A novel electrochemical aptasensor based on layer-by-layer assembly of DNA-Au@Ag conjugates for rapid detection of aflatoxin M₁ in milk samples

Yuanyuan Hui,¹ Haishuai Peng,¹ Fuxin Zhang,¹ Lei Zhang,² Liu Yufang,¹ Aiqing Zhao,¹ Rong Jia,¹ Bini Wang,^{1,3*} and Yuxuan Song^{2*}

¹College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an, Shaanxi 710119, China

²College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China

³Shaanxi Provincial Key Laboratory of Electroanalytical Chemistry, Northwest University, Xi'an, Shaanxi 710069, China

ABSTRACT

Aflatoxin M₁ (AFM₁) is a common toxin in dairy products that causes acute and chronic human health disorders. Thus, the development of a rapid and accurate AFM₁ detection method is of vital importance for food safety monitoring. This work was to develop a novel electrochemical aptasensor for sensitive and specific determination of AFM₁. The dendritic-like nanostructure was formed on the gold electrode surface by layer-by-layer assembly of gold-silver core-shell nanoparticles modified with DNA conjugates. In the presence of AFM₁, the specific recognition between AFM₁ and Apt caused the disassociation of the DNA controlled dual Au@Ag conjugates from the surface of the electrode, causing less methylene blue to bind to the surface and weakening the electrochemical signal. The more AFM₁ there is, the weaker the electrochemical signal. Transmission electron microscope results showed that the successfully synthesized Au@Ag nanoparticles exhibited a core-shell structure with Au as core and Ag as shell, and their average diameter was about 30 nm. Under optimal conditions, the electrochemical aptasensor showed a wide detection ranging from 0.05 ng mL⁻¹ to 200 ng mL⁻¹, and a low detection limit of 0.02 ng mL⁻¹. Moreover, the proposed strategy has been successfully applied to the detection of AFM₁ in cow, goat, and sheep milk samples with satisfactory recoveries ranging from 91.10% to 104.05%. This work can provide a novel rapid detection method for AFM₁, and also provide a new sensing platform for the detection of other toxins.

Key words: electrochemical biosensor, aptamers, Au@Ag nanoparticles, aflatoxin M₁

INTRODUCTION

Aflatoxins (AF), a group of toxins structurally related to secondary metabolites, are produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (Sevim et al., 2019). The most common AF are aflatoxins B₁, B₂, G₁, G₂, M₁ and M₂. Among them, aflatoxin B₁ (AFB₁) has the highest toxicity and strongest carcinogenicity (Feng et al., 2020), and was classified as a group 1 carcinogen by the International Agency for Research on Cancer in 1993 (Jalalian et al., 2018; Zhao et al., 2021). Aflatoxin M₁ (AFM₁), a major hydroxylated metabolite of AFB₁ in the liver (Khodadadi et al., 2018; Fang et al., 2020), is frequently detected in milk of mammals that have ingested AFB₁ contaminated feed so that it is also called “milk toxin” (Zhou et al., 2019; Hamami et al., 2021). During the process of milk pasteurization or other thermal treatments, there is little possibility of inactivation of AFM₁ in milk. Even at a low level of concentration, AFM₁ could cause an enormous and serious threat such as hepatotoxic, neurotoxic, and even immunosuppressive effects to human health (Wang et al., 2016; Gratz et al., 2020). Considering that humans, particularly infants and children, have a great need for dairy products, many countries and regions have implemented regulations to control the content of AFM₁ in dairy products. Both the United States and China set a maximum residue limit in milk at 0.5 µg kg⁻¹, whereas the European Union set a lower permitted level of 0.05 µg kg⁻¹ in milk (European Commission, 2010; Food and Drug Administration 2015; Ministry of Agriculture of China, 2017). Consequently, it is necessary for AFM₁ in milk or dairy products to be monitored and detected.

Common methods for AFM₁ detection mainly include HPLC (Sereshti et al., 2019), liquid chromatography-mass spectrometry (LC-MS; Cavaliere et al., 2007), and ELISA (Jiang et al., 2013). Both HPLC and LC-MS are the most frequently used techniques because of their high accuracy and sensitivity, but the main drawback is that they require expensive equipment,

Received June 28, 2021.

Accepted November 12, 2021.

*Corresponding authors: biniwang@snnu.edu.cn and syx98728@163.com

multiplex pretreatment, and professional operators. Although ELISA is rapid and simple, it may produce both false positive and false-negative results for toxin trace detection. These disadvantages limit their application in real-time and rapid detection of toxins (Jalalian et al., 2021).

