



Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb



Research paper

Ochratoxin A detection platform based on signal amplification by Exonuclease III and fluorescence quenching by gold nanoparticles

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ARTICLE INFO

Article history:

Received 23 June 2017

Received in revised form 19 August 2017

Accepted 23 August 2017

Available online xxx

Keywords:

Ochratoxin A

Aptamer

Exonuclease III

Signal amplification

Gold nanoparticles

ABSTRACT

This study aimed to establish a detection platform for ochratoxin A (OTA), a common mycotoxin contaminant of various food products. The detection platform is based on signal amplification by Exonuclease III (Exo III) and fluorescence quenching by gold nanoparticles (AuNPs). The aptamer recognizes and binds to OTA, thus leading to the formation of duplex DNA between the complementary DNA (cDNA) strand and the signal probe. Exo III then digests the duplex DNA from the 3' blunt terminus of the signal probe to liberate the fluorophore and release cDNA. The released cDNA then hybridizes with other signal probes to initiate a new cleavage reaction. Through this cyclic hybridization–hydrolysis process, an OTA molecule can trigger the cleavage of a large quantity of signal probes. Upon the addition of AuNPs, the fluorophore cannot be adsorbed and quenched, thus notably amplifying fluorescence. The designed aptasensor is highly selective for OTA with a low limit of detection (4.82 nM). The feasibility of the detection procedure and the applicability of the aptasensor were validated through the detection of OTA in spiked red wine without interference from the sample matrix. Results indicated that the aptasensor could be used to verify the effectiveness of mycotoxin-control strategies.

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1. Introduction

Ochratoxin A (OTA) is a naturally occurring mycotoxin produced by *Aspergillus ochraceus*, *Penicillium verrucosum*, and *Penicillium nordicum* [1]. OTA is one of the most abundant mycotoxin contaminants of various food products, such as cereals and cereal products, coffee, beans, milk and milk products, eggs, pork and meat products, wine, and beer [2–4]. OTA has been classified as a possible carcinogen by the International Agency for Research on Cancer (IARC) [5]. This mycotoxin is also weakly mutagenic and exerts immunosuppressive and immunotoxic effects [6,7]. The presence of OTA in food and food products has been reported in different countries. The detection and quantification of OTA in contaminated raw materials are of considerable significance for avoiding OTA consumption [8,9]. OTA is detected through conventional chromatographic methods, such as thin layer chromatography; high-performance liquid chromatography (HPLC); or gas chro-

matography coupled to ultraviolet–visible, fluorescence, or mass spectrometry [10,11]. These methods, however, generally require multiple pretreatment steps, including extraction, extensive sample purification, preconcentration, and analyte derivatization. The execution of these pretreatment steps extends the duration of analysis, adds to costs, and requires trained personnel [12,13]. Therefore, simple, cost effective, and comparatively rapid analytical methods that do not require the use of complex instruments are highly desired for OTA detection.

Aptamers are artificial, single-stranded oligonucleotides of DNA or RNA sequences which are produced by *in vitro* selection process called the systematic evolution of ligands by exponential enrichment (SELEX) [14]. They can recognize specific targets and form defined tertiary structures upon target binding. Compared with antibodies, aptamers possess the significant advantages including small size, high stability, low-cost, and easy synthesis and labeling. Furthermore, aptamers can be selected for a broad range of targets, including small molecules, proteins, nucleic acids, cells, tissues, and even organisms. Although the binding ability of aptamers is similar to that of antibodies, their synthesis, maintenance, and delivery are easier than those of antibodies [15–17]. Thus, aptamers are promising molecular receptors for bioanalytical applications.

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Table 1
Synthesized oligonucleotides(5'-3') used in the experiment.

Name	Sequence (5'-3')
Aptamer	GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACA TTTT
SP	FAM-CGTAAAGGGAGCATCG C
cDNA	GCGATGCTCCCTTTACG CC TTTT

Exonuclease III (Exo III) is an exonuclease that catalyzes the digestion of duplex DNAs from blunt or recessed 3'-hydroxyl termini. Exo III enables the selective hydrolysis of a single stand of duplex DNA and does not attack single-stranded DNA (ssDNA) or oligonucleotides. The selectivity of Exo III digestion can be applied in the digestion of probe DNA. Furthermore, Exo III digestion does not require a specific target DNA sequence for the cyclic enzymatic digestion of DNA [18–20]. Given these advantages, Exo III is a versatile platform for the development of various detection strategies based on cyclic signal amplification.

In recent years, many novel analytical tools have been synthesized from various nanomaterials, such as nanoparticles [21–24], nanotubes [25,26], nanosheets [27–29], nanoislands [30,31], nanochannels [32] and nanowires [33–35]. Gold nanoparticles (AuNPs) are widely used in immunoassays and in biochip and bioassay read-out techniques because of their unique characteristics, including chemical stability; facile synthesis; excellent optical, thermal, and electronic properties; excellent compatibility with biomolecules; extremely high absorption coefficient; and ability to accept energy [36–38].

