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Supplementary information

Catalytic hairpin assembly mediated liposome-encoded magnetic beads for signal amplification of peroxide test strip based point-of-care testing of ricin

Qi Chao Ye[a] , Chen Men[a], Yuan Fang Li [a], Jia Jun Liu [b], Cheng Zhi Huang [b], Shu Jun Zhen *[a]

[a] Key Laboratory of Luminescence Analysis and Molecular Sensing (Southwest University), Ministry of Education, College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, P. R. China.

[b] College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, P. R. China.

*Corresponding author: FAX: +86 2368367257; Tel: +86 23 68254059; E-mail: zsj@swu.edu.cn

Contents

Experimental

Tables

Figures

References

1. Experimental Section

1.1. Materials and apparatus

All the DNA oligonucleotides (Table S1) designed in this study and glucose were purchased from Sangon Biotech (Shanghai, China). RTB was obtained from Vector Laboratories. Streptavidin-coated magnetic beads (MBs, with the size of 1 μm and std dev < 0.5 mm), cholesterol and lysozyme were purchased from Sigma Aldrich (St. Louis, MO). L-α-phosphatidylcholine (PC) and Horseradish per oxidase (HRP) were purchased from Aladdin (Shanghai, China). GOD was purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China).1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethyleneglycol)-2000] (DSPE-PEG) was purchased from Peng Shuo (Shanghai, China). Albumin Fraction V (AFr), pepsase and α-amylase were purchased from Xiya Reagent (Chengdu, China). 10×TNaK buffered solution was prepared by mixing 200 mM Tris-HCl, 1.4 mM NaCl, 50 mM KCl at pH 7.5. All other chemicals were of analytical-reagent grade. Millipore Milli-Q water (18.2 MΩ.cm) was used in all experiments.

Peroxide test strip is from Taobao and Hand-held instrument (H_2O_2 detector) for signal readout is from Millipore (USA). Solutions blended and MBs operated were using a vortex mixer Vortex-6 and Four-dimensional rotary mixer BE-1100 (Kylin-Bell, China), respectively. A thermomixer C was used for controlling temperature. A mini-extruder (Avestin Inc., Canada) was used for preparation of liposome.

1.2. Preparation of GOD-encapsulated liposomes (L_{GOD})

L_{GOD} was prepared according to the literature with slight modifications[1]. First, PC, cholesterol, and DSPE-PEG_{2000} (100:50:5 molar ratio, 0.026 g total) were added into 1 mL chloroform in a round-bottom flask. Then, the chloroform was removed in a rotary evaporator under reduced pressure at 40 °C for 30 min to form a thin lipid film and then, the flask was under vacuum drying for 2 h to remove residual organic solvent traces. Afterward, the dried

lipid film was hydrated with 5 mg/mL GOD (0.01 M PBS, pH 7.4) for 30 min with vigorous shaking at 40 °C to form cloudy suspension, and subsequently passing through 100 nm polycarbonate film to uniform the size of liposome. Thus, 1.757g GOD-encapsulated liposomes (L_{GOD}) was obtained. Finally, the untrapped glucose oxidase was removed by a dialysis bag with 300 kDa MWCO (SpectrumLabs, Rancho Dominguez, CA, USA) under 4 °C in PBS (0.01 M, pH 7.4) with stirring for 24 h, and 1.124g purified L_{GOD} was obtained. The prepared liposomes were stored at 4 °C for further use.

1.3. Prepartion of MBs-aptamer-blocker hybrid probe

First, 30 nM aptamer, 30 nM blocker and 10×TNaK solution was added together, and the mixture was incubated at 95 °C for 5 min. Then, the mixture was cooled to room temperature for 30 min. Subsequently, 200 μL 1 mg mL^{-1} streptavidin-coated MBs (resuspended in 1×TNaK solution) was added to the above mixture and incubated at room temperature for 30 min. After removing the supernatant, MBs was washed three times and finally resuspended in 240 μL 1×TNaK.

1.4. Prepartion of H1-modified MBs and H2-functionalized liposome

First, 40 nM biotinylated H1 was heated to 95 °C for 1 min and cooled to room temperature for 30 min and then, 200 μL 1 mg mL^{-1} streptavidin-functionalized MBs (resuspended in 1×TNaK solution) was added and incubated at room temperature for 30 min with gentle shaking. Subsequently, the resultant MBs-H1 was rinsed with 1×TNaK three times. Finally, the MBs-H1 was resuspended in 400 μL 1×TNaK and stored at 4 °C for further use.

