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Construction of Metal-Ion-Free G-quadruplex-Hemin DNAzyme and Its Application in S1 Nuclease Detection

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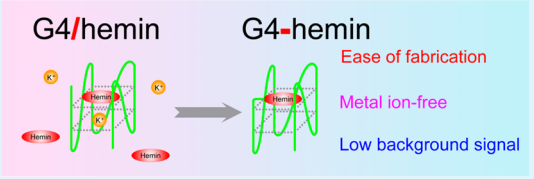
ABSTRACT: In this work, a new kind of peroxidase-mimicking DNAzyme (G-quadruplex-hemin DNAzyme, G4-hemin) was constructed by using hemin-modified G-rich DNA (hemin-G-DNA). Experimental results demonstrated that the G-rich DNA can form a G-quadruplex structure by the inducement of terminally modified hemin, rendering the assembly of hemin and G-quadruplex structure spontaneously and efficiently. As a result, G-hemin revealed higher peroxidase activity than traditional G-quadruplex/hemin DNAzyme (G4/hemin). Besides, different from G4/hemin, G4-hemin was constructed in one step without the participation of metal ions and adscititious hemin. Accordingly, the construction procedure was significantly simplified and the background signal from dissociative hemin was remarkably reduced. In a proof-of-concept trial, according to the colorimetric signals of G4-hemin, a novel biosensor for the detection of S1 nuclease activity was established, which provides a novel perspective for designing peroxidase-mimicking DNAzyme-based biosensors.



KEYWORDS: *peroxidase-mimicking DNAzyme, hemin-modified DNA, metal ion-free, colorimetric biosensor, S1 nuclease*

INTRODUCTION

Since the first discovery of DNAzyme,[1] our understanding of DNA has been extended from the carrier of genetic information to catalytic molecular functions, and various DNAzymes have been discovered.[2−6] In addition to the common advantages of DNAzyme (e.g., thermal stabilization, economic viability, ease of synthesis and modification[7]), G-quadruplex/hemin DNAzyme (G4/hemin) possesses a high peroxidase-mimicking activity,[8] resulting in its widespread application in analytical protocols.[9,10] G4/hemin is capable of catalyzing diverse molecules, resulting in colorimetric,[11] fluorescent,[12] electrochemical,[13] chemiluminescent,[14] electrochemiluminescent,[15] and photoelectrochemical signals.[16] Therefore, G4/hemin can be adopted as an ideal signal element in diverse biosensors.

G4/hemin is typically formed by G-rich DNA in the presence of metal ions and hemin.[17] Metal ions can induce G-rich DNA to form a G-quadruplex structure that is crucial to further assemble with the catalytic center (hemin).[18,19] Therefore, both the metal ions and hemin are required to obtain the activity of G4/hemin. Although G4/hemin has been well-studied, the preparation of outstanding peroxidase-mimicking DNAzyme remains challenging. First, the construction of G4/hemin should be divided into two steps as follows: formation of the G-quadruplex structure and assembly of the catalytic center. Therefore, the entire procedure is relatively complicated and time-consuming. Second, G4/hemin is a metal ion-dependent DNAzyme. Therefore, superfluous metal ions and hemin should be added to obtain maximum activity of G4/hemin. However, high concentration of metal ions may lead to undesirable influence in DNAzyme applications. In addition, hemin itself possesses inherent catalytic activity, which results in a high background signal. Therefore, contamination from excess reagent is unavoidable for G4/hemin. By overcoming these challenges, the sensing performances of peroxidase-mimicking DNAzyme-based biosensors can be significantly enhanced.

In addition to the applications involving assembling hemin and DNA, some studies have demonstrated the advantages of directly linking hemin at the terminal end of DNA. For example, relying on the interaction between hemin and some enzymes, hemin-linked DNA has been used to reconstitute and produce an active enzyme.[20] In addition, hemin that is modified at the terminal end of DNA retains its catalytic property and could generate fluorescent or photoelectrochemical signals for target DNA sensing.[21−23] The aforementioned studies mainly focus on the inherent properties of hemin at the terminal end of common DNA. However, the relationship between hemin and specific DNA sequence is rarely reported.[24] To facilitate the applications of hemin-modified DNA, more researches on linking hemin to specific DNA sequences are needed.

