Biosensors and Bioelectronics 70 (2015) 181–187

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

A novel colorimetric triple-helix molecular switch aptasensor for ultrasensitive detection of tetracycline

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ARTICLE INFO

Article history:
Received 24 December 2014
Received in revised form
26 February 2015
Accepted 16 March 2015
Available online 17 March 2015

Keywords:
Aptamer
Triple-helix molecular switch
Tetracycline
Colorimetric biosensor
Gold nanoparticles

ABSTRACT

Detection methods of antibiotic residues in blood serum and animal derived foods are of great interest. In this study a colorimetric aptasensor was designed for sensitive, selective and fast detection of tetracycline based on triple-helix molecular switch (THMS) and gold nanoparticles (AuNPs). As a biosensor, THMS shows distinct advantages including high stability, sensitivity and preserving the selectivity and affinity of the original aptamer. In the absence of tetracycline, THMS is stable, leading to the aggregation of AuNPs by salt and an obvious color change from red to blue. In the presence of tetracycline, aptamer binds to its target, signal transduction probe (STP) leaves the THMS and adsorbs on the surface of AuNPs. So the well-dispersed AuNPs remain stable against salt-induced aggregation with a red color. The presented aptasensor showed high selectivity toward tetracyclines with a limit of detection as low as 266 pM for tetracycline. The designed aptasensor was successfully applied to detect tetracycline in serum and milk.

1. Introduction

Tetracyclines are broad-spectrum antibiotics which are used in human and veterinary therapy (Gan et al. 2014; Zhao and Wang 2013). Excessive use of tetracyclines in veterinary medicine as antibiotics and growth promotors could lead to antibiotic residues in food products (Asadollahi-Baboli and Mani-Varnosfaderani 2014; Tan et al. 2013b). Residues of tetracyclines in food products could result in undesirable side effects on human health and emergence of bacterial resistance to antibiotics in both veterinary and human (Moreira et al. 2010; Tan et al. 2013b). Allergic reactions, yellowing of teeth and liver damage are among side effects of tetracyclines (Mu et al. 2012).

Therefore, selective, fast and sensitive methods for detection of tetracyclines are of great interest. Current methods for detection of tetracyclines are: immunoassays (Aga et al. 2003; Jeon and Rhee Paeng 2008), high performance liquid chromatography (HPLC) (Cinquina et al. 2003; Shariati et al. 2009), microbiological methods (Idowu et al. 2010; Kurittu et al. 2000), chemiluminescence (Townshend et al. 2005) and capillary electrophoresis (CE) (Kowalski 2008; Tong et al. 2009). Generally these approaches require sophisticated and expensive instruments and need lengthy turnaround time (Chen et al. 2014; Tan et al. 2013b).

Aptamers are single-stranded oligonucleotides, obtained by an in vitro process named SELEX (systematic evolution of ligands by exponential enrichment) (Lian et al. 2015). They react to their targets ranging from small substances to proteins and even cells with high specificity and affinity (Huang et al. 2014; Zhao et al. 2015). Aptamers possess advantages over antibodies like low cost, excellent thermal stability, ease of production and modification, and lack of toxicity and immunogenicity (Bai et al. 2013; Lian et al. 2015; Luo et al. 2014; Shan et al. 2014). Owing to these unique properties, aptamers have attracted substantial attentions for application in biosensors (Evtugyn et al. 2013; Taghdisi et al. 2014).

Recently gold nanoparticles (AuNPs) have been broadly used in the development of colorimetric and fluorescence quenching aptasensors, because of their unique characteristics such as chemical stability, high sensitivity, ease of synthesis and extremely high absorption coefficient (Gopinath et al. 2014; Liu et al. 2014; Tan

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http://dx.doi.org/10.1016/j.bios.2015.03.040
0956-5663/

Table 1
Oligonucleotide sequences used in this study. The boldface type is the aptamer and the underlined sequences indicate the bases that form a triplex.

Entry	Sequence
Signal transduction probe	5′- GAGAGAGAGAGAGA- 3′
Tetracycline-binding aptamer	5′-CTCTCTCGGTGGTGTCTCTC-3′

et al. 2013a; Zhang et al. 2013).

