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Highly sensitive and selective aflatoxin B_1 biosensor based on Exonuclease I-catalyzed target recycling amplification and targeted response aptamer-crosslinked hydrogel using electronic balances as a readout

Linyue Tang[a,1], Yaying Huang[a,1], Cuiying Lin[a,**], Bin Qiu[a], Longhua Guo[a], Fang Luo[b], Zhenyu Lin[a,*]

[a] Ministry of Education Key Laboratory for Analytical Science of Food Safety and Biology, Fujian Provincial Key Laboratory of Analysis and Detection for Food Safety, College of Chemistry, Fuzhou University, Fuzhou, Fujian, 350116, China
[b] College of Biological Science and Engineering, Fuzhou University, Fuzhou, Fujian, 350116, China

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ABSTRACT

The biosensors based on aptamer based stimuli-responsive hydrogels have the characters of high specificity, good stability, portability. Electronic balance is one of the most accurate equipment and can be reached nearly in all labs. Aflatoxin B_1 (AFB_1) is highly toxic and carcinogenic to humans and animals, it is necessary to develop simple and convenient detection method to apply in resource limited area. In this study, a novel strategy for quantitative detection of AFB_1 has been developed by combining the high selectivity and convenient of target-responsive hydrogel and the simple of using electronic balance as readout devices. The AFB_1 target responsive double crosslinked hydrogel has been constructed using linear hyaluronic acid grafted single-stranded DNA complex as the backbone, AFB_1 aptamer and polyethyleneimine as crosslinkers. And platinum nanoparticles (PtNPs) had been embedded in the hydrogel firstly. The present of AFB_1 can bind with the aptamer with high affinity and cause the releasing of aptamer from hydrogel. The addition of Exo I can specifically recognize and cleave the aptamer in AFB_1-aptamer complex, resulting in the releasing of AFB_1, which can react with the hydrogel again, thereby achieving the target cycle. By this means, the hydrogel will collapse and many pre-embedded PtNPs can be released. The transferring of the released PtNPs to a drainage device which contains H_2O_2 can results in the increasing of the internal pressure since the production of oxygen through the catalytic decomposition of H_2O_2 by PtNPs has low solubility. Which will cause the discharging of water from the system and this can be collected and weighed by an electronic balance easily. The weight of water has a linear relationship with AFB_1 concentration. Under 30 min catalytic time, the linear range is 31.2 μg/kg – 6.2 mg/kg with the detection limit of 9.4 μg/kg (S/N = 3). The proposed method was successfully applied to the detection of AFB_1 in peanut samples.

1. Introduction

DNA/aptamer cross-linked DNA polymer hybrid stimuli-responsive hydrogels have attracted widespread attention due to the high specificity of aptamer, good stability, portability and easy storage [1,2]. The indicator embedded in the hydrogel can be released after the stimulation by specific target [3,4]. This character had been coupled with different readout techniques, such as colorimetric [5–7], microfluidic chips [8,9], glucometer [10], pressure meter [11], pH meters [12], and so on, to develop sensitive biosensors for different targets. Electronic balances are weighing tools that are specifically designed to weigh the amount of the substance, which can be founded nearly in all chemical labs. It can also work as an accuracy readout equipment in the biosensors. Recently, our group had coupled different recognition techniques, such as antigen-antibody recognition, DNAzyme, and controlled released system, with the convenient of using electronic balances as readout to develop biosensors for thrombin [13], lead ions [14] and hyaluronidase [15]. It is necessary to couple the high specificity of aptamer based stimuli-responsive hydrogels with easy reach of electronic balances to develop simple biosensors to different targets.

* Corresponding author. Tel./Fax: +8659122866135; +861370591250 (Mobile).
** Corresponding author. Address: 2 Xue Yuan Road, University Town, Fuzhou, Fujian, PR China.
E-mail addresses: lcuiying@fzu.edu.cn (C. Lin), zylin@fzu.edu.cn (Z. Lin).
[1] These authors contributed equally to this work.