Aptamers (**Apts**) are single-stranded DNA or RNA molecules that can bind to proteins, cells, metal ions and small molecules with high affinity and specificity (Ellington and Szostak, 1990; Tuerk et al., 1993). As a novel targeted recognition molecule, aptamers have another name, chemical antibodies. However, compared with antibodies, Apts still have some distinct advantages, such as low cost, excellent stability, convenient preservation, easy synthesis, and high affinity. Considering the advantages of aptamers, researchers have developed various aptamer-based sensors (aptasensors), including colorimetric aptasensors (Qiao et al., 2021; Zhang et al., 2021), surface plasmon resonance aptasensors (Kotlarek et al., 2020), surface-enhanced Raman spectroscopy aptasensors (He et al., 2020), fluorescence aptasensors (Bi et al., 2021; Shi et al., 2021) and electrochemical aptasensors (Jalalian et al., 2018). Owing to the simple operation, rapid detection and high sensitivity and selectivity, electrochemical aptasensors have been widely used in food analysis, clinical testing, environmental monitoring and life science. Nanomaterials with high conductivity are commonly used as signal amplifiers for electrochemical aptasensors, and gold nanoparticles (**AuNP**), a class of typical and representative nanomaterials, have been widely adopted to construct the electrochemical sensing interface. Chen et al. (2018) constructed 3 different electrochemical aptasensors (dual AuNP conjugate, single AuNP, conjugate and the traditional method) based on the specific recognition ability of aptamers and the superior physical and chemical properties of AuNP (Chen et al., 2018). Results found that the use of DNA controlled dual AuNP conjugate could greatly amplify the electrochemical signal and improve the sensing performance significantly compared with single AuNPs conjugate or without the involvement of AuNP. However, compared with AuNP, the gold-silver core-shell nanoparticles (**Au@Ag**) not only exhibit better conductivity because of the synergistic effects of individual nanomaterials (Xu et al., 2015), but can also compensate for the deficiencies of individual nanomaterials and provide an excellent platform for the further immobilization of oligonucleotide (Amouzadeh Tabrizi et al., 2017; Song et al., 2018).

In this work, we developed a simple electrochemical aptasensor for highly sensitive and selective detection of AFM₁. The Apts-Au@Ag and cDNA2-Au@Ag conjugates were assembled onto the electrode interface

sequentially by specific base pairing, forming DNA controlled dual Au@Ag conjugates. The presence of AFM₁ led to the dissociation of DNA controlled dual Au@Ag conjugates from the electrode interface. Methylene blue (**MB**) was used as the electroactive substance and intercalated on the double-stranded DNA to produce the electrochemical signal. The larger the concentration of AFM₁, the weaker the electrochemical signal. The developed electrochemical aptasensor provides a promising strategy for the rapid and accurate detection of AFM₁.

MATERIALS AND METHODS

Reagents and Materials

All oligonucleotides (HPLC purified) used in this work were synthesized by Shanghai Biotechnology Co. Ltd., and the oligonucleotide sequences are as follows:

- cDNA1: 3'-ATGTGGAACTATCTAGCAGT-(CH₂)₆-SH-5'
- AFM₁ aptamers (Apt): 3'-ACTGCTAGAGATTTTCCACATTTCTGTGTGT-(CH₂)₆-SH-5'. The underlined portion of sequence is aptamer, which was designed according to the literature (Jalalian et al., 2018).
- cDNA2: 3'-ACACACAGAAATGTGGAAACTATCTAGCAGTGTCTCGGTGT-(CH₂)₆-SH-5'.

Oligonucleotides were dissolved in 20 mM Tris-HCl buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1 mM CaCl₂. Aflatoxin M₁, aflatoxin M₂, AFB₁, aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂), ochratoxin A (**OTA**), and fumonisin B₁ (**FB**₁) were purchased from Sigma-Aldrich. Hydrogen tetrachloroaurate trihydrate (HAuCl₄·3H₂O), Tris (2-carboxyethyl) phosphine hydrochloride (**TCEP**), silver nitrate (AgNO₃), 6-mercaptohexanol (**MCH**), and MB were ordered from Aladdin Chemistry Co. Ltd. Other reagents were of analytical grade and used without further purification.