In this work, we investigated the feasibility of constructing a novel peroxidase-mimicking DNAzyme (named G-quadruplex-hemin DNAzyme or G4-hemin) with hemin-modified G-rich DNA (hemin-G-DNA). After being directly linked at the terminal end of the G-rich DNA sequence, hemin induced the formation of a G-quadruplex structure and enhanced the binding affinity with the G-quadruplex structure. Therefore, G4-hemin can be assembled in one step without the participation of metal ions and adscititious hemin, which greatly simplified the construction procedure and substantially

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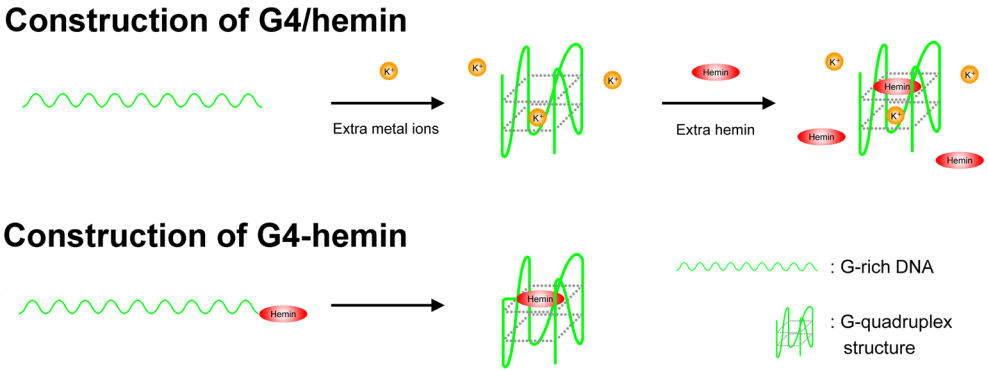
Scheme 1. Schematic Representation of the Construction Procedures for G4/Hemin and G4-Hemin



reduced the background signal. On the basis of these results, this peroxidase-mimicking DNAzyme was employed in a proof-of-concept assay to detect S1 nuclease activity. According to the colorimetric signals of G4-hemin, quantification of S1 nuclease activity was conveniently, sensitively and selectively accomplished. This approach provides a novel perspective for designing peroxidase-mimicking DNAzyme-based biosensors.

EXPERIMENTAL SECTION

Chemicals and Materials. S1 nuclease was purchased from Promega Corporation. Pepsin, bovine serum albumin (BSA), fetal bovine serum, was obtained from Sunshine Biotechnology (Nanjing) Co., Ltd. 3,3′,5,5′-Tetramethylbenzidine (TMB) liquid substrate system, chloro(protoporphyrinato)iron(III) (hemin), tris-(hydroxymethyl)-amino-methane (Tris), potassium chloride (KCl), sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), zinc chloride ($ZnCl_2$) were purchased from Sigma-Aldrich (Shanghai) Co., Ltd. All other chemicals used in this work were of analytical grade and directly used without additional purification.

Oligonucleotides (hemin-G-DNA: 5′-hemin-GGGAAAGG-GAAAGGGAAAGGG-3′; G-DNA: 5′-GGGAAAGGGAAAGG-GAAAGGG-3′) were obtained and purified with HPLC by TaKaRa Co., Ltd. (Dalian, China).

All oligonucleotides were dissolved in 10 mM Tris-HCl buffer (pH 8.0). Hemin was dissolved in 0.1 M NaOH as the stock solution and then diluted to the required concentration with Tris-HCl buffer. All solutions were prepared with ultrapure water (18.2 MΩ cm) from a Milli-Q purification system (Bedford, MA).