Colorimetric assay is a common technique for analytical applications, since the target recognition event can be determined only by the naked eye (Gopinath et al. 2014).

In this study a colorimetric triple-helix molecular switch (THMS) system was designed for the first time for detection of tetracyclines based on aptamer and AuNPs. THMS presents unique advantages over known double-helix DNA molecular switches and molecular beacon-based signaling aptamers including high sensitivity and stability and preserving the specificity and affinity of the original aptamer (Zheng et al. 2011). THMS system usually consists of a label-free target specific aptamer sequence with two arm segments and a dual-labeled oligonucleotide as a signal transduction probe (STP) (Verdian-Doghaei et al. 2014; Zheng et al. 2011), but in this study a label-free STP was used which is economical and does not need any special instrument. In this project a truncated 8-mer ssDNA aptamer, which selectively binds to different tetracyclines (Kwon et al. 2014), was used as targeting agent.

2. Materials and methods

2.1. Materials

The sequences of tetracycline-binding aptamer flanked at the 5′- and 3′- ends with two arm segments (Apt) and signal transduction probe (STP) were purchased from Bioneer (South Korea) (Table 1). Plasma from rat, Clindamycin, tetracycline, doxycycline, amoxicillin, ciprofloxacin and sodium tetrachloroaurate (III) ($HAuCl_4$) were obtained from Sigma (USA). Milk was purchased from Razavi (Iran).

2.2. Synthesis of water resuspended gold nanoparticles

AuNPs were prepared by the classical citrate reduction of $HAuCl_4$, based on the previously published protocol (Storhoff et al. 1998). The synthesized AuNPs solution was centrifuged at 15000*g* for 20 min at 4 °C. The supernatant was discarded and AuNPs were resuspended in ultrapure water. The size and zeta potential of AuNPs were measured using particle size analyzer (Malvern, UK). AuNPs concentrations were calculated based on Extinction coefficient of $2.7 \times 10^{8}\ M^{-1}\ cm^{-1}$ at $\lambda = 520$ nm for 15 nm AuNPs.

2.3. Preparation of triple-helix molecular switch

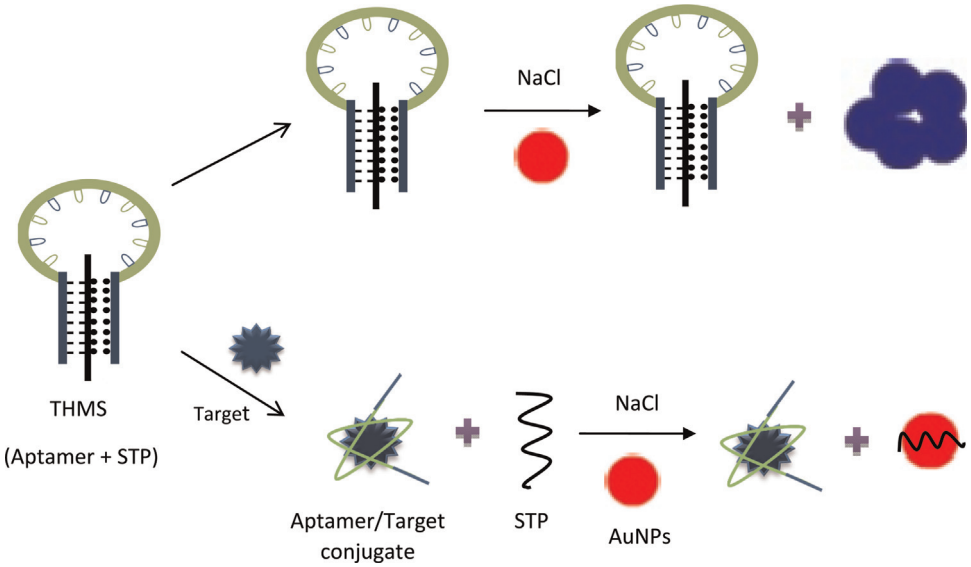
The THMS was prepared by mixing the Apt (20 μM final concentration) and STP (20 μM final concentration) in binding buffer (20 mM Tris–HCl, pH 7.5). The mixture was incubated at room temperature for 60 min. Formation of THMS was investigated by 3% agarose gel electrophoresis.

