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Research Article

High-sensitivity detection of oxytetracycline using light scattering agglutination assay with aptasensor

We present an aptamer-based biosensor (aptasensor) for rapid and high-sensitive detection of oxytetracycline (OTC) antibiotic in PBS inside a Y-channel PDMS microfluidic device. The detection was made by real-time monitoring of the agglutination assay of ssDNA aptamer-conjugated polystyrene latex microspheres with proximity optical fibers. The agglutination assay was performed with serially diluted OTC antibiotic solutions using highly carboxylated polystyrene particles of 920 nm diameter conjugated with OTC-binding ssDNA aptamer. Proximity optical fibers were used to measure the increase in 45° forward light scattering of the aggregated particles by fixing them around the viewing cell of the device with stable angle and distance to the detector. The detection limit was around 100 ppb for the current aptasensor system with the detection time less than 3 min.

Keywords:
Agglutination assay / Aptamer / Microfluidic device / Oxytetracycline / Static light scattering
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1 Introduction

Antibiotics are natural compounds of low molecular weight that can be produced by fungi or bacteria. They possess antibacterial activity; therefore, killing or inhibiting the growth of many microorganisms [1, 2]. In animal husbandry, antibiotics are mainly used to prevent bacterial infections and to increase growth rate. If these antibiotics were to be improperly administrated, however, their residual or degradation products may be present in the final products, which cause harmful effects on consumers. In particular, a trace amount of antibiotic compounds in milk can foster the development of antibiotic-resistant bacteria [3]. To address this issue, many countries have set definitive maximum residue limits in food products, for which suitable analytical methods need to be developed to control the concentration of these compounds below the maximum residue limit level [2].

Oxytetracycline (OTC) has been licensed for use in a variety of food producing animals including cattle, sheep, goats, poultry, and fish, as an additive in feed or drinking water to maintain optimal health. For this reason, many researchers have made extensive efforts to develop a rapid, accurate, and sensitive detection method for the determination of antibiotic contents [4]. Several methods have been introduced on the basis of fluorescein-assisted bioassays, but they usually lack sensitivity and specificity [5, 6].

Antibodies provide a fertile ground for molecular recognition for a wide range of applications and thus have made substantial contributions toward the advancement of diagnostic assays [7]. However, the use of antibodies in multiple detection methods or in the analysis of very complex samples could encounter limitations mainly from the nature and synthesis of their protein receptors. In order to circumvent some of these drawbacks, other recognition molecules have been explored as an alternative [8]. Of these, the systematic evolution of aptamer by Systematic Evolution of Ligands by Exponential Enrichment process has made possible the isolation of oligonucleotide sequences with an ability to recognize virtually any class of target molecules with high affinity and specificity [9].

Here, our interest lies in the combination of the aptamer-based biosensing with light scattering detection within a microfluidic channel. As is known, light scattering offers advantages such as small size, low cost, and ease of integration [10, 11]. Although there have been a number of optical approaches with microfluidic devices including surface enhanced Raman scattering, interferometry, and particle beam emission spectroscopy [12–14], little effort has been made

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Abbreviations: LSPIA, light scattering particle immunoagglutination assay; **OTC,** oxytetracycline; **PS,** polystyrene

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toward the development of light scattering detection inside a microfluidic device [15, 16]. In this sense, the so-called "light scattering particle immunoagglutination assay" (LSPIA) could be a new, alternative method for rapid and high-sensitivity detection of proteins, viruses and bacteria [17–19].

In this study, we report on the development of a highly specific, antibiotic aptasensor for the OTC antibiotic, which can detect the agglutination of OTC-binding ssDNA aptamer-conjugated polystyrene (PS) latex microspheres. As described shortly, the aptamer-conjugated PS particles demonstrate a strong binding affinity to OTC with high specificity and selectivity due to the intrinsic binding ability of the aptamer to its target. Therefore, the LSPIA, when combined with a microfluidic device, allows for a versatile platform for the detection of various residual or degradation products of antibiotics in a simple, accurate, rapid, and high-sensitivity manner.

