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A Novel Reflectance-based Aptasensor Using Gold Nanoparticles for the Detection of Oxytetracycline

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We present a novel reflectance-based colorimetric aptasensor using gold nanoparticles for the detection of oxytetracycline for the first time. It was found that the reflectance-based measurement at two wavelengths (650 and 520 nm) can generate more stable and sensitive signal than absorbance-based sensors to determine the aggregation of AuNPs, even at the high AuNP concentrations. One of the most common antibacterial agents, oxytetracycline (OTC), was detected as low as 1 nM in both a buffer solution and a tap water, which was 25-fold higher sensitive, compared to the previous absorbance-based colorimetric aptasensors. This reflectance-based colorimetric aptasensor using gold nanoparticles is considered to be a better platform for portable sensing of the small molecule detection using aptamers.

Introduction

Antibiotics contained in foods and drinking water has potentially serious effects on human health. Overuse of antibiotics can contribute to the development of antibiotic resistance such as evolving drug-resistant bacteria. Thus, there is a growing demand for on-site diagnosis of antibiotic residues in foods and drinking water. Oxytetracycline (OTC) is one of the antibiotics of tetracycline groups, and used to treat infections caused by Mycoplasma organisms and Chlamydia, preventing infections and diseases of livestock and poultry via intramuscular or oral administration [1]. It can damage calcium-rich organs such as bones and teeth, causing gastrointestinal and photosensitive allergic reactions.

Diverse analytical methods have been investigated to realize rapid on-site detection of antibiotic residue such as OTC from small amount of food samples. Y-channel microfluidic device using latex microspheres, electrochemical aptasensor, and aptasensor using gold nanoparticles have been reported for the application [2-5]. One of the most promising sensors among them is colorimetric aptasensors using gold nanoparticles (AuNPs) because of its simple operation and easy detection with the naked eyes [4]. The previously reported colorimetric aptasensors use either AuNPs modified by partly complementary oligonucleotides for hybridizing with aptamers [6, 7] or unmodified AuNPs on which single stranded DNA (ssDNA) aptamers can be physically adsorbed [8]. The aptasensors using unmodified AuNPs do not require any pre-treatment, and thus it is considered as a better approach for on-site sensing applications. When the target is added to the aptasensor, the target molecules will bind to the aptamers, resulting in the detachment of the adsorbed aptamers from AuNPs. The AuNPs will then aggregate, leading to the color change from red to purple (the absorbance peak shifts from 520 to 650 nm) because the localized surface plasmon resonance (LSPR) is changed [9]. This absorbance peak shift can be detected by measuring peak shift on reflectance signal. [10].

However, all of the previously reported colorimetric aptasensors using AuNPs are based on the indirect absorbance measurement, in which a spectrophotometer has been used [11]. The typical measurement platform for colorimetric sensors using AuNPs is a spectrophotometer [11]. The polychromic light that penetrates through the sample can be scanned over a specific wavelength range. A primary drawback of this method is significant signal loss due to light scattering in the presence of AuNPs (particularly, at high AuNP concentrations). Thus, only a low range of AuNP concentrations could be used in the

previous studies, resulting in relatively high limit of detection (LOD) (25 nM) [4].