2.4. Optimizing signal transduction probe concentration

Increasing concentrations of STP (0–4 μM final concentrations) were added to 5 nM AuNPs (final volume 100 μl). Mixtures were incubated for 30 min at room temperature. NaCl to final concentration of 50 mM was added to each well and after incubation for 5 min, A_{620}/A_{520} was recorded using a Synergy H4 microplate reader (BioTeK, USA).

2.5. Effect of pH on the formation of THMS

The THMS was prepared by mixing the Apt (20 μM final concentration) and STP (20 μM final concentration) in binding buffer with different pH values (5.5–8.5). The mixtures were incubated at room temperature for 60 min. 5 nM AuNPs were added to each well containing THMS (2 μM final concentration, final volume 105 μl) and incubated for 30 min. NaCl to final concentration of 50 mM was added to each well and after incubation for 5 min, A_{620}/A_{520} was recorded.



Scheme 1. Schematic description of tetracycline detection based on colorimetric THMS. In the absence of tetracycline, THMS (Aptamer+STP) is stable, resulting in the aggregation of AuNPs by salt and an obvious color change from red to blue. In the presence of target, aptamer binds to its target, STP leaves the THMS and adsorbs on the surface of AuNPs. So the well-dispersed AuNPs remain stable against salt-induced aggregation with a red color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

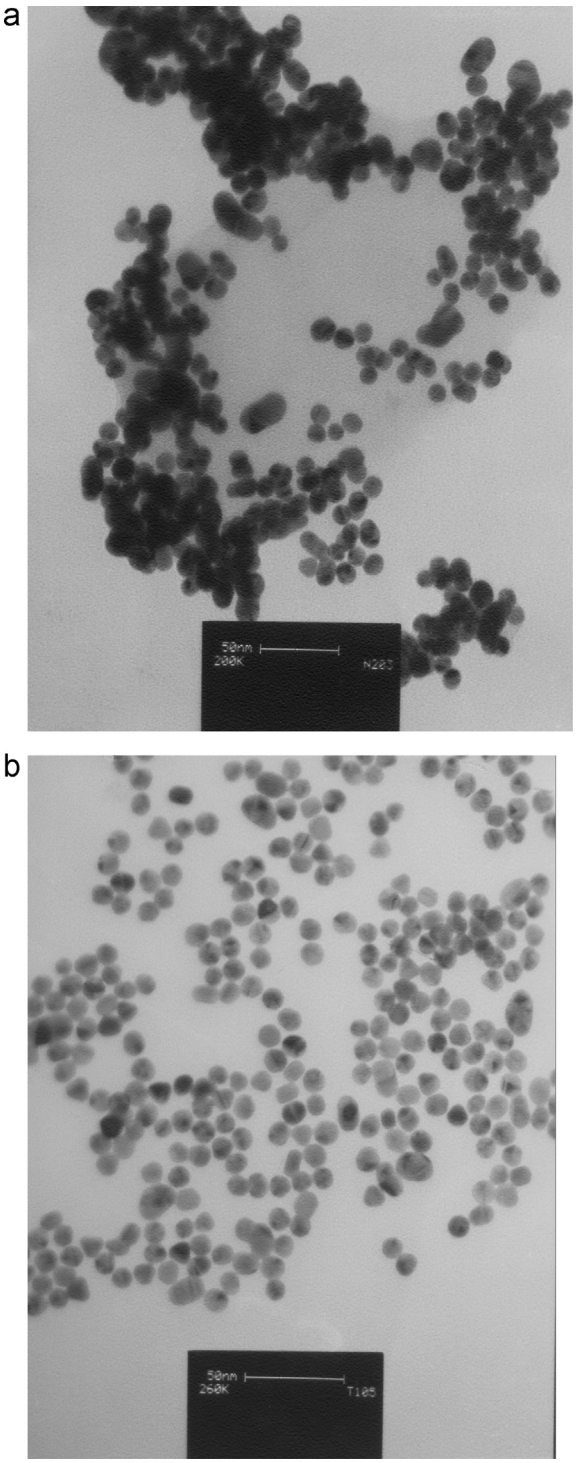


Fig. 1. TEM images of AuNPs (a) in the absence of tetracycline and (b) after addition of tetracycline (10 nM).