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Aflatoxin B_1 (AFB_1) is a derivative of dihydrofuran oxaphthalene containing a difuran ring and an oxaphthalenone (coumarin) [16,17]. It is highly toxic and carcinogenic to humans and animals. It mainly pollutes peanut, corn, rice, wheat, peanut oil and other cereals and oils [18–20]. The allowable amount of AFB_1 in corn, peanut and peanut oil is ≤ 20 μg/kg according to the regulation of Chinese Food Hygiene Standard [21]. Many methods, such as thin-layer chromatography (TLC) [22], high-performance liquid chromatography (HPLC) [23], enzyme-linked immunosorbent assay (ELISA) [24], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [25–27], electrochemiluminescence (ECL) [28], fluorescence [29,30] and the like [31,32], had been applied for AFB_1 detection. Many Point-of-care testing (POCT) methods had been developed for AFB_1 detection [19,33–36] also. For example, Yang's group [37] used AFB_1 response hydrogel as recognize unit and distance as readout signal for AFB_1 detection. Our group [12] used pH as readout signal for AFB_1 detection. To the best of our knowledge, no report about the application of electronic balance as readout to detect trace amount of AFB_1 in food samples had been reported.

In this work, a simple method for AFB_1 detection had been designed which combines the advantages of versatility of aptamer based target stimulate response hydrogel system and convenient of using electronic balance as readout. An AFB_1 target responsive double crosslinked hydrogel has been constructed from linear hyaluronic acid grafted single-stranded DNA complex as the backbone, AFB_1 aptamer, and polyethyleneimine as crosslinkers. Platinum nanoparticles (PtNPs) had been embedded in the hydrogel firstly. The present of AFB_1 can bind with the aptamer with high affinity and cause the releasing of aptamer from the hydrogel. The addition of Exo I then specifically recognizes and cleaves the aptamer in AFB_1-aptamer complex, resulting in the releasing of AFB_1, which can react with the hydrogel again to cause the releasing of aptamer for the hydrogel again and thereby achieving the target cycling. By this means, the hydrogel will collapse and much amount of PtNPs can be released. The released PtNPs react with H_2O_2 in the drainage device to cause a pressure difference between the inside and the outside, thereby discharging the water, and the weight of the water can be accurately weighed by a simple electronic balance and which is related to the amount of AFB_1 added. The proposed method has been applied in the detection of AFB_1 in peanut samples.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt (HA), 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), polyethyleneimine (PEI) and methanol were obtained from Sigma Aldrich Co., Ltd. (American). Sodium chloride, potassium chloride, calcium chloride, tris (hydroxymethyl) aminomethane and other reagents were obtained from Aladdin Chemistry Co. (Shanghai, China). Exonuclease I (Exo I) and 10 × Exo I buffer (pH 7.5) were purchased from Thermo Fisher Scientific (Shanghai, China). AFB_1, other toxins and an AFB_1 ELISA Kits were obtained from Sangon Biotechnology Co. (Shanghai, China). The peanuts were purchased from Yonghui supermarket (Fuzhou, China). All other reagents were analytical grade and used as received. Deionized water obtained from a Millipore water purification system (Milli-Q, Millipore, resistance of 18.2 MΩ cm) was used all throughout the project.

All oligonucleotides were synthesized by Sangon Biotechnology Co. (Shanghai, China). Their sequences are shown below:

SA: 5′-NH_2-TTTTGTGGGCCTAGCGA-3′

SB: 5′-NH_2-TTTACACGTGCCCAAC-3′

Apt-linker: 5′-GTTGGGCACGTGTTGTCTCTCTGTGTCTCGTGCCCTTCGCTAGGCCCACA −3′ (the sequence for AFB_1 aptamer [12]).