H2 was functionalized on the surface of liposomes by inserting the cholesterol molecules into the lipid bilayer. Briefly, 120 μL 1 μM cholesterol modified H2 and liposome were mixed in 40 μL HEPES (100 mM NaCl, 10 mM HEPES, pH 7.4) at 25 °C with gentle shaking for 1 h. Then, unembedded H2 was removed through a dialysis bag with 300 kDa MWCO (SpectrumLabs, Rancho Dominguez, CA,

USA) in PBS at 4 °C for overnight.

1.5. CHA assembly mediated liposome-encoded MBs for RTB detection

MBs-aptamer-blocker probe was mixed with RTB and 1×TNaK, after incubation at room temperature for 40 min, the supernatant (trigger DNA, t-DNA) was obtained by magnetic separation. Then, 10 μL 0.5 mg mL^{-1} MBs-H1 and 16.4 μL H2-L_{GOD} was incubated with 21.6 μL t-DNA and 2 μL 1×TNaK at 37 °C for 1 h. After, the unreacted component was removed and followed washing the MBs-H1-H2-L_{GOD} complex for three times with 1×PBS. Subsequently, 10 mM glucose solution (1% Triton X-100, 10 mM PBS, pH 5.5 for the most suitable pH of GOD) was added into MBs-H1-H2-L_{GOD} complex. After incubation for 40 min at 55 °C, liposomes were broken and GOD was released to catalyze the oxidation of glucose to produce sufficient H_2O_2, which can be captured by PTS with hand-held detector for achieving simultaneous naked-eye observation and quantitative detection

1.6. Preparation of DNA-GOD Conjugate.

DNA-GOD conjugate was prepared by the maleimide-thiol reaction using heterobifunctional linker sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) according to the reported methods. Briefly, 30 μL of thiol-DNA (1 mM), 2 μL of 1 M sodium phosphate buffer (pH 5.5), and 2 μL of TCEP (30 mM) were mixed and incubated at room temperature for 1 h. Then the mixture was purified using tubular ultrafiltration membranes (10 K) prior to washing with 0.1 M NaCl and 0.1 M sodium phosphate buffer PBS (buffer A, pH 7.3) by 8 times. Concurrently, 1 mg of sulfo-SMCC was added to 200 μL of GOD (5 mg/mL) in buffer A, followed by vortexing for 5 min and then shaking for 1 h at room temperature. The purified sulfo-SMCC-activated GOD was then incubated with the purified thiol-DNA at room temperature for 48 h. The solution was purified using tubular ultrafiltration membranes (30K) and washed with buffer A 8 times in order

temperature. The mixture was purified using tubular ultrafiltration membranes (10 K) and then washed with buffer A 8 to remove unreacted thiol-DNA and GOD[2-4].

1.7. Catalytic hairpin assembly single signal amplification

MBs-aptamer-blocker probe was mixed with RTB and 1×TNaK, after incubation at room temperature for 40 min, the supernatant (trigger DNA, t-DNA) was obtained by magnetic separation. Then, 10 μL 0.5 mg mL^{-1} MBs-H1 and 16.4 μL H2-GOD was incubated with 21.6 μL t-DNA and 2 μL 1×TNaK at 37 °C for 1 h. The unreacted component was removed and followed by washing the MBs-H1-H2-GOD complex for three times with 1×PBS. Subsequently, 10 mM glucose solution (1% Triton X-100, 10 mM PBS, pH 5.5 for the most suitable pH of GOD) was added into MBs-H1-H2-GOD complex. After incubation for 40 min at 55 °C, liposomes were broken and released GOD to catalyze oxidation glucose and produce sufficient H_2O_2, which can be captured by PTS with hand-held detector.

1.8. Liposome-encoded magnetic bead single signal amplification

MBs-aptamer-blocker probe was mixed with RTB and 1×TNaK, after incubation at room temperature for 40 min, the supernatant (trigger DNA, t-DNA) was obtained by magnetic separation. Then, 10 μL 0.5 mg mL^{-1} MBs-cDNA and 16.4 μL pDNA-L_{GOD} was incubated with 21.6 μL t-DNA and 2 μL 1×TNaK at 37 °C for 1 h. The unreacted component was removed and followed washing the MBs-cDNA-blocker-pDNA-L_{GOD} complex for three times with PBS. Subsequently, 10 mM glucose solution (1% Triton X-100, 10 mM PBS, pH 5.5 for the most suitable pH of GOD) was added into MBs-cDNA-blocker-pDNA-L_{GOD} complex. After incubation for 40 min at 55 °C, liposomes were broken and released GOD to catalyze oxidation glucose and produce sufficient H_2O_2, which can be captured by PTS with hand-held detector.

