J Korean Soc Appl Biol Chem (2013) 56, 165–171
DOI 10.1007/s13765-013-3019-7

ORIGINAL ARTICLE

Potential of Fluorophore Labeled Aptamers for *Pseudomonas aeruginosa* Detection in Drinking Water

Lan Hee Kim · Hye-Weon Yu · Yang-Hoon Kim · In S. Kim · Am Jang

Received: 24 January 2013 / Accepted: 16 February 2013 / Published Online: 30 April 2013

Abstract *Pseudomonas aeruginosa* has been considered as a representative pathogenic bacteria in drinking water. In order to detect *P. aeruginosa*, aptamers were utilized in this study. In particular, fluorescein isothiocyannate (FITC) and quantum dot (QD) were used for aptamer labeling. FITC-labeled aptamers showed higher binding capacity with optimal incubation time of 30 min compared to QD-labeled aptamers. However, incubation speed did not have any effect on the binding capacity of FITC-labeled aptamers to bacteria. Aptamer-binding capacity was measured according to varying cell concentrations of 0, 10, 100, and 1000 cells/mL. As a result, the limit of detection, limit of quantification, and limit of linearity of *P. aeruginosa* were 5.07, 5.64, and 100 cells/mL, respectively. The low detection limit shows the fluorophore-labeled aptamer potential to detect *P. aeruginosa* labeling in the field.

Keywords aptamer · biosensor · fluorescein isothiocyannate · *Pseudomonas aeruginosa* · quantum dot

L. H. Kim · I. S. Kim
School of Environmental Science and Engineering, Gwangju Institute of Science and Technology (GIST), 261 Oryong-dong, Buk-gu, Gwangju, 500-712, Republic of Korea

H.-W. Yu
Department of Soil, Water and Environmental Science, College of Agriculture and Life Sciences, University of Arizona, Tucson, Arizona, United States

Y.-H. Kim
Departmet of Microbiology, Chungbuk National University, 52 Naesudong-Ro, Heungduk-Gu, Cheongju 361-763, Republic of Korea

A. Jang (✉)
Department of Civil and Environmental Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea
E-mail: amjang@skku.edu

Introduction

With the lack of drinking water, the risk of water-related diseases has become a significant problem in the world. According to the World Water Council (2006), billions of people worldwide do not have access to safe water. In addition, everyday, an estimated number of 3,900 children under the age of five die from water-related diseases (e.g. diarrhea). For these reasons, nations have imposed strict regulations on pathogenic bacteria in drinking water. Among pathogenic bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococci* spp., *Cryptosporidium* spp., and *Legionella* spp. were reported in the drinking water standards of China, Europe, and the United States as the most prevalent pathogens found in contaminated drinking water (Zhou et al., 2011). In particular, *P. aeruginosa* is the most detrimental pathogenic bacterium that causes enteric infections to humans (Pang et al., 2007). Because some bacteria, such as *P. aeruginosa,* are difficult to control due to their widespread habitats (Carnazza et al., 2008), early detection is required to ensure the safety of water.

P. aeruginosa can be detected by the conventional culture-dependent method; however, this method is labor-intensive and time-consuming (3-7 days) (Paoli, 2006). On the other hand, biosensors enable quick detection of analytes and even allow on-site field monitoring (Rodriguez-Mozaz et al., 2006).

The general type of biosensors for *P. aeruginosa* detection includes a unique combination of receptors for molecular recognition (DNA or antibody) which can be detected by electrochemical or optical methods (Kim et al., 2004; Cai et al., 2011) (Table 1). However, there are some limitations for DNA and antibody biosensors. Although the detection limit of DNA biosensors is low (1.5×10^2 CFU/mL), the PCR process takes a long time (5 h) to complete (Cai et al., 2011). In addition, antibody biosensors have selectivity limitations in the differentiation of *P. aeruginosa* and *Xanthomonas* spp. (Kim et al., 2004).

