Electronic Supplementary Information

Superior G-quadruplex DNAzyme through Functionalized Modification of Hemin Cofactor

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Materials and Methods

Materials

Chemicals used in this study, such as hemin, 2,2'-azinobis(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS), 3,3',5,5'-tetramethylbenzidine (TMB) and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red, AR), were purchased from either Sigma-Aldrich or Alfa Aesar. The original hemin was dissolved in DMSO and filtered through 0.22-micron filter to get a clear solution. The exact concentration was determined by measuring the UV–Vis spectra in 0.1 N NaOH aqueous solution and calculated using the molar extinction coefficient of 58,400 cm^{-1} $(mol/L)^{-1}$ at 385 nm.[1]

All the oligonucleotides were purchased from Sangon Biotech (Shanghai, China), and the sequence information was listed in Table S1 and Table S2. All DNA/RNA samples were dissolved in ultrapure water and quantified by OD260 nm using the calculated molar extinction coefficient based on the sequence information. To form G-quadruplex, the oligonucleotides were heated in the designated buffer at 95°C for 5 min, followed by cooling down in ice for 5 min, and then maintained at room temperature for over 2 hr to pre-form the G-quadruplex structure.

Synthesis and characterization of modified hemin cofactors

N,N'-diisopropylcarbodiimide (213 μL, 1.38 mmol), 1-hydroxybenzotriazole (310 mg, 2.3 mmol) and the original hemin (30 mg, 0.046 mmol) were dissolved in 15 mL dry DMF. Amine derivatives, dimethylamine (2 mol/L in dry THF, 1.15 mL, 2.3 mmol), piperidine (210 μL, 2.3 mmol), 2-aminoethanol (138 μL, 2.3 mmol), N,N-dimethyl-1,2-ethanediamine (250 μL, 2.3 mmol), or histamine (255 mg, 2.3 mmol) were added into the mixed solution. The reaction mixture was then heated overnight at 60 °C. After reaction, the reaction mixture was filtered to remove the undissolved residue and purified by semi-preparative HPLC using Agilent Infinity 1260 system through Agilent ZORBAX SB-C18 column (9.2 mm×250 mm). HPLC conditions were described as followings: the ratios of mobile phase A (CH_3CN) over mobile phase B (0.1% HCOOH in H_2O) were 25% in 0~5 min, then gradually raised to 50% from 5 to 20, and further increased to 95% from 20 to 25 min; the column temperature was maintained at 25 °C, and the flow rate was 5.0 mL/min. The HPLC factions were collected and lyophilized to obtain the pure products. The analytic yields of modified hemin compounds were 92.1% for H-1, 69.6% for H-2, 49.2% for H-3, 71.8% for H-5, and 34.8% for H-H.

For synthesis of H-4, N,N'-carbonyldiimidazole (22.3 mg, 0.138 mmol) was mixed with hemin (30 mg, 0.046 mmol) in 15 mL dry DMF, and the solution was stirred at room temperature for 2 hr before dropwise addition of ethylenediamine (153 μL, 2.3 mmol). After stirred overnight at room temperature, the reaction mixture was processed and the produced

was purified following the same procedure as described above. The analytic yield of H-4 was 54.5%.

All these modified hemin cofactors were confirmed by high-resolution mass spec:

H-1: mass spectral analysis for $[C_{38}H_{42}N_6O_2Fe]^+$, (cal. 670.2719, found 670.2715);

H-2: mass spectral analysis for $[C_{44}H_{50}N_6O_2Fe]^+$, (cal. 750.3345, found 750.3340);

H-3: mass spectral analysis for $[C_{38}H_{42}N_8O_4Fe]^+$, (cal. 702.2617, found 702.2618);

H-4: mass spectral analysis for $[C_{38}H_{44}N_8O_2Fe]^+$, (cal. 700.2931, found 700.2931);

H-5: mass spectral analysis for $[C_{42}H_{52}N_8O_2Fe]^+$, (cal. 756.3563, found 756.3570);

H-H: mass spectral analysis for $[C_{44}H_{46}N_{10}O_2Fe]^+$, (cal. 802.3155, found 802.3148).

Purified hemin cofactors were dissolved in DMSO as stock solutions for following experiments, and the exact concentrations were determined by UV-Vis absorbance.

Measurement of enzymatic activity of hG4 DNAzyme

Kinetics experiments were performed using the Epoch 2 microplate spectrophotometer. Original or modified hemin cofactors (300 nM) were incubated with or without different sequences of G-quadraplex DNA (200 nM) in MES-NH_4OH buffer or other designated buffers (pH 6.0, 20 mM MES or other designated buffer components, 50 mM KCl and 1% DMSO) at room temperature for 0.5 hr to form the DNAzyme complex. Reductive substrate ABTS (2 mM) was pre-mixed with the DNAzyme complex before addition of H_2O_2 (2 mM). The reactions were initiated upon addition of H_2O_2, and the absorbance at 414 nm was monitored as a function of time. For the TMB substrate, the absorbance at 650 nm was monitored. For the AR substrate, the absorbance at 570 nm was monitored, and the percentage of DMSO in the solution was increased to 1.25% due to the low solubility of AR. Kinetic rates of peroxidase activities were calculated by fitting the absorbance increase in the first 30 s using the linear fitting equation by GraphPad Prism. Error bars were derived from at least three independent replicates.