2 Materials and methods

2.1 Materials and aptamer-conjugated latex microspheres

The OTC-binding ssDNA aptamer was reported earlier [20]. It is composed of a 76-mer size with a molecular weight of 23.7 kDa and functionalized with amino group at the 3′ terminal for immobilization on latex microspheres. A random ssDNA library of 11.25 μg or 10^{15} molecules with the following sequence: 5′-CGTACGGAATTCGCTAGC-N_{40}-GGATCCGAGCTCCACGTG-3′ chemically synthesized and purified with PAGE column separation (Genotech, Dajon, Korea) was used in this study [20]. The amino modified ssDNA aptamer was dissolved in 100 mM phosphate saline buffer (pH 7.5) solution and incubated for 10 min at room temperature and stored at −20°C prior to use. OTC, gentamicine, penicillin G, enrofloxacine, and sulfametazine were purchased from Sigma-Aldrich and used without further treatment. Highly carboxylated PS submicron (920 nm) latex microspheres were purchased from Bangs Laboratories (Fishers, IN, USA). The microspheres were coated with the OTC aptamer by covalent coupling method, resulting in the full surface coverage of aptamer to the microspheres [16]. The concentration of microspheres was 0.04% v/w.

2.2 Fabrication of microfluidic device

The microfluidic device was fabricated using standard soft lithography with PDMS molding technique [21, 22]. The PDMS (Dow corning, Midland, MI, USA) microchannel had the dimension of 200 μm (wide) × 100 μm (depth). The Y-shaped microfluidic channel was designed in such a way that a sample solution flows through the viewing cell from the bottom and over the top in order to prevent the generation of air traps, which is detrimental to light scattering detection methods [17]. Figure 1 shows a schematic illustration of the fabricated Y-channel microfluidic device with four channel bubble traps.

2.3 Light scattering detection

A miniature, portable spectrometer (Ocean Optics, Dunedin, FL, USA) was used to measure forward light scattering intensity collected by the same type of multi-mode fiber. The two optical fibers for lighting and detection had 600 μm core diameter and 30 μm cladding with optimal transmission in the UV–visible wavelengths. The fiber optic cables were 1.0 m in length with SMA-905 connectors on each end. The numerical aperture of these optical fibers and probes was 0.22 with an acceptance angle of 25°. The 380 nm wavelength UV LED supplied 45 μW of power to the optical fiber assembly. Figure 2 shows that the light source and detector fibers were positioned below and over the microfluidic device, respectively, with the detection probe positioned at a 45° angle to the incident light (Edmund Optics, Barrington, NJ, USA).

The collected scattered light at 45° to incident beam would optimize the assay in terms of intensity and S/N within the range of 30–90°, whereas avoiding any of the direct incident light beam. Small angles (<30°) prevented the detector probe from collecting scattered light for the given microfluidic device configuration. Angles larger than 90° created a reflection problem on the bottom of the microfluidic device [23]. To inject the microspheres conjugated with OTC-binding aptamer and target solutions to the Y-junction microchannel, a syringe pump (KD Scientific, Holliston, MA, USA) was used. Two teflon tubes (0.79 mm od) connected two 500 μL gastight syringes (Hamilton, Reno, NV, USA) to the top openings of the microfluidic device. A total of six different dilutions were tested with the concentrations ranging from 1 to 10^5 ppb of OTC. In the experiments, two controls were used: the first is the blank solution with the PS microspheres (control) and the second is the one with a mixture of gentamicine, penicillin G, enrofloxacine, and sulfametazine (negative control). Light intensity was measured ten times in the interval of 1 s and the data were stored in a storage module.

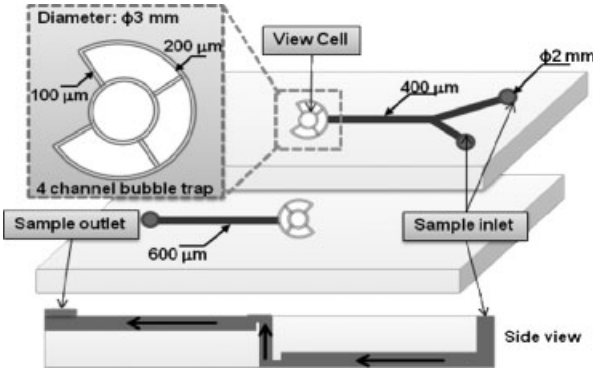

Figure 1. Schematic illustration of Y-shape microfluidic device with four channel bubble traps.