Apparatus

Electrochemical measurements including square wave voltammetry (**SWV**), cyclic voltammetry, electrochemical impedance spectroscopy (**EIS**), and differential pulse stripping voltammetry (**DPV**) were carried out on an electrochemical workstation (CH Instruments) with a 3-electrode system composed of a gold electrode as the working electrode, a platinum wire electrode as the counter electrode, and Ag or AgCl as the reference

electrode. Transmission electron microscope (TEM) images were collected using a HT-7700 transmission electron microscope (Hitachi) with an accelerating voltage of 80 kV. The UV-visible absorption spectra were obtained using a UV300 spectrophotometer (InsMark).

Preparation of DNA (Apts and cDNA2)-Au@Ag Conjugates

The AuNP were synthesized by the classic Turkevich citrate reduction method (Frens, 1973). Briefly, 100 mL of HAuCl₄ solution (0.01%, wt/vol) was heated to boiling, and then 1.5 mL of trisodium citrate (1%, wt/vol) was immediately added to the solution and mixed thoroughly. Then the mixture was heated until it changed to deep wine red. Thereafter, the obtained AuNPs solution was cooled to room temperature and then stored at 4°C in a brown glass bottle for further use.

The Apts-Au@Ag conjugates was synthesized according to previously reported methods with slight modifications (Amouzadeh Tabrizi et al., 2017; Yao et al., 2017). To activate sulfhydryl groups, 100 µL of 10 µM thiolated Apts was added to 100 mL of 10 mM TCEP and the mixture was kept at room temperature for 2 h. Subsequently, 3 mL of the AuNP solution was added to the mixture and stored at 4°C for 24 h to form the Apts-Au conjugates through Au-S bond. The obtained conjugates were centrifuged at $5,600 \times g$ for 20 min at room temperature to remove unbound Apts, and then redispersed in distilled water. Afterward, 60 µL of ascorbic acid (10 mM) and 100 µL of AgNO₃ solution (0.01 mM) were added to the conjugates dropwise and gently stirred for 5 min to form Apts-Au@Ag conjugates. After centrifugation at $5,600 \times g$ for 20 min at room temperature, the obtained Apts-Au@Ag conjugate sediments were dispersed in distilled water for further use. The preparation process of cDNA2-Au@Ag conjugates was the same as that for Apts-Au@Ag conjugates except for the cDNA2.

Fabrication of Electrochemical Aptasensor

Figure 1 shows the construction process of the electrochemical aptasensor. The bare gold electrode (GE) was first polished with 0.05-µm alumina powder and washed ultrasonically in ethanol and water (1:1) for 5 min. After that, GE was immersed in a freshly prepared piranha solution (98% H₂SO₄:30% H₂O₂ = 3:1, vol/vol) for 10 min, rinsed thoroughly with ultrapure water, and dried under a stream of nitrogen.

The cleaned GE was immersed in 20 µL of SH-cDNA1 solution (3.0 µM) for 4 h at room temperature, and then washed gently with Tris buffer solution. To block the nonspecific binding site, 10 µL MCH was dropped

on the surface of cDNA1/GE (the slash indicates a combination or layering of the components). After drying, the cDNA1/GE was incubated in 30 µL Apts-Au@Ag conjugates for 1 h at room temperature to make Apts-Au@Ag conjugates bind to the cDNA1 by specific base pairing. Then, the Apts-Au@Ag/cDNA1/GE was immersed in 30 µL of cDNA2-Au@Ag conjugates for 1 h at room temperature, and free Apts on the Apts-Au@Ag conjugates were combined with cDNA2-Au@Ag conjugates to form DNA controlled dual Au@Ag conjugates. The fabricated aptasensor was stored at 4°C for future use.

Electrochemical Characterization

To verify the successful fabrication of the electrochemical aptasensor, EIS, and SWV characterizations of bare GE, cDNA1/GE, Apt-Au@Ag/cDNA1/GE, cDNA2-Au@Ag/Apt-Au@Ag/cDNA1/GE, and AFM₁/cDNA2-Au@Ag/Apt-Au@Ag/cDNA1/GE were performed, respectively. The electrolyte solution used in electrochemical characterization was 5 mM [Fe(CN)₆]^{3-/4-} solution containing 0.1 M KCl, and [Fe(CN)₆]^{3-/4-} worked as the redox indicator. Electrochemical impedance spectroscopy was carried out at an initial potential of 0.1 V, an amplitude of 0.005 V and a scanning range from 1 to 10⁵ Hz. The SWV scanned from -0.3 to 0.4 V with an amplitude of 0.025 V and a frequency of 15 Hz.