2.2. Preparation of platinum nanoparticles

The platinum nanoparticles (PtNPs) were synthesized according to our previous method [14]. The synthesized PtNPs were centrifuged and resuspended in water and finally concentrated to 6.2 nM. The diameter of the synthesized PtNPs by TEM (Tecnai G2 F20 S-TWIN, FEI, U.S.A.) was about 50 nm ± 10 nm.

2.3. Preparation of HA-DNA hydrogel

EDC (50 μL 28.8 mg) and NHS (50 μL 5.8 mg) were added into the 1% HA solution for 15 min. Next, the activated HA (0.4 mL) has been reacted with 100 μL SA or SB solution (500 μM) for 12 h to produce HA-SA and HA-SB.

To prepare the AFB_1-responsive hydrogel, HA-SA (3 μL, 100 μM), HA-SB (3 μL, 100 μM), Apt-linker (1 μL, 300 μM), PtNPs (2 μL, 6.2 nM) had been mixed and incubated with 4 h at 37 °C under shaking. After this, the mixture was added into the solution contains 1 μL EDC (4.8 mg 50 μL) and 1 μL NHS (2.3 mg 200 μL), and then stirred with 15 min at 37 °C. Finally, 2 μL PEI was added to the mixture, mix and incubate at 37 °C for 6 h. By this means, a double cross-linked hydrogel with encapsulated PtNPs can be obtained.

2.4. Procedures for AFB_1 detection

20 U Exo I and a solution containing different amount of AFB_1 were added into the prepared hydrogel (final volume 63 μL), then incubated 1 h at 37 °C. 35 μL of the supernatant was collected and added to a glass bottle (5 mL) containing H_2O_2 (1 mL), and then placed in a water-filled plastic bottle with a catheter (10 mL), then immediately capped the bottle and left react for 30 min. Then, the discharged water was weighed by an electronic balance (Sartorius, BS124S, China). All tests were performed at room temperature.

2.5. Procedure for actual sample processing

The moldy peanut samples were prepared from fresh peanuts in an environment of 28–32 °C and a relative humidity of 85% or more for two weeks. Then weighing 1.0 g peanut and crushing, add 5 mL 50% (v/v) methanol-water solution and vortex to mix. Place them in ultrasonic treatment for 30 min, centrifuge at 6000 r/min for 10 min, and take the supernatant for later use. Add 5 mL 50% (v/v) methanol-water solution and repeat the above steps, and then filter through 0.22 μm filter. The obtained sample was added to the column, activated, rinsed, and after the loading step, methanol (1 mL) was added to obtain a sample. By this means, AFB_1 in the samples can be extracted. Fresh peanut sample extraction was identical to moldy peanut sample extraction. The sample was then diluted with deionized water and then AFB_1 had been detected according to the above-mentioned procedures.

3. Results and discussion

3.1. Principle of the proposed system for AFB_1 detection using electronic balance as readout

Fig. 1A illustrates the principle of the proposed method for AFB_1 determination based on target-responsive aptamer-cross-linked hydrogel using electronic balance as a readout. In the presence of AFB1, the aptamer will combine with AFB_1 in lieu of dsDNA in the hydrogel since AFB_1 has high affinity with the aptamer. This results in the breakdown of the cross-linking of the aptamer-HA-SA-HA-SB duplex. The addition of Exo I can specifically recognize and cleave the aptamer in AFB_1-aptamer complex, resulting in the releasing of AFB_1 (Since Exo I recognizes only single-stranded DNA, it does not cleave the aptamer in the hydrogel). The released AFB_1 competes again for the aptamer in the hydrogel to achieve the target cycle amplification. By this means, the