These limitations of absorbance-based detection can be overcome by employing reflectance configuration. Unlike absorbance-based aptasensors in which the absorbance of AuNPs is analyzed indirectly from the spectrum transmitted, reflectance-based measurement is a direct measurement of the reflected spectrum from surface plasmon resonance of AuNPs (**Fig. 1**). In this approach, high AuNP concentrations are actually more desirable, since they can amplify the signal, potentially improving the sensitivity and LOD. In addition, since the reflectance-based measurement can be configured with flexible optical fibers, the system can be constructed on diverse platforms such as flow cells and microfluidic channels [2, 12, 13]. However, there is also a challenge in reflectance-based measurement. The challenge arises from the fact that there are two types of reflections: specular reflection at the surface of solution with no transmission into the solution and diffused reflection in which the radiation penetrates into the solution and is reflected at the surface of particles with partial absorbance and scattering [14]. When the measurement of diffused reflection from particles suspended in a solution is desired, the specular reflection from solution or container surface must be minimized. This would require optimization of incident angle and sample container. So far, few studies have been reported using reflectance configurations as biosensors. Gold nanoparticle thin film modified with biotin was reported for monitoring biotin-avidin interaction and they used a fixed incident angle [10]. There have been no reports using any reflectance configurations together with aptamers and AuNPs. This study presents, for the first time, a novel reflectance-based colorimetric aptasensor using gold nanoparticles for the detection of oxytetracycline for the first time. It was found that the reflectance-based measurement at two wavelengths (650 and 520 nm) can generate more stable and sensitive signal than absorbance-based sensors to determine the aggregation of AuNPs, even at the high AuNP concentrations. OTC was detected as low as 1 nM in both a buffer solution and a tap water, which was 25-fold higher sensitive, compared to the previous absorbance-based colorimetric aptasensors. Moreover, the sample does not need to be contained in the standard cuvettes and thus the sample loading platform can be miniaturized in portable and on-site diagnosis sensors. This novel reflectance-based aptasensor approach is believed to address the drawbacks of the absorbance-based sensors and thus has a great potential into a portable sensor system for the on-site diagnosis applications.

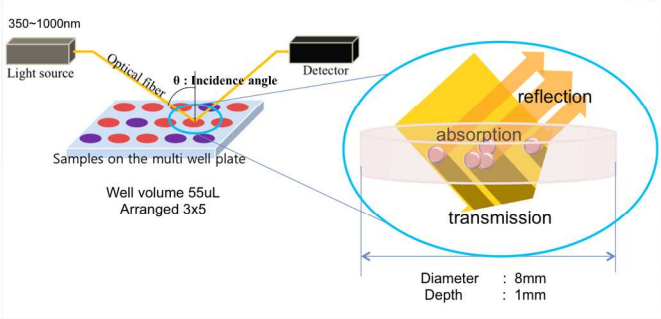

Figure 1. The scheme of the reflectance-based aptasensor and the multi well plate and the colorimetric aptasensor using AuNPs.

Materials and methods

Materials and DNA aptamer

All the chemicals were purchased from Sigma-Aldrich, unless specified otherwise. OTC binding ssDNA aptamer (OBA) (5' - CGTACGGAATTCGCTAGCACGTTGACGCTGGTGCCCG GTTGTGGTGCGAGTGTTGTGTGGATCCGAGCTCCACG TG - 3') was synthesized by Genotech Inc. (Korea) and sorted after dissolving in distilled water [15]. AuNPs were synthesized by citrate reduction of $HAuCl_4$ as reported formerly [16].

Experimental Set-up for Reflectance Measurement

The experimental set-up consisted of a light source (HL-2000-FHSA, Ocean Optics), a spectrometer (USB4000, Ocean Optics), and optical fibers (all from Ocean Optics, Dunedin, FL). Optical fibers were fixed in a holder to control the angle between the optical fibers and the well plate that contains the sample. Multi-well plates were made of acrylic plastic by machining. We custom designed wide but shallow (8 mm in diameter and 1 mm in depth) 15-multiwell plates arranged in a 3 x 5 format for our study. The wide well was designed to obtain strong reflectance intensity. In order to decrease the required sample volume, we designed the well to be shallow. The internal volume of the well was approximately 50 ul. The well size was also designed to be slightly larger than the diameter of the projected light (3-6 mm). The internal volume of the well was approximately 50 ul. The USB4000 measured light spectrum reflected by AuNPs and the aggregation of AuNPs was analyzed by the ratio of reflectance intensity at 520 nm and 650 nm wavelengths. All devices were set up on a probe station and remained horizontal except the optical fiber.

