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# Specific detection of oxytetracycline using DNA aptamer-immobilized interdigitated array electrode chip

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ABSTRACT

An electrochemical sensing system for oxytetracycline (OTC) detection was developed using ssDNA aptamer immobilized on gold interdigitated array (IDA) electrode chip. A highly specific ssDNA aptamer that bind to OTC with high affinity was employed to discriminate other tetracyclines (TCs), such as doxycycline (DOX) and tetracycline (TET). The immobilized thiol-modified aptamer on gold electrode chip served as a biorecognition element for the target molecules and the electrochemical signals generated from interactions between the aptamers and the target molecules was evaluated by cyclic voltammetry (CV) and square wave voltammetry (SWV). The current decrease due to the interference of bound OTC, DOX or TET was analyzed with the electron flow produced by a redox reaction between ferro- and ferricyanide. The specificity of developed EC-biosensor for OTC was highly distinguishable from the structurally similar antibiotics (DOX and TET). The dynamic range was determined to be 1–100 nM of OTC concentration in semi-logarithmic coordinates.



## 1. Introduction

Tetracyclines (TCs) are a group of antibiotics containing a general structure composed of four condensed aromatic rings. Most commonly used TCs are oxytetracycline (OTC), doxycycline (DOX) and tetracycline (TET). They are widely used for the treatment of infectious diseases in fodder animals. TCs are designed to act very effectively at low doses and to be completely excreted from the body after a short time of residence. Extensive use of TCs in veterinary medicine for their application as antibiotics and growth promoters have lead to their accumulation in food products, such as such as meat [1,2], milk [3], and eggs/chicken [4] and causes serious threat to human health. The most persistent and frequently found contaminated among the TCs is the OTC because of its effective antimicrobial properties [5–7].

Specific detection of OTC is difficult by conventional techniques including HPLC because of their close structural similarities among TCs. For example, OTC, TET, and DOX differ in minor functional groups, such as –H and –OH at $X_1$ and $X_2$ positions of 5th and 6th carbon atoms on rings B and C, respectively, in a tetracycline backbone (Fig. 1). Hence, there is a constant effort to develop a sensitive and selective biosensor system for detection of these antibiotics in contaminated food products and pharmaceutical preparations. Recently, studies showed that TCs can be detected in serum, meat and milk samples by dipstick colorimetric method [8]. An improved amperometric detection method for TCs by using multi-wall carbon nanotube modified electrodes has been reported and the detection limits determined to be in sub-micromolar range (0.09–0.44 μM of OTC) [9]. However, these detection methods are not sensitive enough to detect low concentrations of TCs in contaminants, which is present in nanomolar range. Sensitive detection techniques for TCs are often expensive, such as immune-based colorimetric methods employing ELISA which utilize antibodies and offers detection of TCs in ng $mL^{-1}$ range, but this method do not show a high selectivity for TCs detection because of structural similarities [10].

In recent years, numerous studies have been reported for the development of ssDNA or RNA aptamers that bind to specific target molecules [11–13]. These aptamers can bind to various targets with high affinity and specificity by various interactions, such as ionic interaction, hydrogen bond, and van der Waals' forces. One of the major applications of aptamers is that they rival antibodies. This is because of their specificity and affinity to their target molecules. They can be chemically synthesized, have high thermal stability and easy to modify and immobilize for biosensor applications unlike the antibodies. Several studies on selection of aptamers for small organic molecules have been reported [13–16]. Antibiotics belonging to the class of TCs are considerable potential targets for the development of aptamers. Recently, aptamers have been developed that selectively bind to OTC with strong affinity [16]. Application of this aptamer for detection of OTC can be of great advantage for screening and analysis in contaminated products. One possible application is by the use of an electrochemical system in which

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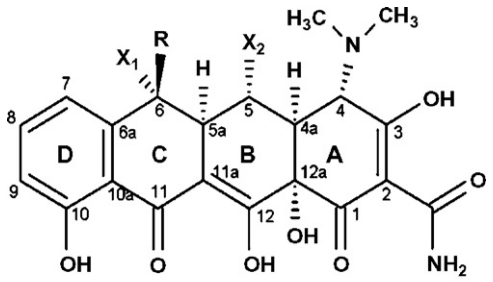


| Chemical | R | $X_1$ | $X_2$ |
|---|---|---|---|
| Oxytetracycline | $CH_3$ | OH | OH |
| Tetracycline | $CH_3$ | OH | H |
| Doxycycline | $CH_3$ | H | OH |

**Fig. 1.** General structure of tetracyclines (TCs). The inset table shows the unique functional groups on different positions on tetracycline backbone. The letters A, B, C, and D, respectively, denote the rings from right to left and the numbers represent the positions of carbon atoms.

an aptamer specific for OTC serves as a bio-receptor component. Because of these advantages of aptamers, a number of aptamer-based biosensor for proteins [17–19] and small organic molecules [20,21] have been developed.

