A Simple Colorimetric Analytical Assay for the Determination of Tetracyclines based on In-situ Generation of Gold Nanoparticles Coupling with a Gold Staining Technique

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Abstract

The development of simple and sensitive detection methods for tetracyclines (TCs) is crucial for their routine detection. The present study developed a colorimetric method for the detection of TCs based on the in-situ generation of AuNPs which is subsequently coupled with a gold staining reaction. Briefly, TCs containing phenolic groups reduce HAuCl₄ to form gold nanoparticles (AuNPs) as gold seeds. In the gold staining process, the gold seeds catalyze the reduction of HAuCl₄ by NH₂OH to form gold atoms which deposit on the surface of AuNPs resulting in the enlargement of AuNPs. Sensitive detection of TCs was achieved by employing the gold staining technique. As low as 14 nM, 18.9 nM, and 1.98 nM of oxytetracycline (OTC), tetracycline (TC), and doxycycline (DC) could be sensitively detected. The proposed method also exhibited good repeatability and specificity, and then was applied to the determination of OTC in milk samples.
Introduction

Tetracyclines (TCs) containing linear fused tetracyclic nucleus are a class of broad-spectrum antibiotics that have good activity on a variety of both Gram-positive bacteria and Gram-negative bacteria. Clinically, TCs are mainly used for bacterial dysentery, trachoma, pneumonia, suppurative meningitis, skin infection and so on. Long-term and overdosage use of TCs causes damage to the liver and kidney, gastrointestinal disorder, and bone deposition. Nowadays, TCs are also employed in animal husbandry to promote the growth of animals, thus, may cause undesirable residues in food and environment. The residue of TCs can accumulate in human body via the food chain, which may induce antibiotic resistance cases. According to WHO, the maximum allowable level in human food is 0.1 mg L$^{-1}$. Thus, developing simple and rapid methods for the detection of TCs is significantly important to the routine detection of TCs.

Until now, analytical methods developed for the determination of TCs include microbial assays, high-performance liquid chromatography, capillary electrophoresis, fluorescent, electrochemical, and chemiluminescent methods. Although most of these methods can achieve sensitive detection of TCs. However, they are often suffered from requiring expensive instruments, needing professional staff, time-consuming, etc. Colorimetric method is convenient because the signal can be read by the naked eye or be monitored by a simple microplate reader. Several colorimetric methods have been developed for the detection of TCs. For example, Wang’s group developed a colorimetric method for TCs determination based on the trimethylbenzene (TMB) chromogenic reaction catalyzed by Fe$_3$O$_4$ magnetic nanoparticles.
Gold nanoparticles (AuNPs) possess unique optical properties and have a surface plasmon resonance (SPR) absorption band around 520 nm, which depends on the size, shape, and dielectric environment of AuNPs. AuNPs have been widely used for the development of colorimetric assays for the determination of pesticides, biomolecules, inorganic ions. Also, AuNPs have been employed for the colorimetric detection of TCs. For example, Qi’s group employed aptamer to recognize TC and developed a colorimetric method for TC detection based on salt-induced aggregation of AuNPs. Yuan’s group took the advantage of the catalytic activity of graphene@AuNPs hybrid toward the TMB chromogenic reaction and established a colorimetric sensing platform for TCs based on the recognition of aptamer. These methods are simple and sensitive methods for the detection of TCs. However, most of these methods should employ biomolecules such as aptamer and antibody in the detection, which leads to a high price of the detection. Besides, nanoparticles or nano-composites should be prepared before the experiment leading to a time-consuming pre-experiment process.

