Supplementary Materials for

A novel CRISPR/Cas14a-synergized wood-based platform for ultrasensitive detection of aflatoxin B1

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Materials

Balsa wood was purchased from a store (Changsha, China). Bovine serum albumin (BSA) was supplied by Dingguo Biological Products (Beijing, China). Polyethyleneimine (PEI) and Glutaraldehyde (50% aqueous solution) were obtained from Beijing J&K Scientific Ltd. Aflatoxin B1 (AFB1) and horseradish peroxidase (HRP) were supplied by Macklin Biochemical Co., Ltd. (Shanghai, China). Streptavidin (SA) was supplied by Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) was purchased by Bepharm Science&Technology Co., Ltd. (Shanghai, China). Cas14a1 was supplied by Hongming Biotechnology Co., Ltd. (Nanjing, China). All DNA and RNA oligonucleotides used in this work were synthesized and purified by GenScript Biotech Co., Ltd. (Jiangsu, China). The sequences were listed in Table S3. All other chemicals were at least of analytical reagent grade and all buffer solutions were prepared with ultrapure or sterile water.

Phosphate buffered (PB, pH 7.4) was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. The washing buffer was PB (10 mmol/L, pH 7.4) containing 0.5% (w/v) Tween 20 (PBT). Blocking buffer was PB (10 mmol/L, pH 7.4) containing 10% (w/v) BSA and 0.1% (w/v) Tween 20 (PBBT). The chromogenic substrate was prepared by mixing 10 mmol/L TMB (dissolved in anhydrous ethanol) with 20 mmol/L H_2O_2 solution (dissolved in acetic acid-sodium acetate buffer at pH 5).

Instruments

Electric Thermostatic Water Bath (DZKW-0-1); Vacuum Drying Oven (LC-10N-60A, Beijing Yongguangming); Electronic Balance (JF1004, Ningbo Jinnuo); pH Meter (PHs-25, Shanghai Leici); Scanning Electron Microscope (TESCAN MIRA LM, Czech Republic); Fourier Transform Infrared Spectrometer (Thermo Fisher Scientific

Nicolet iS5, USA); Transmission Electron Microscope (JEM-2100Plus, Japen), and other instruments.

Experimental methods

Surface modification of wood chip.

Firstly, the balsa wood was cut into dimensions of 3 mm × 2 mm × 1 mm with a cutting machine. The cut surface was marked with a marker pen, using the opposite side of the marked surface as the test area (longitudinal section). The wood chip was washed with ultrapure water and then placed in a 1% NaOH solution at 70 ℃ for 30 minutes. It was rinsed repeatedly with ultrapure water five times. Subsequently, the wood chip was placed in PEI solution at 70 ℃ for 30 minutes, followed by three rinses with ultrapure water. Finally, it was immersed in a 2.5% glutaraldehyde solution in the dark at room temperature for 2 hours. After being rinsed with ultrapure water and dried at room temperature for a certain period.

Fixation of wood chip.

A white plastic plate (5 cm × 3 cm) was prepared. Three equally spaced fixing points were marked in parallel on the plastic plate, which was then swiftly dipped into the paraffin solution and removed, allowing the paraffin to solidify at room temperature. The wood chip was gently held with tweezers, and its base was sealed by dipping into 5 μL wax solution. Subsequently, 1.0 μL of adhesive was added to each fixing point. The wood chip was slowly pressed onto the plate until the glue had set.

Preparation of SA-AuNPs-HRP nanoprobe.

Firstly, sodium citrate reduction method was used to prepare AuNPs. 100 mL $HAuCl_4 \cdot 4H_2O$ (0.01%) was heated to boiling, and then 1.1 mL trisodium citrate (1%) was quickly added to this solution under magnetic stirring, in which the stirring rate was constant during the heating process. When the solution color changed from light yellow to deep wine red, heating was continued for 20 minutes. Subsequently, the AuNPs was cooled to room temperature and stored in brown glass bottles at 4 °C for

further use. And then 20 μg of SA solution was added to 5 mL of AuNPs solution and allowed to react at room temperature for 1 hour. Subsequently, 50 μL of HRP solution (1 mg/mL) was added and the mixture was incubated in the dark at room temperature for 1 hour. Finally, the mixture was centrifuged at 10,000 rpm for 5 minutes, after which 2.5 mL of 1% PBB solution was added and stored at 4 °C for subsequent use.