1.9. No signal amplification strategy for Point-of-care testing of ricin B-chain.

MBs-aptamer-blocker probe was mixed with RTB and TNaK, after

incubation at room temperature for 40 min, the supernatant (trigger DNA, t-DNA) was obtained by magnetic separation. Then, 10 μL 0.5 mg mL^{-1} MBs-cDNA and 16.4 μL pDNA-GOD was incubated with 21.6 μL t-DNA and 2 μL 1×TNaK at 37 °C for 1 h. The unreacted component was removed and followed washing the MBs-cDNA-blocker-pDNA-GOD complex for three times with PBS. Subsequently, 10 mM glucose solution (1% Triton X-100, 10 mM PBS, pH 5.5 for the most suitable pH of GOD) was added into MBs-H1-H2-GOD complex. After incubation for 40 min at 55 °C, liposomes were broken and released GOD to catalyze oxidation glucose and produce sufficient H_2O_2, which can be captured by PTS with hand-held detector.

1.10. Ricin detection in real samples

In order to verify the practicability of our method, we exploited it to the detection of ricin castor. First, a 34 g shelled castor beans sample was exactly weighed and then grinded into pulverization and transferred to a mortar. Second, 68 mL 200 mM PBS (pH 7.2) was added until ground to pulpiness. After lixiviating at 4 °C for 12 h, the mixture was placed into the 50 mL centrifuge tube. Third, the centrifuge tube was centrifuged at 4 °C for 30 min (4000 rpm/min), the middle liquid was collected after discarding the upper oil and lower pellet, this process was repeated two times. Finally, 40 mL extract solution was obtained after removing the residue by a filter membrane (0.22 μm).

1.11. Calculation of the number of GOD-encapsulated liposomes (L_{GOD})

The lipid molecules (N_{tot}) number composed of L_{GOD} was calculated according to equation (1):

$$N_{tot} = \frac{[4\pi\left(\frac{d}{2}\right)^2 + 4\pi\left[\frac{d}{2} - h\right]^2]}{\alpha_L} \quad (1)$$

Where d is the hydrodynamic diameter, h is the bilayer thickness, and α_L is the average head group surface area per lipid. The lipid bilayer thickness was assumed to

be 4 nm and α_L value for phosphatidylcholine, phosphoethanolamine and cholesterol were 0.65 ± 0.01 nm^2, 0.52 ± 0.01 nm^2, and 0.41 nm^2, respectively. Rely on those values and the molar fraction of each component, the α_L value obtained for our produced liposomes was 0.6 nm^2/lipid. The N_{tot} was calculated to be 2.0 $\times 10^5$ lipid molecules per liposome. The number of liposomes per milliliter (N_{lipo}) can be derived from the lipid concentration with Avogadro's number as shown in equation (2):

$$N_{lipo} = \frac{M_{lipid} \times N_A}{N_{tot} \times 1000} \quad (2)$$

Where M_{lipid} is the molar concentration of lipid and N_A is Avogadro's number. The number of GOD-encapsulated liposome was calculated based on the number of lipid molecules in a liposome and the lipid concentration used to compose the liposome solution. Given that the total concentration of lipid used to compose the liposomes is 13 mM, the number of liposomes N_{lipo} was calculated to be 3.9×10^{13} liposomes per mL.

During the fabrication process, the liposome solution lost during extrusion and dialysis step was estimated to be around 10 ±3% and 40.8±2%, respectively. The data were evaluated preparing similar liposomes with fluorescent phosphatidylcholine lipids and analyzed with UV-vis spectrometry. Thus, liposome recovery number after preparation is calculated to be 1.62×10^{12} liposomes per mL.

The number of glucose oxidase molecules encapsulated in one liposome was calculated according to equation (3):

$$N_{GoD} = \frac{M \times N_A}{M_r \times N_{lipo}} \quad (3)$$

Where M is enzyme mass, and Mr is the relative molecular mass according to the aforementioned equation. Therefore, the loading capacity was obtained 998 molecules/liposome.