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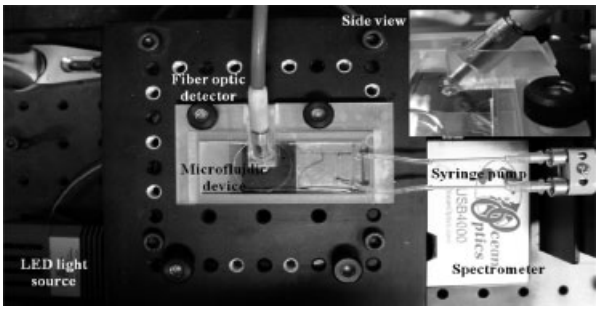

Figure 2. Photograph of the experimental setup, consisting of the microfluidic aptasensor with insertion tray and optic fiber fixing module and optical fibers with portable spectrometer and a UV (380 nm) light source for optical fiber detection.

3 Results and discussion

3.1 Agglutination assay with OTC-binding aptamer-conjugated PS microspheres

Antibody–antigen binding causes the microspheres to form agglutination complexes, which can easily be detected by static or dynamic light scattering method. LSPIA adopts the principle of antibody–antigen reaction and determines an antigen concentration by measuring the light scattering intensity as a result of agglutination of specific antibody immobilized latex microspheres. In this agglutination assay, an increase in the antigen concentration can be interpreted with an increase in the aggregated particle size. It is noted here that specific detection of OTC is difficult with conventional techniques including HPLC and immunoassay partly because small molecules such as antibiotics are much smaller than antibody (height 122 Å, width 139 Å). Therefore, the reaction between antibody and small molecules hardly occurs [24, 25]. Aptamer is a small-sized molecule (25 Å high and 21 Å wide) and it is a conserved sequence motif that forms a major portion of the main stem and loop in its secondary structures. It was previously demonstrated that such a conserved sequence of OTC-binding aptamer exhibited a strong affinity to OTC at several binding regions in a sandwich immunoassay format [20].

We first investigated that ssDNA aptamer can bind with OTC through an agglutination of PS latex microspheres using microscopic images (Fig. 3). As shown in Fig. 3A, 920 nm PS latex microspheres (0.04% v/w) in PBS solution mostly existed as singlets, whereas the ones coated with ssDNA aptamers existed in multiple forms of singlets, doublets, triplets, or more clusters (Fig. 3B). The reason for the difference in comparison to the pristine PS latex microspheres is that the ssDNA aptamers bound to the surface might induce additional protein–protein interactions. Figure 3C shows the microscopic image of clustered particles when the microspheres were mixed with 1000 ppb OTC solution, suggesting that a significant agglutination takes place in the presence of strong binding reaction between the microspheres and OTC. These results collectively show that the ssDNA aptamer-conjugated PS microspheres can be used as an effective aptasensor in the current agglutination assay.