Titration of UV-Vis spectra

Original or modified hemin cofactors (2.5 μM) were diluted into the MES-NH_4OH buffer (pH 6.0, 20 mM MES, 50 mM KCl and 1% DMSO). Different concentrations of structured G-quadruplex DNA (G4S-1 sequence) was added into the hemin solution to form the binding complex. All the mixed solutions were incubated at room temperature for 0.5 hr before measurements of UV-Vis spectra by Shimadzu UV-2600 spectrometer.

Calculation of binding constants

The binding affinity of hemin to G-quadruplex DNA was determined by plotting absorbance changes in the Soret band (404 nm) as a function of DNA concentration. The dissociation constant (K_d) was calculated by fitting the titration plot with the following equation by GraphPad Prism[2]:

$[DNA] = K_d\ A/(A_\infty - A) + [H]_0\ A/A_\infty$

where [DNA] is the concentration of DNA; $[H]_0$ is the initial concentration of hemin; A represents change of hemin absorbance at 404 nm in the presence of different concentrations of DNA. A_∞ represents the maximum change with saturated DNA concentrations.

Characterization of H_2O_2 mediated formation of peroxidation Intermediate

Original or modified hemin cofactors (2.5 μM) were incubated with structured G-quadruplex DNA (G4S-1 sequence, 2.5 μM) to form the DNAzyme complex in MES-NH_4OH buffer (pH 6.0, 20 mM MES, 50 mM KCl and 1% DMSO) at room temperature for 0.5 hr. Upon addition of H_2O_2 (2 mM), the absorption spectra of DNAzyme complex was recorded by Shimadzu UV-2600 spectrometer at various time points under the room temperature.

Kinetic assays for pH response of peroxidase activity

To investigate the pH response of DNAzyme activity, the phosphate buffer was utilized as reaction buffer to minimize the component variation during pH change. In addition, AR was selected as the reductive substrate since the oxidized product of AR was stable to pH change. H-0 and H-5 cofactors (300 nM) were incubated with two sequences of G-quadraplex DNA (G4S-1 and EAD2, 200 nM) in phosphate buffer (20 mM NaH_2PO_4/Na_2HPO_4 with different pH values, 50 mM KCl and 1.25% DMSO) at room temperature for 0.5 hr to form the DNAzyme complex. After mixing with AR, the absorbance at 570 nm was monitored by Epoch 2 microplate spectrophotometer upon addition of H_2O_2 (2 mM). Kinetic rates of peroxidase activities were calculated by fitting the absorbance increase in the first 30 s using the linear fitting equation by GraphPad Prism. Error bars were derived from at least three independent replicates.

Supporting Figures

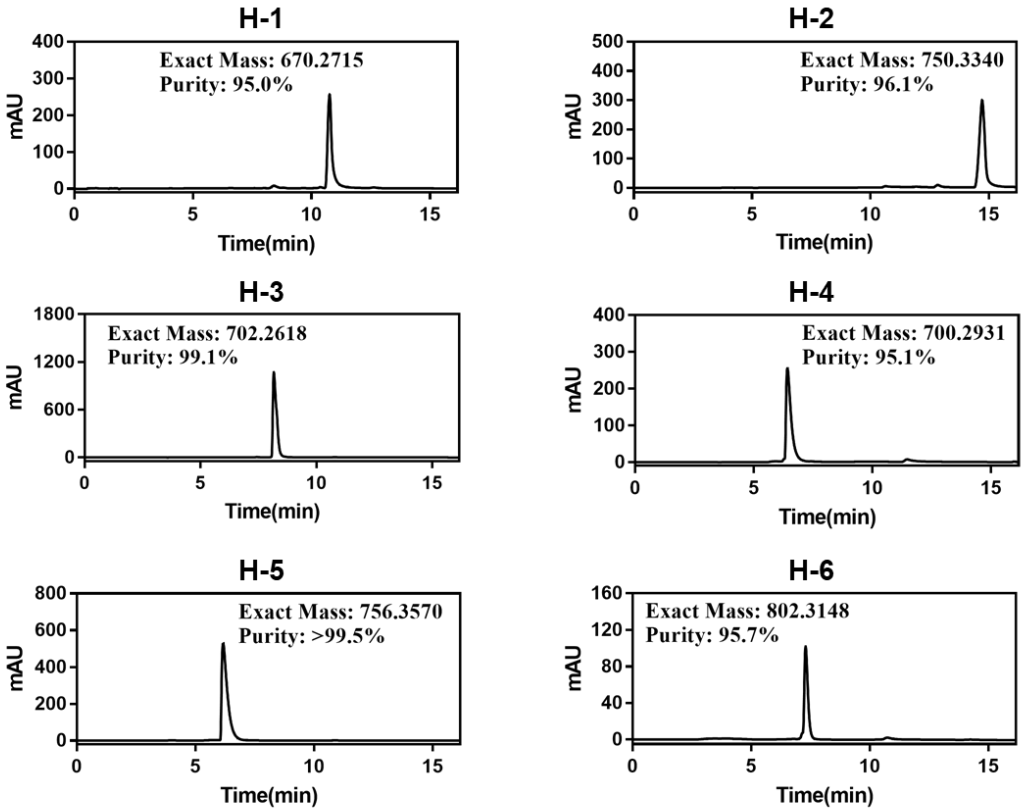

Figure S1. HPLC analysis of tail-modified hemin cofactors. HPLC was analyzed by the Poroshell 120 column (4.6 mm×150 mm) using Agilent Infinity 1260 system. HPLC conditions were described as followings: the ratios of mobile phase A (CH_3CN) over mobile phase B (0.1% HCOOH in H_2O) were gradually raised from 30% to 95% in 15 min, the column temperature was maintained at 40 °C and UV absorbance at 400 nm (characteristic absorption area of hemin structure) was monitored.