In order to develop a biosensor, it is very important to generate a measurable signal from the target–probe interaction [22]. Numerous aptamer-based biosensors have been developed with different signal transducer formats such as an optical method, mass-dependant measurement, colorimetric detection and an electrochemical analysis [23]. Electrochemical system is an attractive sensing platform because it is simple, rapid, cost-effective and can be easily miniaturized, which is necessary for a high-throughput system and on-site applications [24,25]. Therefore, many electrochemical aptasensors have been reported based on labeling with electroactive compounds such as methylene blue (MB) or ferrocine [26,27], enzyme-based sandwich assay [28] and ion selective field-effect transistor (ISFET) [29]. ISFET-based aptasensor is a label-free system and yields electrochemical signal, i.e., a change in the current produced directly from the interaction between the target compound and the aptamer in this system. Recently, there have been continuous efforts to develop aptamer-based electrochemical biosensors for detection of a variety of target molecules including proteins and small organic molecules mainly because of its sensitivity, easy to fabricate the integrated sensor chip for multiplexing and on-site applications.

We have previously reported on selection of a few high affinity aptamers that selectively bind OTC [16]. In this study, we report on the development of an electrochemical biosensor for specific detection of OTC by employing one of the previously reported ssDNA aptamers specific for OTC. OTC-binding aptamer (No. 5) that strongly bind OTC with a $K_d$ = 11.13 nM was immobilized on a gold interdigitated array (IDA) electrode for the development of an electrochemical detection system preserving native specificity and selectivity of aptamer to bind its target. The use of aptamer-immobilized IDA electrode chip offered high sensitivity and consistency of electrochemical detection of OTC by cyclic voltammetry (CV) and square wave voltammetry (SWV) analysis.

## 2. Experimental

### 2.1. Reagents and DNA aptamer

The OTC-binding ssDNA aptamer have been developed in our previous study [16]. It is composed of a 76-mer size with a molecular weight of 23.7 kDa. The dissociation constant ($K_d$) of this aptamer with OTC was 11 nM. The following ssDNA aptamer sequence was chemically synthesized (GenoTech, Daejon, Korea): 5′-GGAATTCGCTAGCACGTTGACGCTGGTGCCC GGTTGTGGTGCGAGTGTTGTGTGGATCCGAGCTCCACGTG-3′–$C_3$–SH. Aptamer was functionalized with a thiol group at the 3′ terminal for immobilization on gold electrode, and the three carbon linker was used as a spacer between aptamer and gold electrode surface. The thiol-modified DNA aptamer was dissolved in 1 M dithiothreitol (DTT, Sigma–Aldrich Co.) solution to allow free-sulfhydryl group of aptamer remain in reduced form and incubated for 10 min at room temperature and stored at −20 °C until use. The structurally similar target molecules, such as OTC, TET and DOX were purchased from Sigma–Aldrich. TET and DOX are structural analogues of OTC that were used for the specificity test in this study.

### 2.2. Immobilization of aptamer on IDA gold electrode

The OTC-specific and thiol-modified ssDNA aptamer was immobilized on gold electrode based on the covalent chemistry. The detail procedure for immobilization is as follows: initially, the bare gold electrode was washed with 10 mM $H_2SO_4$ under electric potential within a range of 1 to −0.2 V. The thiol-modified aptamer suspended in DTT solution was purified using Microcon YM30 columns (Millipore Co., USA) for five times right before use and removed all traces of DTT. The aptamer was denatured by heating at 90 °C for 10 min, quickly cooled at 4 °C for 15 min and incubated at 25 °C for 5 min to allow renaturation of aptamer to attain its most stable conformation, which is a perquisite condition for its binding to target molecule (as described in Ref. [16]). Then, 5 μL of various concentrations of thiol-modified ssDNA aptamer (1, 10, 100 nM and 1 μM) was added on to the working electrode and incubated for 30 min at room temperature. Finally, the electrode was treated with 5 μL of 3,3′-dithioldipropionic acid (1 mM) for 30 min. All incubation steps were performed in a closed chamber to prevent the evaporation of solution. The immobilization procedure for thiol-modified aptamer on gold electrode was analyzed by cyclic voltametry (CV) and square wave voltametry (SWV).