Preparation of CRISPR reporting mix (CRM).

According to the manufacturer's instructions, Cas14a1 (500 nmol/L, 0.3 μL) and corresponding sgRNA (3.125 μmol/L, 0.36 μL) were mixed in the 10×HM buffer for Cas14a1 (0.6 μL) at room temperature. And the volume of the solution was supplemented with sterile water to 15 μL for 10 minutes. Then, the ssDNA reporter modified by a biotin at both ends (B-B reporter) was added to the solution and mixed in an ice bath for 1 hour. In the final solution, the concentrations of Cas14a1, sgRNA and B-B reporter were 10, 75, and 400 nmol/L, respectively.

Procedures for AFB1 detection by using HARRY (highly sensitive aptamer-regulated Cas14 R-loop for bioanalysis).

1 μL of 10×HM buffer was mixed with 5 μL of aptamer (20 nmol/L), followed by adding 1.25 μL of the AFB1 and 2.75 μL sterile water. After incubating at 37 ℃ for 10 minutes, 15 μL of CRM was added and mixed gently, and then incubated at room temperature for 1.5 hour in a dark place for subsequent processing. This solution was named CRAM (CRISPR reporting and AFB1 mix).

Sample preparation.

A simple method was employed to extract AFB1 from peanut oil. 1 g of the certain brand peanut oil was weighed into a 10 mL test tube, and 2.5 mL of methanol was added. The mixture was vortexed for 5 minutes. Subsequently, it was centrifuged at 2000 rpm for 5 minutes, and the supernatant was transferred to another test tube for subsequent use in the spike recovery experiment.

The analysis of AFB1 based on wood and CRISPR/Cas14a.

A typical sandwich-type determining method was used to quantify AFB1. Firstly, 20 μL of 10 μg/mL SA (dissolved in 0.01 mol/L PB, pH 7.4) was immobilised onto

each wood chip and incubated at room temperature for 1 hour. After discarding the solution, the wood chip was washed 2 times with 350 μL PBT. Unbound sites were then blocked with 150 μL PBB at room temperature for 1.2 hour, then removed the liquid. Subsequently, 100 μL of 10 μmol/L ethanolamine was added and incubated at room temperature for 40 minutes, followed by washing the wood chip 2 times with 350 μL PBT. Next, 20 μL of CRAM standard or peanut oil sample was applied and allowed to react at room temperature for 30 minutes. After washing 2 times with 350 μL PBT, 20 μL of SA-AuNPs-HRP nanoprobe was added and incubated at room temperature for 30 minutes. The wood chip was then washed many times with 1000 μL PBT and gently blotted with filter paper. Finally, 20 μL of a mixed chromogenic solution (5 mmol/L TMB and 10 mmol/L H_2O_2 in acetate buffer, pH 5.0) was added and incubated in the dark for 3 minutes at room temperature. After incubation, the wood chip turned from yellow to blue, enabling visual qualitative detection of AFB1.

Data acquisition.

All images were captured using a smartphone placed in a fixed holder at a distance of 20 cm from the wood chip surface. A lighted photo studio was used to maintain consistent light intensity, angle, and color temperature across all measurements. Auto-flash and HDR were disabled, and manual focus was applied to ensure image sharpness. These standardized conditions ensured reproducible image acquisition for subsequent analysis using ImageJ software.

Results

Optimization of the ssDNA reporter concentration.

The ssDNA reporter played a crucial bridge role in the colorimetric reaction. As shown in Fig. S1, when the concentration of the ssDNA reporter was 400 nmol/L, the blank signal was minimal and the AFB1 detection signal reached its maximum. Therefore, 400 nmol/L was selected as the optimal reaction condition for the ssDNA reporter.

Optimization of capture probe concentration.