Preparation of G4/Hemin and G4-Hemin. Hemin-G-DNA and G-DNA were first dissolved in Tris-HCl buffer at a final concentration of 10 μM. G4/hemin was prepared according to previously reported literature with a minor modification.[25,26] Specifically, 16 μL of 10 μM G-DNA and 2 μL of 500 mM K^+ ions were mixed. After that, the mixture was heated up at 90 °C for 5 min, and then cooled to the room temperature slowly in 2 h. Finally, 2 μL of 80 μM hemin was added to the solution and kept at room temperature for 30 min. On the contrary, preparation of G4-hemin was accomplished in one step. That is, 20 μL of solution containing 16 μL of 10 μM hemin-G-DNA and 4 μL of Tris-HCl buffer was heated up at 90 °C for 5 min, and then cooled to the room temperature slowly in 2 h.

Kinetic Study of Peroxidase Activity of G4/Hemin and G4-Hemin. 180 μL TMB substrate solution was mixed with 20 μL as-prepared solution that contained G4/hemin or G4-hemin. The absorption value at 653 nm of different solutions was recorded for 15 min by a Varian Cary 5000 UV−vis spectrophotometer (Varian, America). The obtained time-dependent curves were used to kinetic analysis. In addition, the final color of different solutions in 10 min was also photographed by a Nikon digital camera.

Circular Dichroism (CD) Study. The procedure of sample preparation was similar to the preparation of G4/hemin and G4-hemin as described above, but higher concentrations were used. Specifically, 50 μL of 10 μM G-rich DNA was mixed with 10 μL of 1 M K^+ ions. CD measurements were carried out on a digital Circular Dichroism Spectrometer (Britain, Applied Photophysics) using quartz cells and a 1 nm bandwidth, 1 nm step size, 10 mm path length. The scan range was from 400 to 200 nm. All the experiments were performed at room temperature.

Detection of S1 Nuclease Activity with Hemin-G-DNA and G/Hemin. Five microliters of different concentrations of S1 nuclease, 5 μL of 50 mM Zn^{2+} ions, and 10 μL of 10 μM hemin-G-DNA were mixed in 30 μL of Tris-HCl buffer, and incubated at 37 °C for 30 min. Then, the mixture was heated at 90 °C for 5 min, and gradually cooled to room temperature. After that, 150 μL of TMB substrate solution was added and the mixture turned blue gradually. Fifteen minutes later, 200 μL of 2 M H_2SO_4 was added to stop the reaction and the color of mixture became yellow. Finally, UV−vis absorption spectra of the solution were recorded to quantify S1 nuclease activity. The inactive S1 nuclease was obtained by heating at 95 °C for 10 min.[27] For comparison, G/hemin was assembled by using 10 μL of 10 μM G-DNA and 10 μL of 10 μM hemin, and was used in quantifying the activity of S1 nuclease.

RESULTS AND DISCUSSION

Procedures of the Construction of G4/Hemin and G4-Hemin. In this study, two types of peroxidase-mimicking DNAzymes (i.e., G4/hemin and G4-hemin) have been constructed (Scheme 1). G4/hemin was fabricated using a conventional strategy. Specifically, extraneous metal ions were first mixed with G-rich DNA to form a G-quadruplex structure. Then, additional hemin was equipped with the G-quadruplex structure to obtain an active DNAzyme. Because G-rich DNA and hemin can be considered as a pair of aptamer and its target molecule,[28] we designed another peroxidase-mimicking DNAzyme (G4-hemin) using hemin modified G-rich DNA. There are two apparent differences between these two DNAzymes. First, extraneous metal ions and hemin are necessary for G4/hemin, but can be avoided in the preparation of G4-hemin. Second, the entire procedure is simplified from two steps to one step. Therefore, construction of G4-hemin is more straightforward and efficient.

Comparison of the Peroxidase Activity of G4/Hemin and G4-Hemin. TMB is a standard molecule to characterize the peroxidase activities of enzyme,[29] ribozyme,[30] DNAzyme,[31] and nanomaterials.[32] Because of oxidation by peroxidase-mimicking DNAzyme, the TMB solution gradually changes from colorless to blue, accompanied by the emergence of a characteristic absorption peak at 653 nm.[33] By monitoring the time-dependent changes of absorption intensity at 653 nm, kinetic information regarding the catalytic reaction can be obtained, which may provide insight into the catalytic procedure. Therefore, to investigate the peroxidase activity in

