Article

A Novel Fluorescent Sensor Based on Aptamer and Qpcr for Determination of Glyphosate in Tap Water

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Abstract

Glyphosate (GLYP) is a broad-spectrum, non-selective, organic phosphine post emergence herbicide registered for use on many food and non-food field. Herein, we developed a biosensor (Mbs@dsDNA) based on carboxylated modified magnetic beads incubated with NH_2-polyA and then hybridized with polyT-glyphosate aptamer and complementary DNA. Afterward, a quantitative detection method based on qPCR was established. When the glyphosate aptamer on Mbs@dsDNA specifically recognized glyphosate, a complementary DNA is released and then enters the qPCR signal amplification process. The linear range of the method was 0.1-5 μg/mL, and the detection limit was set at 0.1 μg/mL. The recoveries in tap water were ranged from 103.4 ~ 104.9%, and the relative standard deviations (RSDs) were < 1%. The aptamer proposed in this study has a good potential for recognizing glyphosate. The detection method combined with qPCR might have a good application prospect in detecting and supervising other pesticide residues.

Keywords : Glyphosate; Aptamer; qPCR; SYBR Green I; sensor;

1.Introduction

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Glyphosate (GLYP), with a chemical name of N-(phosphorylmethyl) glycine, is a non-selective, broad-spectrum organic phosphine herbicide used in agriculture to eliminate various annual and perennial weed (Benbrook et al. 2016; Gill et al. 2017; Jimenez-Lopez et al. 2020; Liu et al. 2019) around the globe. Following application, it is evenly distributed and rapidly translocated to regions of active growth within the plant causing death. However, due to the high herbicidal activity, it led to the abuse of glyphosate (Chang et al. 2016; Duke et al.2008). Many studies have proved that glyphosate has toxic effects and potentially harms the ecological environment and human health (Prasad et al. 2014; Regiart et al. 2020; Singh et al. 2020). Further, residues have been found in soil, even groundwater as a result of extensive usage. Moreover, recent studies have shown that glyphosate accumulation in the environment may cause a certain degree of reproductive toxicity, teratogenicity, mutagenicity and carcinogenicity to humans (Xu et al. 2019; Bringolf et al. 2007). To reduce the impact of glyphosate residues on food safety and human health, detection of glyphosate residual levels is crucial and essential.

As concerns and studies on the behavior of glyphosate in plant and the environment are growing, several methodologies, including high-performance liquid chromatography (HPLC) (Khrolenko et al. 2005; Araujo et al. 2003), gas chromatography-mass spectrometry (GC-MS) (Kudzin et al. 2002; Connolly et al. 2020; Morgan et al. 2019), LC–tandem mass spectrometry (LC-MS/MS) (Bressan et al. 2021; Gormez et al. 2021), liquid chromatography (LC)(Jost et al. 2020; Thompson et al. 2019), ion chromatography(IC) (Geerdink et al. 2020; Schutze et al. 2021), capillary electrophoresis (CE) (Wimmer et al. 2020; Cao et al. 2014), enzyme-linked immunoassays (ELISA) (Byer et al. 2008; El-Gendy et al. 2018), fluorescence detection (Guan et al. 2021; Sasaki et al. 2020) and electrochemical luminescence methods (Habekost 2017; Sahoo et al. 2018) have been developed to detect its residues. Although the sensitivity and specificity of these methods are relatively

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high, their shortcomings are also apparent. They normally required expensive instruments, professional operators, time-consuming sample pretreatment and high testing cost, with certain limitations for glyphosate detection. Therefore, it is very important to develop a rapid, simple, low-cost, high sensitivity and specificity sensor for the quantitative detection of pollutants.