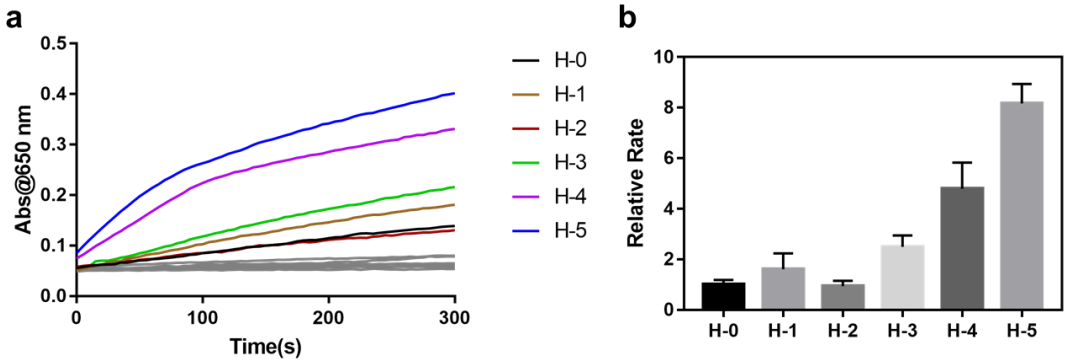

Figure S2. Peroxidase activity of G4S-1 hG4 DNAzyme using TMB as substrate. (a) Representative kinetic curves of increasing absorbance at 650 nm. (b) Relative kinetic rates with different modified hemin cofactors. The G-quadruplex forming sequence was G4S-1.

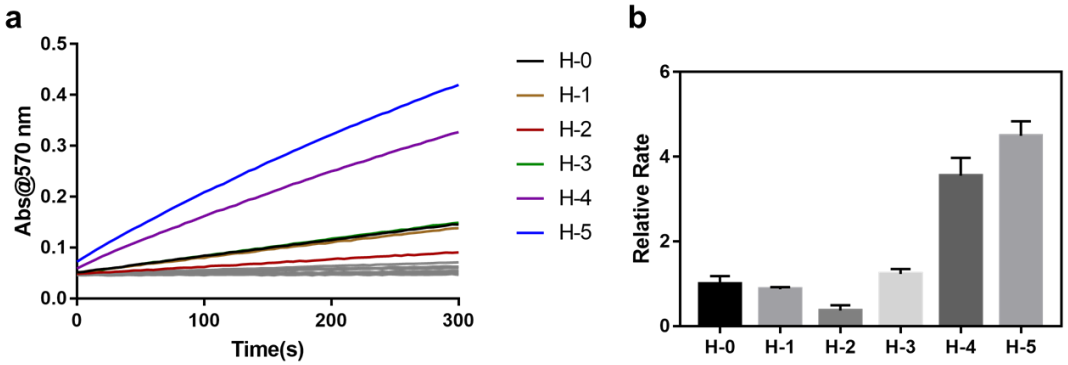

Figure S3. Peroxidase activity of G4S-1 hG4 DNAzyme using AR as substrate. (a) Representative kinetic curves of increasing absorbance at 570 nm. (b) Relative kinetic rates with different hemin cofactors. The G-quadruplex forming sequence was G4S-1.

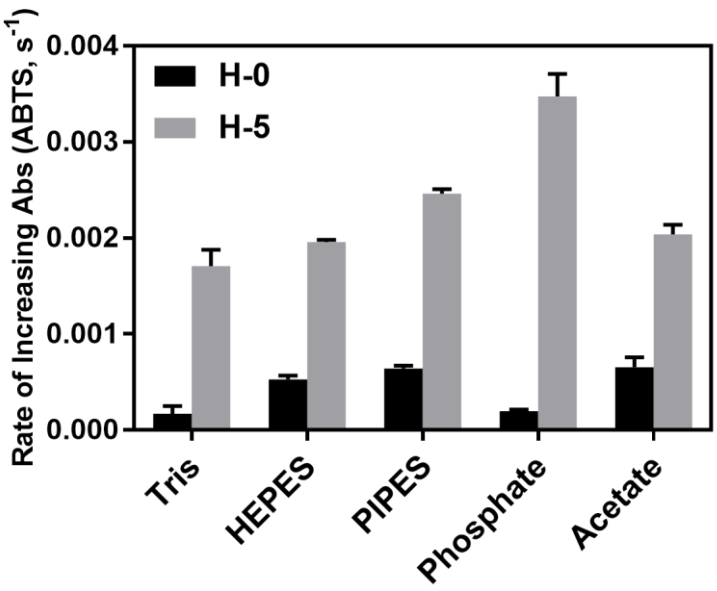

Figure S4. The enhancing effects of amine tail under different buffer conditions in G4S-1 DNAzyme. H-5 was selected as representative to compare with unmodified H-0. Five buffer conditions were Tris-HCl, HEPES-NH_4OH, PIPES-NH_4OH, sodium phosphate and sodium acetate. All these buffers were maintained at the same pH (6.0) and concentration (20 mM) as the MES buffer. The tail-modified H-5 exhibited a greatly enhanced peroxidase activity than the original H-0 under all the conditions of five different buffers, suggesting the generalized enhancement for the amine tail.