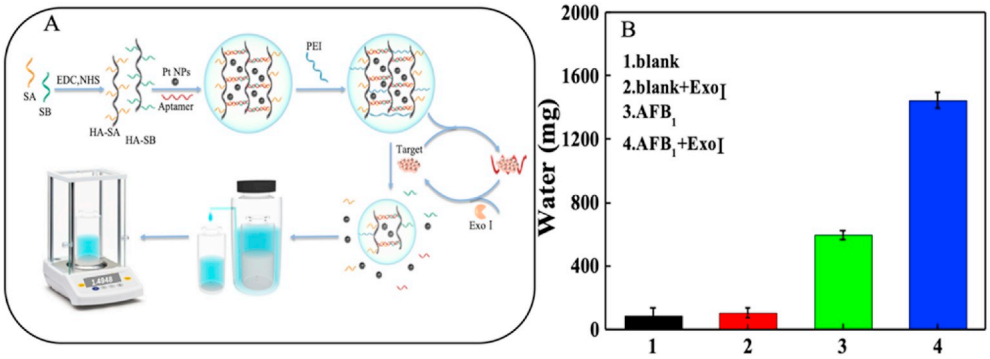

Fig. 1. (A) The principle AFB_1 biosensors based on aptamer based stimuli-responsive hydrogels using electronic balance as a readout; (B) Changes of overflowed water weight under different conditions. 1. Without the addition of AFB_1 and Exo I; 2. [Exo I] = 20 U; 3. [AFB_1] = 3.1 mg/kg; 4. [Exo I] = 20 U, [AFB_1] = 3.1 mg/kg.

hydrogel will collapse and a large amount of PtNPs can be released into the supernatant. A certain amount of supernatant was collected and added to the reaction bottle of the drain (containing 1 mL of H_2O_2). PtNPs can catalyze the decomposition of H_2O_2 and produce O_2. Since the solubility of O_2 is very low, thereby cause the increasing of the internal pressure in the drainage device, which results in the discharging of the water from the system because of the pressure difference. The discharged water can be collected and weighed by an electronic balance, which has a certain relationship with the amount of AFB_1. In this way, a simple portable sensor for AFB_1 detection can be developed.

Control experiment has been performed to verify the feasibility of the proposed system firstly. As shown in Fig. 1B, almost no water had been detected without the addition of target and Exo I, which also indicated that nearly no leakage of PtNPs from the hydrogel. When Exo I is added to the system, the background signal will increase slightly, which maybe lies in the fact of no specific hydrolysis by Exo I and cause the releasing of a small amount of PtNPs. When only AFB_1 present, the aptamer binds to AFB_1, resulting in cross-linking decomposition of the aptamer-HA-SA-HA-SB duplex, and some PtNPs are released from the hydrogel into the supernatant. So relatively large signal had been detected. When AFB_1 and Exo I were simultaneously added to hydrogel, the detected signal increased greatly because of the target recycling amplification. All the results confirm the principle of the hypothesis.

3.2. Optimization of the reaction conditions

To improve the performance of the method, several reaction conditions were optimized. As shown in Fig. 2A, Hyaluronic acid DNA complexes (HA-SA/HA-SB), AFB_1 aptamers can hybrid with each other and form a hyaluronic acid DNA hydrogel, because the hyaluronic acid DNA hydrogel will swell in the water even no target present, which will cause the leakage of PtNPs and results in a large background signal detected. Fortunately, PEI has been added as a second crosslinking agent, which can prevent the probe from leaking and the background signal reduced greatly .

As a crosslinking agent, aptamers play an important role in the formation of hydrogels. Cross-linked hydrogels with low concentrations of aptamers have high sensitivity because since the produced hydrogels are more susceptible to collapse, but background signal will be big because the leakage of PtNPs. However, excess amount of aptamer requires will cause more reaction time to disrupt the hydrogel. Therefore, the amount of aptamer used to prepare the hydrogels is optimized. The red bar in Fig. 2B indicates that the weight signal decreases as the aptamer concentration increases from 10 μM to 50 μM and the background signal decreases as the aptamer concentration increases from 10 μM to 50 μM. This indicates that a high concentration of aptamer will results in the releasing of relatively few PtNPs while the background signal is also low. Low concentrations of aptamer can result in the releasing of more PtNPs and the background signal is also high. We have to choose an intermediate concentration to make the background signal lower and the target response signal higher. Therefore, the concentration of 30 μM was taken as the optimal condition.