Aptamers are nucleic acid molecules synthesized *in vitro* by a process known as Systematic Evolution of Ligands Exponential Enrichment (SELEX), which has unique binding properties to different targets, such as proteins, small molecules metal ions, and whole cells (Kalra et al. 2018; Shaban et al. 2021; Sharma et al. 2017; Tuerk et al. 1990). As a new type of molecular probes, it can be synthesized with high purity in vitro. And the cost is lower, which is a point of superiority compared with antibody. The aptamer has high thermal stability, low immunogenicity, and chemical stability. In addition, the preparation process of antibody is usually very long, while the synthesis time of aptamer is usually shorter. At the same time, it can meet the experimental design requirements of modification by chemical labels, such as nanoparticles, fluorescent groups and functional groups, and will not affect the affinity between them and target molecules. (Li et al. 2018; Ojha et al. 2021; Ren et al. 2020). They are in general more stable under harsh conditions than antibodies (Shi et al. 2011; Dong et al. 2018; Zhang et al. 2017). After thermal denaturation, the aptamers usually return to their native state. Further, they can be used as effective recognition elements in biosensors and are widely used in the field of detection as biosensing. .

SYBR Green real-time qPCR can monitor the amplification process in real-time by detecting the fluorescent signal emitted by dsDNA specific dye (SYBR Green I). In real-time fluorescent quantitative PCR amplification, the fluorescent group can monitor the amplification products in each cycle of PCR in real-time, and the operation is convenient and straightforward. Additionally, DNA amplification and data analysis can

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be carried out in a closed system under the same conditions so that samples and products can not be polluted. Electrophoresis confirmation of standard PCR products can be directly omitted. As far as the author knows, there are few studies on the detection of glyphosate based on the combination of aptamers and qPCR. Herein, the aptamer-SYBR Green I-based sensing technology was established for rapid detection of glyphosate by qPCR using the single-stranded DNA released from the aptamer after recognizing glyphosate. The recognition process of the aptamer to glyphosate was converted to the cycle threshold (CT) value of qPCR.

2. Experiment

2.1 Materials and instruments

All the DNA sequences were synthesized and purified by Sangon Biotech (Shanghai) Co. Ltd. The sequences of the DNA strands are shown in the supporting information (Table S1). Carboxyl magnetic beads was procured from Sangon Biotech (Shanghai) Co. Ltd. Power Up™ SYBR™ the green premix was acquired from Thermo Fisher Scientific(USA). Glyphosate, acetamiprid, chlorpyrifos, dimethoate, trichlorfon, methomyl, and propoxur (purity greater than 98%) standards were provided by Dr Ehrenstorfer Gmbh (Augsburg, Germany). Other chemical reagents were purchased form Sinopharm Group Co., Ltd.(Beijing). All the purchased chemicals were used as received without further purification. The water involved in the experiments was purified by Milli-Q (U.S.A) (with resistivity of 18.2 MΩ cm).

UV-2600 (SHIMADZU(CHINA) Co., Ltd); TECAN Infinite 200 PRO Multifunctional Enzyme Reader (Switzerland); 7500 real-time fluorescence quantitative PCR instrument, Thermo Fisher Scientific(USA); HM100-Pro, Dragon Laboratory Instruments Limited(Beijing) were used in this study.

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Acetamiprid, chlorpyrifos, dimethoate, trichlorfon, methomyl and propoxur were prepared into 1000 μg/mL with methanol, diluted into 10 μg/mL with deionized water, glyphosate was prepared into 1000 μg/mL with deionized water, and diluted into 5, 2, 1, 0.5, 0.2, 0.1 μg/mL with deionized water gradient.

2.2 Magnetic bead activation

The procedure used to activate magnetic beads was performed according to the manufacturer's protocols (Sangon Biotech Co. Ltd., Shanghai, China). After the carboxyl magnetic beads were whirled for 1 min, a 100 μL was taken into a 2 mL centrifuge. Next, the supernatant was removed after magnetic separation, and then 100 μL 25 mM MES solution was added and whirled for 15 s. After magnetic separation, the supernatant was removed and washed three times with deionized water. After that, 500 μL 10 mg/ml 1-Ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDC) and 100 μL 10 mg/ml N-Hydroxysuccinimide (NHS) were quickly introduced into the centrifuge tube. After mixing, the mixture was shaken slowly at room temperature for 30 min and then stored at 4 ℃.