Electrochemical Detection of AFM₁

The determination of AFM₁ was performed as follows: The fabricated aptasensor was incubated with 20 µL of different concentrations of AFM₁ (0.05, 0.1, 1, 10, 50, 100, and 200 ng mL⁻¹) for 45 min at room temperature. After thoroughly capturing, the aptasensor was carefully rinsed with PBS to remove specific conjugates of AFM₁-Apts-Au@Ag/cDNA2-Au@Ag. Thereafter, the aptasensor was further incubated with 20 µL of MB (4 mM) for 40 min at room temperature, and the electrochemical signals were recorded using DPV. The DPV measurements were carried out in 0.1 M PBS (pH = 7.0) containing 0.02 M KCl with a scan range of 0.1 to 0.3 V, a pulse amplitude of 0.05 V, and a pulse width of 0.05 s.

Milk Sample Preparation

Cow, goat, and sheep milk samples were obtained from China Resources Vanguard supermarket (Shaanxi, China), the Redstar Meiling Dairy Co., Ltd. (Shaanxi, China), and the Gansu Yuansheng Agriculture and Animal Husbandry Technology Co., Ltd. (Gansu, China),

respectively. They were treated according to the reported literature with minor modifications (Jiang et al., 2020). In brief, the milk samples (25 mL) were heated in a water bath (65°C) for 30 min and centrifuged at $1,510 \times g$ for 20 min at room temperature. The upper fat layer was discarded, and the aqueous phase at the middle of the centrifugal tube was carefully recovered. The recovered liquid was spiked with various concentrations of AFM₁. Afterward, 2 mL of recovered liquid was mixed with 6 mL of methanol (HPLC grade). The mixture was ultrasonically treated for 30 min before being filtered through a disposable syringe filter (0.22 μ m). Finally, for spiked recovery experiments, 0.2 mL of filtered supernatant was diluted 1,000 times with PBS and spiked with different concentrations of AFM₁.

RESULTS AND DISCUSSION

The Sensing Principle for Determination of AFM₁

Figure 1A exhibits a schematic illustration of the synthesis of DNA-Au@Ag conjugates. First, the DNA

(Apt or cDNA2) modified with SH at the 5' end was immobilized on the AuNPs surface via Au-S bonds. Then the DNA-AuNP are encapsulated with silver. During this process, DNA might be embedded partly into the silver shell layer and lose the bio-recognition features. To avoid the problem, the base sequences of Apt (TTCTGTGTGT) and cDNA2 (GTCTCGGTGT) were used to protect the bio-recognition part.

Figure 1B illustrates the principle of the electrochemical aptasensor for determination of AFM₁. The SH-cDNA1 was immobilized on the surface of GE through Au-S bond. Then cDNA1/GE was treated with MCH. The MCH not only blocks the remaining active sites and avoids nonspecific adsorption, but also maintains the linear structure of cDNA1 and increases its hybridization efficiency. The Apts-Au@Ag and cDNA2-Au@Ag conjugates could be attached to cDNA1/GE via specific base pairing, successively, eventually forming DNA controlled dual Au@Ag conjugates. When AFM₁ was absent, MB, as an electroactive substance, could intercalate into double-stranded DNA, binding specifically to guanine and producing a strong electrochemical

Figure 1. Schematic of the fabrication of the electrochemical aptasensor for the detection of aflatoxins in milk. (A) Illustration of the synthesis of DNA-Au@Ag conjugates; (B) illustration of the principle of the electrochemical aptasensor for determination of aflatoxin M₁ (AFM₁). SH = sulfhydryl group; AuNP = gold nanoparticles; cDNA1 and cDNA2 = oligonucleotide sequences; MCH = mercaptohexanol; Au@Ag = gold-silver core-shell nanoparticle; Apt = aptamer; MB = methylene blue.

signal. But in the presence of AFM₁, AFM₁ preferentially bonded to Apt due to the high affinity between AFM₁ and Apt, causing dissociation of DNA controlled dual Au@Ag conjugates from the surface of cDNA1/GE, which in turn resulted in less accumulation of MB on the surface of GE and a decrease of electrochemical signals. There is an inverse proportional relationship between electrochemical signals and AFM₁ concentrations, and the higher the AFM₁ concentration, the weaker the electrochemical signal.