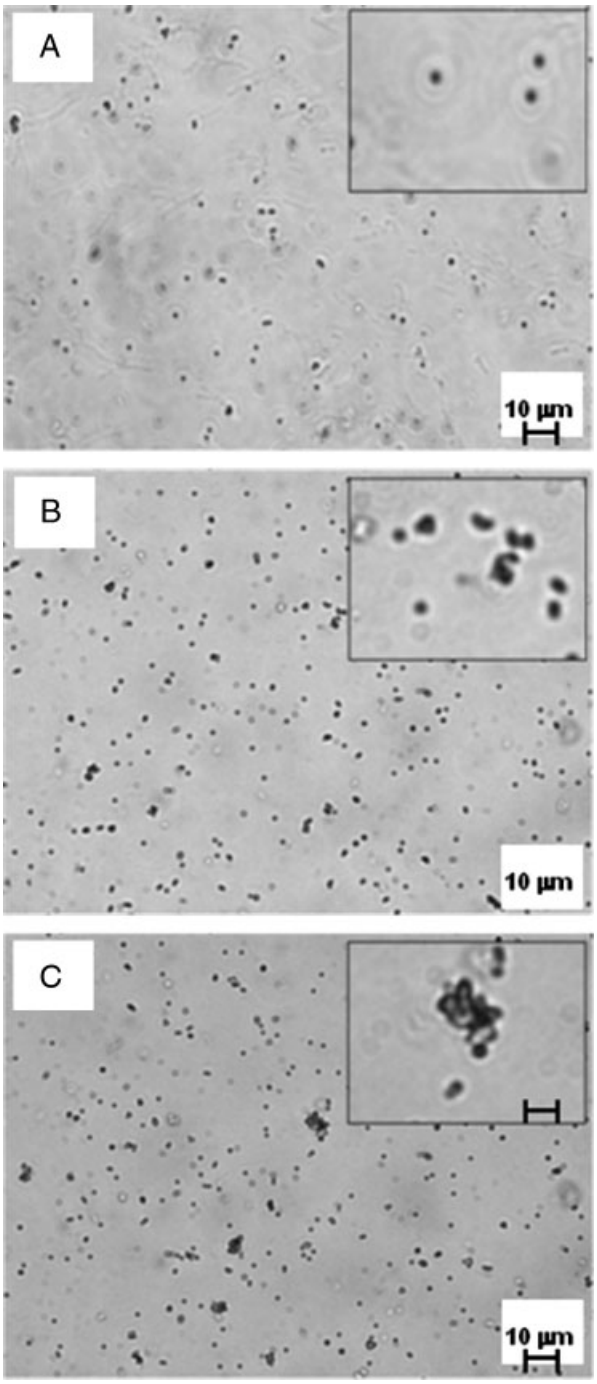

Figure 3. Light microscopic images of 0.04% v/w PS latex microspheres with PBS (A), OTC-binding ssDNA aptamer-conjugated PS microspheres (B), and the same microspheres in (B) after treatment with 100 ppb solution (C).

3.2 Microfluidic aptasensor for detection of OTC

To demonstrate how aptasensor signals are changing in the presence of the OTC antibiotic inside a microfluidic device, the light scattering intensities were monitored for 6 min at 100 ppb OTC concentration. To compare, the data for

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negative control are also shown by adding a mixture of other antibiotic solutions instead of OTC (Fig. 4). As can be seen from the figure, there was no change of signals until the sample solution reached the viewing reservoir up to 120 s. In the presence of 100 ppb OTC, the intensity was linearly increased for the duration up to 60 s, and then maintained with a relatively stabilized signal around 480 000 (a.u.) even after 5 min. From the negative control, however, no appreciable signal changes were observed throughout the sample flow. Interestingly, this result is quite different from the previous work of ours, in which an immobilized antibody was attached onto PS latex microspheres for the detection of *micoplasma pheumonia* [17]. In the case of the antibody-conjugated PS microspheres, the light scattering intensity gradually increased, whereas the microspheres were being introduced into the viewing reservoir until 60 s and then started decreasing thereafter. We hypothesized that the ssDNA aptamer-conjugated PS latex microspheres presented here are significantly smaller than the antibody-conjugated ones and thus would not disturb the emission of light scattering from the view cell. Therefore, the presence of a peak can be used as an indicator that the agglutination process takes place.

3.3 Detection limit and dynamic range of aptasensor agglutination assay

In the experiment, the positions of light source and detector were controlled using an optical positioning stage. However, it turned out to be difficult to maintain the distance between light source and detector constantly at an exact position. For example, it was observed that the initially positioned detector underwent a slight position change with repeating measurements and gave rise to a substantial experimental error in the data output. To overcome this problem, we developed a system tray to maintain the light source and detector at a given position irrespective of repeating multiple measurements. Figure 5 shows a schematic illustration of the tray with corresponding geometry information (A) and an assembled module to maintain the constant measurement angle and distance to the detector (B). As the light scattering intensity is heavily affected by the measurement angle, we have tested for three different angles of 40, 45, and 50° and found that the angle of 45° was optimal and thus used in this study.