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detail, the increase in the absorption intensity at 653 nm was observed for the first 15 min (Figure 1). Because of the lack of a catalytic center, G-DNA itself does not exhibit peroxidase activity (curve G-DNA in Figure 1). However, the absorption intensity of hemin gradually increased (curve Hemin in Figure 1), which reflects the inherent peroxidase activity of hemin. G4/hemin, which is a DNAzyme with hemin as the catalytic center, possesses higher activity than hemin. Therefore, the time-dependent absorption curve of G4/hemin exhibited a larger gradient than that of hemin (curve G4/hemin in Figure 1). In comparison to G4/hemin, G4-hemin possessed much higher activity (curve G4-hemin in Figure 1). The absorption intensity of G4-hemin was at least five times larger than that of G4/hemin at any time point during the assay period. Similarly, before reaching a maximum at 10 min, the gradient of G4-hemin curve was much larger that of G4/hemin curve. These results were confirmed by the results captured in the corresponding photographs (inset of Figure 1). Therefore, a novel DNAzyme with higher peroxidase activity was successfully constructed by directly linking hemin at the terminal end of G-rich DNA.

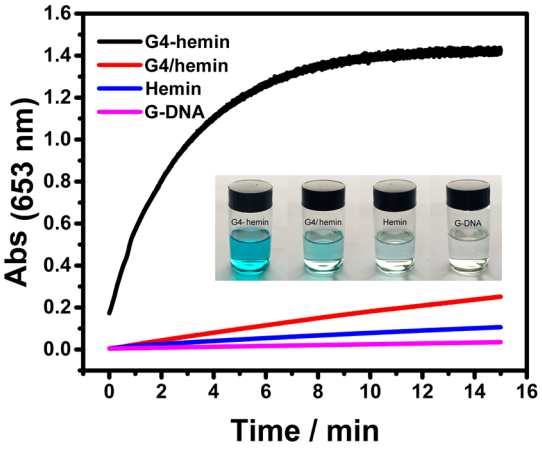

Figure 1. Time-dependent curves of the UV−vis absorption intensity at 653 nm for G-hemin, G/hemin, hemin, and G-DNA in a TMB solution. Inset: the corresponding photographs of G-hemin, G/hemin, hemin, and G-DNA in a TMB solution after 10 min.

Effort of K^+ Ions on the Peroxidase Activity of G4/Hemin and G4-Hemin. K^+ ions have a significant influence on the peroxidase activity of G4/hemin. Due to the suitable size of the cavity of the G-quadruplex structure, K^+ ions may stabilize the G4/hemin.[18,34] As shown in Figure 2A, as the amount of K^+ ions increased, the peroxidase activity of G4/hemin increased. However, it is important to note that to significantly enhance the activity of G4/hemin, the required amount of K^+ ions was quite large (in the range of submolar/100 mM). The effort of K^+ ions on the peroxidase activity of G4-hemin was further investigated. Similar to G4/hemin, the activity of G4-hemin increased as the amount of K^+ ions increased (Figure 2B). However, a low concentration of K^+ ions (1 mM) substantially enhanced the peroxidase activity, which reduced a plateau from about 10 to 8 min, which was different from G4/hemin. More importantly, although the peroxidase

