Nanomed. J. 6(2): 105-111, Spring 2019

RESEARCH PAPER

A colorimetric aptasensor for selective detection of oxytetracycline in milk, using gold nanoparticles and oxytetracline-short aptamer

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ABSTRACT

Objective (s): In light of misuse of antibiotics in animal husbandry and their side effects on human health, there is an argent need to develop simple and rapid methods for determining the quantification of antibiotics in biological systems.

Materials and Methods: In this work a facile and ultrasensitive colorimetric aptasensor was reported for detection of oxytetracycline (OTC) in water and milk samples employing OTC-short aptamer and gold nanoparticles (AuNPs).

Results: In the presence of OTC, the interaction between OTC and its aptamer leads to the separation of OTC aptamer from the surface of AuNPs which is followed by the aggregation of AuNPs by salt, showing an evident color change from red to blue. On the contrary, in the absence of OTC, the attachment of aptamer on the surface of AuNPs can protect AuNPs against salt-induced aggregation with a wine-red color. The proposed aptasensor exhibits excellent sensitivity for detection of OTC with linear range between 20 to 2000 nM with limit of detection (LOD) as low as 10 nM. Furthermore, this strategy was applied to detect OTC in spiked milk samples and presented satisfying linear range from 25 to 1500 nM with the LOD of 20 nM.

Conclusion: Owing to demonstrating appropriate sensitivity and selectivity, the designed biosensor can be considered as a promising tool to be applied in the field of biomedicine and food safety.

Keywords: *Aptasensor, Colorimetry, Gold nanoparticle, Oxytetracycline*

How to cite this article

Kazerooni H, Bahreyni A H, Ramezani M, Abnous Kh,Taghdisi S M. A colorimetric aptasensor for selective detection of oxytetracycline in milk, using gold nanoparticles and oxytetracline-short aptamer. Nanomed J. 2019; 6(2): 105-111. DOI: 10.22038/nmj.2019.06.0004

INTRODUCTION

Antibiotics have been extensively applied in medicine and food industry as well as animal agriculture intended for both prevention and treatment of various infectious diseases caused by several bacteria [1, 2]. It has been proven that incorrect and uncontrolled application of antibiotics trigger their accumulation in food chain and water, putting great challenge to human health and the environment safety globally [3, 4]. Furthermore, overuse of antibiotics can engender the super pathogenic organisms which are resistant to typical antibiotics and decline the efficiency of disease treatment significantly [5, 6]. Therefore, owing to long-term adverse effects of antibiotics residues in food and environment, it is exigent to develop practical procedures for monitoring them effectually [7]. Currently, a number of techniques

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Note. This manuscript was submitted on December 20, 2018; approved on February 29, 2019

including high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry, immunoassay, chemiluminescence, electrochemical and capillary electrophoresis have been used for analysis of diverse antibiotics [3, 8]. In spite of being highly sensitive, these approaches show some downsides such as time-consuming, sophisticated instrumentation and technical trainers [9]. To develop novel methods for rapid and convenient monitoring of antibiotics more efforts should be made. Among innovative procedures, colorimetric methods have attracted tremendous attentions due to possessing outstanding features namely rapidness, stability and cost effective Analysis [2].

Oxytetracycline (OTC), one of the most commonplace member of the broad-spectrum tetracycline (TC) group of antibiotics, has been widely employed as an antimicrobial agent, in addition to growth enhancer in animal feeding industry [10, 11]. According to WHO, maximum acceptable residues of OTC in human food is 0.1 mg L^{-1} [12].

Aptamers are short single-stranded DNA or RNA oligonucleotides, which are typically obtained through a combinatorial selection process named systematic evolution of ligands by exponential enrichment (SELEX) [13, 14]. Since the discovery of first aptamer, numerous of them have been designed and developed which can bind a broad range of targets from small molecules to whole cells with extraordinary selectivity and specificity [15, 16]. Due to demonstrating prominent features including low in vitro synthesis cost, high room temperature stability as well as sustainability to repeated denaturation and renaturation compared with antibodies, aptamers have been widely applied as recognition elements in designing different types of biosensors [17, 18].

In recent years, gold nanoparticles (AuNPs) are considered as an effective optical sensing tool for a variety of targets, since they present quite high surface area-to-volume ratio and appropriate biocompatibility [19, 20]. Developing colorimetric aptasensors applying gold nanoparticles have become popular in biosensing field in light of its easy preparation and simple operation together with detection of its colorimetric signal with the naked eye [10].