Table 1 Biosensors for *P. aeruginosa* detection

Detection techniques		Recognition element	Detection limit	Label	Detection time	Reference
Electro chemical	Piezoelectric sensor	DNA	10^{-4} g /L (DNA)	Label-free	<3 h	(He and Liu, 2004)
		Antibody	1.3×10^7 CFU/mL	Label-free	8 min	(Kim et al., 2004)
		N/A[a]	10^3 cells/mL	Label-free	Real time	(Pang et al., 2007)
		DNA	1.5×10^2 CFU/mL	Gold nanoparticle	5 h	(Cai et al., 2011)
	Electro chemical	N/A	76 CFU/mL	Label-free	N/A	(Carpani et al., 2008)
		16S rRNA	0.012 pg/L (HRP)	Label-free	2 h	(Liu et al., 2011)
	SPR sensor	chitosan-alginate	1×10^7 CFU/mL	Label-free	7 min	(Park et al., 2007)
Optical		Pyoverdine (siderophore)	10^2 cells/mL	3,3'-Dioctadecyloxacarbocyanine perchlorate	N/A	(Doorneweerd et al., 2010)
		DNA	5.07 cells/mL	FITC	30 min	This study

[a]N/A: not available

Park et al. (2007) applied self-assembled chitosan-alginate multilayers to detect *P. aeruginosa* cells in a solution. However, its detection limit (1×10^7 CFU/mL) was higher than that of other research (Table 1). Therefore, although some methods have been developed to detect pathogenic bacteria, their accuracy, speed, and sensitivity are still the problems to solve.

Alternatively, whole cell-based biosensors have been studied for rapid and sensitive detection. Aptamers are highly specific single-stranded DNA or RNA oligonucleotide ligands with high affinity to a number of targets such as small molecules, proteins, nucleic acids, cells, and microorganisms through folding into specific three-dimensional structures (Reinemann et al., 2009; Tennico et al., 2010). Aptamers can recognize and bind to their target by molecular shape complementarities, stacking of aromatic rings, electrostatic or van der waals interactions, and hydrogen bonding (Reinemann et al., 2009). Their small size (<30 kDa), high affinity, and specificity for targets enable specific detection of bacteria simply and rapidly via electrochemical, optical, and mass-sensitive bioassay methods (Song et al., 2008). In particular, optical biosensors enable on-site detection of multi targets (Ligler, 2009).

Among the optical assay formats, fluorescent detection is widely employed due to the ease of labeling aptamers with fluorescent dyes. Fluorescein isothiocyannate (FITC) has been generally used in optical DNA biosensors for hybridization or immobilization (Sassolas et al., 2008). Recently, instead of using organic fluorescent dyes, quantum dot (QD) has been employed to improve optical assay performance for their advantages such as high photostability, quantum yield, and broad absorption with narrow emission spectra. In addition, QD offers brighter and prolonged fluorescent lifetimes (Song et al., 2008; Tennico et al., 2010).

In the present study, QDs and FITC-labeled aptamers were compared on sensitivity and rapid detection of *P. aeruginosa* in drinking water. A suitable labeling method was established and aptamer binding capacity was investigated in *P. aeruginosa* whole cell. This is a first study reporting the characterization of aptamer binding on *P. aeruginosa* whole cell.

Materials and Methods

***P. aeruginosa* growth property.** *P. aeruginosa* (ATCC10145) was purchased from Korean Collection for Type Culture (Korea). To observe the growth property of *P. aeruginosa*, 200 μL of cultured cells ($O.D._{600}$=1.6) were inoculated in 500 mL bottles containing 100 mL nutrient broth (BD, USA) and were incubated at 35±2°C in a rotary shaker (150 rpm). Two hundred microliters of cultured cells were collected at selected time points (from 0 to 21 h) from each of the three bottles and then measured for optical density and cell number. Optical density was determined by a UV spectrophotometer (Shimadzu Corp., Japan). Total cell number and cell size distribution were observed by confocal laser scanning microscopy (Zeiss, Germany) equipped with a PC with the LSM software (PASCAL) for control of all system components after staining with diamidino-2-phenylindole (DAPI; 0.1 mg/mL).