Process Optimization Study and Assay Protocol

All the experiments were conducted by using the same amount of solutions: 51.3 µl of AuNPs compounds, 2.85 µl of ssDNA aptamer, 2.85 µl of target compounds, and 3 µl of NaCl solution. The OTC target was dissolved in binding buffer (NaCl 100 mM, $MgCl_2 \cdot 6H_2O$ 2 mM, KCl 5 mM, $CaCl_2$ 1 mM, $C_4H_{11}NO_3$ 20 mM). The total volume of solution introduced to individual multi-wells for optical analysis was 60 µl. At first, AuNPs were mixed with ssDNA aptamers by shaking at 200 rpm for 30 min. Then the target compounds were pipetted to the mixture of AuNPs and ssDNA aptamers. After another 30 min of mixing, 1 µl of NaCl (0-1.5 M) were injected 3 separate times because 3 µl of NaCl injection at one time can cause excessive electrostatic instability. The target compounds, oxytetracycline, was replaced with buffer solution in all optimization process. The target concentrations tested in binding assay were: 1 nM to 25 µM in both buffer and tap water.

Results and discussion

Optimization of reflectance-based colorimetric aptasensor

In the reflectance-based aptasensor using unmodified AuNPs, the optimization of the incidence and reflection angles is critical because the portion of light reflected from AuNPs surface varies with the incidence angle. In our experiment, the diffused reflection at the surface of AuNPs is our primary interest and thus incidence angle and a type of container were

carefully selected through experimental characterization in order to minimize the specular reflection. Though the incidence angle is important factor in reflectance-based approaches, the effect of incidence angle has been disregarded in previous research. Other critical factors in this type of aptasensor using umAuNPs that influence the performance of the sensor significantly are salt concentrations, the ratio of AuNPs to aptamer, and AuNPs concentrations. These parameters were optimized through experimental characterization as well.

Effect of incidence angle

We designed the reflectance-based aptasensor to change the incidence angles. As the incidence angle change, the portion of the reflected light also was changed. The light can be reflected from the surface of solution and AuNPs. To increase the portion of the reflected light on the AuNPs surface, we performed the measurement of samples at the 20°, 30°, 40° and 50° of the incidence angles from the optical fibers (**Fig. 2**). While the peak shifting from 520 nm to 650 nm was observed for all four angles investigated as AuNPs aggregation occurred, the reflectance peak at 520 nm wavelength was the sharpest at 50° incidence angle, and the ratio of R650/R520 was lowest. It means that an amount of the reflected light on the AuNPs surface was the highest at 50° angle. We performed all other experiments at this optimal incidence angle.

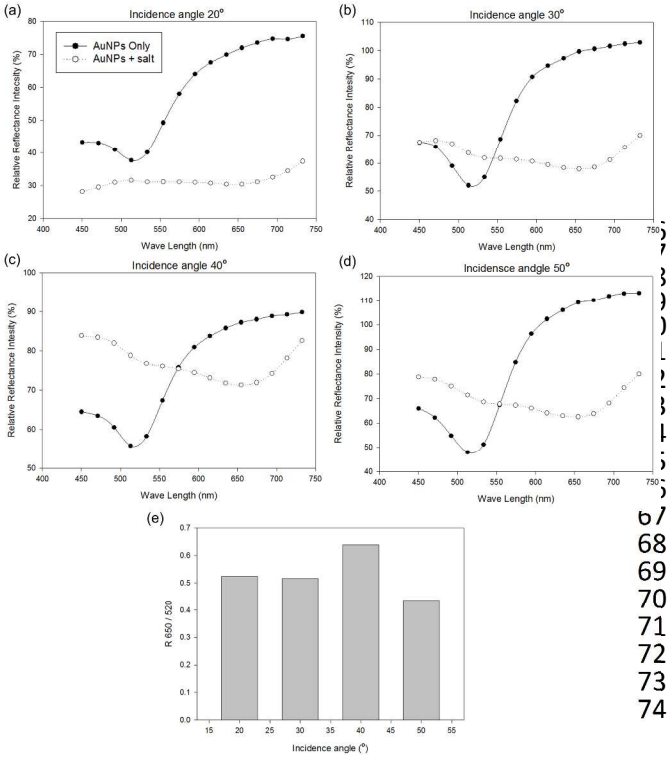

Figure 2. Relative reflectance intensity at 520 and 650 nm of the light wavelengths for different incidence angles:(a) 20°, (b) 30°, (c) 40°, (d) 50°, (e) R 650/520 of AuNPs at different incidence angles.