2.6. Function study of triple-helix molecular switch

The interaction of THMS and tetracycline was analyzed by measuring the absorbance of AuNPs. 5 μl tetracycline (10 nM final concentration) was added to the mixture containing THMS (2 μM final concentration) and 5 nM AuNPs (final volume 105 μl). After 30 min, NaCl to final concentration of 50 mM was added and after 5 min, the absorbance was recorded.

Also the function of THMS was assessed by 3% agarose gel electrophoresis.

2.7. Tetracycline detection based on colorimetric technique

A range of tetracycline concentrations, 0–50 nM final concentrations, were added to THMS (2 μM final concentration) and 5 nM AuNPs (final volume 105 μl) and incubated for 30 min. NaCl to final concentration of 50 mM was added to each well and after incubation for 5 min, the absorbance was measured.

2.8. Selectivity

The selectivity was analyzed in the presence of 10 nM tetracycline, clindamycin, doxycycline, ciprofloxacin and amoxicillin as measured above.

2.9. Tetracycline detection in milk and serum

To investigate the application of the designed aptasensor in serum and milk samples, increasing concentrations of tetracycline (0–50 nM) were spiked to rat serum and milk. Proteins of serum and milk were removed using acetonitrile. 125 μl of cold acetonitrile was added to 50 μl of serum and milk, with gentle mixing. The mixtures were incubated for 60 min at 4 °C. The samples were centrifuged at 9500*g* for 10 min at 4 °C. The supernatant was collected and tetracycline concentrations were measured.

3. Results and discussion

3.1. Sensing scheme

The presented colorimetric aptasensor is based on target-induced release of STP from Apt, strong interaction of ssDNA (STP) with water resuspended AuNPs, and no or very less interaction of THMS with AuNPs. It has been shown that the removal of sodium citrate by water resuspended AuNPs could enhance the sensitivity of colorimetric aptasensors (Liu et al. 2014). So in this study water resuspended AuNPs were used.

Two arm segments of the Apt bind to the sequence of STP through Watson–Crick and Hoogsteen base pairings, leading to formation of THMS.

As shown in Scheme 1, in the absence of tetracycline, THMS is stable. THMS could not protect AuNPs against salt-induced aggregation, owing to its very rigid structure. It has been demonstrated dsDNA provides no or little stabilization to the AuNPs against salt-induced aggregation because of its rigid structure (Gopinath et al. 2014; Smith et al. 2014; Wei et al. 2007), while in this study we used THMS, a triple-helix structure, which has a more rigid structure than dsDNA. NaCl addition caps the repulsion between unmodified negatively AuNPs, resulting in the aggregation of AuNPs (Fig. 1(a)). The color of AuNPs obviously change from wine-red to blue or light blue, when their aggregation is triggered (Wen et al. 2013).

Addition of tetracycline induces a conformational change and formation of aptamer/target conjugate. So that, THMS is disassembled and released STP adsorbs on the surface of AuNPs by electrostatic interaction between the positively charged bases of STP and the negatively charged AuNPs (Chang et al. 2013; Liu et al. 2014). So that, AuNPs are stabilized by the STP against salt-induced aggregation and remain red (Fig. 1(b)).