Characterization of the Au@Ag Nanoparticles

To confirm the successful syntheses of Au@Ag nanoparticles, the nanoparticles were characterized by TEM, high-angle annular dark field-scanning TEM-energy-dispersive X-ray spectrometry (HAADF-STEM-EDX), and UV-vis spectroscopy. The structural characterization of the synthesized Au@Ag nanoparticles was analyzed by TEM and images are shown in Figure 2A; it can be seen that nanoparticles, with a size of approximately 30 nm, were relatively regular and possessed a core-shell nanostructure. The EDX spectrum of the Au@Ag nanoparticles demonstrated that the elements of Au and Ag were distributed homogeneously within the core-shell nanostructure (Figure 2B and 2C). The superimposed image of Au@Ag nanoparticles shows a clear boundary between the Au and Ag segments (Figure 2D), further confirming the Au@Ag core-shell nanostructure. To further study the optical characterization of AuNPs, Au@Ag nanoparticles and DNA-Au@Ag conjugates, we analyzed their UV-vis spectra to confirm the successful formation of these nanocomposites. It can be seen from Figure 2E that AuNP illustrated a strong absorption peak in the UV region around 525 nm (curve a). When the Au@Ag nanoparticles were formed by adding AgNP, the absorption peak of Au disappeared, and only the characteristic absorption peak of Ag was clearly observed at 420 nm, suggesting that the Au@Ag core-shell structure was successfully formed with the AgNPs coating the outside (curve b). The UV-vis spectra confirmed that the synthesized Au@Ag nanoparticles had only one absorption band of Ag at 420 nm, indicating that the Au@Ag core-shell structure was successfully formed with the AgNPs coating the outside. When thiolated DNA (Apts or cDNA2) was combined with AuNPs through Au-S bond, the addition of AgNPs resulted in the disappearance of the absorption peak of AuNP at 525 nm, with 2 absorption peaks at 257 nm (DNA) and 420 nm (AgNP) observed (curve c). These results indicated that AuNP were completely covered by AgNP but DNA was not, which further proved that DNA-

Au@Ag conjugates were successfully formed with DNA extending outside for the binding of another DNA-Au@Ag conjugate via specific base pairing.

To further explore the reasons for the disappearance of the absorption peak of Au after the formation of Au@Ag nanoparticles, the UV-vis absorption spectra of Au@Ag nanoparticles containing different amounts of silver nitrate was also characterized. As illustrated in Figure 2F, with the increasing amount of silver nitrate added, the absorption intensity of the mixture gradually increased, and the color gradually changed from wine red to yellow. Only when 50 μ L of AgNO₃ was added did 2 absorption peaks of AuNP and AgNP appear simultaneously, and the absorption peaks of AuNP showed a blue shift compared with that of pure AuNP. The main reason might be that the encapsulation of Ag⁺ changed the dielectric constant of the Au and scattering ability of light at different wavelengths. These obtained results were in accordance with the previously published results on Au@Ag nanoparticles (Mao et al., 2017; Zhao et al., 2018), which also further demonstrated the successful synthesis of Au@Ag nanoparticles.

Electrochemical Characterization of Different Electrodes

Electrochemical impedance spectroscopy was used to monitor the fabrication process of the electrochemical aptasensor. For EIS, the semicircle diameter can be used to evaluate the electron transfer resistance (R_{et}) on the surface of the electrode using [Fe(CN)₆]^{3-/4-} as the redox probe, and the increase in semicircle diameter signifies the increase in the R_{et} value. As shown in Figure 3A, the R_{et} of the bare GE was relatively low (curve a). When the SH-cDNA1 was self-assembled onto the surface of the GE by Au-S bond, an obviously increased R_{et} value was observed (curve b). This is because the negatively charged phosphate backbone of cDNA1 hindered electron transfer, which also demonstrated that the cDNA1 was successfully immobilized on the electrode surface. After the successive immobilization of the Apts-Au@Ag (curve c) and the cDNA2-Au@Ag conjugates (curve d) onto the GE, the R_{et} were significantly enhanced. This could be caused by the increase in negative charge density of the double-helical DNA structure. It was noticeable, however, that the R_{et} value obviously decreased when incubating with 10 ng mL⁻¹ AFM₁ (curve e). This suggested that the preferential binding of Apt toward AFM₁ resulted in the disassociation of Apts-Au@Ag from the surface of the GE. Then the R_{et} value continued to reduce when the GE was immersed in 4 mM MB solution (curve f).

Figure 2. Transmission electron micrographs of (A) gold-silver core-shell nanoparticles (Au@Ag); high-angle annular dark field-scanning TEM-energy-dispersive X-ray spectrometry (HAADF-STEM-EDX) elemental mapping of (B) Ag, (C) Au, and superimposed image of (D) Au@Ag; (E) UV-visible (Vis) spectra of AuNPs (a), Au@Ag (b), and DNA-Au@Ag (c); the amount of AgNO₃ added was 350 μ L; and (F) UV-Vis spectra of Au@Ag; the amount of AgNO₃ added was (curves 1 to 6): 0, 50, 150, 250, 350, and 450 μ L.