1.12. Calculation of the encapsulation efficiency in L_{GOD}

Encapsulation efficiency (EE) was determined based on the ratio of the amount

of encapsulated GOD in the liposome to the GOD amount added in the preparation of the liposome (equation 4). According to the glucose can be catalyzed oxidation by GOD to generate H_2O_2, which can be quantitative measured by PTS and Hand-held instrument. Therefore, the number of H_2O_2 was used to determine the amount of GOD. The procedure has three steps. First, GOD calibration curve was obtained (Fig. S1) by measuring the produced H_2O_2 of the GOD, the linear equation is that PTS signal (mg/L)=0.51879 m ($\times 10^{-5}$ mg)-0.67167. Second, the liposome was lysed by Triton X-100. Finally, 50.3% of encapsulation efficiency was acquired by using total encapsulated GOD mass (mg) divide the added mass (10 mg).

$$EE = \frac{encapsulated\ GOD}{total\ added\ GOD} \times 100\ \% \qquad (4)$$

1.13. The enzyme activity influenced by surfactant

The influence of Triton X-100 on GOD activity was investigated because of released GOD activity was directly related to the sensitivity of the sensing strategy. Due to GOD can catalyze glucose to generate H_2O_2, which can be quantitative measured by PTS and Hand-held instrument. Therefore, the amount of H_2O_2 was used to evaluate GOD activity influenced by Triton X-100. The definite process is: 3×10^{-4} mg GOD, 2 mM glucose and 1 % Triton X-100 were added together and incubated at 55 °C for 5 min. PBS solution was used to replace Triton X-100 as a control. The result is that H_2O_2 has slight difference in the present of Triton X-100 (Fig. S2). It was confirmed that Triton X-100 has no influence on GOD activity and thus guarantee the sensitivity of the sensing strategy.

2. Table Section

Table S1. The list of oligonucleotide sequences

Name	Sequence (5′ to 3′)
aptamer	Biotin-TTTTTACACCCACCGCAGGCAGACGCAACGCCTCGGAGACTAGCC
blocker	GGCTAGTCTCCGAGCGCACAA

H1-1	TTGTGCGCTCGGAGACTAGCCCCATGTGTAGAGGCTAGTCTCCGAGT$_{15}$-biotin
H1-2	TTGTGCGCTCGGAGACTAGCCCCATGTGTAGAGGCTAGTCTCCGAGT$_{20}$-biotin
H1-3	TTGTGCGCTCGGAGACTAGCCCCATGTGTAGAGGCTAGTCTCCGAGT$_{5}$-biotin
H1-4	TTGTGCGCTCGGAGACTAGCCCCATGTGTAGAGGCTAGTCTCCGAGT$_{10}$-biotin
H2	ACTAGCCTCTACACATGGGGCTAGTCTCCGAGCCATGTGTAGAT$_{20}$-cholesterol
H2-SH	ACTAGCCTCTACACATGGGGCTAGTCTCCGAGCCATGTGTAGAT$_{20}$-SH
cDNA	Biotin-T$_{20}$ACTTGTGCGCTCG
pDNA	GAGACTAGCCTAT$_{20}$-cholesterol
pDNA-SH	GAGACTAGCCTAT$_{20}$-SH

Table S2. Characterizations of GOD-free liposome and L_{GOD}.

Sample	mean diameter(nm)[a]	Polydispersiy index[a]	Zeta potential(mV)[a]
GOD-free Liposome	120±7.9	0.257±0.028	-7.4±0.8
L_{GOD}	142±9.5	0.294±0.019	-10.3±1.2

[a]Mean ± standard deviation, n=3; Mean diameter is the hydrodynamic diameter; Polydispersity index indicates the quantification of dispersity; Zeta potential indicates the average surface charge. Mean diameter, polydipersity index and surface charge were obtained by a Malvern Zetasizer instrument.