### 2.3. Electrochemical detection of OTC

The electrochemical measurements were performed at room temperature using an electrochemical analyzer PGSTAT 30 Autolab (Ecochemi, Utrecht, Netherlands). The InterDigitated Array (IDA) gold electrode chip was purchased from ALS Co. (Tokyo, Japan). The working, reference and counter electrodes are integrated on a single glass chip, especially a working electrode (2 $mm^2$) is composed of 65 pairs of gold electrodes (10 μm × 2 mm) as shown in Fig. 2A and B. The DNA aptamer (1 μM) immobilized gold IDA electrode chip was thoroughly washed with distilled water and 5 μL of binding buffer (100 mM NaCl, 20 mM Tris–HCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6), which was used for the selection of OTC-binding aptamer [16], containing various concentrations of OTC, DOX and TET (0.1, 1, 5, 10, 50, 100 and 1000 nM) was dropped on working electrode and incubated for 30 min. The chip was thoroughly washed with distilled water and 10 μL of 5 mM $K_3Fe(CN)_6$ solution containing 100 mM KCl was added on gold IDA electrode chip until all three electrodes are covered. Then, cyclic voltammogram was recorded under electric potential ranging −0.6 to 0.6 V with a scan rate of 20 mV $s^{-1}$ and a step potential of 2 mV. The SWV was performed under electric potential ranging −0.6 to 0.6 V with a frequency of 8 Hz, step potential of 5 mV, and amplitude of 2 mV. After the electrochemical measurement, the working electrode was treated with 2 M NaCl for 10 min to regenerate the aptamer-immobilized IDA gold electrode. The high concentration of sodium chloride causes disruption of the hydrogen bonds and electrostatic interactions responsible for the aptamer–target association, subsequently it enables unfolding of aptamer and the bound

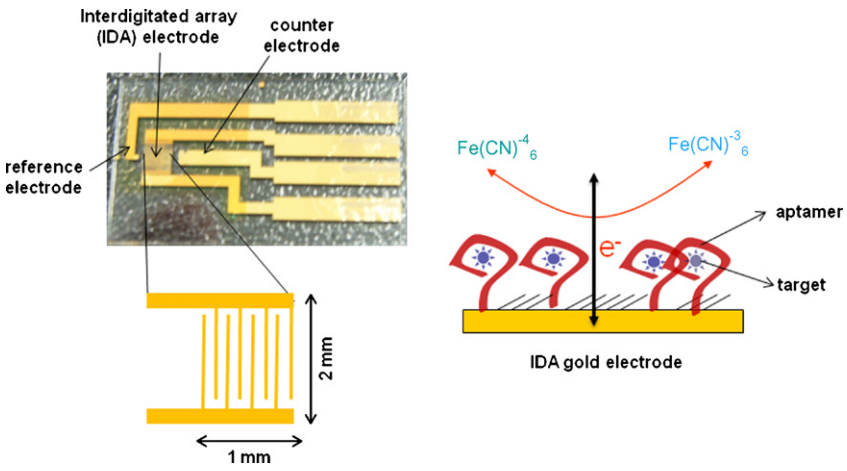


**Fig. 2.** Schematic diagram of electrochemical detection system for oxyteracycline (OTC) using aptamer-immobilized inter digitated array (IDA) gold electrode chip. (A) A photograph of an IDA gold electrode chip, (B) schematic diagram of IDA gold electrode showing magnified region of IDA, and (C) schematic diagram of a typical electrochemical reaction occurring after aptamer binds to its target molecule on a gold IDA electrode chip.

OTC is released from aptamer [30]. After each regeneration step, the electrochemical signal was evaluated to confirm the efficiency of regeneration method and that data was used for the estimation of current change after following sample treatment. The ferricyanide solution was used as a mediator to generate the electron flow between bulk solution and working electrode as shown in Fig. 2C. The current from the electrochemical system decreases by the binding of micro- or macromolecules to aptamer immobilized on gold electrode. The electrochemical data analysis was carried out and the decreasing percent of currents before and after the sample treatment ($\Delta I = (I_0 - I_1)/I_0 \times 100$) was measured. Where $\Delta I$ is relative current change, $I_0$ and $I_1$ represent the current before and after sample treatment, respectively.