Kim et al have developed a colorimetric system for specific detection of OTC using gold nanoparticles and 76-mer size of OTC binding ssDNA aptamer merely in buffer sample [21]. However, developing simple and sensitive aptasensor for OTC analysis in real samples is required. In this study, a simple and highly sensitive as well as more economical colorimetric aptasensor based on AuNPs and OTC-short aptamer was designed and developed for time-efficient monitoring of OTC in both water and milk samples. This procedure relies on AuNPs aggregation by means of aptamer–OTC interaction.

MATERIALS AND METHODS

Materials

Oxytetracycline aptamer 5′-CGA CGC ACA GTC GCT GGT GCG TAC CTG GTT GCC GTT GTG T-3′ was obtained from Bioneer (South Korea) (http://www.bioneer.com). OTC, tetracycline, cephalexin, ciprofloxacin, ampicillin, kanamycin and tetrachloroauric (III) acid ($HAuCl_4$) were purchased from Sigma-Aldrich (USA) (https://www.sigmaaldrich.com). Milk was obtained from Salamat (Iran).

Synthesis of AuNPs

Classical citrate reduction method was employed to synthesize AuNPs [22]. In brief, under vigorous stirring, sodium citrate solution (10 mL, 38 mM) was quickly added to a boiling $HAuCl_4$ solution (100 mL, 1 mM), triggering to an obvious color shift of the reaction solution from light yellow to wine red. Solution was additionally boiled for 15 min. Subsequently, the reaction was gradually cooled down to room temperature and stored at 4°C before use. The synthesized AuNPs solution was centrifuged at 17000 × g for 20 min at 4°C. The supernatant was removed and AuNPs were resuspended in ultrapure water. The nanoparticle concentration was determined based on Extinction coefficient of 2.4 at 520 nm for 15 nm AuNPs [23].

The size and dispersion of AuNPs were analyzed using both dynamic light scattering (DLS) and transmission electron microscopy (TEM) (CM120, Philips, Holland).

Formation of OTC aptamer-modified AuNPs

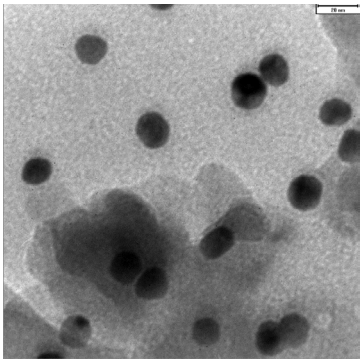
OTC aptamer-modified AuNPs was formed through mixing 25 µL OTC aptamer (final concentration was 250 nM) and 7 nM AuNPs. The solution was incubated at room temperature for 1 hr. Preparation of OTC aptamer-modified AuNPs was assessed through 2.5% agarose gel electrophoresis.

Assay procedure in water

Various concentration of OTC (0-5000 nM) were added into OTC aptamer-modified AuNPs (final concentration of aptamer 250 nM) at the final volume of 100 µL. All the mixtures were incubated at room temperature for 1 hr. Then, NaCl at the final concentration of 40 mM was added into the mixture. This was followed by another 5 min of incubation. At the end, absorbance of AuNPs (A_{620}/A_{520}) was recorded using a Synergy H4 microplate reader (BioTek, USA). Data are means ± SD, n = 3.

Monitoring of OTC in real sample

To determine the accuracy of the designed aptasensor, this approach was utilized to detect OTC in water samples. To achieve this aim, three determined concentrations of OTC, 20, 200 and 1000 nM, were added into water and the aptasensor was used to monitor OTC according to aforementioned method.



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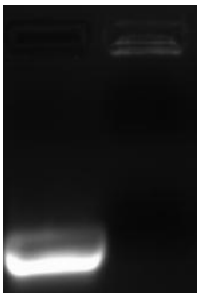


Fig 1 (a). TEM image of synthesized AuNPs. (b). Assessment of OTC aptamer attachment on the surface of AuNPs. Lane 1: OTC aptamer, lane 2: OTC aptamer-modified AuNPs

Assessment of procedure for detection of OTC in milk

Increasing concentration of OTC (0- 3000 nM) were added to solutions containing OTC aptamer-modified AuNPs (final concentration of aptamer 250 nM) at the final volume of 100 µL. In order to analysis of OTC in milk, milk samples were primarily diluted 1:50. After 1 hr incubation, NaCl at the final concentration of 40 mM was added into the mixtures and solutions were left at room temperature for 5 min. Eventually, absorbance of AuNPs (A_{620}/A_{520}) was recorded using a Synergy H4 microplate reader (BioTek, USA). Data are means ± SD, n = 3.