2.3 Preparation of Mbs@dsDNA and pre-experiment

Take 600 μL of activated magnetic beads, discard the supernatant after magnetic response, add 600 μL of 10 μM polyA solution, thoroughly mix, and incubate in a 37 ℃ water bath overnight. The follow day, that supernatant was removed after magnetic separation and redissolved in 600 μL deionized water, stored at 4 ℃. To obtain the purified Mbs@polyA, after magnetic separation, polyA that is not bound to the magnetic beads were removed from the supernatant. And 600 μLof 2 μmol polyT-aptamer solution was added in Mbs@polyA solution and denatured at 90 ℃ for 2 min. After vortex oscillation for 2 min, the supernatant was removed

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and washed three times with deionized water to remove the polyT-Aptamer that is not hybridized with Mbs@polyA in supernatant of solution. Next, 600 μL of 2 μmol complementary DNA(C-DNA) solution was added and denatured at 90 ℃ for 2 min. After vortexed for 2 min, it was shaken slowly at room temperature for at least 30 min. Then the magnetic separation was carried out to remove the C-DNA not hybridized in the supernatant. After washed with deionized water for three times, the obtained Mbs@dsDNA was suspend with deionized water and set it at 4 ℃ for standby.

20 μL of 0.1~10 μg/mL glyphosate and blank samples were added into the the prepared Mbs@dsDNA system respectively and incubated at room temperature for 3 h. After magnetic separation, the supernatant was taken for qPCR and photographed under a UV lamp.

2.4 Specificity detection

A series of centrifugation tubes each containing 60 μL Mbs@dsDNA solution were prepared. After magnetic separation, the supernatant was removed, and 60 μL of different solutions, including 10 μg/mL acetamiprid, chlorpyrifos, dimethoate, trichlorfon, methomyl, propoxur and 1 μg/mL glyphosate, were added to each tube and incubated at room temperature for 3 h. After incubation, the supernatant was separated by magnetic separation, and the released C-DNA in the system was identified by UV spectrophotometer and then detected by qPCR. The qPCR reaction was carried out at a total volume of 20 μL in the quantitative study. The conditions of amplification reactions are shown in the supporting information (Table S2).

2.5 Determination of glyphosate

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A series of centrifugation tubes with 60 μl of Mbs@dsDNA in each tube were prepared. After magnetic separation, the supernatant was removed, and 60 μl glyphosate with different concentrations of 5, 2, 1, 0.5, 0.2 and 0.1 μg/mL was added into each tube.

After 3h incubation, the supernatant was separated by magnetic separation and detected by UV spectrophotometry and qPCR, respectively.

2.6 Actual sample testing

Tap water of the City of Beijing was taken as the actual sample, and glyphosate with the concentration of 1.4 μg/mL, 0.7 μg/mL and 0.35 μg/mL was added according to the national standard of China (GB/T 5749-2006). Each sample was tested 3 times. The relative standard deviation (RSD) of the test results was used to evaluate the method's precision.

3. Results and discussion

3.1 Experimental principle

In this paper, a method based on qPCR signal amplification to detect glyphosate was proposed. With fabricated Mbs@dsDNA, the interference of matrix in the sample system can be quickly eliminated by magnetic separation. Fig. 1 (A) shows the formation of Mbs@polyA by connecting the polyA sequence with the magnetic bead. Then, aptamer with modification of polyT (polyT-aptamer) was matched with Mbs@polyA by forming A-T pairs. Subsequently, C-DNA was added to match with aptamer forming Mbs@dsDNA. As shown in the figure, the redundant polyT- Aptamer and C-DNA without double-strand formation can be eliminated by magnetic separation. As Fig. 1 (B) shown, when glyphosate is present in the system, the aptamer can specifically recognize glyphosate and form Mbs@Aptamer@GLYP. as the ligand. The binding force between aptamer and glyphosate is stronger than that between aptamer and C-DNA. With the help of a external

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magnetic field, the C-DNA and fMbs@Aptamer@GLYP were separated. The supernatant contained C-DNA was extracted and utilized in qPCR as templates. The fluorescent signal obtained from qPCR with SYBR Green I was recorded to set up the linear equation. And then the amount of glyphosate was calculated. When other interfering pesticides and blank are added to the system, C-DNA will not be separated from the Mbs@dsDNA, and the signal will not appear in qPCR.