The mass ratio of PEI to HA is also a key factor. A low mass ratio will make the hydrogel structure less dense, which will cause the leakage of PtNPs from the hydrogel, resulting in a high background signal. Long reaction time is need at high mass ratio. As shown in Fig. 2C, the detected weight decreases as the mass ratio increases from 0.05 to 0.35, and the background signal also decreases. When M (PEI/HA) is 0.1, the background signal is observed to be low and the target response signal is relative large. Therefore, M (PEI/HA) was chosen to be 0.1 for subsequent measurements.

Exo I is less active under acidic and alkaline conditions and has higher activity under neutral conditions. The effect of pH value on performance of the system had been optimized. The results showed that at pH 7.5, the response of the biosensor is best, so which had been shown as the optimized condition. Then the effect of enzyme dosages was investigated. The weight signal increases as the Exo I dose increases from 0 to 20 U and then increase little after 20 U (Fig. 2D). When the amount of Exo I is greater than 20 U, the background signal increases also, which may be due to non-specific cleavage of the enzyme. Therefore, 20 U is chosen as the best condition. The action time of Exo I has been optimized too (Fig. 2E). The weight of the discharged water increases as the digestion time increases, when the digestion time is longer than 60 min, the further increasing is not significant but the background signal enhanced. Therefore, 60 min was chosen for subsequent measurements.

The time for PtNPs to catalyze H_2O_2 was also optimized (Fig. 2F). The weight signal increased as the time increases from 5 min to 60 min and the background signal did not change significantly from 5 min to 60 min. When the catalytic time is 30 min, the detection limit of the proposed method can reach the requirements of real sample detection. So 30 min has been chosen in this work.

3.3. Performance of the proposed sensor

Under the optimized experimental conditions, the weight of water collected after the stimulation by different concentration of AFB_1 were recorded. There has a good linear relationship between the weight of the discharged water and the AFB_1 concentration in the range of 31.2 μg/kg – 6.2 mg/kg (Fig. 3A). The regression equation is:

$$W = 0.36C + 143.27 (R^2 = 0.9962)$$

where W is the weight of discharged water, C is the AFB_1 concentration, and R is the correlation coefficient. The limit of detection (LOD) was found to be 9.4 μg/kg based on three times standard deviation over the blank response (S/N = 3).

Compared with other AFB_1 detection methods (as shown in Table S1), the proposed method has a wider linear range, and the LOD is comparable to some fluorescence and POCT methods [12,38]. Although the LOD of this work is not as good as some methods using large instruments [28,30,31,39], it has already met China's allowable content of AFB_1 in corn, peanut and peanut oil ≤20 μg/kg. If a lower LOD is