Selectivity assay for OTC detection in milk samples

To investigate the selectivity of proposed approach, the response of the OTC aptamer-modified AuNPs aptasensor to other antibiotics including tetracycline, cephalexin, ciprofloxacin, ampicillin and kanamycin was evaluated (the final concentration of each antibiotics was 500 nM). Data are means ± SD, n = 3.

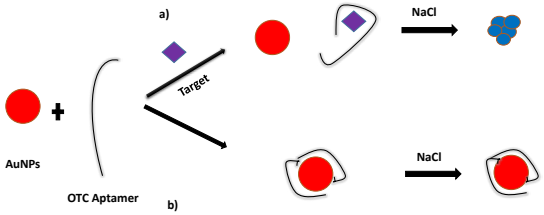
Analysis of OTC in real sample

To further evaluate the feasibility of the developed aptasensor, the recovery experiments have been performed. After adding three known concentrations of OTC into diluted milk samples (50, 400 and 600 nM), the proposed method was used to analyze the level of OTC.

RESULTS

Characterization of AuNPs

The synthesized AuNPs was characterized by DLS and TEM. The size and zeta potential of AuNPs were 14.2 ± 2 nm and -27 ± 2.3 mV, respectively, that was in consistent with the results acquired from TEM mage (Fig 1 (a)). These results verified that prepared AuNPs are appropriate for constructing sensing systems.



Scheme 1. Illustration of the fabricated aptasensor. (a) Through introduction of OTC, the interaction between OTC aptamer and its target, results in separation of OTC aptamer from the surface of AuNPs. Hence, OTC aptamer is not able to protect AuNPs against salt-induced aggregation. The color of the reaction mixture evidently alters from wine-red to blue or light blue due to the aggregation of these nanoparticles. (b) In the absence of target (OTC), OTC aptamer remains attached on the surface of AuNPs, stabilizing AuNPs against salt-induced aggregation

Gel retardation assay

Gel retardation assay validated the successful construction of OTC aptamer-modified AuNPs via the electrostatic interaction among the bases of ssDNA and AuNPs [21]. As can be seen in Fig 1 (b), the band of OTC aptamer-modified AuNPs was retarded in comparison to the band of OTC aptamer, proving the binding of OTC aptamer on the surface of AuNPs and formation of OTC aptamer-modified AuNPs.

The design strategy

The working principle of fabricated sensor is schematically represented in Scheme 1. Through the introduction of OTC to aptasensor, the interaction between OTC aptamer and its target, results in separation of OTC aptamer from the surface of AuNPs. Hence, OTC aptamer is not able to protect AuNPs against salt-induced aggregation [24, 25]. NaCl addition caps the repulsion between unmodified negatively AuNPs, resulting in the aggregation of AuNPs. The color of the reaction mixture evidently alters from wine-red to blue or light blue in light of the aggregation of these nanoparticles [26]. In the absence of target (OTC), OTC aptamers remain attached on the surface of AuNPs, stabilizing AuNPs against salt-induced aggregation [27].

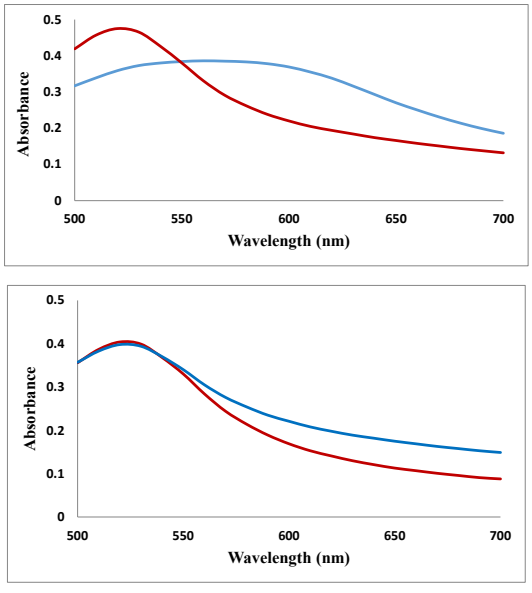


Fig 2. (a). Absorbance spectra of aptasensor in the presence (blue curve) and absence of OTC (red curve) in water sample. (b). Absorbance spectra of aptasensor in the presence (blue curve) and absence of OTC (red curve) in milk sample