Table S3. Comparison of different ricin detection methods

Method	Type	Intrument	Detection limit	Detection time
Colorimetric[5]	antibody	eye	10 ng/mL	10 min
Aptamer arrays biosensor assay[6]	aptamer	micromachined chips	320 ng/mL	6 h
SPR[7]	aptamer	AFM	1.5 ng/mL	160 min
MS[8]	antibody	MS	2.5 ng/mL	16 h
Fluoroimmunoassay[9]	antibody	Fluorometer	1 μg/mL	12 h
Colorimetric[10]	aptamer	Microplate reader	20 ng/mL	1h
SPR biosensor[11]	antibody	hand-held SPR device	200 ng/mL	20 min
Immunochromatographic assay[12]	antibody	Chromatographic test	50 ng/mL	3 h
Electrochemiluminescent assay[13]	antibody	electrochemilumines cence	0.05 ng/mL	2.5 h

This work	aptamer	PTS	450 pg/mL	140 min

3. Figures

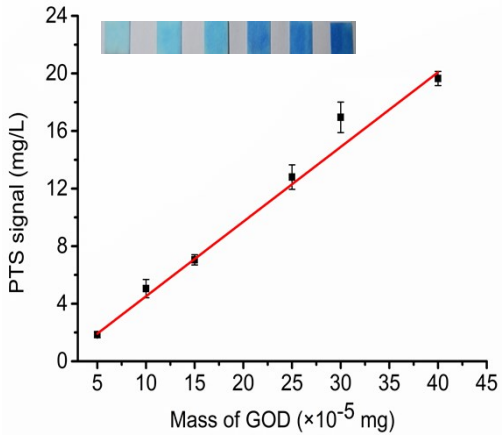


Fig. S1 PTS siganl intensity vs different mass of GOD. The experiments were operated in 2 mM glucose solution (1% Triton X-100, 0.01 M PBS, pH 7.4), inculated at 55 °C for 5 min. Inset shows the color of PTS gradually deepen with the increase of GOD.

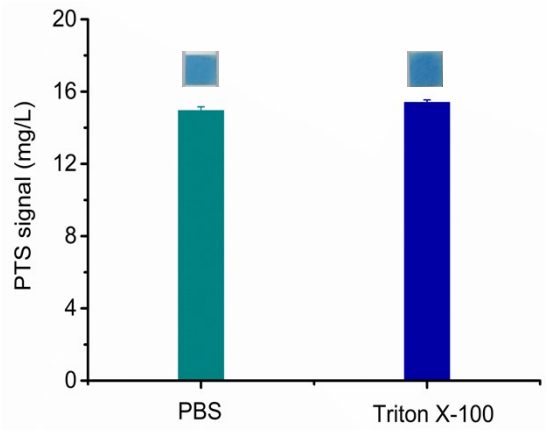


Fig. S2 Effect of Triton X-100 on GOD. The experiments were operated in 2 mM glucose solution (1% Triton X-100, 0.01 M PBS, pH 7.4), inculated at 55 °C for 5 min. Inset shows the color of PTS under different conditions.

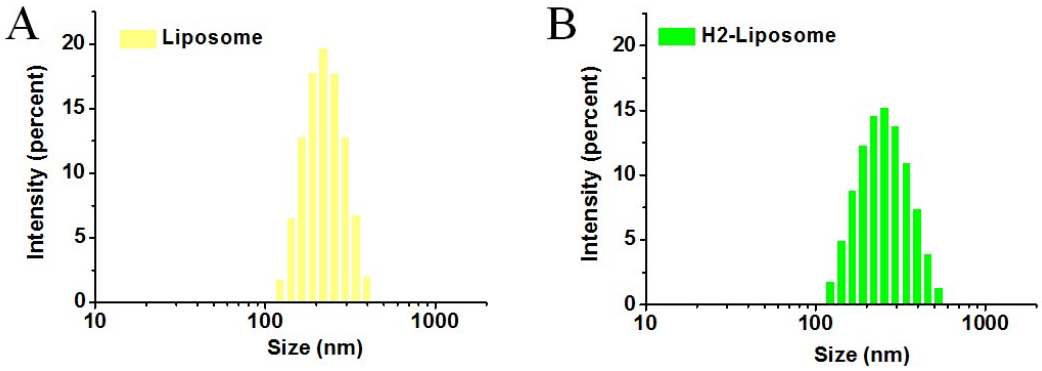


Fig. S3 The size of liposome (A) and H2-liposome (B), respectivity.