To improve the detection limit, fiber optic probes were installed in proximity to the viewing cell, so that a small change in the scattering intensities can be detected. In detail, a light source fiber probe was installed in perpendicular contact to the bottom of the viewing cell, whereas a detection fiber optic probe was installed above a microfluidic device with a 45° angle. The detection fiber probe was positioned exactly at 0.5 mm gap above the microfluidic device. Experimentally, this was accomplished by placing a plastic film of 0.5 mm in thickness on the microfluidic device, bringing the detection probe in close contact with the film, and removing the film to make the distance of 0.5 mm.

The detection system measures the change of light scattering intensity due to a change in the size of aggregated particles. Therefore, it is necessary to differentiate targeted light scattering resulting from agglutination by specific interactions and from the binding with non-target antibiotics (non-specific binding). For this purpose, the intensity was measured with pure 920 nm latex microspheres (control), OTC-binding ssDNA aptamer-conjugated ones, and the same ones with a mixture of other antibiotics

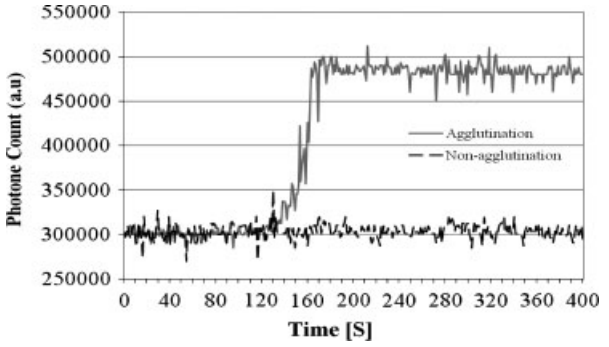

Figure 4. Comparison of light scattering intensity between agglutination and non-agglutination inside the microfluidic device. The light scattering intensities were monitored over a time span of 6 min with an OTC concentration of 100 ppb together with negative control (a mixture sample with gentamicine (1000 ppb), penicillin G (1000 ppb), enrofloxacine (1000 ppb), and sulfametazine (1000 ppb)).

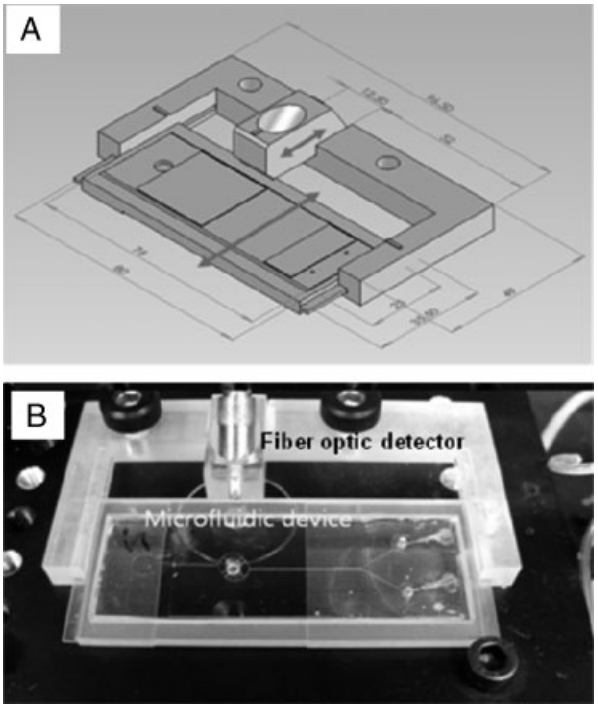

Figure 5. (A) Schematic illustration of the tray with corresponding geometry information and (B) photograph for an assembled module to maintain the constant measurement angle and distance to the detector.