Figure 3. Electrochemical impedance spectroscopy (A) and square wave voltammetry (B) of different modification steps of the aptasensor in 5 mM [Fe(CN)₆]^{3-/4-} containing 0.1 M KCl: (a, a') bare GE; (b, b') cDNA1/GE; (c, c') Apt-Au@Ag/cDNA1/GE; (d, d') cDNA2-Au@Ag/Apt-Au@Ag/cDNA1/GE; (e, e') AFM1/cDNA2-Au@Ag/Apt-Au@Ag/cDNA1/GE; and (f, f') MB/AFM1/cDNA2-Au@Ag/Apt-Au@Ag/cDNA1/GE. GE = gold electrode; cDNA1 and cDNA2 = oligonucleotide sequences; Apt = aptamer; Au@Ag = gold-silver core-shell nanoparticle; MB = methylene blue. Slashes indicate layers of the aptasensors.

The reason could be attributed to the fact that MB is an electroactive substance that could intercalate into the double-stranded DNA, promoting the transfer of electrons. These results proved that the aptasensor was successfully fabricated.

The SWV measurements were also recorded to further prove the results of EIS. As shown in Figure 3B, the bare GE showed a strong current signal (curve a'). The current signal was reduced after the cDNA was assembled (curve b'). When Apts-Au@Ag and cDNA2-Au@Ag conjugates were immobilized on the surface of electrode, a clearly declining current was observed (curve c' and d', respectively). However, the addition of AFM₁ resulted in an increasing current (curve e'). Subsequently, the current signal was further enhanced when the electrode was immersed in MB solution (curve f'). These results were consistent with those obtained by EIS experiments above, indicating the feasibility of our scheme for AFM₁ detection.

Optimization of the Experimental Conditions

To acquire the optimum analytical performance, several experiment parameters that could affect the electrochemical signal of aptasensor were optimized by the cyclic voltammetry method. The Au@Ag nanoparticles plays an important role in the construction of the electrochemical aptasensor. Thus, the effect of the addition amount of AgNO₃ on the electroconductivity of Au@Ag nanoparticles was explored. Different amounts of AgNO₃ (50, 150, 250, 350, and 450 μ L) were added in the preparation of Au@Ag nanoparticles. Then, 8 μ L of Au@Ag nanoparticles with different AgNO₃ additions were dropped on the surface of GE and left overnight at room temperature. The electrochemical signals were recorded using SWV. Results were presented in Figure 4A. The electrochemical signal first increased with the increasing amount of AgNO₃. This could be ascribed to the excellent electron transfer performance of Au@Ag nanoparticles. But when the amount of AgNO₃ exceeded 350 μ L, the electrochemical signal gradually decreased, probably because too much AgNO₃ increased the thickness of the shell of the Au@Ag core-shell structure, which may further affect the conductivity of Au@Ag nanoparticles. Hence, 350 μ L was selected as the optimal amount of AgNO₃.

The incubation time of AFM₁ with Apts could also affect the performance of the electrochemical aptasensor. The cDNA2-Au@Ag/Apt-Au@Ag/cDNA1/GE was incubated with 20 μ L of AFM₁ (100 ng mL⁻¹) for different times (15, 25, 35, 45, and 55 min) at room temperature and the electrochemical signals were measured using SWV. As shown in Figure 4B, the electrochemical signal was enhanced with increasing

Figure 4. Optimization of the experimental conditions of (A) amount of AgNO₃ added, (B) incubation time of AFM₁ with aptamers (Apt), and (C) incubation time of methylene blue (MB). The data depict the average results of 3 experiments.

Table 1. Comparison of the analytical performance with other reported methods for aflatoxin M₁ detection

Detection method	Linear range, ng mL ⁻¹	Detection limit (LOD), ng mL ⁻¹	Reference
LC-MS	0.1–20	0.05	Fan et al., 2015
ELISA	2.5–80	1	Rosi et al., 2007
Electrochemiluminescence aptasensor	10–200	0.05	Khoshfetrat et al., 2018
Optical aptasensor	0.3–75	0.03	Jalalian et al., 2021
Electrochemical aptasensor	0.006–0.06	0.00198	Nguyen et al., 2013
Electrochemical aptasensor	0.01–80	0.002	Jahangiri-Dehaghani et al., 2020
Electrochemical aptasensor	0.05–200	0.02	This work

incubation time, and then almost kept stable after 45 min. This indicated that the combination between AFM₁ and its Apts was completed, and the Apt-AFM₁ complex formed on the surface of the electrode might have reached saturation. Thus, 45 min was selected as the optimum incubation time.