Fluorophore-labeled aptamer bioprobes. The following three types of fluorophore-labeled bioprobes were designed: biotinylated aptamer at their 5' ends directly conjugated with QD (Fig. 1A), spacer 9 conjugated with aptamer bound to QD (Fig. 1B), and FITC-labeled aptamer (Fig. 1C).

An aptamer that was reported to combine specifically with *P. aeruginosa* oligonucleotide (5'-CCC CCG TTG CTT TCG CTT TTC CTT TCG CTT TTG TTC GTT TCG TCC CTG CTT CCT TTC TTG-3') (Wang et al., 2008) was employed. Biotinylated and FITC-labeled aptamers were purchased from Xenotech (Korea).

QD_{655} (Q10121MP) conjugated with streptavidin functional groups were acquired from Invitrogen Inc. (USA). QD-streptavidin conjugate is composed of nanometer-scale crystals of semiconductor material (CdSe), coated with an additional semiconductor shell (ZnS) and further coated with a polymer shell that allows the materials to be conjugated to biological molecules and to retain their optical properties. The excitation and emission wavelengths of QDs were 488 and 655 nm, respectively. FITC had a molecular weight of 332.31 and released light at 521 nm wavelength (λ_{ex}, 494 nm).

Conjugation of QD-aptamer. To confirm the conjugation of QDs

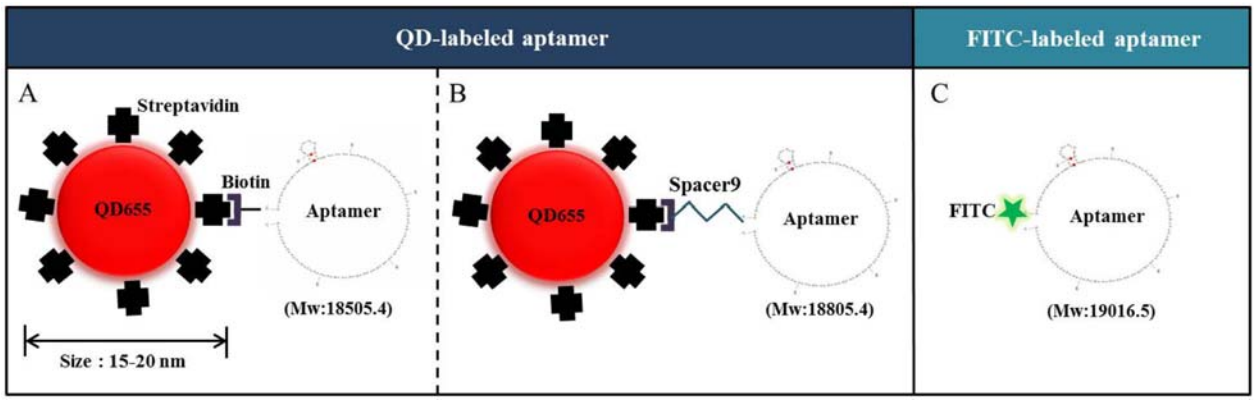

Fig. 1 Schematic diagram of the fluorophore-labeled aptamers with QD and FITC (A) conjugation of QD and aptamer without spacer and (B) with spacer (C) FITC-labeled aptamer.

to biotinylated-aptamers, FITC-labeled aptamer-complementary ssDNA was purchased from Xenotech. In an eppendorf tube, two sequences were reacted by heating at 95°C for 5 min and then cooling down to 20°C for 10 min. The unbound DNA strands were removed by filtration (50 K MWCO filter) and washed out three times with 1×PBS buffer. QD-aptamer binding was confirmed by measuring fluorescence intensity (λ_{ex}, 488 nm). In addition, QD conjugation with aptamers was verified by gel electrophoresis. QDs and QD-labeled aptamers were loaded on a 1.5% agarose gel. The gel was illuminated with an ultraviolet transilluminator (Vilber Lourmat, France).