Effects of salt concentration and AuNP to aptamer ratio

There are three important parameters in this reflectance-based colorimetric aptasensor, such as AuNPs concentration, salt concentrations, and the ratio of AuNPs to aptamers. These parameters are interdependent to each other, and therefore it should be optimized in the proper order. First, we fixed the AuNPs concentration at 2.5 nM and optimize the salt concentration. When the AuNPs are aggregated, the reflectance peak shifts from 520 nm to 650 nm wavelength in spectra graph and the ratio of R650/R520 increase (**Fig. 3 (a), (b)**). These color change and peak shift were saturated at a specific salt concentration (~ 1.25 M), as shown in Fig. 2a and 2b, which is considered to be optimal.

The ssDNA aptamers can be adsorbed on the AuNPs surface by the electrostatic forces. The aptamers attached on the AuNPs surface makes a stable state, even at the high salt concentrations. The ratio of AuNPs to aptamers is also a key parameter in this step. For finding an appropriate AuNPs to aptamer ratio, we performed experiments with the ratios of 1:0, 1:75, 1:100, 1:125, 1:150 and 1:200. As the aptamer concentration is increasing, the AuNPs were getting more stable (**Fig. 3 (c), (d)**). Therefore, the ratio of AuNPs to aptamers was determined to be 1:125 as an optimum.

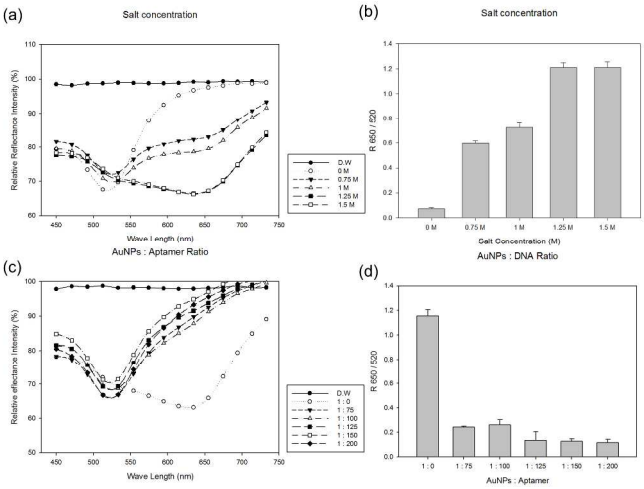

Figure 3. The optimization of salt concentration and AuNP to DNA ratio: (a) the relative reflectance intensity at 0 ~ 1.5 M salt concentrations, (b) the R 650/520 at each salt concentrations, (c) the relative reflectance intensity at 1:0 ~ 1:200 AuNPs to aptamer ratios, (d) R 650/520 at each AuNP to aptamer ratio.

Assessment of Specificity for OTC

Oxytetracycline is tetracycline group antibiotics. Oxytetracycline, tetracycline and doxycycline have similar structures, except just one functional group. It has been already reported that the specificity of OTC binding aptamer was good [3], and the specificity of this reflectance-based aptasensor configuration was confirmed again for 50 μM of tetracycline, doxycycline and diclofenac, respectively. In fact, in spite of the similar structure of tetracyclines, the peak shift occurred only with oxytetracycline, not with other similar tetracyclines. (**Fig. 4**)