3.2. AuNPs characterization

Particle size and zeta potential of AuNPs were 14.7 ± 0.7 nm and -35.6 ± 1.7 mV, respectively. Particle size of AuNPs after treatment with 10, 25, 50 and 100 mM NaCl were 64.7 ± 4.7 nm, 181.2 ± 10.9 nm, 312.5 ± 18.2 nm and 327.6 ± 15.6 nm, respectively. Zeta potential of AuNPs after treatment with 10, 25, 50 and 100 mM NaCl

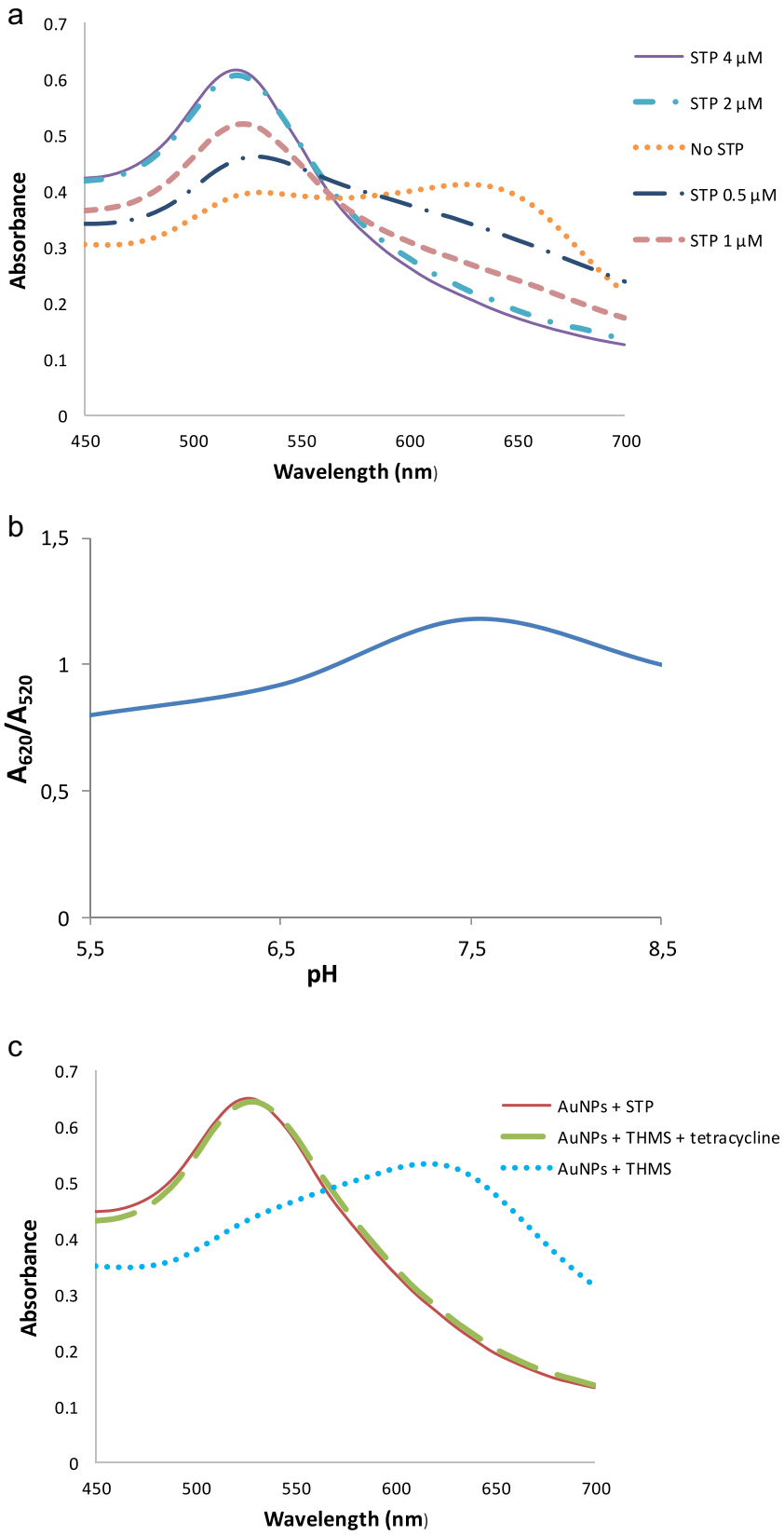


Fig. 2. (a) Absorbance of AuNPs in in the presence of 50 mM NaCl and various concentrations of STP (from bottom to top 0, 0.5, 1, 2 and 4 µM). (b) Relative absorbance of AuNPs in the presence of THMS in binding buffer with different pH values (5.5–8.5). (c) Absorbance of AuNPs+STP (red), AuNPs+THMS (blue) and AuNPs+THMS+tetracycline (green) in the presence of 50 mM NaCl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