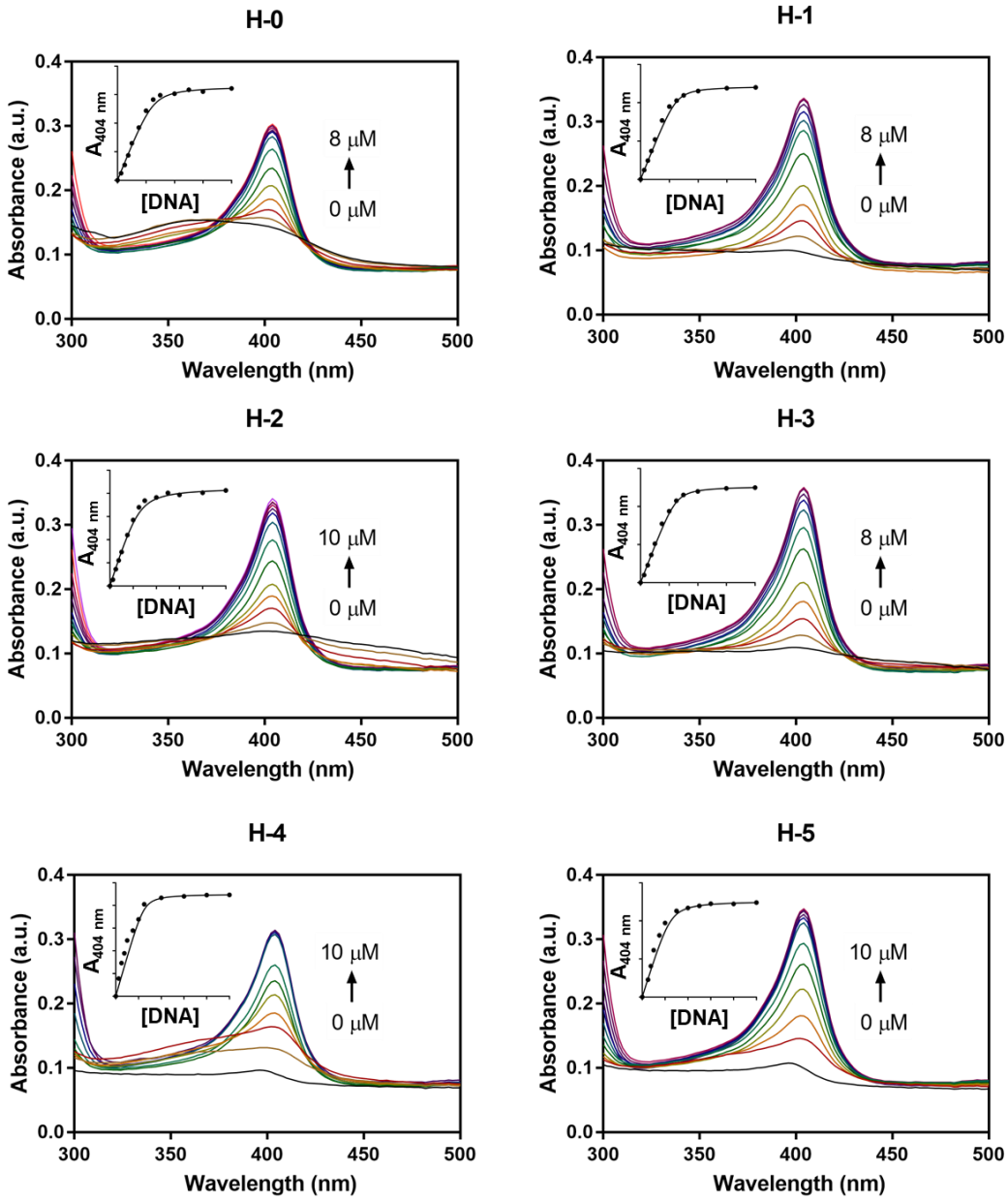

Figure S5. Binding affinity analysis of H-0, H-1, H-2, H-3, H-4, and H-5 with G4S-1 G-quadruplex DNA.