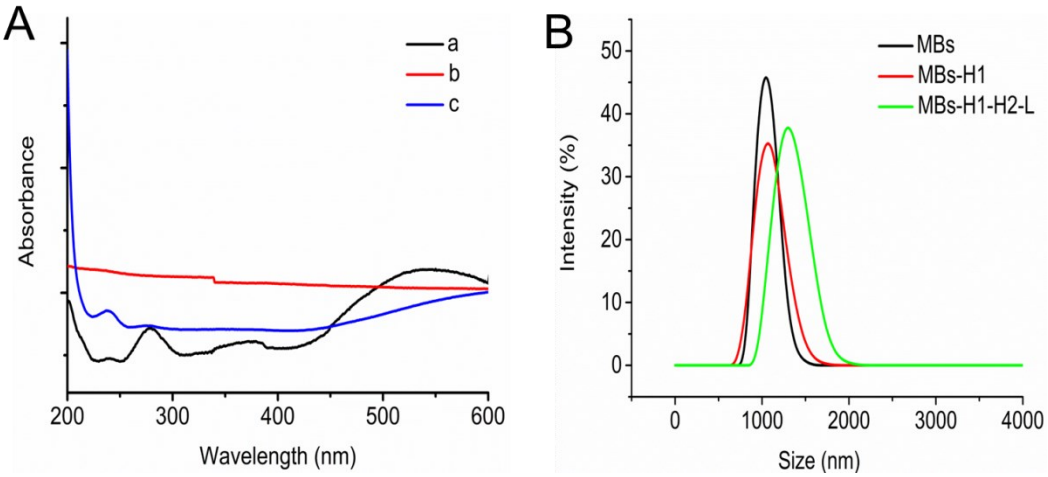


Fig. S4 (A) the absorbance of (a) MBs, (b) MBs-H1 and (c) MBs-H1-H2; (B) The size of MBs before and after modification of H1 (MBs-H1) and reaction with H2-Liposome (MBs-H1-H2-L).

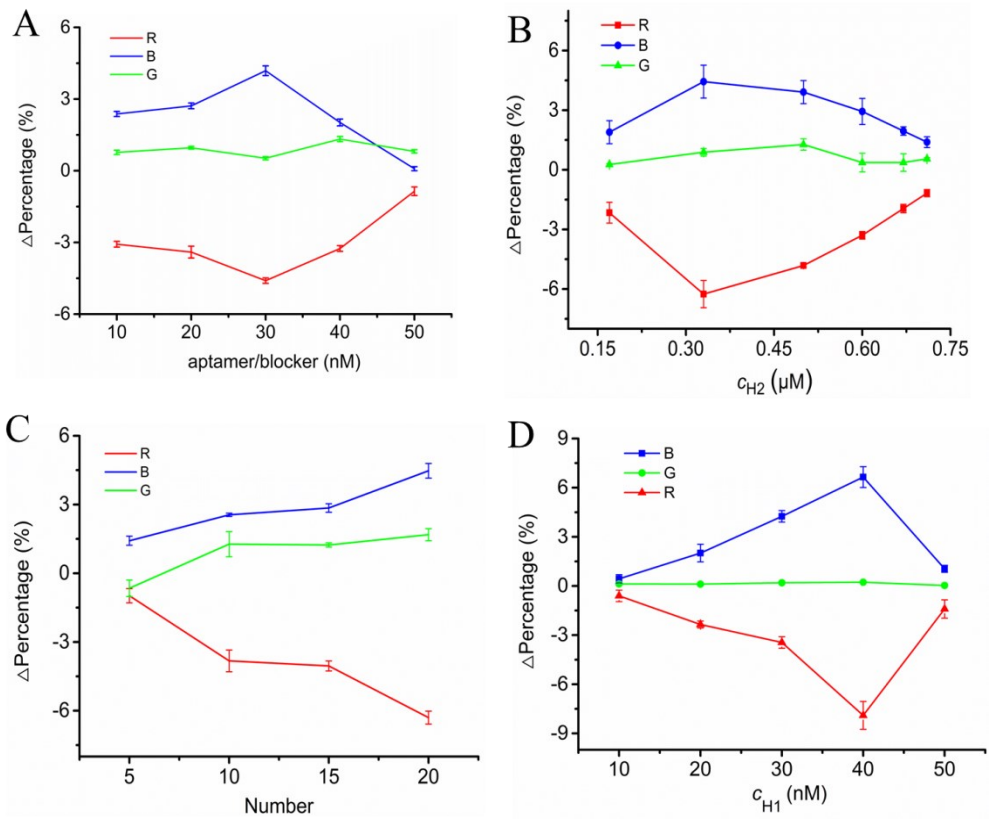


Fig. S5 The effect of different conditions on RGB analysis. (A) the concentration of aptamer/blocker; (B) the concentration of H2; (C) the number T bases on H1; (D) the concentration of H1.