In addition, the effect of incubation time of MB on the aptasensor was also investigated. The AFM₁/cDNA2-Au@Ag/Apt-Au@Ag/cDNA1/GE was incubated with 20 μ L of MB (4 mM) for different times (10, 20, 30, 40, and 50 min) at room temperature. The SWV was used to monitor the signal of each electrode. From Figure 4C, it can be seen that the electrochemical signal gradually increased with an increase in the incubation time from 10 to 50 min. When the incubation time was greater than 40 min, the increasing signal was not significant. The reason could be explained by the enough MB adsorbed in the double strand DNA. Therefore, 40 min of MB incubation time was used in the following experimental study.

Analytical Performance of the Fabricated Electrochemical Aptasensor

Under the optimal experimental conditions, the DPV responses of the aptasensor at different concentrations of AFM₁ (0.05, 0.1, 1, 10, 50, 100, and 200 ng mL⁻¹) were detected. As shown in Figure 5A, the electrochemical signal gradually decreased with increased AFM₁ concentrations. Figure 5B shows a linear relationship between the variation value of the electrochemical signal relative to blank (ΔI) and the logarithm of AFM₁ concentrations ($\log c_{\text{AFM}_1}$) from 0.05 ng mL⁻¹ to 200 ng mL⁻¹. The linear regression equation obtained was $\Delta I (\mu\text{A}) = 9.2441 \log c + 21.8880$ with a correlation coefficient of 0.9973, and the detection limit was estimated to be 0.02 ng mL⁻¹ using the equation $\text{LOD} = 3s/n$ (Skoog et al., 1998), in which s is standard deviation for the blank solution and n is the slope of the calibration curve (9.2441). Compared with other previously reported methods of AFM₁ detection (Table

Figure 5. (A) Differential pulse stripping voltammetry (DPV) response of aflatoxin M₁ (AFM₁) at different concentrations (from a to h): 0, 0.05, 0.1, 1, 10, 50, 100, and 200 ng mL⁻¹. (B) Linear relationship between the current signal change (ΔI) and the logarithmic value of the AFM₁ concentration. Error bars represent the SD of the triplicates.

1; Rosi et al., 2007; Nguyen et al., 2013; Fan et al., 2015; Khoshfetrat et al., 2018; Jahangiri-Dehaghani et al., 2020; Jalalian et al., 2021), the electrochemical aptasensor based on Au@Ag nanomaterials and redox probe MB demonstrates a wider linear range (4 orders of magnitude). Its performance was superior to some detection methods, particularly LC-MS and ELISA. The sample pretreatment of the aptasensor was not only simple, but also linear range and detection limit, perfectly satisfying the current detection requirement. This could be attributed to the following factors: First, the excellent electrical conductivity, high surface area and good biocompatibility were beneficial for charge transport and DNA immobilization of DNA, thus increasing the sensitivity of aptasensor. Moreover, using aptamers as the recognition element avoided the shortcomings of antibodies, such as poor stability and complicated preparation procedures, therefore, the sensitivity and selectivity of detection could be improved. In addition, MB was introduced into the sensing system and used as signal amplification element, which further improved the detection sensitivity. However, the approaches reported by Nguyen et al. (2013) and Jahangiri-Dehaghani et al. (2020) have higher sensitivity than our method. The reason could be explained by the fact that these approaches use nanomaterials with better conductivity, such as Fe₃O₄ incorporated polyaniline (Fe₃O₄/PANi) film and platinum nanoparticles loaded on Fe-based metal-organic frameworks [PtNP/MIL-101(Fe)]. Thus, utilizing some novel nanomaterials with good conductivity was very meaningful to further improve the sensitivity of detection in further study.

Reproducibility, Stability, and Selectivity of the Fabricated Electrochemical Aptasensor

The reproducibility of aptasensor is another key factor to consider. To estimate the reproducibility, 5 electrodes prepared under the same conditions and steps were used to detect AFM₁ of 100 ng mL⁻¹. As shown in Figure 6A, these electrodes exhibited the closely current response, and their relative standard deviation is 6.35%. The result demonstrated that this aptasensor has good reproducibility. Besides, the stability of the aptasensor was evaluated by long-term storage. After being stored for days at 4°C, the electrochemical signal could remain at 92.9% of its initial signal (Figure 6B), indicating the aptasensor possessed desirable stability.