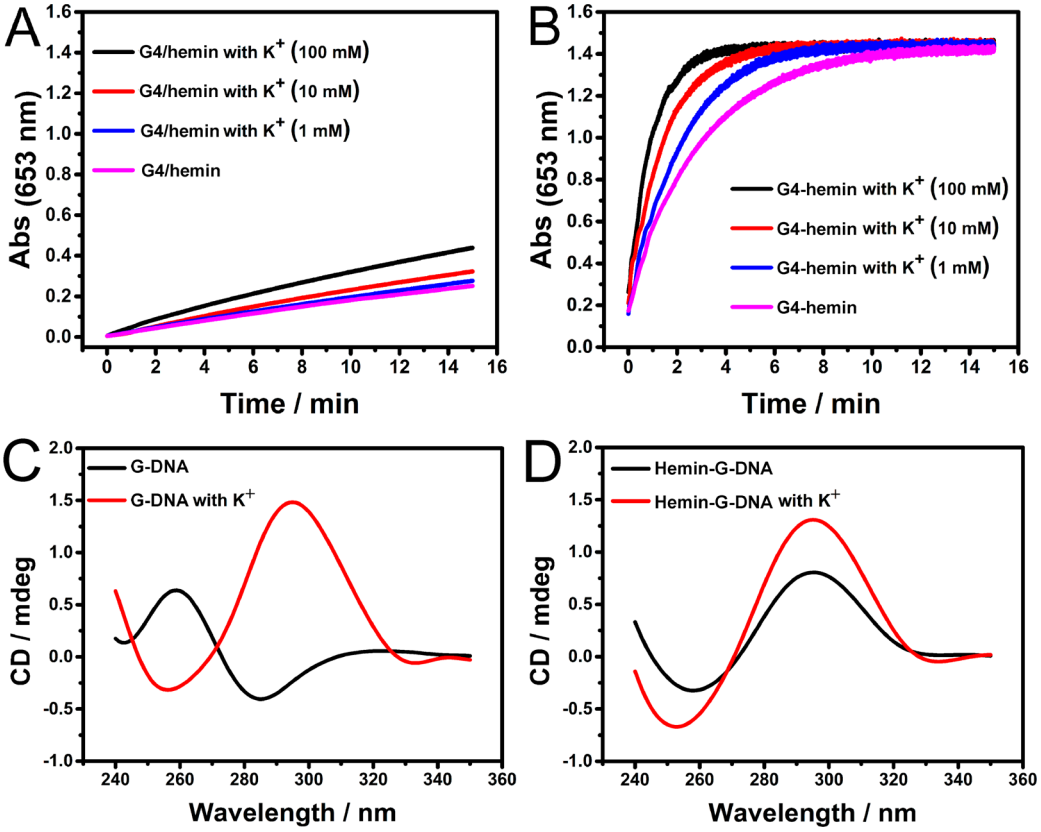

Figure 2. Time-dependent curves of the UV−vis absorption intensity at 653 nm for (A) G4/Hemin and (B) G4-Hemin at various K^+ ion concentrations: 0, 1, 10, and 100 mM (from bottom to top). CD spectra of (C) G-DNA with and without K^+ ions as well as (D) hemin-G-DNA with and without K^+ ions.

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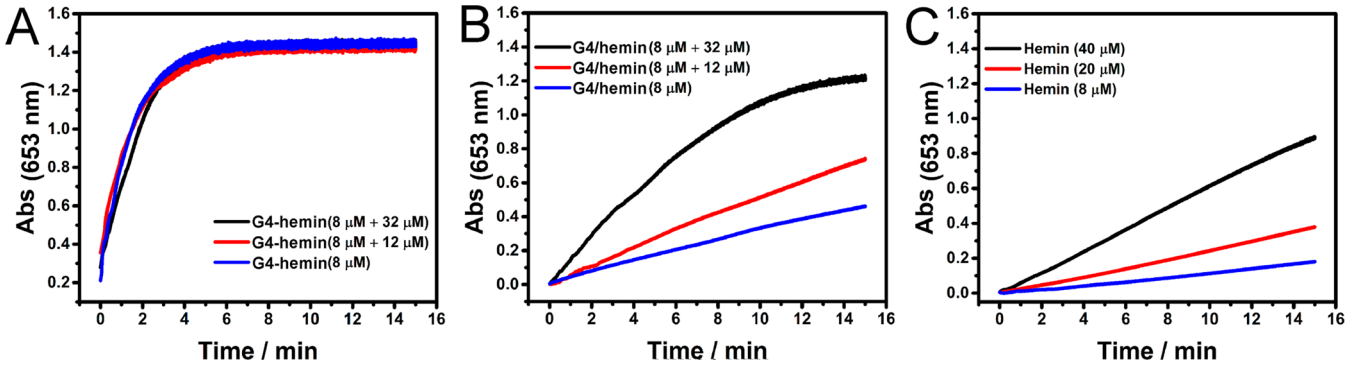

Figure 3. Time-dependent curves of the UV−vis absorption intensity at 653 nm for (A) G-hemin, (B) G/hemin, and (C) hemin. Blue, red, and black curves represent 8, 20, and 40 μM of total hemin in solution, respectively.