The amount of capture probe immobilized on the wood chip also influenced the sensitivity and stability of detection, so the concentration of SA was examined. As illustrated in Fig. S2, the blank signal remained essentially unchanged with increasing SA concentration, whereas the detection signal gradually increased in the presence of AFB1 until it declined at 20 nmol/L. So, 10 nmol/L was chosen as the optimal coating concentration for SA.

Effect of methanol dilution ratio on AFB1 detection.

Furthermore, the methanol content in CRAM may exerted a certain influence on this system. To achieve the best detection performance, five different dilution ratios, which were 1:0(methanol), 1:1, 1:3, 1:5, and 0:1 (sterile water) were evaluated (Fig. S3). Results indicated that signal intensities across different methanol-to-water ratios did not differ significantly. However, it was noted that the signal intensity was relatively higher at a methanol-to-water ratio of 1:5. Therefore, this ratio was selected as the optimal reaction condition.

Table S1 Results of the spiked recovery experiment for AFB1 in peanut oil.

Sample	S_0	S_1	S_2	S_3
Added (μg/kg)	0.00	2.00	4.00	8.00
Found (μg/kg)	4.00	5.85	8.24	12.9

Recovery (%)	/	92.5%	106%	111%

Table S2 Comparison of analytical performance of the developed assay with other methods for detection of AFB1.

System	Method	Linear range	Detection limit	Ref.
Cas12a	Fluorimetric	0.001-50 ng/mL	0.84 pg/mL	[1]
Cas12a	Colorimetric	1.0-20 nmol/L	0.32 nmol/L	[2]
	Fluorimetric	0.5-20 nmol/L	0.24 nmol/L	
Cas12a	Fluorimetric	0.01-100 fg/mL	0.00257 fg/mL	[3]
Cas12a	Electro-chemiluminescence	0.0001-500 ng/mL	0.044 pg/mL	[4]
Cas12a	SERS Technology	0.001-1.5 ng/mL	3.55 pg/mL	[5]
Cas14a	Isothermal Amplification Colorimetric	0.05-10 ng/mL	31.90 pg/mL	[6]
Cas14a	Fluorimetric	20-3000 nmol/L	16 nmol/L	[7]
/	ELISA	0.08-0.65 ng/mL	0.04 ng/mL	[8]
Cas14a	Colorimetric	0.001-1 pmol/L	0.67 fmol/L	This work

Table S3. The nucleic acid sequences used in this research.

Names	**Sequences (from 5' to 3')**
sgRNA	5'-GGC ACG AGA CAC AGA GAG AGG UUG CAU UCC UUC AUU CUU UCA AAU GAA UUU GUU UCG AGG GUU ACU UUC CGA ACA AGA CAC UUC UCG ACA UUA GGC UGA UUC AAG CAG GCC ACC UUC AUC CAA GUU CUA AUC CCC UAA GGG ACA GCU UUU GGU GAA CGG GUU CUC CAC UUU AUC AGU GAA G-3'
ss-DNA	5' biotin-TEG-TTTTTTTTTTT-TEG-biotin 3'
AFB1 Aptamer	5'-GTT GGG CAC GTG TAG TCT CTC TGT GTC TCG TGC CCT TCG CTA GGC CCA CA-3'