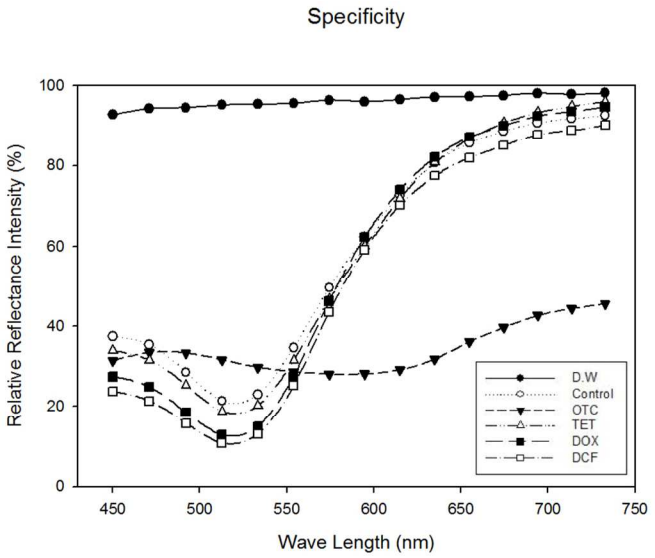

Figure 4. Specificity of the reflectance-based colorimetric aptasensor. The final concentrations of the oxytetracycline, tetracycline, doxycycline, diclofenac are 50 μM.

Effect of AuNP concentrations and dose-dependent assay

The main text of the article should go here with headings as appropriate. We performed dose dependent binding assay at different AuNP concentrations for optimizing AuNPs concentration and measuring the limit of detection (LOD) (**Fig. 5**). In our previous work based on an absorbance system, the optimal AuNPs concentration was 0.2 nM and the LOD was 25 nM [4, 17].

In this reflectance-based method, the reflectance light from AuNPs, not scattering light, at a certain angle is measured. So, higher the AuNP concentrations, stronger the reflectance light obtained. Therefore, in this study we have used higher AuNP concentrations than that used in other method such as absorbance-based colorimetry, in which the absorbance signal is more decreased at higher AuNP concentrations. In this novel reflectance-based aptasensor system, better results were obtained at higher AuNPs concentrations even if the same aptamer sequence was used. With AuNP concentration of 10 nM, the LOD was 1 nM, which is 25-fold smaller than the previous result obtained by absorbance system. In addition, even though the AuNPs concentration (10 nM) on this novel reflectance-based method was higher than the previous method (0.2 - 2nM), the amount of sample required was decreased about 0.75 times because the experiments were conducted with only 60 μl solution.

In order to be used for on-site applications, this sensor should be functional in any sample solutions. The detection of oxytetracycline was attempted in tap water solution for this purpose (**Fig. 6**). The OTC was dissolved in tap water at different concentrations. All other conditions were the same as the binding assay described in Fig. 3. Even in tap water, the LOD remained as low (1 nM) as in buffer solution.

Table 1 shows comparison of the current reflectance-based aptasensor with absorbance-based aptasensor and other biosensors (cantilever sensor, light scattering agglutination assay, and indirect competitive assay) with regard to sensor performance for the detection of OTC. LOD, limit of quantification (LOQ), linear dynamic range, and EC50 values of the sensors were obtained from literature [2, 4, 18, 19]. The linear range of reflectance-based aptasensor was 0–10nM. Reflectance-based aptasensor shows lower LOD/LOQ and does not require high sample volume or pre-treatment. Therefore, it seems to be suitable for on-site analysis of low concentration target such as OTC. The more complicated food sample tests could be a subject in the further study of this work.