TCs containing phenolic groups is capable of reduce HAuCl₄ to form AuNPs. Liu's group employed the HAuCl₄-TCs redox reaction and developed a colorimetric method for the detection of TCs based on the generation of AuNPs. The colorimetric detection of TCs based on the in-situ generation of AuNPs is simple. However, the detection sensitivity is poor. Gold staining technique, a commonly used amplification strategy, uses reductant such as ascorbic acid, hydroxylamine, etc to reduce Au³⁺ ions to Au atoms which deposit on the surface of gold seeds leading to the enlargement of small AuNPs. Gold staining technique has been employed to improve the detection sensitivity of
sequence-specific DNA, proteins, and metal ions.\textsuperscript{29,30} To the best of our knowledge, the gold staining technique has not been employed for the sensitive detection of TCs yet. Thus, the present study tried to couple the HAuCl\textsubscript{4}-TCs reduction reaction with the gold staining technique to improve the detection sensitivity for TCs. As depicted in Scheme 1, HAuCl\textsubscript{4} is reduced by TCs to form AuNPs that are the gold seeds in the gold staining step. Hydroxylamine is employed as the reductant in the gold staining process, in which the HAuCl\textsubscript{4} is reduced by hydroxylamine to gold atoms depositing on the surface of gold seeds. The large AuNPs with a much higher excitation coefficient are produced in the gold staining step, thus, a higher sensitivity for TCs can be achieved by coupling the in-situ generation of AuNPs with a gold staining technique.

(Scheme 1)

**Experimental**

**Reagents and chemicals**

All chemicals were of analytical reagent grade and were used as received. Distilled water (18.2 MΩ cm\textsuperscript{-1}) was used throughout the current study. Oxytetracycline (OTC), tetracycline (TC), hydrogen tetrachloroaurate (III) tetrahydrate (HAuCl\textsubscript{4}·4H\textsubscript{2}O), and streptavidin (SA) were purchased from Heowns Biochem Technology Co., Ltd. (Tianjin, China). Doxycycline (DC) was purchased from Meryer Chemical Technology Co., Ltd. (Shanghai, China). Hydroxylammonium chloride (NH\textsubscript{2}OH·HCl) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). NaOH, K\textsubscript{2}HPO\textsubscript{4}, and metal salts including NaCl, KCl, MgCl\textsubscript{2}, CuCl\textsubscript{2}, MnCl\textsubscript{2}, FeCl\textsubscript{3}, and CaCl\textsubscript{2} were purchased
from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Phenylalanine (Phe), histidine (His), arginine (Arg), valine (Val), and isoleucine (Ile) were purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) and Calmodulin (CaM) were purchased from Sigma-Aldrich (Beijing, China). Other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

A stock solution of HAuCl₄ (1%, w/v) was prepared by dissolving 1 g HAuCl₄ in 100 mL of ultrapure water and stored at 4°C. 0.1 M of McIlvaine-EDTA buffer (pH 4) was prepared by dissolving 5.9 g trisodium citrate dihydrate, 13.8 g Na₂HPO₄·12H₂O, and 18.6 g Na₂EDTA·12H₂O in 500 mL of ultrapure water.

Apparatus

The UV-Vis spectra and absorbance of formed AuNPs were recorded on a multifunctional microplate reader (SpectraMax M₂, Molecular Devices Corporation, USA). TEM images of the formed AuNPs were obtained on an FEI Tecnai G2 F20 high-resolution transmission electron microscope (FEI, USA). The hydrodynamic diameters of generated AuNPs were determined on Zetasizer Nano ZS90 (Malvern, UK).

Colorimetric method for the detection of tetracyclines

25 µL of 1 mM HAuCl₄ was added to the well of a 96-well plate and mixed with 25 µL of OTC with different concentrations. The mixtures were incubated at 70°C for 30 min. After cooling to room temperature, 50 µL of 0.5 mM NH₂OH was added to the mixture. The redox reaction was kept at room temperature for
30 minutes. Finally, the absorbance of the mixture at 550 nm was measured by using the SpectraMax M2 microplate reader. Each sample was detected three times parallely.

**Determination of OTC in a milk sample**

The liquid milk samples were purchased from a local supermarket in Tianjin university. The milk samples were pretreated according to method reported previously. Briefly, 0.6 mL of 20% trichloroacetic acid in acetonitrile and 3 mL of 0.1 M Mcllvaine-EDTA buffer (pH 4) was introduced to 1 mL liquid milk to precipitate proteins, then an hydrophile lipophile balance-solid phase extraction (HLB-SPE) column was employed to extract OTC in the milk sample. The extraction process was carried out according to the product manual. Briefly, the HLB-SPE column was activated with 5 mL of methanol, and was equilibrated by 10 mL of ultrapure water under gravity. The sample extracts were allowed to pass through and column was washed with 5 mL of 30% methanol. The retained analyte was eluted with 7 mL of methanol-ethyl acetate (V: V=1:9). The elution was collected in a clean glass test tube. The organic reagent was removed by nitrogen blowing at 40°C. The final product was dissolved in 100 µL of ultrapure water. Then, 25 µL of the extract was mixed with 25 µL of 1 mM HAuCl₄, and the colorimetric assay was carried out as described above. Each sample was detected three times parallely.