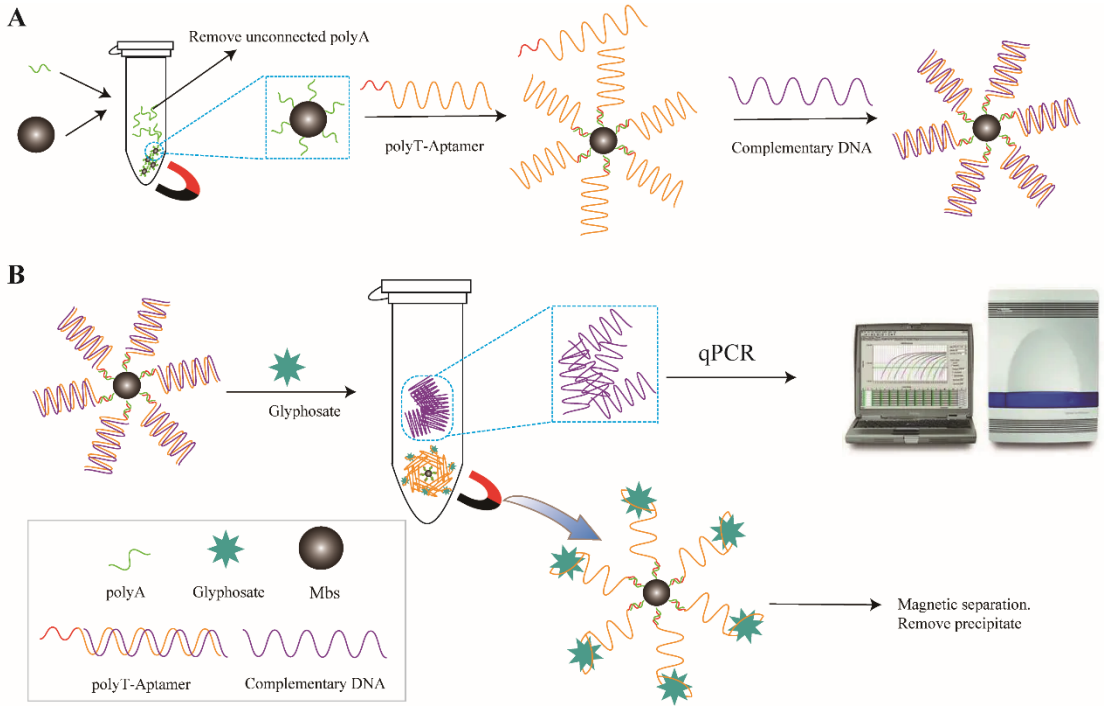

Fig. 1. (A) Forming dsDNA compound. (B) Detection of glyphosate by qPCR

3.2 Characterization of Mbs@dsDNA and pre-experiment

UV spectrophotometer was used to characterize the chemical structure changes in the glyphosate sensing procedures. As shown in Fig. 2 (A), the supernatant was tested after the assembly of magnetic beads and polyA to measure the free polyA in supernatant which were not assembled on the magnetic beads. Compared with the total polyA, the absorption peak at 260 nm of free polyA in the system is significantly decreased, indicating that polyA is successfully bound to the magnetic beads to obtain the composite Mbs@polyA. As

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shown in Fig. 2 (B), the polyT-Aptamer solution is added into Mbs@polyA solution, after denaturing hybridization and incubation, polyT-Aptamer, which is not bound to Mbs@polyA, in the supernatant is collected. And the absorption peak value is measured at 260 nm, compared with the total polyT-aptamer added.. The absorbent signal of free polyT-Aptamer in the system is much smaller, indicating that most of the polyT-Aptamer is successfully assembled on the Mbs@polyA to obtain a complex Mbs@aptamer. As shown in Fig. 2 (C), C-DNA solution was added to the Mbs@Aptamer. After denaturing hybridization incubation, the C-DNA not assembled to the Mbs@Aptamer in the supernatant was collected, and its absorption peak was measured at 260 nm. Compared with the total amount of C-DNA, the amount of free C-DNA in the system decreased, indicating that the complex Mbs@dsDNA was successfully assembled. As shown in Fig. 2 (D), SYBR I can embed the double helix structure of DNA and emit fluorescence; And the qPCR reaction cannot be carried out when there is no glyphosate in the system, so SYBR Green I cannot embed the double helix structure of DNA and does not emit fluorescence under UV light. On the contrary, when glyphosate is present in the system, the aptamer binds explicitly with glyphosate and releases C-DNA into qPCR reaction. SYBR I is embedded into the formed DNA double helix structure and emits fluorescence under ultraviolet lamp excitation.

