTA ATC GTG ATA GGG GT	**30**
D1	g1, c*, b*a*	GGGTGGGTGGACTGTCTTGGGTACTGGCTTGGGTACTGTC	**41**
D2	b, c, g2	ACCCAAGCCAGTACCCAAGACAGTGTGGGT	**30**

with computational validation. The design process began with target DNA identification, followed by the strategic development of complementary oligonucleotide strands, including specialized hairpin structures and G-quadruplex DNAzyme sequences. These designs facilitated the intended toehold-mediated strand displacement (TMSD) reactions and subsequent signal generation as detailed in Table 1.

Each oligonucleotide was engineered with specific functional domains to enable controlled hybridization, strand displacement, and catalytic activities in the autocatalytic circuit. The theoretical designs were rigorously evaluated using NUPACK software, which provided computational predictions of structure formation probabilities, thermodynamic stability parameters, and the energetic feasibility of the intended TMSD reactions. This computational approach, as described by Fornace et al. (2022), allowed for the optimization of sequence compositions and reaction conditions prior to experimental implementation. The software analysis identified potential secondary structures, unintended interactions, and energetic barriers that might affect the performance of the autocatalytic assembly circuit (7).

Oligonucleotide Synthesis and Sample Preparation

All oligonucleotide strands employed in this study were chemically synthesized and purified by Metabion (Germany). Stock solutions were prepared at a concentration of 100 µM by dissolving the lyophilized oligonucleotides in nuclease-free deionized water under sterile conditions. Working solutions at 25 µM and 50 µM concentrations were subsequently prepared through serial dilution of the stock solutions.

To study the autocatalytic assembly circuit, several reaction mixtures were prepared in microtubes, each containing different combinations of oligonucleotide strands corresponding to distinct stages of the assembly process. The prepared samples were subjected to a specific thermal cycling program designed to promote controlled hybridization and subsequent structure formation according to the computational design parameters. The thermal cycling protocol included an initial denaturation step followed by gradual cooling to allow proper annealing and formation of the designed DNA nanostructures, thereby facilitating the intended toehold-mediated strand displacement reactions.

Electrophoretic Analysis of DNA Nanostructures

Agarose gel electrophoresis was employed as a primary analytical technique to visualize and confirm the formation of the desired DNA nanostructures within the autocatalytic assembly circuit. The prepared samples containing various oligonucleotide combinations were loaded onto a 3% agarose gel prepared in 1× TBE buffer. Electrophoretic separation was conducted at a constant voltage of 95 V for 50 minutes.

Following the electrophoretic separation, the DNA bands were visualized using the Gel Doc imaging system under ultraviolet illumination. The migration patterns observed in the gel provided valuable information regarding the size, conformation, and structural integrity of the assembled DNA components. The successful formation of the intended structures was confirmed by comparing the experimental band patterns with theoretical predictions based on the oligonucleotide designs and computational models, thereby validating the assembly of the autocatalytic circuit components.

Colorimetric Detection Methodology

The functional performance of the autocatalytic assembly circuit was evaluated through a colorimetric detection method that leveraged the peroxidase-mimicking activity of G-quadruplex DNAzyme structures formed during the assembly process. In this detection system, the successful assembly of the autocatalytic circuit in the presence of target DNA leads to the formation of G-quadruplex structures that, when complexed with hemin, exhibit peroxidase-like catalytic activity. This enzymatic activity catalyzes the oxidation of ABTS by hydrogen peroxide, resulting in a colorimetric change that can be quantified spectrophotometrically. The oxidation of ABTS was monitored by UV-Vis

spectroscopy using a spectrophotometer (Model CARY-50). Specifically, absorbance at 416 nm, corresponding to the oxidized form of ABTS, was measured to track the colorimetric changes catalyzed by the G-quadruplex DNAzyme-hemin complex in the presence of hydrogen peroxide.

Optimization of Reaction Parameters

An optimization approach was used to determine the optimal conditions for the autocatalytic assembly circuit and subsequent colorimetric detection. Four critical parameters were investigated: TMSD reaction time, reaction temperature, hemin concentration, and hemin incubation time. The optimization process for each parameter involved maintaining all other conditions constant while varying the parameter of interest, followed by evaluation of system performance through colorimetric readout. For each set of conditions tested, parallel experiments were conducted with and without target DNA to assess both the signal intensity and the signal-to-background ratio. The colorimetric response was quantified by measuring the absorbance at 416 nm using the spectrophotometer, and the optimal conditions were determined based on maximum signal intensity in the presence of target DNA combined with minimal background signal in its absence.