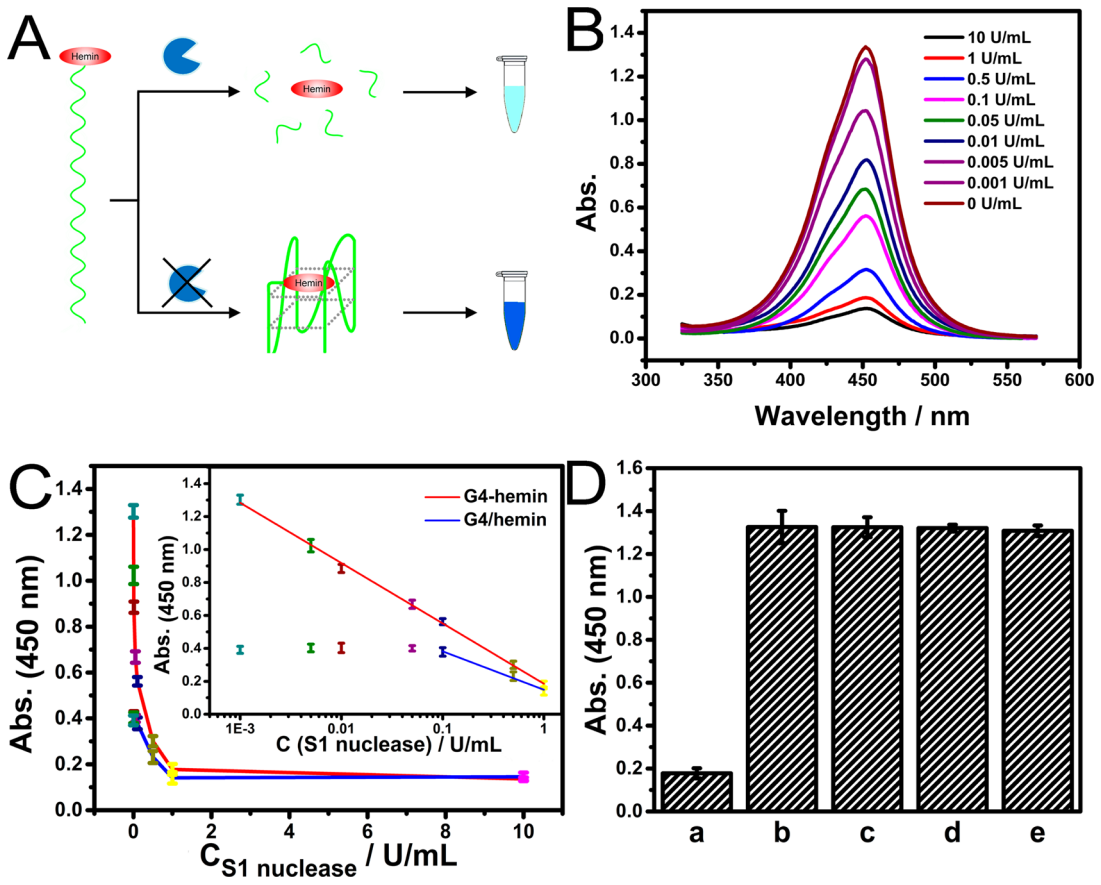

Figure 4. (A) Schematic representation of a S1 nuclease biosensor based on G4-hemin. (B) UV−vis absorption spectra of G4-hemin treated with S1 nuclease at concentrations ranging from 0 to 10 U/mL. (C) Absorption intensity (450 nm) of G4-hemin and G4/hemin as a function of the S1 nuclease concentration, which ranged from 0.001 to 10 U/mL. Inset: the corresponding calibration plot of the concentration of S1 nuclease as a function of the absorption intensity (450 nm) of G4-hemin and G4/hemin. (D) Selectivity of G4-hemin detection system toward (a) S1 nuclease, (b) S1 nuclease without Zn^{2+} ions, (c) inactive S1 nuclease, (d) 1 mg/mL pepsin, and (e) 1 mg/mL BSA. The error bars represent the standard deviations of three parallel tests.

activity was enhanced by the addition of a high concentration of K^+ ions, the peroxidase activity of G4/hemin was much lower than that of G4-hemin prepared without K^+ ions. As a result, using hemin-G-DNA, a peroxidase-mimicking DNAzyme can be constructed in the absence of metal ions.