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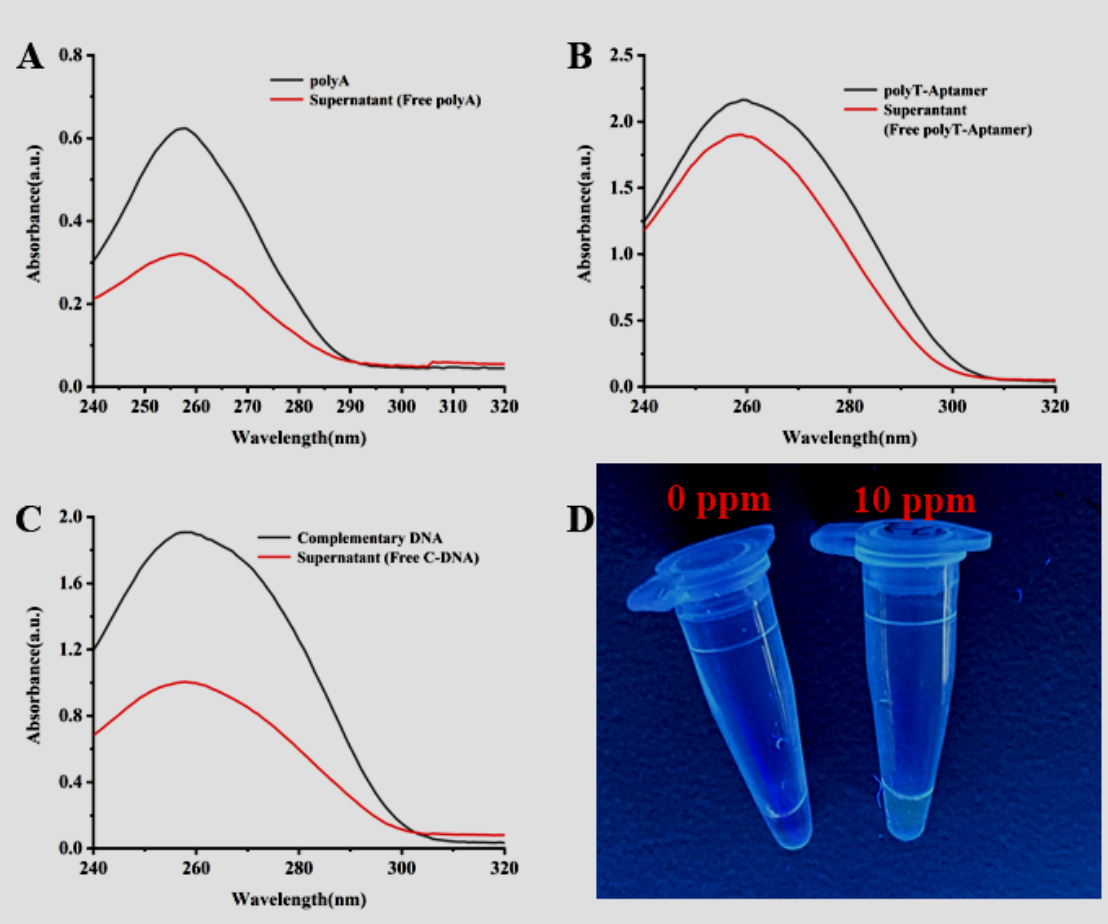

Fig. 2 (A) UV-Vis absorption spectrum: polyA (black), supernatant (free polyA) (red), (B) UV-Vis absorption spectrum: polyT-Aptamer (black), supernatant (free polyT-Aptamer) (red), (C) UV-Vis absorption spectrum: complementary DNA (black), supernatant (free C-DNA) (red), (D) Fluorescent photos under 365nm UV lamp

3.3 Specificity

According to the pre-experiment, the feasibility and specificity of the method is verified. Based on the experimental steps, different pesticides including acetamiprid, chlorpyrifos, dimethoate, trichlorfon, methomyl, propoxur and glyphosate are added into the prepared Mbs@dsDNA system. And after room temperature shaking incubation and magnetic separation, the supernatant is subjected to ultraviolet absorption photometry and fluorescence signal detection. As shown in Fig. 2 (A), when these pesticides were added, the aptamers could not recognize them, thus the C-DNA could not be released. And the subsequent qPCR