Detection Limit Determination

To evaluate the system's sensitivity, a series of input strand concentrations (0.001, 0.01, 0.1, 1, 10, 100, and 1000 nM) was prepared. Each concentration was analyzed under optimized reaction conditions using the colorimetric detection method. A control sample without the input strand was also included and served as the negative control.

Statistical analyses

All statistical analyses were performed using SPSS software 18.0 (IBM, Chicago, IL, USA). All data were presented as mean ± S.D. (standard deviation). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value less than 0.05 was considered statistically significant.

Results

Circuit Design and Mechanism

Our design incorporates a Y-shaped structure that can hybridize with an external G-quadruplex structure, enabling a colorimetric readout mechanism that transforms molecular recognition events into visible color changes. The structural components were carefully engineered at the domain level to ensure specificity and efficiency in the detection process (Fig. 1). The autocatalytic assembly circuit comprises one input strand and four distinct hairpin structures (H1, H2, H3, and Hp) arranged in a sophisticated reaction network. The mechanism begins with the binding of the input strand to the auxiliary hairpin (Hp), which initiates a cascade of TMSD reactions. This initial interaction

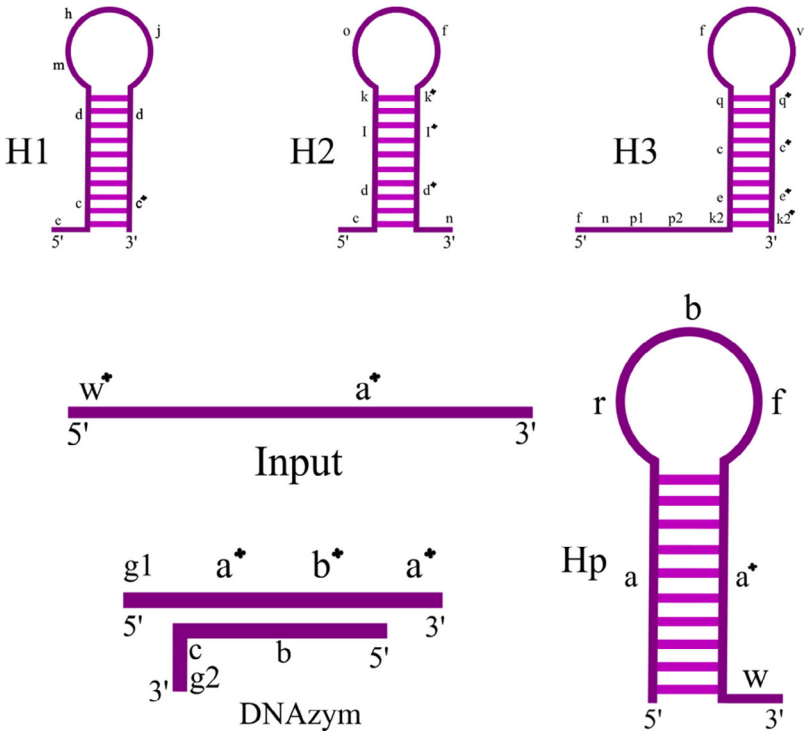

Figure 1. Manual design of oligonucleotides, including the targeted hairpin structures and the G-quadruplex, along with their respective domains.