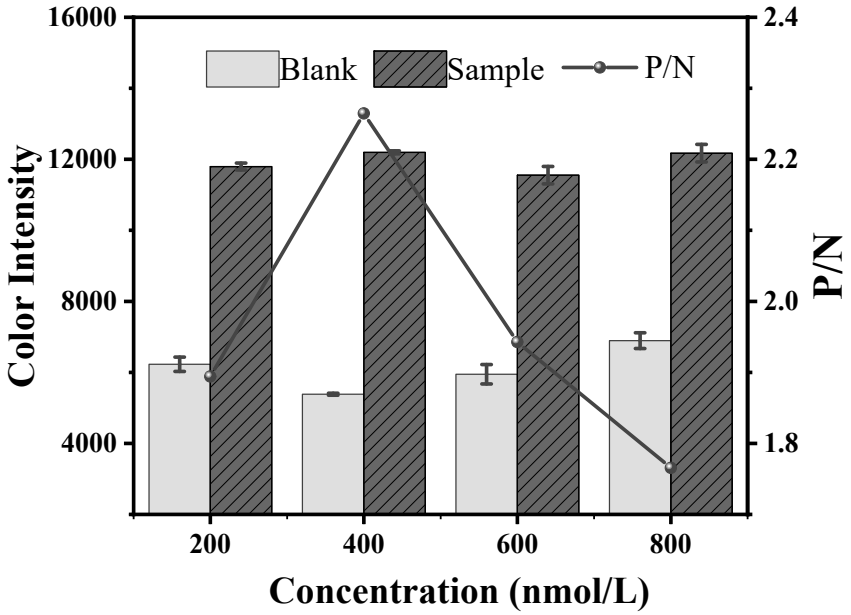

Fig. S1 Detection results of the CWP for AFB1 under different concentration of the ssDNA reporter.

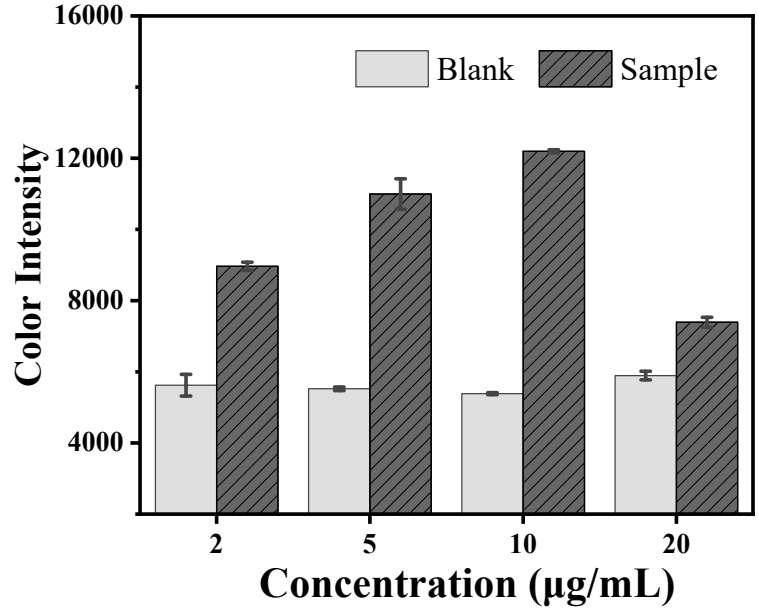

Fig. S2 Detection results of the CWP for AFB1 at different concentrations for SA.

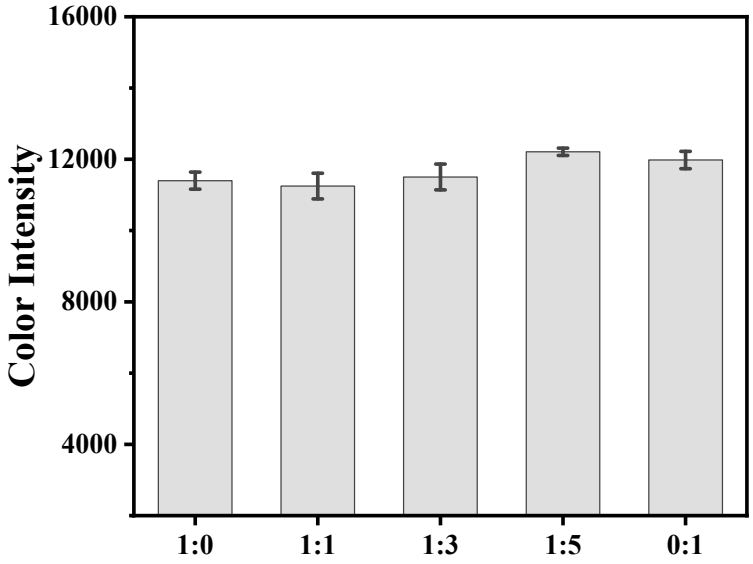

Fig. S3 Detection results of the CWP for AFB1 under different methanol dilution ratios.

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