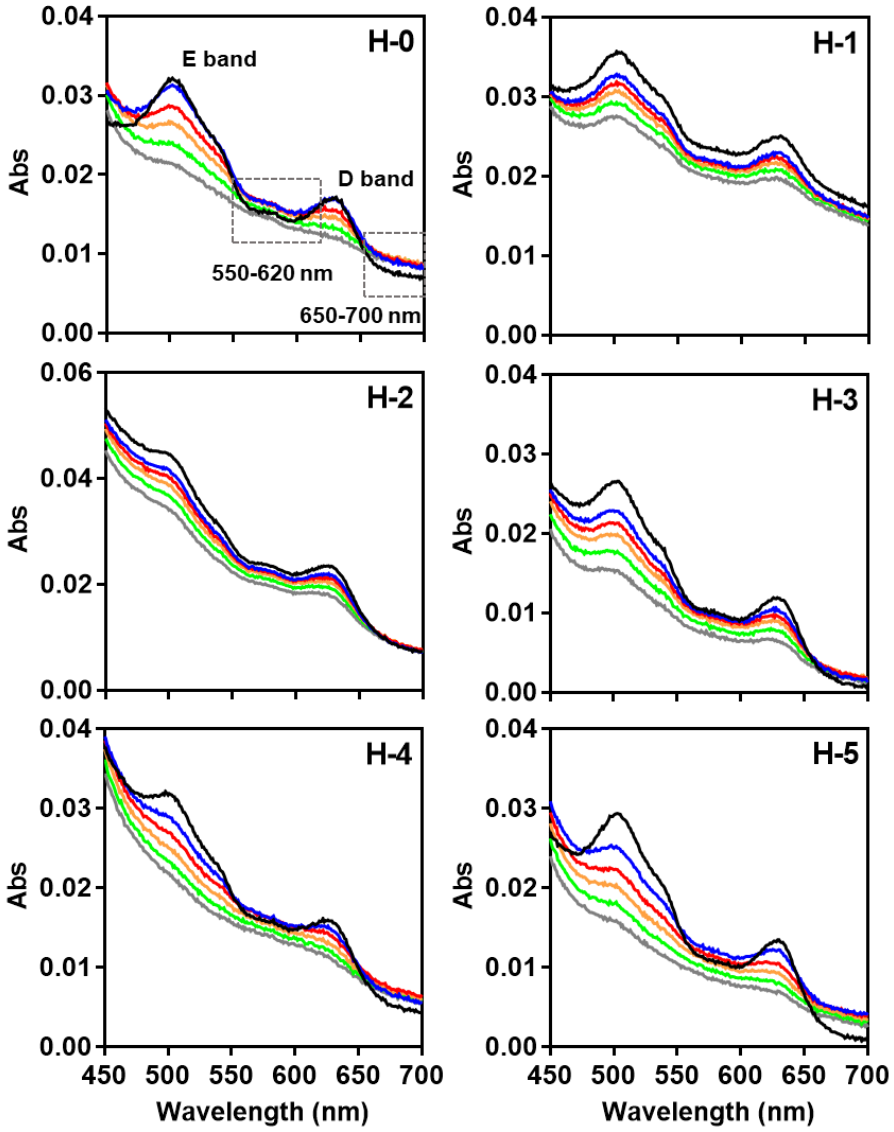

Figure S6. Time-dependent absorption spectra analysis to probe the formation of compound I-like intermediate during H_2O_2-caused oxidation of the hemin/G-quadruplex complex. These spectra were measured with the cofactor H-0, H-1, H-2, H-3, H-4, and H-5, respectively. Time points were 0 s (black), 20 s (blue), 60 s (red), 100 s (orange), 160 s (green), and 240 s (grey). Notably, different hemin derivatives exhibit slightly different absorbance spectra. Besides, modified hemin structures may possess different stabilities against the degradation caused the Compound I. Therefore, instead of comparing the absolute spectra changes between these different hemin structures, relative variations of signature regions for each hemin derivative during the time-dependent analysis may reveal more important insight. Hence, the observation of Compound I was evaluated and evidenced by the relative increase of absorbance over 550–620 and 650–700 nm along with the decreased E band (~500 nm) and D band (~630 nm) as previously reported.[3]

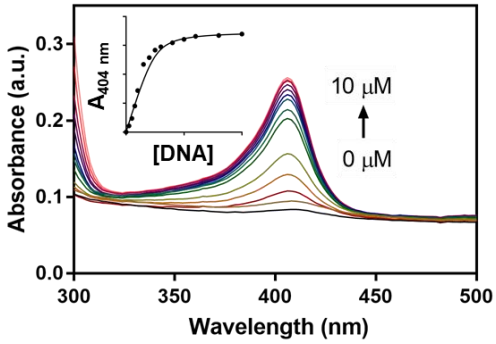

Figure S7. Binding affinity analysis of H-H with G4S-1 G-quadruplex DNA.