## 3. Results and discussion

### 3.1. Quantitative analysis of OTC using aptamer-immobilized gold electrode chip

An electrochemical biosensor for detection of OTC was developed. This electrochemical biosensor was composed of an IDA gold electrode chip functionalized with thiol-modified DNA aptamer, which has high affinity and specificity to OTC, and served as a bio-receptor component of an EC-biosensor. The IDA electrode chip composed of 65 pairs of generator/collector electrodes, which are connected each other that set off electrochemical RedOx cycling continuously. This reaction significantly boosts sensitivity of the electrode. Electrochemical analysis was performed to confirm and optimize the conditions for ssDNA aptamer immobilization on gold surface. Initially, gold chips were immobilized with various concentrations of ssDNA aptamers (1 nM to 1 μM) and the immobilization was confirmed by the current changes in CV and SWV. The current changes were measured before and after immobilization of ssDNA aptamers on gold electrode chips. The current drop with CV and SWV showed a wide dynamic range. The difference in current drop was proportional to the concentration of ssDNA aptamer on the gold electrode (Fig. 3). A sensitive aptamer concentration was determined by using IDA chip immobilized with different concentration of aptamer followed by measurement of current change after OTC treatment.

The CV and SWV analysis was conducted against a series of OTC concentrations (0.1 nM to 1 μM) to probe the binding of OTC using 1 μM aptamer immobilized on IDA gold electrode chip. The interaction of small molecules, such as OTC to the aptamer immobilized on the gold electrode surface was monitored by reduction of the electron flux produced from a redox reaction between ferrocyanide and ferricyanide. The concentration-dependent decrease in current was observed in CV and SWV (Fig. 4). The decrease in current after OTC treatment was based on the specific interaction between aptamer and OTC. The formation of aptamer–OTC complex probably changes the permeability of the layer toward charged ferricyanide ions and hence the rate of their diffusion. Additionally, it is well documented that tetracycline molecules are charged and contain three ionisable groups (tricarbonyl, dimethylammonium, phenolic-dicetone), the p$K$ values of which are 3.3, 7.8, and 9.6 in aqueous solutions, respectively [31]. Therefore, binding of charged small molecule in aqueous solutions, such as OTC to aptamer can also affect electron flow combined with the diffusion rate [23–25]. The difference in the peak potentials $E_a - E_c$ ($\Delta E_p$) on cyclic voltammogram was increased, which was dependent on the OTC concentration. It was observed that 0.164 V of peak separation for control sample was increased to 0.672 V for 100 nM OTC. One possible reason for this increase in peaks separation could be due to the slow kinetics of the charge transfer, including the electron transfer, which is caused by the binding of OTC to aptamer. A linear relationship between the logarithmic OTC concentration and current change ($\Delta I$) was plotted ($R^2 = 0.961$) and dynamic range was determined to be in the range 1–100 nM of OTC (Fig. 5). Although a wide range of OTC concentration was used, but the minimum concentration with which

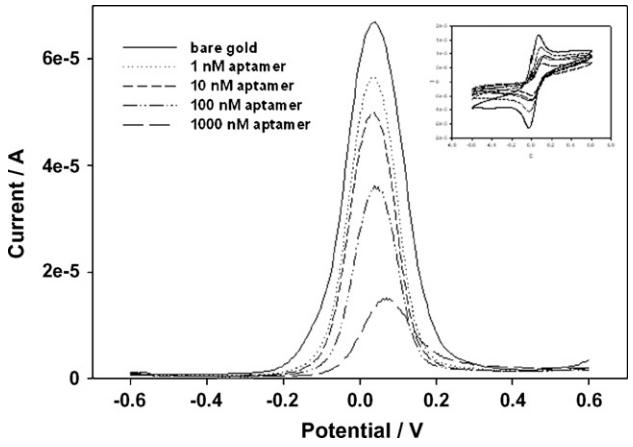


**Fig. 3.** Electrochemical analysis for immobilization of thiol-modified aptamer on IDA gold electrode. The current was decreased depending on the concentration of ssDNA aptamer in square wave voltammogram. The inset figure shows cyclic voltammogram.

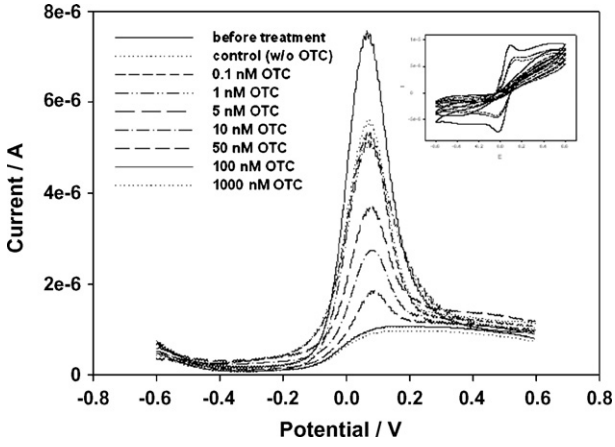


**Fig. 4.** Electrochemical analysis of OTC using aptamer-immobilized IDA gold electrode: The current drop was proportional to the concentration of OTC in a range 1–100 nM from square wave voltammogram. The inset figure shows cyclic voltammogram.

a significant current drop occurred was only at 5 nM OTC and the increasing concentration-dependent changes in current was seen till 100 nM of OTC in SWV. The sensitivity of this system could be enhanced by using more strong affinity of OTC-binding aptamer and well-defined gold electrode chip for reducing the background level. In addition, the coefficient of variation (CV%) of this aptamer-based electrochemical detection system was calculated to be ~4.5%.