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detection experiment could not be performed for lack of enough templates. When glyphosate is added, the aptamer forms Mbs@Aptamer@GLYP after specific recognition with glyphosate. C-DNA were released from the Mbs@dsDNA structure, and subsequent qPCR reaction then can be carried out. With the using of SYBR Green I, the products of qPCR showed a obvious fluorescence signal. The column diagram shown in Fig. 2 (b) is the fluorescence intensity at 520 nm. The fluorescence intensity of the supernatant, which was magnetically separated from the mixture of Mbs@dsDNA and glyphosate or other different pesticides, was detected. As a result, only when glyphosate exists, C-DNA can be released as the PCR template to form a double-stranded DNA structure, and the dye SYBR Green I can be intercalated into the double helix structure. Otherwise, the PCR could not be performed, while the dye SYBR Green I was not embedded in the double helix, without fluorescent signal emitted. As described above, the specificity of the detection method is reasonable.

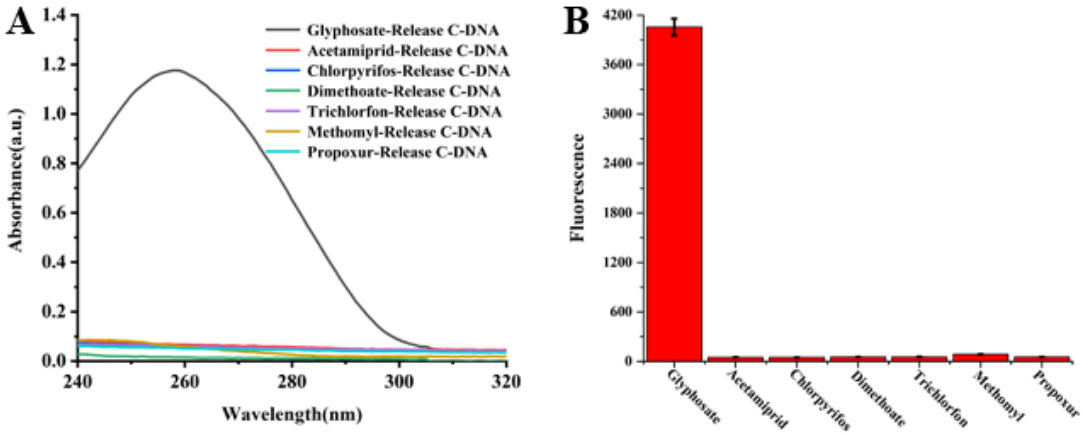

Fig. 3 (A) Different pesticides and Mbs@dsDNA UV-Vis absorption spectrum of C-DNA released after incubation, B) Fluorescence intensity histogram at 520 nm

3.4 Standard curve

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The prepared Mbs@dsDNA systems was added with 5, 2, 1, 0.5, 0.2 and 0.1 μg/mL glyphosate standard solution, respectively. And after incubation and magnetic separation, the supernatant solution was detected by ultraviolet absorbance and qPCR. As shown in Fig.4 (A), with the increase of the concentration of glyphosate, the measured ultraviolet absorption photometric value grew up, which indicates that with the increase of the concentration of glyphosate, more and more C-DNAs released from the Mbs@dsDNA structure and are accessible in the supernatant. However, the change of the ultraviolet absorption value is not particularly obvious. Therefore, the obtained C-DNA was subjected to qPCR amplification and detection, and a linear equation was fitted based on the obtained data. As shown in Fig. 4 (B), the obtained CT value had a good linear relationship with the logarithm of the concentration. The linear equation is y = -4.0799x + 26.20509. The linear correlation coefficient is R^2=0.99742, and the detection limit obtained by the experiment can reach 0.1 μg/mL, which is far lower than the maximum residue limit of glyphosate in the national standard.