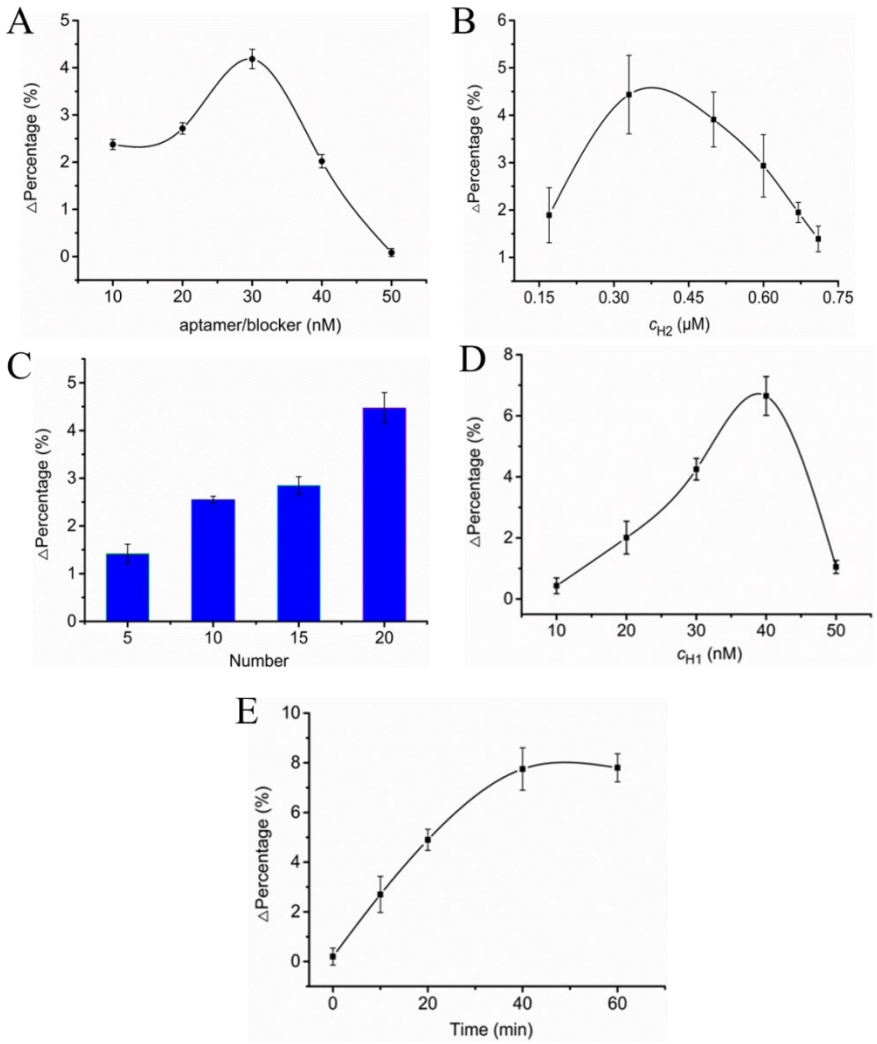


Fig. S6 The influence of different conditions (A) the concentration of aptamer/blocker; (B) the concentration of H2; (C) the number T bases on H1; (D) the concentration of H1; (E) enzyme reaction time.

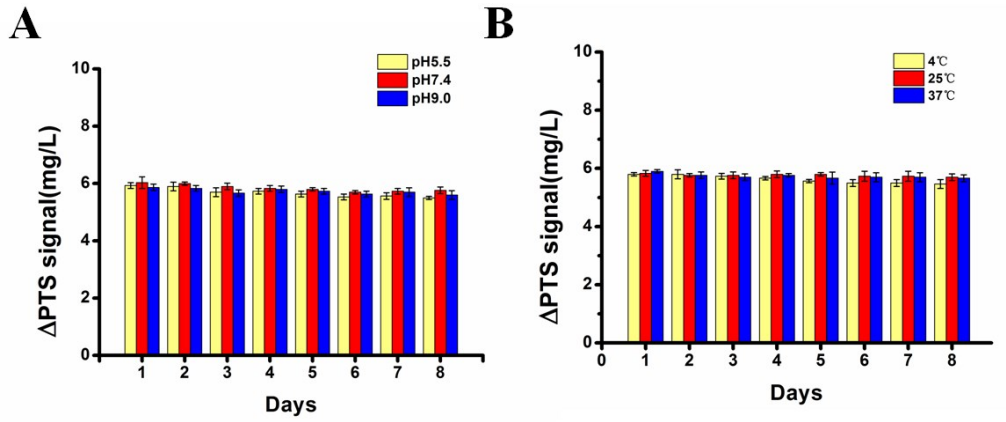


Fig. S7 The stability of L_{GOD} in different pH (A) and temperature (B).