**Results and Discussion**

**The principle of the colorimetric method for the detection of OTC**

HAuCl₄ can be reduced by reductive agents to form AuNPs which is a red
colloidal solution. Fig. S1 (Supporting Information) shows the structures of TCs. Reductive functional groups such as phenolic hydroxyl groups, enol hydroxyl groups, and amino groups are contained in TC molecules. Herein, a colorimetric method is developed for the detection of TCs by taking advantage of the reductive property of TCs. The colorimetric method is based on the in-situ generation of AuNPs with TCs. To further improve the detection sensitivity, a gold staining method based on NH$_2$OH-HAuCl$_4$ reduction reaction is employed. In the reaction process, HAuCl$_4$ is firstly reduced by TCs to form small AuNPs which act as gold seeds in the gold staining step. In the gold staining process, HAuCl$_4$ is reduced by hydroxylamine to gold atoms which deposit on the surface of gold seeds to form large AuNPs leading to a higher detection sensitivity for TCs.

To verify the hypothesis, OTC, a representative TCs, was employed as a model analyte. 6.25 μM of OTC was incubated with 1 mM HAuCl$_4$ at 70°C for 30 min. As shown in the inset of Fig. 1, the resulted product was colorless which was similar to the blank test. The UV-Vis absorption spectra showed that both sample and blank test exhibited low absorption in the range of 500-600 nm. Then, the gold staining was performed by adding 0.5 mM NH$_2$OH into the above mixture. After standing at room temperature for 30 min, the sample test with 6.25 μM TCs exhibited an obvious fuchsia color while the blank test without TCs kept colorless. The UV-Vis absorption spectra of the sample test showed a maximum SPR absorption band around 550 nm indicating that large AuNPs were formed in the gold staining process. To confirm the formation of large AuNPs during the reduction process, TEM images of the sample test after gold staining were obtained. Fig. S3 (Supporting Information) shows the DLS result of the generated
AuNPs, which exhibited that AuNPs with a diameter of 61.0 ± 5.4 nm were formed during the NH$_2$OH-HAuCl$_4$ reduction reaction.

(Fig. 1)

**Optimization of experimental conditions**

To achieve the best assay performance for the colorimetric detection of TCs, experimental conditions including the concentration of HAuCl$_4$ and NH$_2$OH, the incubation temperature and time for the reduction of HAuCl$_4$ by OTC, and gold staining time were systematically optimized by using OTC as the model analyte.

The concentration of HAuCl$_4$, incubation temperature and time for the reduction of HAuCl$_4$ by OTC would affect the formation of gold seeds which act as the nucleus in the gold staining process, thus, influencing the colorimetric detection of OTC. The effect of HAuCl$_4$ concentration in the range of 0.25 to 2 mM on the colorimetric detection of OTC was investigated firstly. The absorbance of blank test (without OTC) at 550 nm refer to $A_0$ and the absorbance of sample test (with OTC) at 550 nm refer to $A$, respectively. Both $A$ and $A_0$ increased with the increase of HAuCl$_4$ concentration ranging from 0.25 to 1 mM indicating that higher concentration of HAuCl$_4$ benefits the formation of AuNPs. However, when the concentration of HAuCl$_4$ was higher than 1 mM, the blank absorbance at 550 nm increased dramatically. As shown in Fig. S4a (Supporting Information), the highest $\Delta A_{550}$ (refers to $A-A_0$) was obtained when the concentration of HAuCl$_4$ was 1 mM. Thus, 1 mM HAuCl$_4$ was employed for the further studies. According to previous reports, the formation of AuNPs can be accelerated with higher reaction temperature. Hence, the influence of reaction temperature of reducing HAuCl$_4$ by OTC on the colorimetric detection of OTC was studied (Fig. S4b, Supporting information). $\Delta A_{550}$ increased gradually when the reaction temperature
increased from 30 to 50°C, and then increased dramatically with the increase of reaction temperature in the range of 50 to 70°C. When the reaction temperature is higher than 70°C, \( \Delta A_{550} \) decreased because of the increased blank absorbance at 550 nm. Thus, the reaction temperature of reducing HAuCl\(_4\) by OTC was set at 70°C. Fig. S4c (Supporting Information) shows that the optimal reaction time of reducing HAuCl\(_4\) by OTC was 30 min when 70°C of reaction temperature was employed.