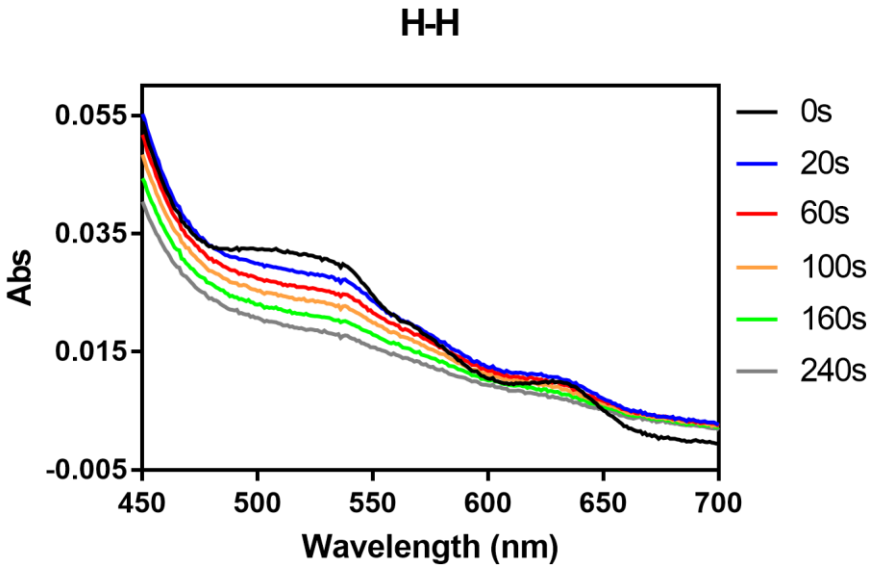

Figure S8. Monitoring the oxidation process in the presence of the modified hemin cofactor H-H with G4S-1 G-quadruplex DNA. Time-dependent absorption spectra analysis during H_2O_2 oxidation of the hemin/G-quadruplex complex. Herein, increase of absorbance over 550–620 and 650–700 nm along with the decreased E band (~500 nm) and D band (~630 nm) suggested detectable Compound I-like intermediate during H_2O_2 oxidation.

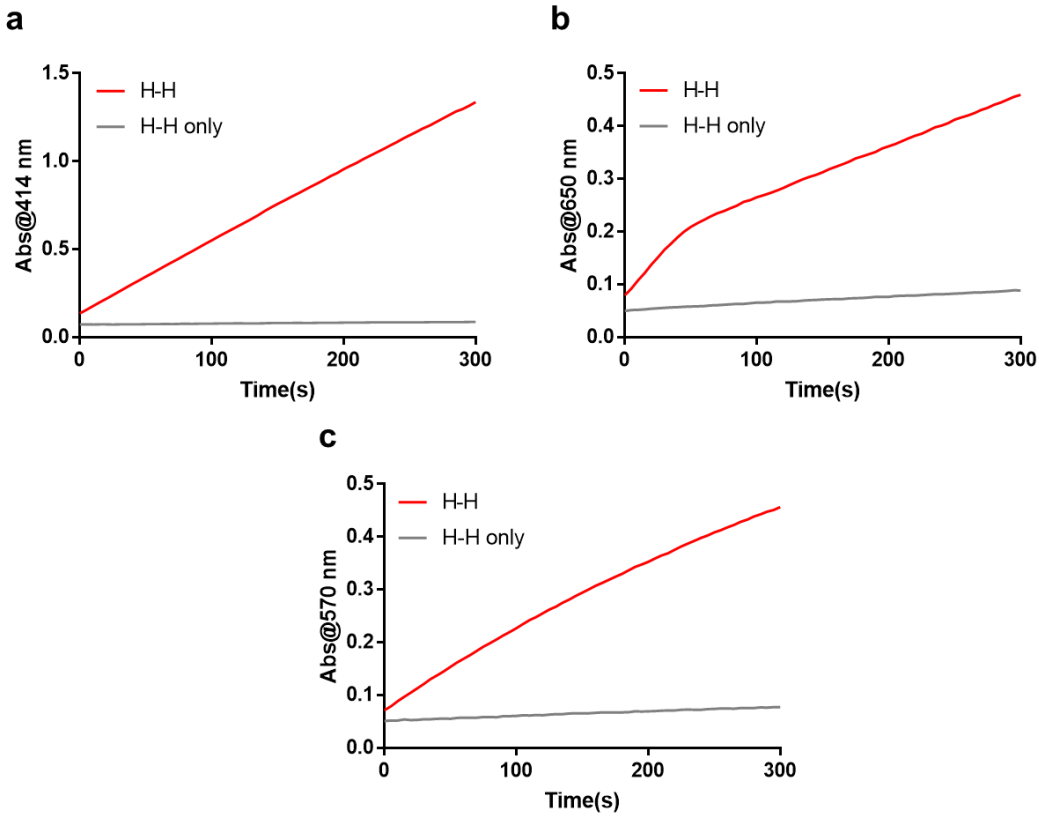

Figure S9. Representative kinetic analysis of peroxidase activity of hG4 DNAzyme with H-H as cofactor using ABTS (a), TMB (b), and AR (c) as substrate, respectively. Red curves indicated the peroxidase activity of the hG4 complex, and the grey curved indicated the intrinsic catalytic activity of H-H.