CD spectrum is a powerful tool for analyzing the G-quadruplex structure. The positive peak at 295 nm is a CD peak that is characteristic of the antiparallel G-quadruplex structure.[35] A comparison of the two CD curves in Figure 2C indicates that G-rich DNA can form an antiparallel G-quadruplex structure only in the presence of K^+ ions. However, in the absence of K^+ ions, hemin-G-DNA still formed an antiparallel G-quadruplex structure (Figure 2D). The adding of K^+ ions to hemin-G-DNA only accelerated the formation. As is well-known that the target molecule can help the formation of secondary structure of aptamer, and hemin can take G-rich DNA as its aptamer. Therefore, hemin at the terminal end of DNA can take the responsibility of K^+ ions to induce the formation of G-quadruplex structure, and the K^+ ions are no longer necessary for G4-hemin. It is worth noting that by

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linking hemin with antiparallel G-quadruplex structure, peroxidase activity will be greatly enhanced, which is consistent with the previous report.[24]

Effort of Hemin on the Peroxidase Activity of G4/Hemin and G4-Hemin. Hemin plays a crucial role in the peroxidase activity.[36] As previously mentioned, the activity of G4-hemin was higher than that of G4/hemin. Therefore, more experiments were performed to gain insight into the effort of hemin. G4-hemin was formed by hemin-G-DNA, and hemin was covalently linked to the terminal end of DNA. Therefore, without the addition of exogenous hemin, the amounts of hemin and DNA in the detection system were the same (8 μM). If more exogenous hemin was added during the preparation of G4-hemin, no conspicuous improvement in the activity was observed in time-dependent curves (Figure 3A), because hemin was modified at the terminal of DNA with a ratio of 1:1 and induced the formation of G-quadruplex structure. This part of hemin was sufficient and preferential to assemble with the G-quadruplex structure as the catalytic center. More exogenous hemin could not further assemble with G-quadruplex structure. Therefore, the activity was not improved. In contrast, the activity of G4/hemin was substantially enhanced as the amount of hemin increased (Figure 3B). Because G4/hemin was fabricated with exogenous hemin in solution, more hemin facilitated its self-assembly with the G-quadruplex structure. Additionally, it is important to note that a large portion of the enhanced activity may be due to the large background signal generated by the hemin in solution (Figure 3C). On the basis of these results, modified hemin has a higher assembly efficiency and lower background than hemin in solution, which benefits the fabrication of biosensors.

Application of G4-Hemin and G4/Hemin in S1 Nuclease Detection. To demonstrate the applicability of this metal ion-free, low background G4-hemin, we designed a colorimetric biosensor for S1 nuclease detection. S1 nuclease is a single-stranded nucleic acid endonuclease that can catalyze ssDNA and ssRNA cleavage to yield small oligonucleotide fragments or mono-oligonucleotide.[37,38] Therefore, in the presence of S1 nuclease, hemin-G-DNA was cleaved into isolated hemin and small DNA fragments, which cannot form G4-hemin and will not catalyze the color change of TMB. However, if S1 nuclease is absent, hemin-G-DNA can form G4-hemin, resulting in a distinct color change of TMB. Based on these principles, S1 nuclease can be quantified (Figure 4A).