Hydroxylamine was employed as the reductant in the gold staining step. According to previous reports, hydroxylamine is capable of reducing Au\(^{3+}\) to form Au\(^0\) and the reduction can be catalyzed by the surface of AuNPs.\(^{26}\) The formed Au atoms can deposit on the surface of AuNPs resulting in the enlargement of small Au seeds to large AuNPs. The concentration of hydroxylamine and gold staining time would affect the growth of Au seeds, thus, influencing the colorimetric detection of OTC. The influence of hydroxylamine concentration ranging from 0 to 9 mM on the detection of OTC was investigated (Fig. S5a, Supporting Information). When the concentration of hydroxylamine increased from 0 to 0.5 mM, \( \Delta A_{550} \) increased dramatically suggesting that high concentration of hydroxylamine accelerated the enlargement of gold seeds to form large AuNPs. However, when the concentration of hydroxylamine was higher than 0.5 mM, \( \Delta A_{550} \) decreased gradually due to decrease of sample absorbance at 550 nm. We assumed that high concentration of hydroxylamine may lead to the formation of AuNPs with too large size which was not stable and precipitated during the gold staining process, thus, causing a decreased sample absorbance at 550 nm. Therefore, 0.5 mM NH\(_2\)OH was selected for the following studies. The time of gold staining also affect the enlargement of Au seeds. As shown in Fig. S5b (Supporting Information), the optimal gold staining time was 10 min when the sample solution containing 6.25 \( \mu\)M of OTC. However, longer gold staining time should be needed for sample solution
containing lower concentration of OTC. To achieve the detection of TCs with higher sensitivity, the gold staining time was set at 30 min.

*Analytical performance for the colorimetric detection of TCs*

The quantitative behavior of the established colorimetric method was evaluated by monitoring the dependence between the absorbance at 550 nm and the concentration of OTC under the optimum conditions. As shown in Fig. 2A, as the OTC concentration increased from 30 nM to 20 µM, the color of the solution gradually changed from light red to fuchsia, and the absorption in the range of 500-600 nm was gradually increased. Specifically, the absorbance at 550 nm increased linearly with the increase of OTC concentration in the range of 30 nM to 20 µM. The regression equation is \( A = 0.2005C - 0.2244 \) with a correlation coefficient of 0.9680, in which \( A \) is the absorbance at 550 nm and \( C \) is the concentration of OTC (µM), respectively. The limit of detection (LOD) was calculated based on \( 3\sigma/S \) rule, where \( \sigma \) is the standard deviation of 8-times parallel measurements of blank tests and \( S \) is the slope of the calibration curve, and the LOD for the colorimetric detection of OTC was calculated to be 14 nM. Besides, the quantitative behavior of the proposed colorimetric method for the detection of other kinds of TCs such as TC and DC was evaluated. As shown in Fig. 2C, the linear range for the detection of TC was 30 nM - 20 µM, and the \( I = 0.2020C - 0.2525 \) with a correlation coefficient of 0.9791. For the colorimetric detection of DC (Fig. 2D), the absorbance at 550 nm was linearly increased with the increase of DC concentration in the range of 10 nM - 6.75 µM. The regression equation is \( I = 0.07603C + 0.0174 \) with a correlation coefficient of 0.9554. The LOD for the detection of TC and DC was calculated to be 18.9 nM, and 1.98 nM, respectively. The colorimetric method based on in-situ generation of Au NPs coupled with a gold staining technique exhibited a competitive detection sensitivity for the detection of TCs (Table S1, Support
The repeatability of the established colorimetric method was evaluated by sixteen repeated measurements with 6.25 μM OTC, TC, and DC, respectively. The relative standard deviation (RSD) for OTC, TC, and DC was 2.6%, 3.7%, and 2.5%, respectively. The results demonstrated that the proposed colorimetric method showed good repeatability for the detection of TCs.