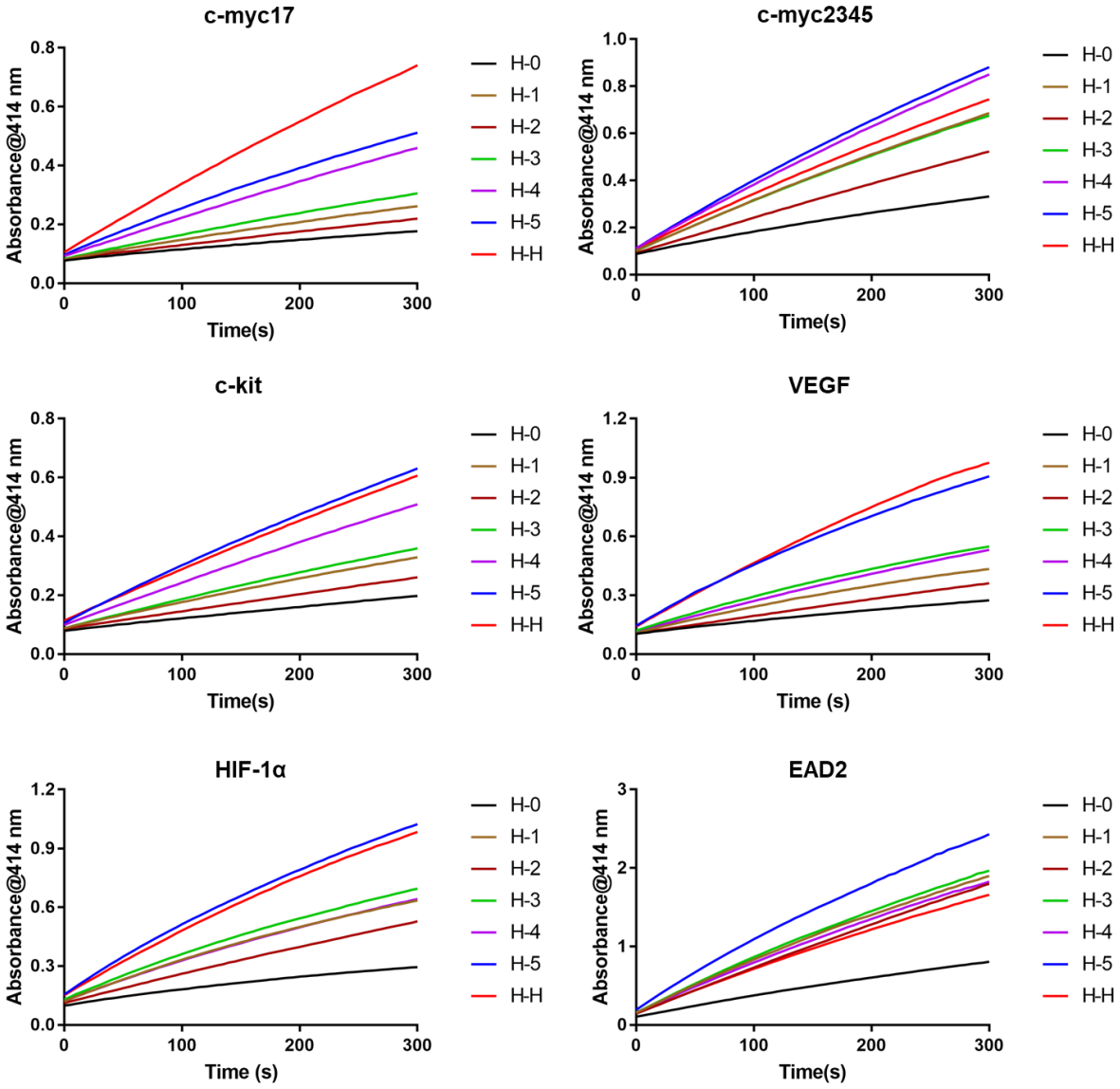

Figure S10. Representative kinetic curves for peroxidase activities of different hG4 DNAzymes with modified hemin cofactors using ABTS as substrate. Absorbance at 414 nm was monitored as a function of time.

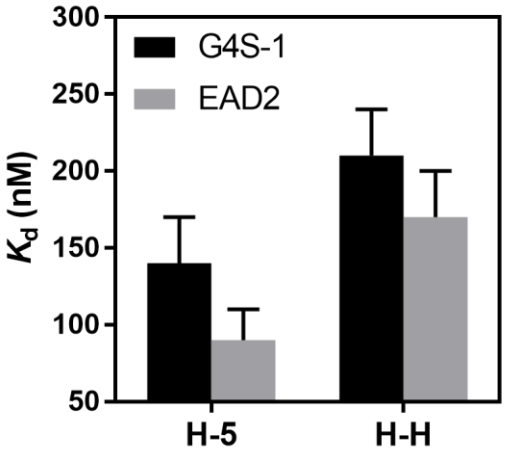

Figure S11. Comparison of dissociation constants (K_d) of H-5 and H-H in different G-quadruplex systems. K_d values of H-5 in G4S-1 and EAD2 systems were 140±30 nM and 90±20 nM, respectively; K_d values of H-H in G4S-1 and EAD2 systems were 210±30 nM and 170±30 nM, respectively. All these values were calculated from three independent measurements. Although both H-5 and H-H exhibited reduced K_d values in the EAD2 system when comparing with G4S-1, the binding enhancement for H-H (~1.2-fold change) is slightly weaker than that of H-5 (~1.5-fold change). This binding difference may partially contribute to the variations of H-H behaviors.

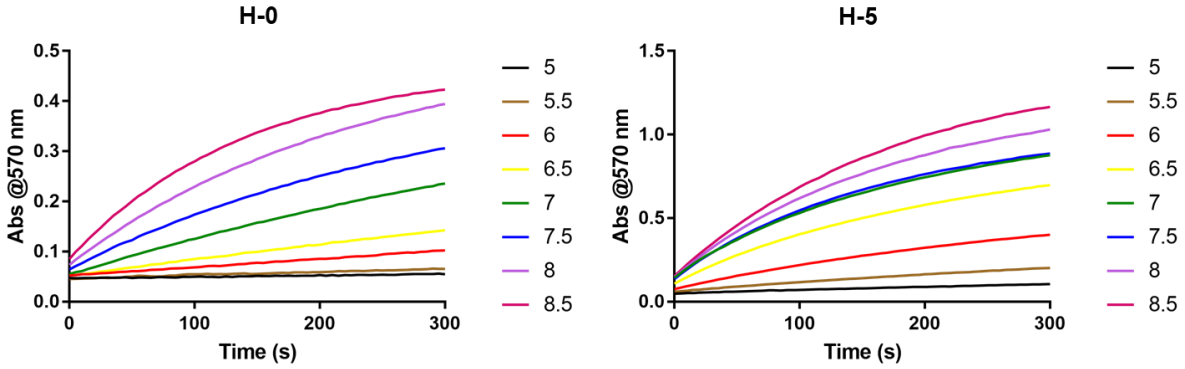

Figure S10. Representative kinetic curves for peroxidase activities of the G4S-1 DNAzyme at different pH conditions with cofactor H-0 and H-5 using AR as substrate. Absorbance at 570 nm was monitored as a function of time.