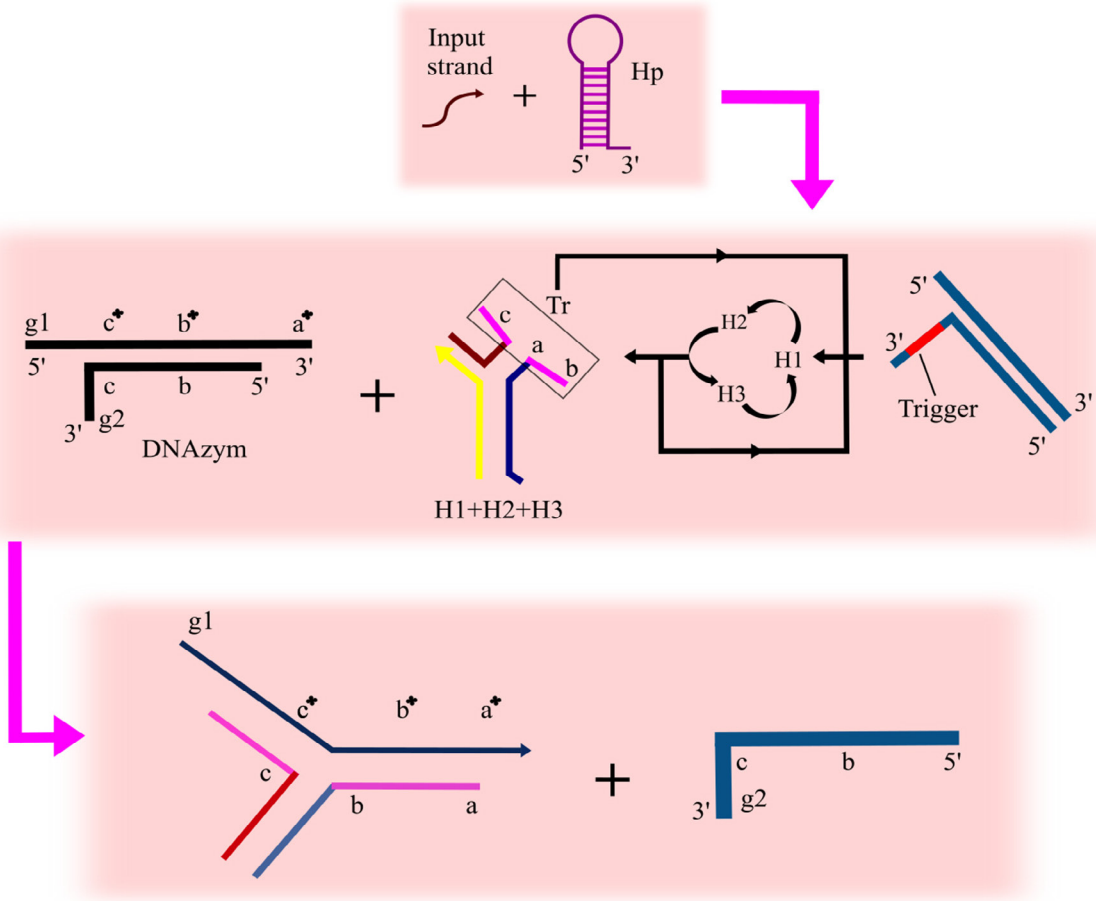

Figure 2. Overall schematic of the autocatalytic assembly circuit, along with the external DNAzyme structure.

serves as the trigger for the sequential incorporation of the three additional hairpin structures (H1, H2, and H3), resulting in the formation of a three-way junction. Critically, this junction structure can then participate in further assembly processes, creating a self-perpetuating cycle that amplifies the detection signal.

The external component of the system involves a DNAzyme structure composed of two oligonucleotide strands, D1 and D2. In this configuration, the guanine bases of the g1 and g2 domains are positioned to face each other, initially activating the signal. However, upon completion of the assembly circuit, the newly formed three-way junction binds to its complementary region in the DNAzyme structure (containing domains a, b, and c), effectively deactivating the signal. This elegant switching mechanism provides the basis for the detection system's functionality and specificity. To validate our design approach, we conducted computational analyses using NUPACK software, which confirmed high probabilities of structure formation at all key steps of the autocatalytic circuit. The predicted Gibbs free energy values and stability indices provided strong evidence for the thermodynamic favorability and robustness of the designed constructs (Fig. 2).

Electrophoresis Analysis

The results confirmed successful hybridization between the input DNA and the Hp hairpin, followed by the progressive incorporation of H1, H2, and H3 hairpins, culminating in the formation of the three-way junction. While the primary products were clearly visible, we also observed some bands suggesting the presence of side products resulting from unintended interactions between the components. These observations highlighted the need for a more direct and easily interpretable readout method to complement the electrophoresis analysis and provide unambiguous detection results (Fig. 3). Minor side bands observed in the electrophoresis gel likely resulted from partial hybridization events or nonspecific interactions between the strands. These bands appeared faint and were significantly less intense than the main product, suggesting that they represent incomplete or off-pathway structures. Notably, they did not contribute to colorimetric signal generation, as signal development requires complete formation of the active DNAzyme complex. Therefore, these side products were excluded from LOD calculations to ensure analytical specificity and accuracy.