Fluorescence quenching occurs when fluorophores are appended onto AuNPs, causing fluorescence resonance energy transfer (FRET) from the organic donor to the AuNPs acceptor [39,40]. Considering that the fluorescent labeling of an aptamer can decrease the binding affinity of an aptamer for its target [41], we fabricated a simple FRET system for OTA detection based on the combination of a label-free aptamer, its complementary cDNA strand, AuNPs, and the catalytic recycling activity of Exo III. The OTA-specific aptamer can fold to form an antiparallel G-quadruplex structure upon exposure to OTA [26], leading to the formation of duplex DNA between the cDNA strand and signal probe (SP). Exo III then digests the duplex DNA from the 3'-blunt terminus of the SP, thus liberating the fluorophore. The released cDNA then hybridizes with another SP to initiate a new cleavage reaction. Through this cyclic hybridization–hydrolysis, an OTA molecule triggers the cleavage of a large quantity of SP. Upon the addition of AuNPs, the fluorophore cannot be adsorbed and quenched, markedly amplifying fluorescence. The developed fluorometric method is inexpensive, highly sensitive, and facile. The assay components are cheap and commercially available. Furthermore, this method is a virtually universal approach because of the exceptional structural selectivity of Exo III, and can be used for the detection of other targets, such as ions, small molecules, and proteins.

2. Materials and methods

2.1. Materials

The OTA aptamer, complementary DNA (cDNA) and SP were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequences of the utilized oligonucleotides are listed in Table 1, and the abbreviations used in this work are listed in Table 2. Exo III was bought from TaKaRa Biotechnology Inc. (Dalian, China). OTA, ochratoxin B (OTB), ochratoxin C (OTC), aflatoxin B1 (AFB1), and zearalenone (ZEN) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gold (III) chloride trihydrate (HAuCl₄·4H₂O) were purchased from Beijing Chemical Reagent Company (Beijing, China). Binding buffer (10 mM Tris, pH 8.0, 120 mM NaCl, 5 mM

Table 2
A list of abbreviations for the work.

Original words	Abbreviations
gold nanoparticles	AuNPs
Exonuclease III	Exo III
Signal probe	SP
Carboxyl fluorescein	FAM
complementary DNA	cDNA
Ochratoxin A	OTA
Ochratoxin B	OTB
Ochratoxin C	OTC
Aflatoxin B1	AFB1
Zearalenone	ZEN

KCl, 10 mM MgCl₂, and 20 mM CaCl₂) was used for the binding reaction between the aptamer and OTA. The OTA stock solution (1 mM) was prepared by dissolving ochratoxin in absolute ethanol and stored at –20 °C. The Changyu rose- red wine used in this work was produced by YanTai ChangYu Pioneer Wine Company Limited (Yantai, China). All other chemicals were of analytical grade and used as received without further purification. Ultrapure water with an electrical resistivity of 18.2 MΩ cm was obtained using a Milli-Q ultrahigh-purity water system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

UV-2600 spectrophotometer (Shimadzu, Japan) was used to quantify the oligonucleotides and to obtain the UV vis spectra of the AuNPs. Hitachi 600 transmission electron microscope (Hitachi, Japan) was used to obtain high-resolution transmission electron microscopy (HRTEM) image of the AuNPs. RF-5301PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) was used to record fluorescence spectra. Meanwhile, emission spectra were recorded within the wavelength range of 500–640 nm upon excitation at 495 nm. Slit widths for excitation and emission were set at 5 nm. All measurements were performed at room temperature unless stated otherwise.

2.3. Synthesis of water-resuspended AuNPs

All glassware was thoroughly cleaned with saturated chromic acid solution, rinsed with ultrapure water, and dried before use. AuNPs were prepared through HAuCl₄ reduction in accordance with a previously reported procedure [42,43]. In brief, 5 mL of 1% sodium citrate solution was rapidly injected into 50 mL of 1% HAuCl₄ solution under magnetic stirring. The mixture was boiled and stirred for 15 min. Then, the heating mantle was removed, and magnetic stirring was continued for an additional 15 min. After cooling down to room temperature, the solution was centrifuged at 10,000 r/min for 20 min. The supernatant was removed, and the AuNPs were resuspended in ultrapure water. The AuNP solution was stored at 4 °C prior to use.

2.4. Characterization of AuNPs

AuNPs formation was monitored using a UV–vis spectrophotometer in the range of 350–800 nm at a resolution of 1 nm. The morphology, size, and shape of AuNPs were analyzed via HRTEM. HRTEM images were obtained in the conventional transmission mode at an accelerating voltage of 200 kV.

2.5. Fluorescence detection of OTA

For the quantitative measurement of OTA, 50 μL of aptamer (0.2 μM) was mixed with 50 μL of OTA solutions with different concentrations. The mixture was incubated for 30 min at room tem-

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