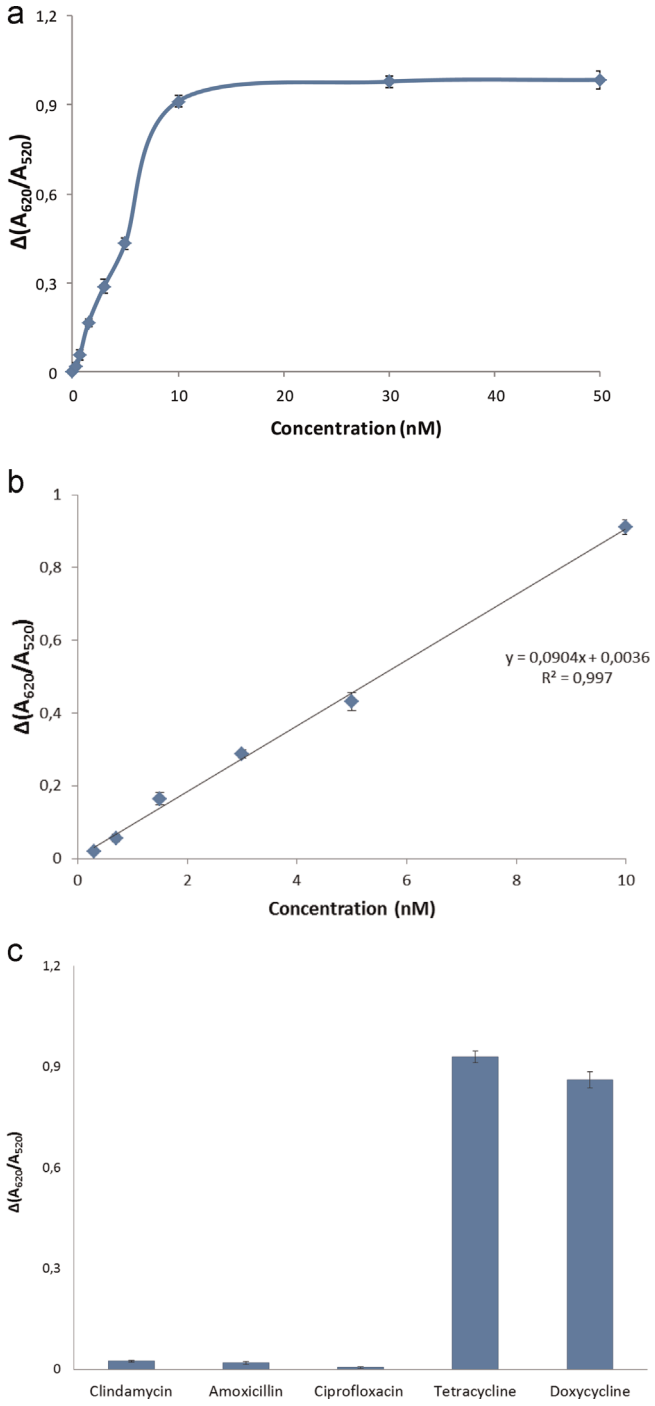


Fig. 3. (a) Relative absorbance of AuNPs as a function of tetracycline concentration. (b) Tetracycline standard curve. (c) Efficiency of absorbance in the presence of various antibiotics.

were -30.6 ± 1.3 mV, -22.1 ± 0.9 mV, -16.4 ± 0.7 mV, -15.8 ± 0.5 mV, respectively. These results confirmed NaCl addition caped the repulsion between unmodified negatively AuNPs.

3.3. Optimum concentration of STP

To obtain the optimum concentration of STP for complete reaction with AuNPs, increasing concentrations of STP were added to a constant concentration of AuNPs. The results indicated the final concentration of 2 μM STP could protect AuNPs against aggregation by 50 mM salt (Fig. 2(a)).