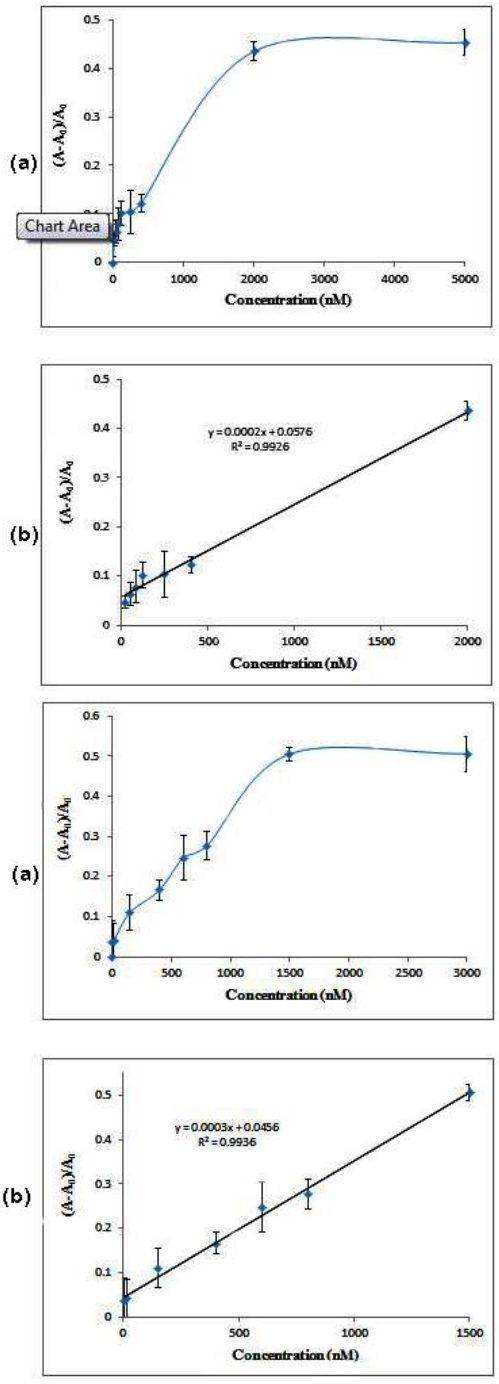


Fig 3. (a). Relative absorbance of aptasensor after interaction with various concentrations of OTC in water. (b) The calibration plot in the presence of various amounts of OTC in water. A0 (A620/A520) and A (A620/A520) are the absorbance intensities before and after addition of OTC, respectively. (c). Relative absorbance of aptasensor after interaction with various concentrations of OTC in milk. (d) The calibration plot in the presence of various amounts of OTC in milk. A0 (A620/A520) and A (A620/A520) are the absorbance intensities before and after addition of OTC, respectively

Table 1. Limit of Detection of Published OTC Sensors

Method	LOD
Label-free aptasensor for the ultrasensitive detection of oxytetracycline residues in aqueous solution environments [28]	17.4 fg/mL
Ultrasensitive SERS aptasensor for the detection of oxytetracycline based on a gold-enhanced nano-assembly [11]	4.35×10^{-3} fg/mL
sandwich-type electrochemical aptasensor based on GR-3D Au and aptamer-AuNPs-HRP for sensitive detection of oxytetracycline [12]	$4.98\times10^{+2}$ fg/mL

Proof of aptasensing concept

In the designed method, an evident color alteration from wine-red to blue can be visualized through the introduction of OTC to aptasensor followed by adding NaCl to samples.

Moreover, the absorbance of AuNPs was enhanced dramatically at 620 nM in both water samples (Fig 2 (a), blue curve) and milk samples (Fig 2 (b), blue curve). However, in the absence of OTC, the color of AuNPs remains red after adding NaCl to samples due to attachment of OTC aptamer on the surface of AuNPs. Additionally, λ_{max} of AuNPs can be detected at 520 nM in both water samples (Fig 2 (a), red curve) and milk samples (Fig 2 (b), red curve).