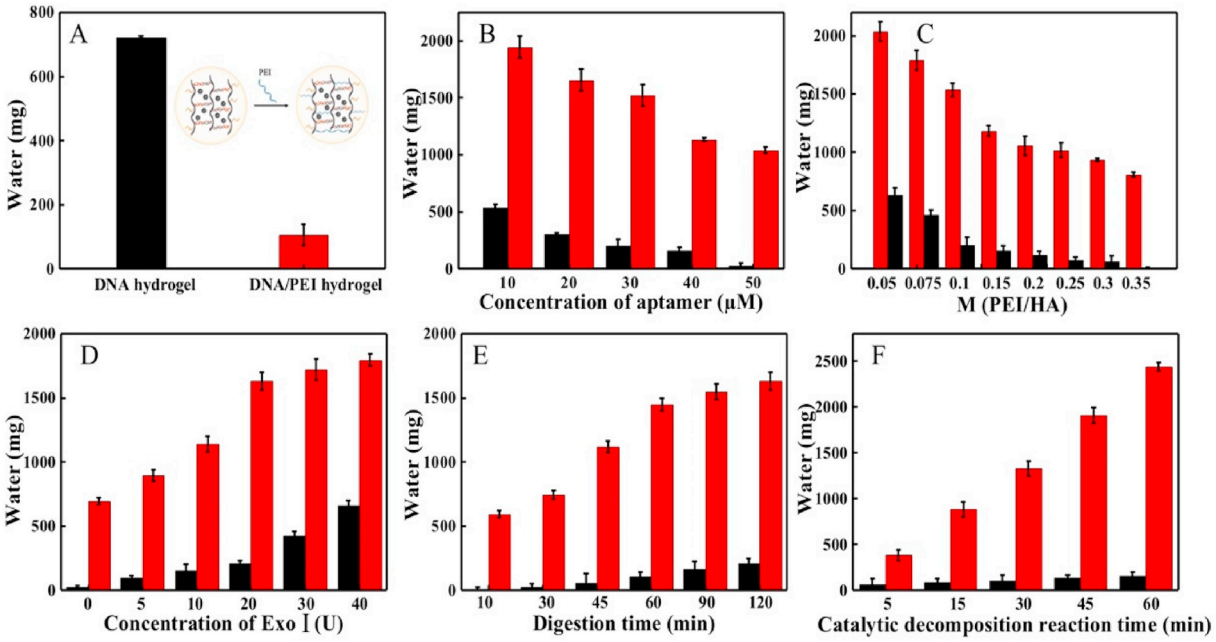

Fig. 2. Effects of experimental conditions on the performance of the biosensors. (A) different DNA hydrogel; (B)aptamer concentration; (C) the mass ratio of PEI to HA; (D) dosage of Exo I; (E) digestion time and (F) catalytic decomposition reaction time. [aptamer] = 30 μM, [M (PEI/HA)] = 0.1, [Exo I] = 20 U, [AFB_1] = 3.1 mg/kg. Black bars were the control group and red bars were the experimental group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

required, which can be achieved easily by prolonging the time of PtNPs catalyzing H_2O_2. As shown in Fig. 3B, when the catalytic decomposition time was prolonged, and the increase rate of the signal of AFB_1 response was greater than that of the background and therefore the signal of noise ratio (S/N) increased. Therefore, when a lower LOD is required, it can be achieved by extending the decomposition time of PtNPs. Compared with other reported methods, the current method does not rely on expensive and precise instruments and professional technicians, and has advantages in application in underdeveloped regions.

To detect the selectivity of the hydrogel sensor, several different toxins were selected as interferences, such as AFG_1, AFB_2, AFG_2 and OTA. The AFB_1 concentration and the other interferents concentration were 0.9 mg/kg and 4.7 mg/kg respectively (also in the mixed sample). As shown in Fig. 3C, in the presence of other toxins, the amount of water discharged is small, which is nearly the same as the blank sample. But significantly signal had been detected if AFB_1 is added. The reason maybe lies in that the hydrogel contains aptamer that specifically recognizes AFB_1, when other interfering substances are present, specific recognition cannot be produced and the hydrogel cannot be broken. The presence of AFB_1 can be specifically recognized and bound by aptamers, resulting in the breakdown of the cross-linking of the aptamer-HA-SA-HA-SB duplex and PtNPs will released from the hydrogel into the supernatant. The released PtNPs can react with H_2O_2 in the drainage device to cause a pressure difference between the inside and the outside, thereby discharging the water. This indicates that the proposed sensor has a strong selectivity. Detection of AFB_1 using a hydrogel prepared 1 month ago (4 °C stored). There was almost no difference compared to the hydrogel prepared in real time, indicating that the prepared hydrogel had good stability.