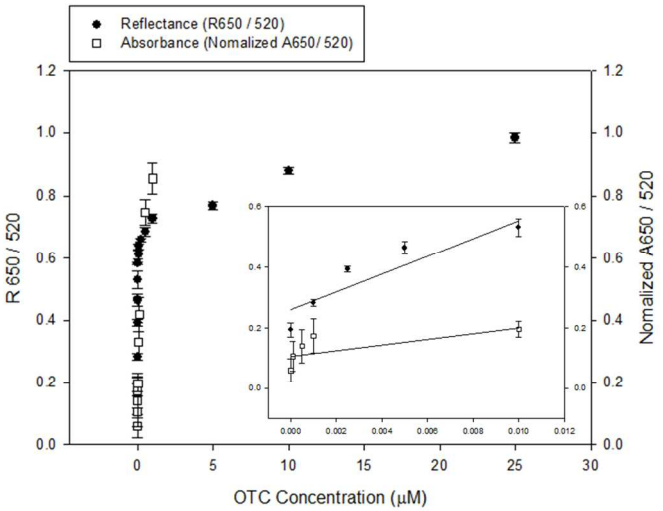

Figure 5. An overlay plot showing a dose-dependence of this reflectance-based aptasensor and absorbance-based aptasensor using unmodified AuNPs for the detection of oxytetracycline. Filled circle indicated reflectance data while empty square indicated absorbance data published in our previous study [17]. Left vertical axis represents R 650/520 for the reflectance intensity ratio and the right vertical axis represents the normalized A650/520 for absorbance intensity ratio.

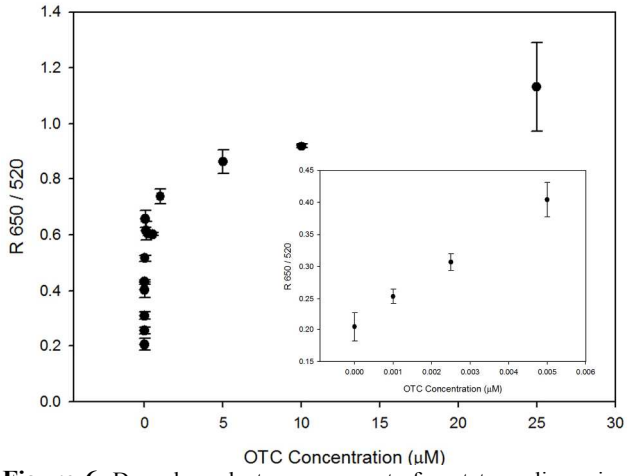

Figure 6. Dose-dependent measurement of oxytetracycline using this reflectance-based aptasensor in tap water.

	pros	cons	LOD/ LOQ	Dynamic Range	EC_{50}	Ref.
Reflectance-based aptasensor	Low LOD Low sample vol. No pre-treatment		1 nM/ 4 nM	1 nM-1 μM	188 nM	This study
Absorbance-based aptasensor	No pre-treatment	High LOD High sample vol.	25 nM/-	0.025-1 μM	313 nM	4
Cantilever sensor	Low LOD	Pre-treatment Long measuring time	0.2 nM/-	1-100 nM	30 nM	18
Light scattering agglutination assay	Real-time monitoring	High LOD	217 nM/-	0.217-21.7 μM	-	2

Indirect competitive assay	High recovery rate in spiked milk	High LOD Pre-treatment	27 nM/ 108 nM	-	-

Table 1. The comparison of biosensing characteristics among various assay platforms for the detection of OTC

Conclusions

In this study, we developed a novel reflectance-based aptasensor using unmodified gold nanoparticles for the detection of oxytetracycline. This reflectance aptasensor can measure the peak shifting of the changing of localized surface plasmon resonance as the AuNPs aggregation in solution state. Compared with the absorbance-based sensors previously reported, this reflectance-based system has significant advantages. First, it can measure the reflectance from AuNPs surface directly, so reflectance signals increase at high AuNPs concentrations. As a result, the sensitivity of aptasensor increased 25-fold. Second, we may be able to use different loading platforms with various shapes, such as well plates, microfluidic channels and disc plates. The reflectance method offers more flexibility in terms of system construction compared with absorbance method. Third, small amounts of sample and reagents are necessary for detection. We believe that our novel reflectance-based aptasensor approach has great potential to be developed into a portable sensor system for the on-site diagnosis applications.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Fig. S1, Fig. S2]. See DOI: 10.1039/b000000x/

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