Colorimetric Readout and Optimization

The colorimetric technique provided visible confirmation of circuit performance through distinct color changes in both positive and negative control conditions. This visual readout system represents a significant advancement toward user-friendly DNA detection methodologies that do not require specialized equipment for result interpretation (Fig. 4).

Reaction Time Optimization

To determine the optimal reaction time for the TMSD reactions, experiments were conducted with three different incubation periods: 1, 1.5, and 2 hours. The results demonstrated that a 1-hour reaction time provided the best signal-to-noise ratio among the tested conditions (Fig. 5). This finding aligns with previous observations by Jiang et al., who reported that shorter reaction times tend to be favorable for achieving good signal amplification in enzyme-free DNA detection systems (8).

Temperature Optimization

System performance was evaluated at three different temperatures: 23°C, 25°C, and 27°C. The results clearly showed that 25°C yielded a significantly more favorable signal-to-noise ratio compared to the other tested temperatures. This empirical finding strongly corroborates the initial design considerations implemented in NUPACK software, which had identified 25°C as the optimal temperature for TMSD reactions and structure formation (Fig. 6).

Hemin Concentration Optimization

A comprehensive optimization was conducted by evaluating five different hemin concentrations: 0.4, 0.5, 0.6, 0.7, and 0.8 µM. The results identified 0.6 µM as the optimal concentration, yielding the most significant difference in signal intensity between positive and negative control samples (Fig. 7). This finding is consistent with previous research by Wang et al., which established that hemin concentration plays a crucial

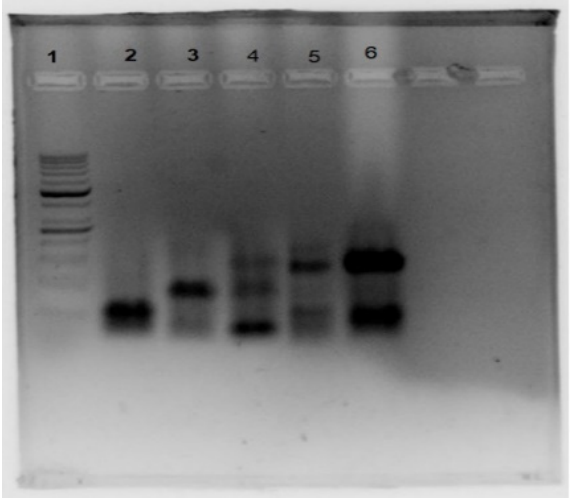

Figure 3. Confirmation of Circuit Functionality via Electrophoresis. Well Contents: 1: Ladder 50, 2: Input + Hp,3: Input+Hp+H1,4: Input+Hp+H1+H2,5: Input+Hp+H1+H2+H3, 6:H1+H2+H3.

Figure 4. Color changes in the colorimetric technique. The visible reduction and alteration in color indicate the confirmation of circuit performance using the colorimetric method in both positive and negative control conditions.

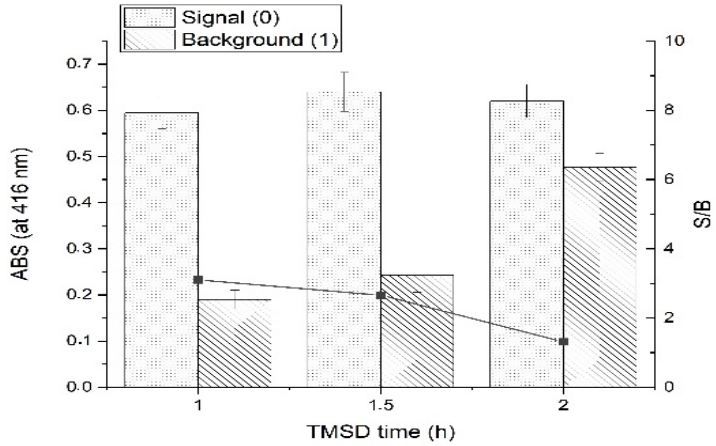

Figure 5. Results related to optimization of TMSD reaction time at three times: 1, 1.5, and 2 hours.