(Fig. 2)

The specificity of the proposed method was investigated by detecting the absorbance response at 550 nm for other interfering substances instead of TCs. These interfering substances include common ions (Mn^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Na^{+}, Ca^{2+}, K^{+}, HPO_4^{2-}), amino acids (His, Arg, Ile, Phe, Val), and proteins (CaM, SA, IgG, BSA), respectively. 6.25 μM OTC was employed as a positive control. Under optimum experimental conditions, only OTC showed a remarkable absorbance at 550 nm while other interfering substances exhibited a negligible signal which was similar to the blank test, even if much higher concentrations of these interfering substances were used.

*Real sample detection*

To evaluate the accuracy of the colorimetric method for the detection of TCs in real samples, a standard addition method was employed by spike OTC in liquid milk samples. The liquid milk was purchased from a local supermarket. Three concentration levels of OTC (0.625, 0.250, 0.125 μM) was spiked into the liquid milk. Then, the liquid milk with spiked OTC was dealt with 0.1 M McIlvaine-EDTA buffer (pH 4) to remove proteins and an HLB-SPE column to remove interfering substances existing in liquid milk samples. The elute from the HLB-SPE column was dried with nitrogen gas. The final products were dissolved in one tenth of the original volume of ultrapure water, and then detected by using
the proposed colorimetric method. The results are summarized in Table S2 (Support Information). The recoveries of 0.625, 0.250, and 0.125 μM OTC were calculated to be 94.0 ± 1.3, 91.9 ± 3.5 and 102.7 ± 3.5%, respectively. The above results demonstrate that this colorimetric method can be used for the detection of OTC in real milk samples with good accuracy.

Conclusions

In summary, a simple and rapid colorimetric method was developed for the detection of TCs based on the in-situ generation of AuNPs with TCs which is subsequently coupled with NH₂OH-HAuCl₄ reduction reaction-based gold staining technique. TCs containing phenolic hydroxyl groups can reduce AuCl₄⁻ into Au⁰ leading to the formation of AuNPs that act as gold seeds. The NH₂OH-HAuCl₄ reduction reaction is employed in the gold staining process. The surface of gold seeds catalyzes the reduction of AuCl₄⁻ by NH₂OH to form Au⁰ which deposits on the surface of Au seed resulting in the enlargement of gold seeds. The detection sensitivity for TCs can be improved by employing the gold staining technique and the results can be monitored by the naked eye directly or microplate reader. Under the optimal experimental conditions, as low as 14 nM, 18.9 nM, 1.98 nM of OTC, TC, DC can be sensitively detected, respectively. Compared to reported colorimetric, FL, CL methods for the detection of TCs, the proposed colorimetric method exhibited a competitive sensitivity for the TCs. In addition, this colorimetric method also exhibited a good specificity toward the detection of TCs. It was applied for the detection of TCs added to milk samples with satisfactory recoveries.
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Supporting Information

The structures of TCs (Fig. S1), the TEM of the AuNPs without gold staining (Fig. S2), size distribution of generated AuNPs (Fig. S3), optimization of experimental conditions (Fig. S4 and Fig. S5), the specificity of this colorimetric method (Fig. S6), comparison of the analytical performance of this colorimetric method with other methods (Table S1) and recovery of OTC spiked in milk samples (Table S2). This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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Figure Captions

Fig. 1  The UV-Vis absorption spectra of blank test (a, b) and sample test (c, d) without (a, c) and with (b, d) gold staining. The inset shows the corresponding photographs and TEM image of sample test with gold staining.

Fig. 2 The UV-Vis absorption spectra with different concentration of OTC (A) and absorbance at 550 nm with different concentration of OTC (B), TC (C), and DC (D). The insets are calibration plots for OTC, TC and DC, respectively. The error bars show the standard deviations for three replicate determination (n=3).

Scheme 1  Schematic mechanism of the colorimetric detection of TCs.
Fig. 1
Fig. 2
Scheme 1
Graphical Index

[Diagram showing the process with labels: Au	extsuperscript{3+}, Au	extsuperscript{5+}, Au	extsuperscript{1+}, TCs, HAuCl	extsubscript{4}, AuNPs, NH	extsubscript{2}OH, 30 min, 70°C, RT, 30 min]