The aptamer-immobilized IDA gold electrode chip was reused. For this, the electrode surface was treated with 2 M NaCl as described previously [30]. To verify the effective regeneration of aptamer-coated electrode, the CV and SWV analysis was performed after each regeneration step. The capture of OTC and regeneration of chip repeated for 30 repeats with the same aptamer-immobilized IDA gold electrode. The results showed that the current signal with each repeat was about 96.0% of the original signal, which followed up till 20 repeats. After 30 repeats, a maximum of 87.9% of the original signal was retained, which is normally acceptable.

### 3.2. Specificity of EC-biosensor to OTC

Specificity test was conducted with 1 μM ssDNA aptamer immobilized on IDA gold electrode chip. Electrochemical analysis was performed against seven different concentrations (0.1, 1, 5, 10, 50, 100 and 1000 nM) of three structurally similar tetracycline group of antibiotics or tetracyclines (TCs), such as OTC, doxycycline (DOX) and tetracycline (TET) (Fig. 1). The result showed that the electrochemical responses displayed considerably higher specificity to OTC (Fig. 5). The current change ($\Delta I$) for DOX and TET was found to be in the range of 32–40%, this was similar to the background signal seen in current change with control sample without any target chemical. This signal was evidently much lower than the current change found with OTC as target chemical (Fig. 5). It is clear from this result that electrochemical response was highly specific for OTC and it was possible to distinguish the responses obtained for OTC from responses with structurally similar antibiotics (DOX and TET). The specificity for OTC was largely dependent on (a) changes in minor functional groups, such as –H and/or –OH on 5th and/or 6th carbon atoms on the tetracycline nucleus (B and C rings) of OTC, DOX, and TET (Fig. 1), (b) because the immobilized aptamer on gold electrode retained its native ability to specifically interact with OTC even after it was immobilized on gold surface. This reflects the potential of aptamer-based electrochemical system for discrimination of structurally related tetracycline antibiotics, which otherwise difficult by any conventional detection systems. Thus the structural differences between OTC and other TCs can be elucidated by electrochemical analysis using the aptamer-immobilized IDA gold electrode chip (Fig. 5).

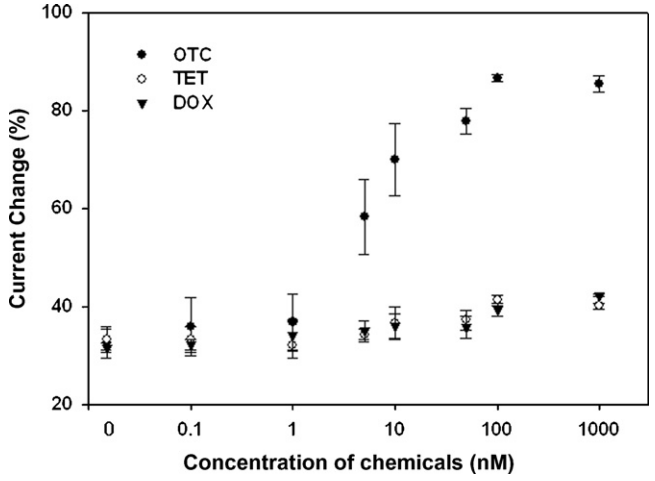


**Fig. 5.** Quantitative and specific detection of OTC. Electrochemical analysis was conducted by applying a series of OTC, TET and DOX concentrations (0.1, 1, 5, 10, 50, 100, 1000 nM) on aptamer-immobilized IDA gold electrode chip.

## 4. Conclusions

We have developed an electrochemical detection system for OTC using DNA aptamer-immobilized IDA gold electrode chip. This aptamer-based biosensor is more sensitive than previous studies designed for OTC detection. It was also shown that the structurally closed tetracyclines (TCs) such as doxycycline (DOX) and tetracycline (TET) can be easily distinguished from OTC, illustrating the specificity of this biosensor system. This result demonstrates that aptamers are suitable bio-receptor for the selective detection of small molecules, especially if target molecule has a structurally closed family in real sample. Finally this biosensor system could be implemented for bioanalytical applications and detection of residual tetracyclines in real filed sample such as contaminated food products and wastewaters after further optimized.

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