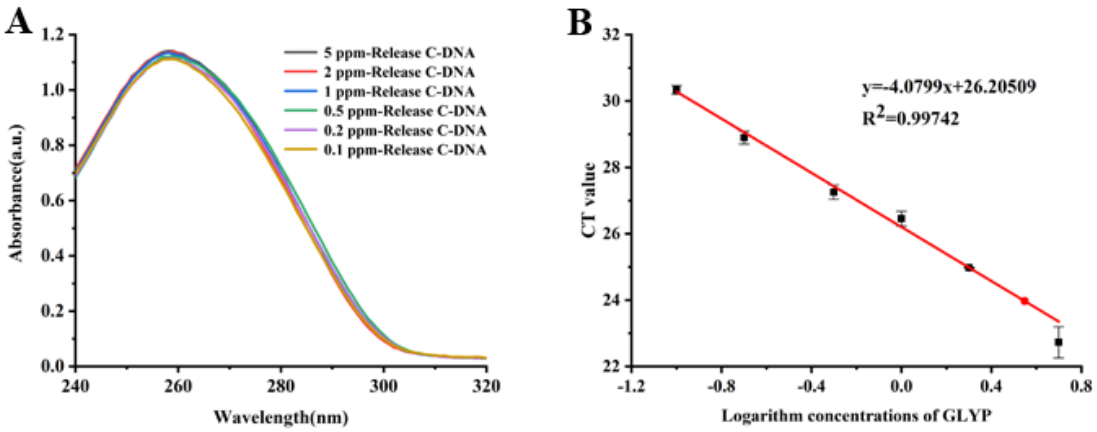

Fig. 4 (A) Different concentrations of glyphosate and Mbs@dsDNA UV-Vis absorption spectra of C-DNA released after incubation,（B) Standard curve of CT value and logarithm concentration of glyphosate

3.5 Real sample analysis

To further verify the practicability of the method in the actual scene, tap water spiked with glyphosate was used as real sample for detection. The validity of the method was confirmed by the reasonable range of

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recovery [103.4% ~ 104.9%] and relative standard deviation [0.4% ~ 0.73%] in the supporting information (Table S3). To illustrate the method's ability of glyphosate detecting, a crosswise comparison was carried out among all sorts of available technologies in Table 1.

Table. 1 Performance evaluation of glyphosate analysis technology

Method	LOD	Recovery	Sample	Reference
GC-MS	0.5 μg/mL	96.7-107.7%	Serum Urine	Saito et al. 2011
ELISA	8 μg/L	87.4-103.7%	River water, Tea, Soil	Wang et al. 2016
HPLC	0.7 μg/L	80.1-109.4%	Natural water	Vu et al. 2021
LC	0.103 μg/mL	80.63-97.11%	Soil	Druart et al. 2011
IC	5 μg/L	80-110%	Honey	Pareja et al. 2019
CE	150 μg/L	89.4-93.7%	Hemp	Lanaro et al. 2015
LC-MS/MS	1 μg/L	97-110%	Breast milk	Steinborn et al. 2016
EC	0.27 μg/L	72.7-98.96%	Cucumber Tap water	Zhang et al. 2017
qPCR	0.1 μg/mL	91.5%-114.4%	Tap water	This work

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4 Conclusion

In conclusion, by utilizing the specific recognition function of the aptamer, a biosensor for recognition of glyphosate based on the aptamer, magnetic beads and qPCR was invented. The linear range of the method is 5 μg/mL to 0.1 μg/mL, and the lowest detection limit detected by the experiment is 0.1 μg/mL. The detection technology can be applied to detect glyphosate in actual samples (such as tap water). The establishment of the detection method has a good application prospect and has reference value for detecting other pesticides.

Declarations

Author contribution: Miao Wang, and Yongxin She conceived and designed the experiments. The experiments were performed by Yong Shao, Run Tian and Jiaqi Duan. Data was analyzed by Fen Jin, Jing Cao, Guangyue Li and Zhen Cao. The paper was written by Run Tian, Jiaqi Duan and Miao Wang. The language of paper was revised and polished by M. Abd El-Aty.

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Declarations

Ethics approval and consent to participate: No applicable

Consent for publication: Not applicable.

Competing interests: The authors declare no competing interests.

Data availability: We declare that the data supporting this study are available within the article.

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Materials availability: We declare that the materials supporting this study are available within the article.

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