3.4. Sample determination

The practicality of the method was demonstrated to detect AFB_1 in fresh and moldy peanut samples. As shown in Table 1, AFB_1 was not detected in fresh peanut samples, but contained approximately 33.16 μg/kg AFB1 in the moldy peanut samples, the recovery of AFB_1 is between 91.5% and 98.1% and the results were basically consistent with the results of the enzyme-linked immunoassay kit (LOD:1 μg/kg). This demonstrates the feasibility of using this sensor for the detection of AFB1 in food samples.

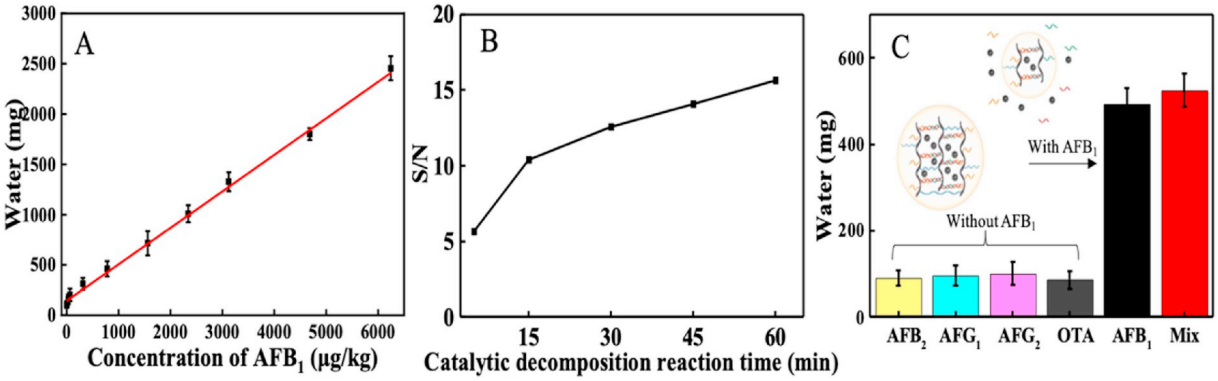

Fig. 3. (A) Linear relationship between the weight of discharged water and AFB_1 concentration; (B) Relationship between S/N and catalytic decomposition reaction time. [AFB_1] = 3.1 mg/kg, (C)Selectivity of the proposed sensor for AFB_1. [AFB_1] = 0.9 mg/kg, [other interferents] = 4.7 mg/kg.

Table 1
Detection of AFB_1 in food samples by the proposed method and ELISA method. (n = 3).

Sample	Added (μg/kg)	The proposed method			ELISA Kit		
		Detected (μg/kg)	Recovery (%)	RSD (%)	Detected (μg/kg)	Recovery (%)	RSD (%)
Moldy peanut	0	33.83	–	4.9	37.69	–	5.3
	50	80.15	92.6	2.7	84.11	92.8	1.9
	100	130.07	96.2	1.3	132.03	94.3	4.2
	200	216.87	91.5	4.2	231.85	97.1	2.5
Fresh peanut	0	–	–	–	–	–	–
	50	47.12	94.3	3.0	48.52	97.0	4.6
	100	95.67	95.7	1.8	97.89	97.9	1.5
	200	196.16	98.1	1.5	195.42	97.7	2.4

4. Conclusion

A simple method for AFB_1 detection had been proposed which binds the high selectivity of aptamer based target stimulate response hydrogel system, high efficiency of Exo I assisted target recirculation amplification strategy, high accuracy and convenient of using electronic balance as readout. The proposed method had been applied to detect AFB_1 in food samples with satisfied results. The method can be further extended to detect different targets by simply changing the aptamer used.

CRediT authorship contribution statement

Linyue Tang: Validation, Formal analysis, Investigation, Data curation, Writing - original draft. **Yaying Huang:** Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Visualization. **Cuiying Lin:** Funding acquisition, Writing - review & editing. **Bin Qiu:** Supervision. **Longhua Guo:** Supervision. **Fang Luo:** Writing - review & editing. **Zhenyu Lin:** Conceptualization, Methodology, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.120862.

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