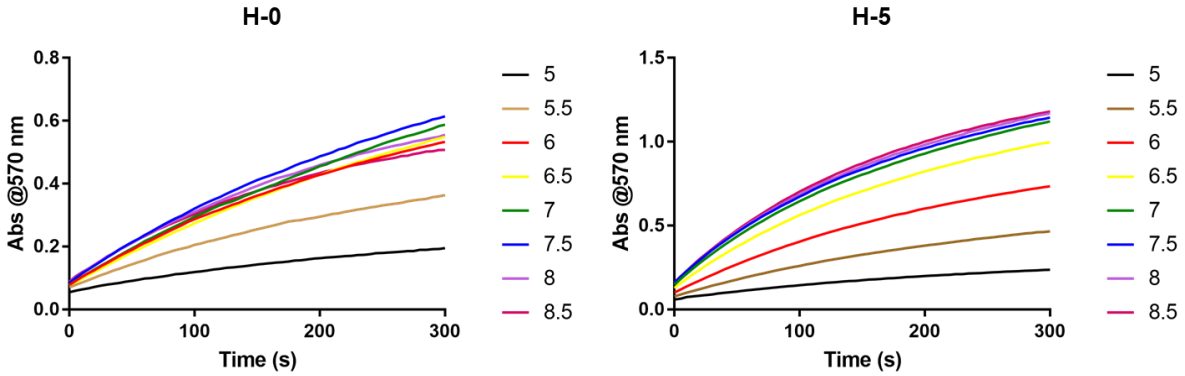

Figure S11. Representative kinetic curves for peroxidase activities of the EAD2 DNAzyme at different pH conditions with cofactor H-0 and H-5 using AR as substrate. Absorbance at 570 nm was monitored as a function of time.

Supporting Table

Table S1. DNA sequences used in this study.

Name	Sequence*
G4S-1	5'-GGGTAGGGCGGGTTGGG-3'
c-myc17	5'-GGGTGGGGAGGGTGGGG-3'
c-myc2345	5'-TGAGGGTGGGGAGGGTGGGGAA-3'
c-kit	5'-GGGCGGGCGCGAGGGGAGGGG-3'
VEGF	5'-CGGGGCGGGCCGGGGGCGGGGT-3'
HIF-1α	5'-AGGGCGGGGGAGAGGGGAGGGGC-3'
EAD2	5'-CTGGGAGGGAGGGAGGGA-3'

*Modified hemin cofactors exhibited distinct boosting effects in different G-quadruplex systems (Figure 4a). The c-my17 sequence can form a compact G-quadruplex similarly as the G4S-1 sequence. As a result, we indeed observed similar effects of these modified hemin cofactors between c-my17 and G4S-1. In contrast, in the sequence of c-myc2345, only a couple of flanking nucleotides were added in the both ends, but we observed varied effects among these modified tails, implying the potential interaction between the tails and the flanking nucleotides. The sequence c-kit is another type of G-quadruplex with a long internal loop, while VEGF, HIF-1α and EAD2 all have different terminal flanking nucleotides. Interestingly, the roles of H-4 and H-H tails were dramatically affected by the non-G-quartet nucleotides when comparing these G-quadruplexes with the compact c-myc17 and G4S-1 (Figure 4a). Previous investigations suggested that the hemin cofactor preferred to bind with the 3'-terminal G-quartet and the flanking nucleotides could affect the enzymatic activity.[4] Therefore, the functionalized tails, particularly for the amine (H-4) and imidazole (H-H) group, had a great probability to interact with those flanking nucleotides through electrostatic interaction and hydrogen bonding.

References:

1. J. R. Brozyna, J. R. Sheldon and D. E. Heinrichs, *Microbiologyopen*, 2014, **3**, 182-195.
2. S. Nakayama and H. O. Sintim, *J Am Chem Soc*, 2009, **131**, 10320-10333.
3. P. Travascio, P. K. Witting, A. G. Mauk and D. Sen, *J Am Chem Soc*, 2001, **123**, 1337-1348.
4. (a) W. Li, Y. Li, Z. Liu, B. Lin, H. Yi, F. Xu, Z. Nie and S. Yao, *Nucleic Acids Res*, 2016, **44**, 7373-7384; (b) Y. Guo, J. Chen, M. Cheng, D. Monchaud, J. Zhou and H. Ju, *Angew Chem Int Ed Engl*, 2017, **56**, 16636-16640; (c) T. Chang, H. Gong, P. Ding, X. Liu, W. Li, T. Bing, Z. Cao and D. Shangguan, *Chemistry*, 2016, **22**, 4015-4021.