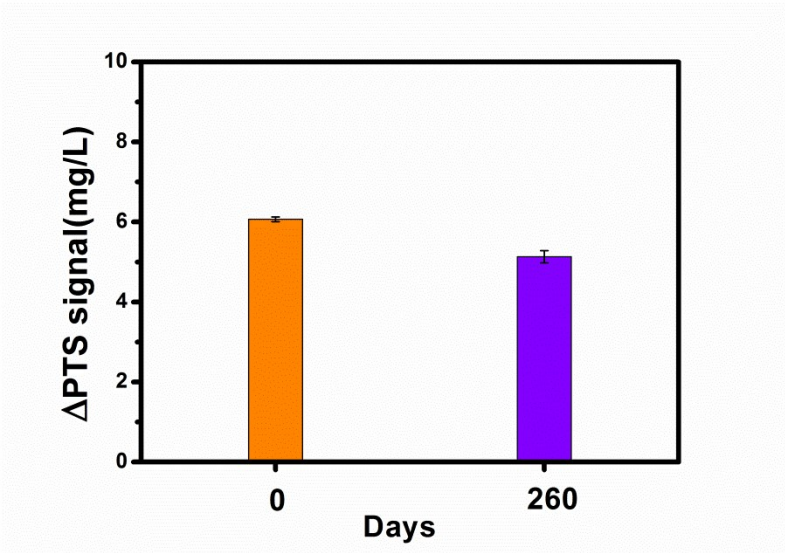


Fig. S8 The PTS signal of L_{GOD} before and after the storage at 4°C for 260 days.

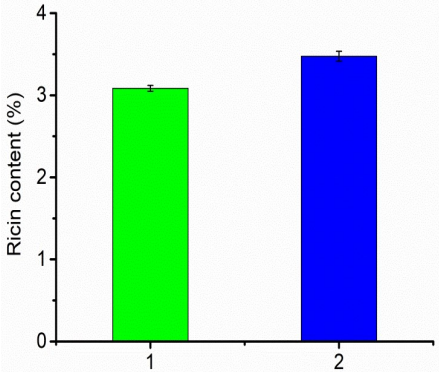
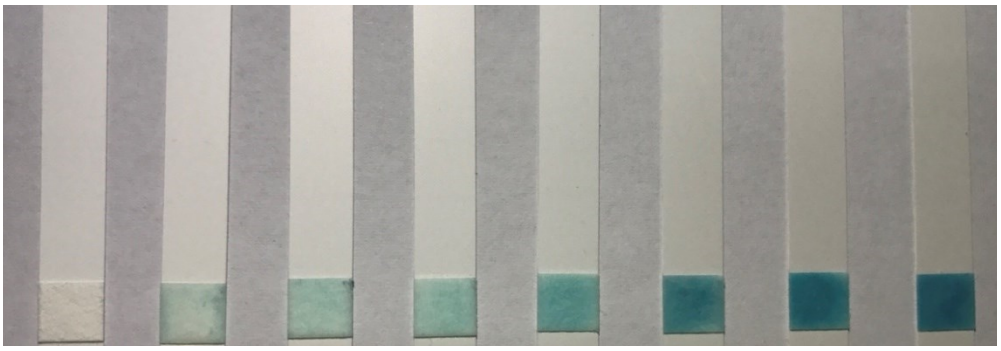


Fig. S9 The ricin content in caster beans. 1, the ricin content tested by ELISA kit, which was 3.09 ± 0.04%; 2, the ricin content tested by our method, which was 3.48 ± 0.06%. The error bars showed the standard deviation of three replicate determinations.



The predicted results (μM)	0	10	30	60	130	260	390	520
The obtained results (μM)	0	10	30	62	128	265	394	518

The predicted results (mM)	0	2.0	4.0	6.0	12.0	18.0	24.0	30.0
The obtained results (mM)	0	1.7	4.4	5.9	11.0	18.2	25.0	30.3

Fig. S10 Comparison between two signal readout systems: PTS is used as portable detector for H_2O_2 and PGM is used as portable device for glucose.

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