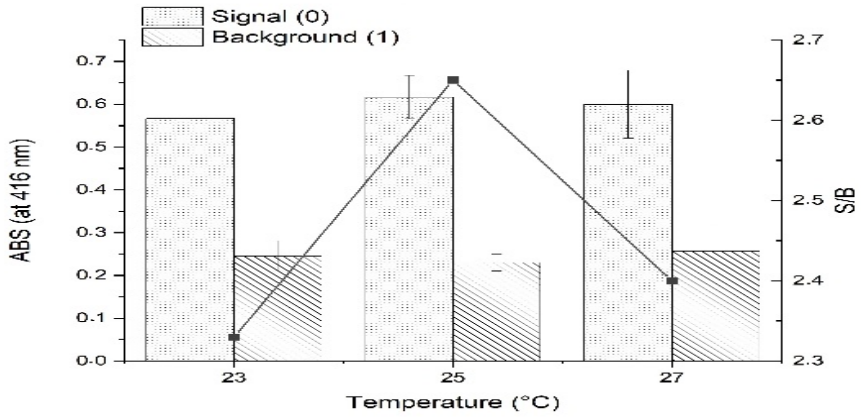

Figure 6. Results of optimization of TMSD reaction temperature at three temperatures: 23, 25, and 27 °C.

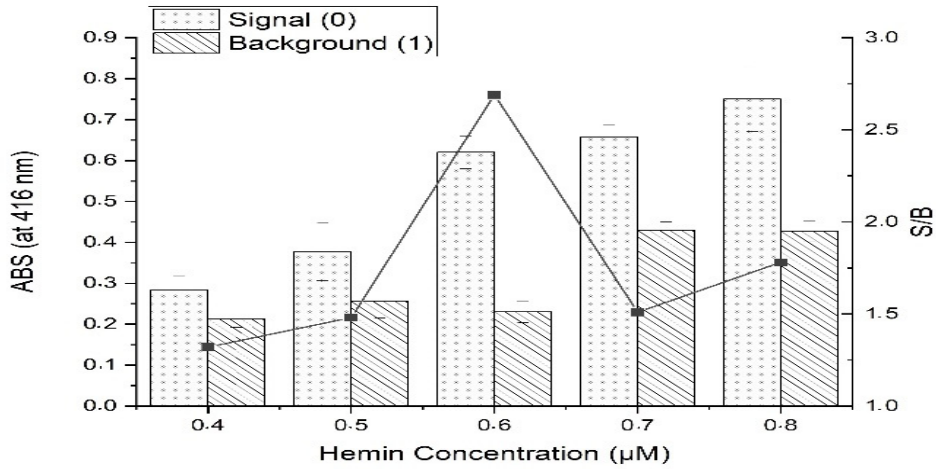

Figure 7. Results related to concentration optimization at five concentrations of hemin: 0.4, 0.5, 0.6, 0.7, and 0.8 μM.

role in enhancing the peroxidase-mimicking activity of G-quadruplex DNAzymes (9).

Hemin Incubation Time Optimization

In addition to concentration, hemin incubation time substantially affects the performance of the G-quadruplex DNAzyme-based colorimetric readout. Three different incubation times—30, 45, and 60 minutes—were evaluated. The results demonstrated that a 60-minute incubation period yielded the highest signal-to-noise ratio. This observation underscores the importance of allowing sufficient time for the G-quadruplex DNAzyme to fully assemble and interact with the hemin cofactor to achieve optimal catalytic activity. The extended incubation time likely promotes more complete formation of the DNAzyme complex and enhances its stability, resulting in improved colorimetric signal generation (Fig. 8).

Detection Limit Determination

After optimizing all relevant parameters, we conducted a sensitivity assessment of our autocatalytic assembly circuit. A series of dilution experiments with target strand concentrations ranging from 0.001 to 1000 nM (including a blank sample as the negative control) was performed. The results demonstrated an inverse correlation between the concentration of the input strand and the intensity of the colorimetric output signal, providing a quantitative basis for target DNA measurement. As the concentration of the input strand increased, a corresponding decrease in the output signal was observed. A linear relationship was established over the tested range, expressed by the regression equation: ABS = –3.0867C + 0.5244, with a high correlation coefficient ($R^2 = 0.99$) (Fig 9).

To calculate the limit of detection (LOD), we used the statistical formula:

Cut-Off = Mean of Negative Controls - 3(SD of Negative Controls).