The selectivity of the aptasensor is an important factor affecting the analysis performance of the aptasensor. To estimate the selectivity of the method, the interfering substances, including AFB₁, AFB₂, AFG₁, AFG₂, aflatoxin M₂, OTA, FB₁, and mixtures (OTA+AFB₁+FB₁+AFM₁), were also detected under

Figure 6. Evaluation of (A) reproducibility of the aptasensor [the different curves are representative of results obtained from 5 electrodes prepared under the same conditions and steps for the detection of 10 ng mL⁻¹ aflatoxin M₁ (AFM₁)]; (B) the stability of the aptasensor (concentration of AFM₁ was 100 ng mL⁻¹); and (C) the specificity of the aptasensor toward different interferences: (a) blank, (b) aflatoxin (AF) B₁, (c) ochratoxin A (OTA), (d) fumonisin B₁ (FB₁), (e) AFG₁, (f) AFG₂, (g) AFM₂, (h) OTA+AFB₁+FB₁+AFM₁ (OTA:AFB₁:FB₁:AFM₁ = 1:1:1:4), and (i) AFM₁. All mycotoxins were used at 100 ng mL⁻¹.

Table 2. The detection results of aflatoxin M₁ (AFM₁) in the spiked pasteurized sheep milk samples with the electrochemical aptasensor (n = 3)

Sample	AFM ₁ (ng mL ⁻¹)		Recovery, %	Relative SD, %
	Added	Detected		
Pasteurized goat milk	150.00	143.72	95.81	7.87
	100.00	93.66	93.66	5.05
	10.00	9.21	92.12	5.24
	1.00	0.94	94.00	4.98
	0.1	0.089	89.00	5.17
Pasteurized sheep milk	150.00	156.07	104.05	5.16
	100.00	98.43	98.43	4.88
	10.00	9.11	91.10	7.02
	1.00	1.03	103.00	6.07
	0.1	0.095	95.00	4.65
Pasteurized cow milk	150.00	142.04	94.69	5.02
	100.00	95.75	95.75	5.77
	10.00	9.38	93.80	6.10
	1.00	0.94	94.00	4.32
	0.1	0.092	92.00	5.89

the same condition. All concentrations of interfering toxins and AFM₁ were 100 ng mL⁻¹. As shown in Figure 6C, compared with the blank groups, a significantly weakened electrochemical signal was observed in the presence of AFM₁, whereas there were no obvious changes in the electrochemical signal in the presence of other toxins. This could be explained by the high specific recognition ability of Apts toward AFM₁, which also suggested that the electrochemical aptasensor possesses excellent selectivity for its target toxins.

Testing of AFM₁ in Milk

To evaluate the potential application of the assay in real samples, the aptasensor was tested in cow, goat and sheep milk samples spiked with various concentrations (0.1, 1, 10, 100, and 150 ng mL⁻¹) of AFM₁. The detection results for AFM₁ were demonstrated in Table 2. The recoveries were in the range of 89.00 to 104.05%, and the relative standard deviations were 4.32 to 7.87%. These results proved that the aptasensor has been successfully applied for AFM₁ detection in milk samples.

CONCLUSIONS

In this study, we successfully demonstrated a novel electrochemical aptasensor based on layer-by-layer assembly of DNA-Au@Ag conjugates for AFM₁ detection. The detection limit was as low as 0.02 ng mL⁻¹, meeting the real-time and rapid detection requirements of government and the food industry. Furthermore, the construction process of the electrochemical aptasensor was straightforward, and the detection time was

85 min. In addition, the specific recognition of Apt toward AFM₁ ensures this aptasensor possesses good repeatability and desirable stability and selectivity. Importantly, this electrochemical aptasensor has been successfully used to detect the AFM₁ in cow, goat, and sheep milk samples with satisfactory results. This aptasensor offers a new method for the rapid and accurate detection of AFM₁ in milk samples, and provides a promising electrochemical platform for determining contamination with other mycotoxins such as OTA, zearalenone, and AFB₁.

ACKNOWLEDGMENTS

We appreciate the Key Industry Innovation Chain Project of Key R & D Program in Shaanxi Province (2019ZDLNY06-06), the Science and Technology Program of Xi'an City (No. 20NYF0014 and 20NYF0018), the Fundamental Research Funds for the Central Universities (GK202003084), and the Project of Cooperating to Build Jinchang Meat Sheep Experimental Demonstration Base A289021806 (K3320219064). The authors have not stated any conflicts of interest.

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