Analytical performance of the proposed sensor

The absorbance of AuNPs in solutions containing various concentrations of OTC (n=3) were measured by the presented aptasensor in water samples (Fig 3 (a)).

A good linear range was obtained through using aptasensor for detection of OTC in water samples (20-2000 nM) (Fig 3 (b)). Also, 10 nM was calculated as the limit of detection (LOD) of aptasensor (3σ/slope, where σ was the standard deviation in blank sample, n=3). In addition to water samples, the absorbance of AuNPs in solutions containing various concentrations of OTC (n=3) were measured by the presented aptasensor in milk samples (Fig 3 (c)). The relative absorbance intensity demonstrates appropriate linearity in the range of 25 nM to 1500 nM OTC in milk samples (Fig 3 (d)).

The detection limit in milk samples was as low as 20 nM (3σ/slope, where σ was the standard deviation in blank sample, n=3).

Thus, those experimental results demonstrated that the presented assay could be a simple and sensitive approach for OTC detection. Although some other procedures provided more appropriate linear ranges and even better LODs, the proposed aptasensor provides more advantages in terms of its simple signal generation, time-efficiency and detection with the naked eye as well as suitable LOD for detection of OTC compared with conventional assays which are summarized in Table 1.

Sensor specificity and selectivity

To evaluate the selectivity of the proposed biosensor that is considered as one of the most significant characteristics in analytical approaches, the response of sensor was tested for OTC and some other antibiotics even those with similar structures such as tetracycline, cephalexin, ciprofloxacin, ampicillin, kanamycin in milk samples.

As shown in Fig 4, introduction of OTC can cause a significant change in absorbance of aptasensor in milk samples.

The experimental results implied that fabricated aptasensor is able to effectively identify OTC.

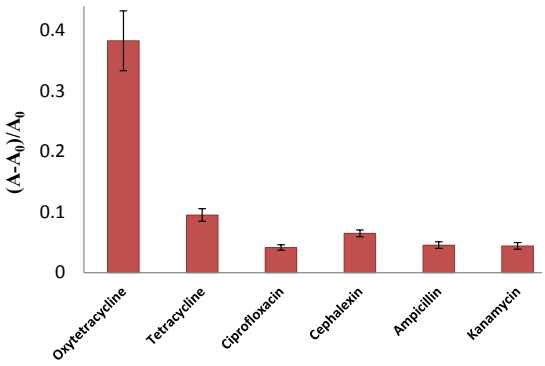


Fig 4. The response of designed aptasensor to OTC and other antibiotics in milk

OTC analysis in real samples (water and milk)

Sensing approach was performed to detect known concentrations of OTC in both water and milk samples in order to assess the reliability and practicality of sensor. It has been demonstrated that the recovery range of aptasensor was between 95.55 and 103.31% in water samples (Table 2) and between 93 and 114.93% in milk

samples (Table 3). These results indicated the accurate applicability of the presented aptasensor.

Table 2. Recovery results for added OTC in water samples acquired by the proposed procedure

Water samples	Added OTC (nM)	Found (nM)	Recovery ± RSD (%)
1	20	19.11 ± 0.004	95.55 ± 0.04
2	200	204.5 ± 5.3	102.08 ± 2.45
3	1000	1033 ± 3.26	103.31 ± 0.33

Table 3. Recovery results for added OTC in milk samples acquired by the proposed procedure

Serum samples	Added OTC (nM)	Found (nM)	Recovery ± RSD (%)
1	50	57.47 ± 0.93	114.94 ± 1.87
2	400	412.5 ± 1.23	103.13 ± 0.31
3	600	558 ± 1.63	93 ± 0.24

CONCLUSION

To sum up, a usensitive label-free colorimetric sensor was designed and fabricated for detection OTC using OTC aptamer-modified AuNPs. In addition to proposing rapid detection, the developed aptasensor presented other excellent features such as high sensitivity and selectivity as well as simplicity. Besides demonstrating satisfying linear ranges in both water and milk samples, the limit detections of this analytical method was shown to be as low as 10 and 20 nM in water and milk samples, respectively, which are much lower than the maximum acceptable residues of OTC in different kinds of samples. These results suggested that not only this procedure is suitable for detection of OTC in various samples, but it also can be extended for the detection of other antibiotics in water or biological fluids by replacement of aptamer sequence.

ACKNOWLEDGMENTS

Financial support of this study was provided by Mashhad University of Medical Sciences.

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