Based on our data, the mean absorbance of negative controls was 0.51, with a standard deviation of 0.028, resulting in a cut-off value of 0.426. Substituting this into

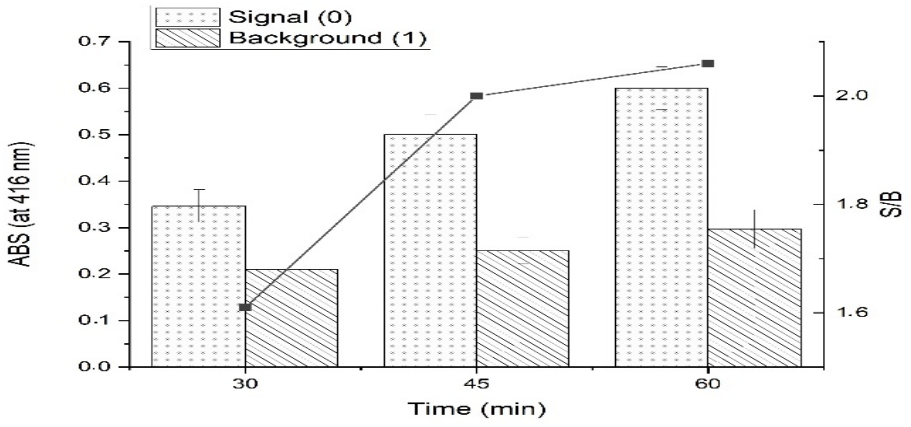

Figure 8. Results of optimization of Hemin incubation time at three concentrations: 30, 45, and 60 minutes.

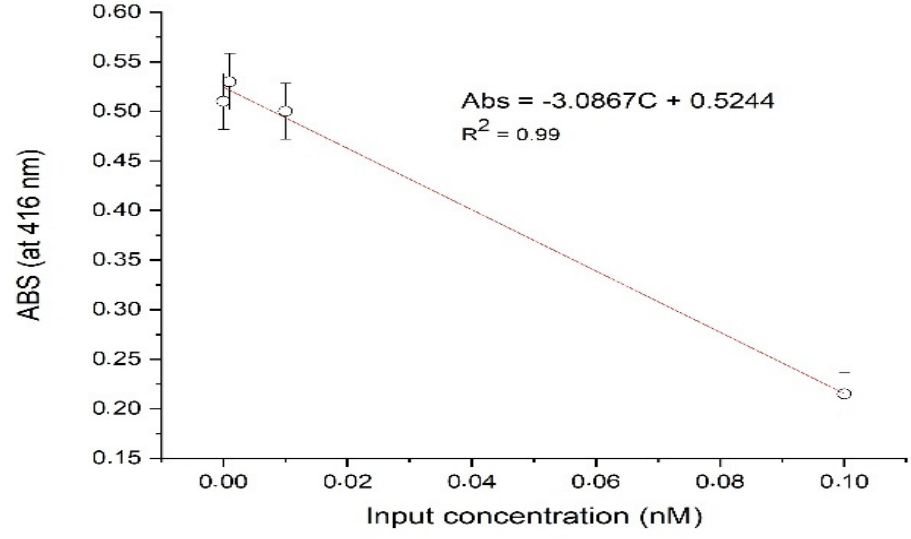

Figure 9. Results from the response to different concentrations of the input strand. As the concentration of the input strand increased, the output signal decreased.

Table 2. Comparative analysis of recent enzyme-free DNA detection systems

Study (Reference No)	Amplification Mechanism	Detection Method	Target	LOD (Limit of Detection)	Enzyme-Free	Reaction Time	Instrument-Free Readout
2	bCHA (branched catalytic hairpin assembly)	Fluorescence	miR-155	2.5 nM	Yes	360 min	No
11	traditional Catalytic Hairpin Assembly (CHA)	Fluorescent, Electrochemical, Colorimetric, SPR, Electrophoretic	Nucleic acids, metal ions, proteins, enzymes, cells	Down to 1 nM	Yes	Minutes to a few hours	Possible
12	Catalytic Hairpin Assembly (CHA), enzyme-free, isothermal	Split Broccoli RNA aptamer, fluorescence activation	Programmable RNA sequences	2.5 nM (in vitro)	Yes	20–60 min (depending on target concentration)	No (requires fluorescence detection)
(13)	AAC (self-stacking autocatalytic circuit)	Fluorescence (FRET)	miR-155	5 pM	Yes	60 min	No
This work	Autocatalytic assembly + Gq DNAzyme	Colorimetric (ABTS)	Input Strand (Nucleic acid)	31 pM	Yes	60 min	Yes

the regression equation, the LOD was calculated to be 0.031 nM (31 pM). This remarkable sensitivity represents a significant advancement compared to previous reports on enzyme-free, DNA-based colorimetric detection platforms, which typically exhibited detection limits in the nanomolar range, as documented in studies by Liu et al. and Karunanayake Mudiyanselage et al.(10, 11).