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(negative control). For negative control, a mixture of four different antibiotic solutions with gentamicine (1000 ppb), penicillin G (1000 ppb), enrofloxacine (1000 ppb), and sulfametazine (1000 ppb) was used. Figure 6 shows the intensities of maximum light scattering *via* real-time monitoring of the viewing cell with three different samples. The mean light scattering intensity of control was $280\,000 \pm 43$, which indicates no self-agglutination. In contrast, the ssDNA aptamer-conjugated microspheres plus the negative control showed the mean light scattering intensity around $300\,000 \pm 109$. This result implies that the self-agglutination reactions slightly increased with the conjugation of the ssDNA aptamer. It is worthwhile noting that the intensity was nearly the same with the negative control, suggesting that such an increase in self agglutination has to do with increased particle–particle interactions, and not with particle–antibiotic interactions. The observed baseline levels were used to calculate the real signals for the agglutination assay.

As shown in Fig. 7, the concentration-dependent variations in light scattering intensity were observed by using a wide range of OTC concentrations from 1 to 10^5 ppb. Figure 7A shows the intensities of maximum light scattering *via* real-time monitoring of the viewing cell with the 920 nm OTC-binding ssDNA aptamer-conjugated microspheres. The data represent average values of triple measurements in which each value was determined by averaging the data (total 100 data) over 30 s around a peak value. When the concentration was higher than ~100 ppb, there was a sudden increase in the signal intensity and a distinct decrease thereafter up to 10^5 ppb (diagonal line bars). When the concentration became too high (~10^5), the signal was hardly distinguishable from that of the negative control (white bar) presumably due to increased interactions of the aggregated particles with incident light and thus reduced scattering. It was found that a good linear relation can be

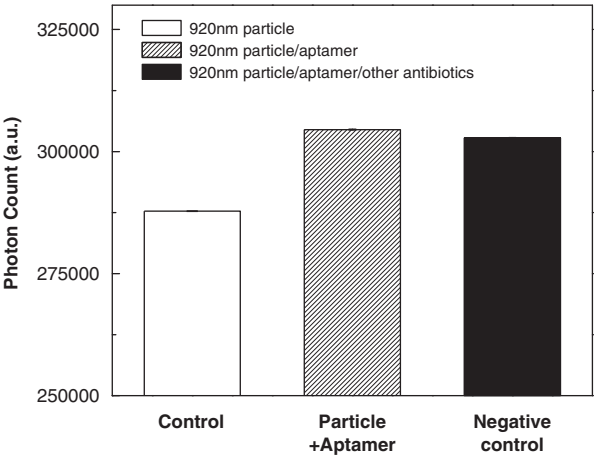

Figure 6. Light scattering intensities of control (white bar; pure PS latex microspheres), particle+aptamer (diagonal line bar; OTC-binding ssDNA aptamer-conjugated PS latex microspheres) and negative control (black bar; mixture sample with gentamicine, penicillin G, enrofloxacine and sulfametazine).