3.4. Optimum pH

The formation of THMS was studied and showed under different pHs (5.5–8.5). The results indicated the maximum formation of THMS achieved at pH 7.5, while at pHs 5.5, 6.5, 8.5 the Apt could not efficiently bind to STP (Fig. 2(b)).

3.5. Monitoring of THMS fabrication and function

The THMS formation and function were confirmed by absorbance measurement of AuNPs (Fig. 2(c)). Unlike STP, the THMS could not protect AuNPs against salt-induced aggregation. This result showed the triple-helix structure, which is a very rigid structure, has formed. A well-defined protection of AuNPs against salt could be observed upon addition of target, tetracycline, demonstrating the formation of aptamer/target conjugate, release of STP from aptamer and good function of THMS system.

Also, the formation and function of THMS was confirmed by gel retardation assay. As shown in Fig. S1 the THMS band was retarded compared to Apt band. Upon the addition of tetracycline to THMS, a conformational change happened and aptamer/target conjugate was formed, leading to the corresponding Apt band shifted to a higher molecular weight position compared to Apt alone.

3.6. Tetracycline analysis

In the colorimetric method, an obvious color change from blue to wine-red could be observed as tetracycline concentrations increased (Fig. S2).

Absorbance of AuNPs at different concentrations of tetracycline were shown in Fig. 3(a). The absorbance increased and reached to plateau at concentration of 10 nM tetracycline. The designed aptasensor showed a well linear range (0.3–10 nM) toward tetracycline (Fig. 3(b)). The limit of detection (LOD) was calculated to be 266 pM (0.127 μg/L), as three times the standard deviation/slope (Fig. S3).

Reported detection limits of tetracyclines in other studies were as following: 0.5–1 μg/L for carrier mediated hollow fiber liquid phase microextraction combined with HPLC-UV (Shariati et al. 2009), 2–35 μg/L for microbiological test (Kurittu et al. 2000), 0.5–1 mg/kg for CE (Tong et al. 2009), 0.19 μg/L for immunoassay (Jeon and Rhee Paeng 2008), 2.4 μg/L for electrochemical aptasensor (Zhou et al. 2012). In comparison with the designed aptasensor most of these approaches are time-consuming and expensive and have higher LODs.

One of the most important characteristics of a practical sensor is selectivity. To investigate the selectivity of the designed aptasensor, other antibiotics including amoxicillin, tetracycline, clindamycin, doxycycline and ciprofloxacin were analyzed by this method. As expected, the absorbance for tetracyclines, doxycycline and tetracycline, were significantly higher than other antibiotics (Fig. 3(c)). The result indicated excellent selectivity of the presented aptasensor for detection of tetracyclines.

3.7. Tetracycline detection in biological samples

The fabricated aptasensor was applied to measure tetracycline concentration in milk and rat serum. Different concentrations of tetracycline were spiked into milk and serum, and LODs were calculated to be 347 (0.166 μg/L) and 393 pM (0.189 μg/L), respectively (Fig. 4). The measured LODs were much lower than the maximum permitted level of tetracycline in milk (0.1 mg/L) (Commission Regulation no. 1999) and the tetracycline toxicity level in blood (30 mg/L) (Schulz et al. 2012). The results showed the presented aptasensor could successfully be applied for detection of tetracycline in serum and milk.