Discussion

The development of sensitive and accessible DNA detection platforms remains a significant challenge in molecular diagnostics and biotechnology. This study presents a novel enzyme-free colorimetric DNA detection system that harnesses the potential of autocatalytic assembly circuits and G-quadruplex DNAzymes. By integrating toehold-mediated strand displacement (TMSD) reactions with autocatalytic assembly principles, we created a system that rapidly amplifies target DNA sequence detection beyond the limitations of linear amplification approaches. The success of this innovative approach relied heavily on computational design and optimization of oligonucleotide components—including hairpin structures and the G-quadruplex DNAzyme—using NUPACK software to evaluate thermodynamic stability and structure formation probabilities.

Experimental validation through agarose gel electrophoresis confirmed the stepwise formation of autocatalytic assembly nanostructures, while also revealing the need for a more direct and interpretable readout method due to the presence of unintended side products. To address this limitation, we integrated a G-quadruplex DNAzyme into the autocatalytic assembly circuit, enabling a visually interpretable, colorimetric detection system. Systematic optimization of key parameters significantly enhanced the sensitivity and reliability of the platform, achieving a detection limit of 31 picomolar for target DNA. This represents a substantial improvement over previous enzyme-free DNA-based colorimetric detection platforms, which typically exhibited detection limits in the nanomolar range (10, 11). The enhanced sensitivity can be attributed to the exponential amplification capabilities of the autocatalytic assembly circuit, as demonstrated in the work by Li et al. (12).

The modular design of our autocatalytic assembly circuit allows for easy adaptation and customization to detect a wide range of DNA targets, expanding its versatility and potential impact. In comparison to previous systems, our work demonstrates several key advantages, including superior sensitivity through autocatalytic amplification, simplicity and user-friendliness of the colorimetric readout, and versatility in target detection. These features set our system apart from earlier studies with nanomolar-range detection limits and complex, instrumentation-based detection methods. Despite the promising sensitivity and simplicity of the proposed enzyme-free system, several limitations should be acknowledged. First, the performance of the platform has not yet been evaluated in complex biological matrices such as serum or plasma, where potential interferences may affect accuracy. Second, the current study focuses on single-target detection, and the system's compatibility with multiplex assays remains to be validated. Finally, long-term stability and storage

conditions of the DNA components were not addressed, which may impact real-world applicability.

Future directions for improving this detection platform include the development of multiplexing capabilities to simultaneously detect multiple targets in a single assay. In addition, adapting the system into a lateral flow format could enable rapid, instrument-free, point-of-care testing. Finally, evaluating the long-term stability of the DNA components under various storage conditions would be essential for potential commercialization and field deployment.

As shown in Table 2, our enzyme-free detection system offers a unique combination of visual colorimetric readout, competitive sensitivity (LOD of 31 pM), and short reaction time (60 min), distinguishing it from previously reported methods. While fluorescence-based systems often achieve lower LODs, they typically require expensive instrumentation and are not visually accessible. The simplicity, portability, and enzyme-free nature of our platform make it highly suitable for point-of-care and field-based diagnostic applications (Table 2).

Conclusion

In summary, we have developed a novel enzyme-free colorimetric DNA detection system that leverages the power of autocatalytic assembly circuits and G-quadruplex DNAzymes. By harnessing the elegance of toehold-mediated strand displacement (TMSD) reactions and the self-reinforcing nature of autocatalytic cycles, the designed system rapidly amplifies target DNA sequence detection with remarkable sensitivity. The achieved detection limit of 31 picomolar represents a significant improvement over previous enzyme-free, DNA-based colorimetric detection systems. This enhanced sensitivity can be attributed to the exponential amplification capabilities of the autocatalytic assembly circuit, making it a powerful tool for addressing the challenges associated with low-abundance targets (13). The simplicity, sensitivity, and versatility of the developed system position it for practical application in molecular diagnostics, environmental monitoring, and biotechnology.

Conflict of interests

The authors declare no conflict of interest.

Funding

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