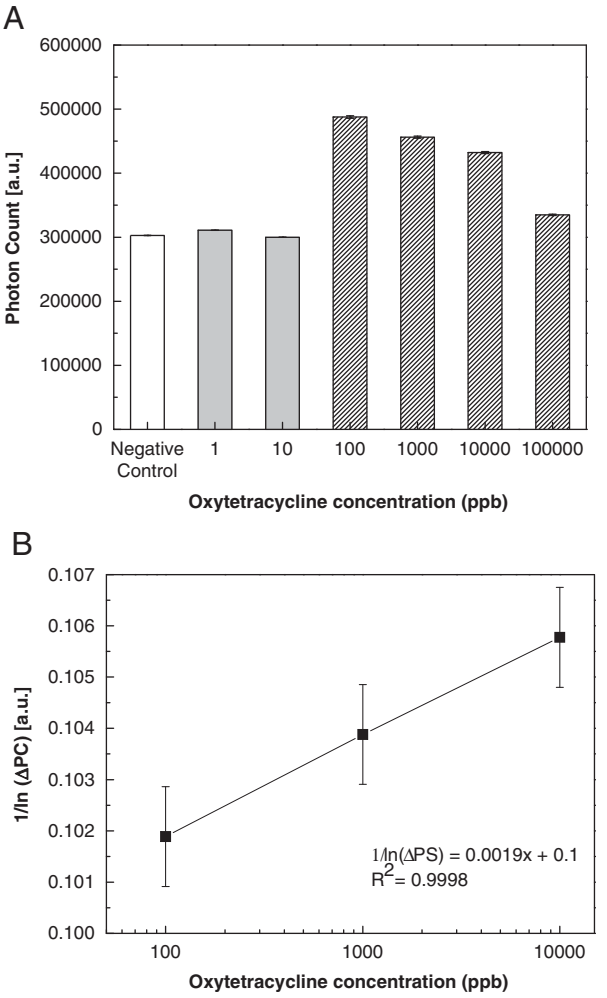

Figure 7. (A) Light scattering intensities of aptamer agglutinated OTC solutions in PBS at a series of dilutions (1–10^5 ppb) using aptasensor. Gray and diagonal line bars indicate significant differences ($p < 0.05$) as compared to negative control. (B) Linear relation between the light scattering intensity (x) and the OTC concentration within the dynamic range from 10^2 to 10^4 ppb.

established with the dynamic range between 100 and 10^4 ppb ($R^2 = 0.9998$), which yields $1/\ln(\Delta PC) = 0.0019x + 0.1$, where ΔPC is the difference between the signal intensity and the baseline measured in Fig. 7B and x is the concentration of OTC. The detection limit was approximately 100 ppb, which was determined by performing *t*-tests between the negative control and each dilution sample solution. It is clear from this result that the microfluidic aptasensor presented here is highly specific for detecting the OTC antibiotic and it is possible to distinguish the responses obtained for OTC from those with other antibiotics (negative control). Each standard deviation appeared to vary proportionally with the OTC concentration, *i.e.* the higher the OTC concentration, the higher the standard deviation.

A few comments follow as to why the intensity decreases with the increase in the concentration after a sudden increase around 100 ppb. The growth of aptamer–antibiotic

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aggregates may be considered to take place in two stages. The first is the primary combination, in which specific determinants of the antibiotic molecules are combined with receptors of the aptamer by specific bonds. In the second stage, the aggregates formed in the first stage further grow progressively to form larger aggregates by the same specific bonds between aptamer receptors and antibiotic molecules and possibly by forming a loop among the aggregates. For higher concentrations (excess of antibiotic molecules), however, the aptamer receptors would be rapidly passivated with the binding molecules and thus there would be a reduced supply of bridging aptamer molecules with respect to the amount of antibiotic in solution. Ultimately, this leads to inhibition of aptamer–antibiotic binding. It is noted that a similar behavior was observed with an electrochemical detection system for OTC using aptamer-immobilized gold electrode chip [26].

Another possibility is related to the increased interactions of the aggregated particles with incident light. It is well known that light can interact with the particle over its cross-sectional area according to the Mie scattering. The polydispersity of the aggregated particles would increase with increasing the concentration, which in turn decreases scattering of the incident light from the viewing reservoir.

4 Concluding remarks

We have demonstrated real-time detection of the OTC antibiotic through agglutination assay of ssDNA aptamer-conjugated PS latex microspheres inside a microfluidic device with proximity optical fibers. The microfluidic device was carefully designed with four air trap channels to avoid undesirable light scattering from the sample reservoir. Also, a simple fixing tray was devised to maintain the stable measurement angle and distance to the detector. The method is essentially one-step and requires no sample pretreatment such as fluorescent labeling or cell culture.

Our experimental data demonstrated that the detection limit was as low as 100 ppb, which appears to be the lowest level among previous detection results of the OTC antibiotic performed in a microfluidic device. The dynamic range was 100–10^4 ppb ($R^2 = 0.9998$), with a linear relation of $1/\ln(\Delta PC) = 0.0019x + 0.1$. Due to reduced light scattering at higher concentrations, the intensity converged to the baseline level for contraptions higher than 10^5 ppb. The combination of a microfluidic device with unique aptasensor agglutination could serve as a new platform for detecting antibiotics in a simple, rapid, and high-sensitivity manner. A further study would be required to improve S/N as well as high linearity in the standard curve over a wider dynamic range.

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The authors have declared no conflict of interest.

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