By mixing oxidized TMB with acid, the color of solution changes from blue to yellow.[26] The yellow solution is much steadier and thus chosen to quantify the S1 nuclease in our studies. As the amount of S1 nuclease increased, more hemin-G-DNA was degraded. Therefore, the color of obtained solution decreased correspondingly, as shown in the UV−vis absorption spectra (Figure 4B). The absorption intensities at 450 nm ($A_{450\ nm}$) were correlated with the concentrations of S1 nuclease ($C_{S1\ nuclease}$) in the range from 0.001 to 10 U/mL (Figure 4C). Additionally, a linear dependency between $A_{450\ nm}$ and $C_{S1\ nuclease}$ in the range from 0.001 to 1 U/mL was obtained (inset of Figure 4C). The regression equation was $A_{450\ nm} = 0.186-0.366\ \lg C_{S1\ nuclease}$ (U/mL), $R = 0.995$. The calculated detection limit for this biosensor was 5.9×10^{-4} U/mL (S/N = 3). The linear range and detection limit were better than or at least comparable to S1 nuclease biosensors based on G4/hemin and several typical S1 nuclease biosensors, which have been summarized in Table 1.[39−43] which may be due to the desirable properties of G4-hemin, such as high activity, free of metal ions and low background signal. Because S1 nuclease is a Zn^{2+}-dependent enzyme, the cleavage assay of hemin-G-DNA without Zn^{2+} ions was performed. In addition, several other proteins (i.e., inactive S1 nuclease, and pepsin and BSA) were also tested (Figure 4D). The satisfactory results confirm the excellent selectivity of this colorimetric biosensor.

To confirm the feasibility for real sample detection, we tested this S1 nuclease biosensor in serum. Different concentrations of S1 nuclease were spiked in 10% fetal bovine serum followed by detection with G4-hemin and G4/hemin. Although there were complex components in fetal bovine serum, the recovery percentage and relative standard deviation (RSD) obtained from G4-hemin was much better than that from G4/hemin, suggesting that G4-hemin is reliable in real sample detection (Table 2).

Table 1. Comparison of the Performance of Several Typical S1 Biosensors[a]

method	detection limit (U/mL)	linear range (U/mL)	ref
fluorescence	5×10^{-4}	5×10^{-3} to 2	39
colorimetry	4.3	NR	40
fluorescence	4×10^{-2}	4×10^{-2} to 4×10^{-1}	41
photoluminescence	1.4×10^{-3}	4×10^{-3} to 1.6×10^{-2}	42
fluorescence	3×10^{-1}	1 to 5	43
colorimetry	5.2×10^{-2}	1×10^{-1} to 1	this work
colorimetry	5.9×10^{-4}	1×10^{-3} to 1	this work

[a]NR represents not reported.

Table 2. Serum Sample Tests of S1 Nuclease with G4-Hemin and G4/Hemin[a]

sample	methods	added (U/mL)	found (U/mL)	recovery (%)	RSD (%, $n = 3$)
sample 1	G4-hemin	0.50	0.52	104.0	3.67
	G4/hemin	0.50	0.54	108.0	5.78
sample 2	G4-hemin	0.10	0.094	94.40	2.23
	G4/hemin	0.10	0.12	120.0	9.52
sample 3	G4-hemin	0.05	0.047	93.60	3.89
	G4/hemin	0.05	N/A	N/A	N/A

[a]N/A represents not applicable.

CONCLUSIONS

In this work, a novel kind of peroxidase-mimicking DNAzyme, i.e. G4-hemin, was constructed using hemin-modified G-rich DNA. Because hemin can efficiently induce the formation of G-quadruplex structure, G4-hemin can be fabricated in one step without the aid of metal ions, which simplifies the construction procedure of peroxidase-mimicking DNAzyme and removes the interference from metal ions. Moreover, hemin modified at the terminal of G-rich DNA exhibited an enhanced capability of assembling with G-quadruplex structure. Therefore, the addition of exogenous hemin can be avoided, which substantially reduce the background signal from excess hemin. It should be noted that G-rich DNA linked with hemin can still be operated by enzymic catalysis, metal ions-induced mismatch,

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molecular recognition, etc., which is of great importance of fabricating diverse biosensors. In addition, a facile S1 nuclease biosensor was designed as an example to confirm the applicability of G4-hemin. The excellent results demonstrated that enhanced analytical performances can be easily accomplished by G4-hemin, which provides an alternative approach for developing advanced peroxidase-mimicking DNAzyme-related biosensors.

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Notes

The authors declare no competing financial interest.

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