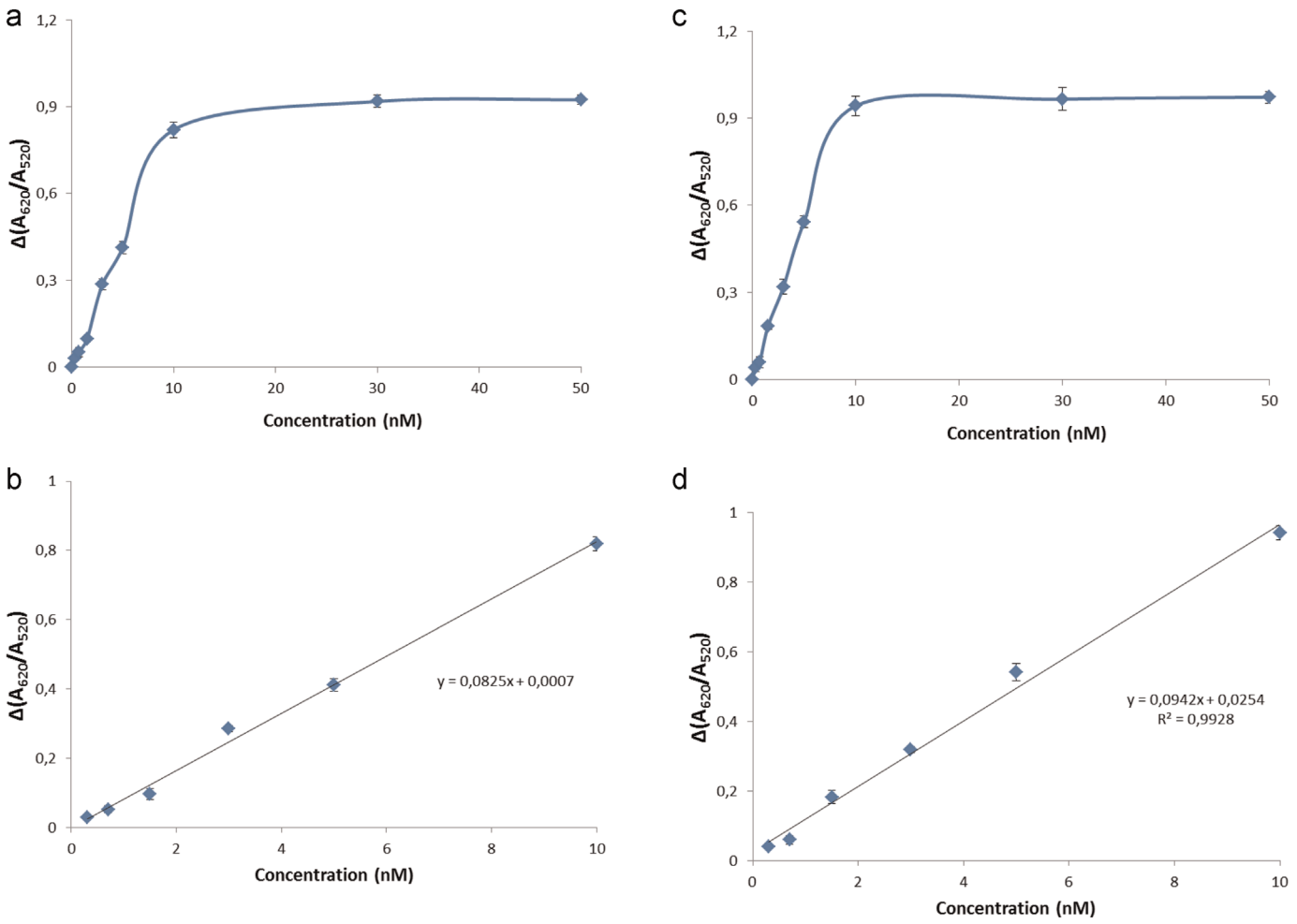


Fig. 4. (a) Relative absorbance of AuNPs upon the addition of various concentrations of tetracycline in serum. (b) Tetracycline standard curve in serum. (c) Relative absorbance of AuNPs upon the addition of various concentrations of tetracycline in milk. (d) Tetracycline standard curve in milk.

4. Conclusion

In summary, we introduced an easy-to-build colorimetric aptasensor based on THMS and AuNPs for the selective, sensitive and fast detection of tetracycline. The presented aptasensor showed high selectivity toward tetracyclines. The limit of detection for tetracycline was calculated as low as 266 pM. Moreover, this aptasensor could well detect tetracycline in serum and milk. It is expected this method could be extended for detection of other drugs and biomolecules in clinical practice, because of its simplicity and high affinity. Moreover, the results readout is possible by visual observation without any need for specialized analytical equipment.

Conflict of interest

There is no conflict of interest about this article.

Acknowledgment

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

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.03.040.

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