Selection of probes. To compare the labeling efficiency between QD and FITC, probe injection volumes were calculated (Equation 1). The ratio of bacteria to aptamer numbers was $1:10^8$ with a cell number of 10^6 cells and 100 pmole of FITC-labeled aptamers, 0.187 μM of QD-labeled aptamers with a spacer, and 0.212 μM without a spacer aptamers. Thus, according to equation 1, 1.66 μL of FITC-labeled aptamer was injected and aptamers with a spacer was 888 μL and aptamers without a spacer was 784 μL.

$$N_{aptamer}=\frac{100\ pmole}{\mu L}\times\frac{6.02\times10^{23}}{mole}\times\frac{mole}{10^{12}pmole}\times(X)\mu L$$
$$N_{QD}=\frac{1\ pmole}{L}\times\frac{6.02\times10^{23}}{mole}\times\frac{mole}{10^{6}pmole}\times(Y)\mu L \quad (1)$$

Reaction between bacteria and probes. Aptamers were treated thermally at 95°C for 5 min to denature the pre-existing 3-D structures that may interfere with target binding, followed by cooling for 10 min using the method of Tennico et al. (2010). Prior to use, bacteria were inactivated based on the method of Wang et al. (2011). To conjugate aptamers and bacteria, 10^6 inactivated *P. aeruginosa* cells were incubated with FITC-labeled aptamer in 2× binding buffer (1 M NaCl, 40 mM Tris-HCl, 2 mM $MgCl_2$ in 1 L distilled water, pH 7.5–7.6) for 3 h and centrifuged (4,000 g, 10 min) with a 0.2 μm Nanosep MF membrane filter (Pall Life Sciences, USA).

Aptamers that were not bound to bacteria were thrown out, and the bacterium-aptamer complex on a membrane with 2× binding buffer was washed three times. Bound aptamer-bacteria were suspended with 2× binding buffer, and its fluorescence intensity was measured by a fluorescence spectrophotometer (Hitachi, Japan).

Optimization of aptamer binding conditions. To evaluate the effect of incubation time on aptamer binding, incubation was carried out from 20 min to 4 h. Incubation temperature and speed were 37°C and 90 rpm, respectively. Cell concentration was 10^6 cells/mL. Subsequently the effects of incubation speed were investigated at 30, 90 and 150 rpm. Other conditions were maintained throughout the incubation period of 3 h, at 37°C, and cell concentration was 10^6 cells/mL.

Dose-response curve. To obtain the value of limit of detection (LOD), limit of quantification (LOQ), and limit of linearity (LOL), a dose-response curve was drawn. Bacteria were inoculated at 10, 10^2, 10^4 cells, and 1.66 μL of FITC-labeled aptamers were added ($N_{bac}:N_{apt}=1:10^8$). The reaction was performed for 3 h at 37°C at an incubation speed of 90 rpm. Fluorescence intensity was measured using a fluorescence spectrophotometer (Hitachi).

Results and Discussion

***P. aeruginosa* growth properties.** Different growth stages of bacteria may result in different phases of infectious diseases (Cao et al., 2009). In addition, due to the target-conformation specific property of aptamers, it is important to use the target in its most stable conformation (Torres-Chavolla and Alocilja, 2009). Fig. 2 shows the growth curve of *P. aeruginosa* and cell size distribution at different growth stages including lag, exponential, stationary, and death phases. In the exponential phase, bacteria morphology varies, because many cells are continuously dividing (Akerlund et al., 1995). Lag and exponential phases of cells showed variable cell size distribution; however, in the stationary phase, 1–2 μm of cell size were usually observed. The maximum concentrations of

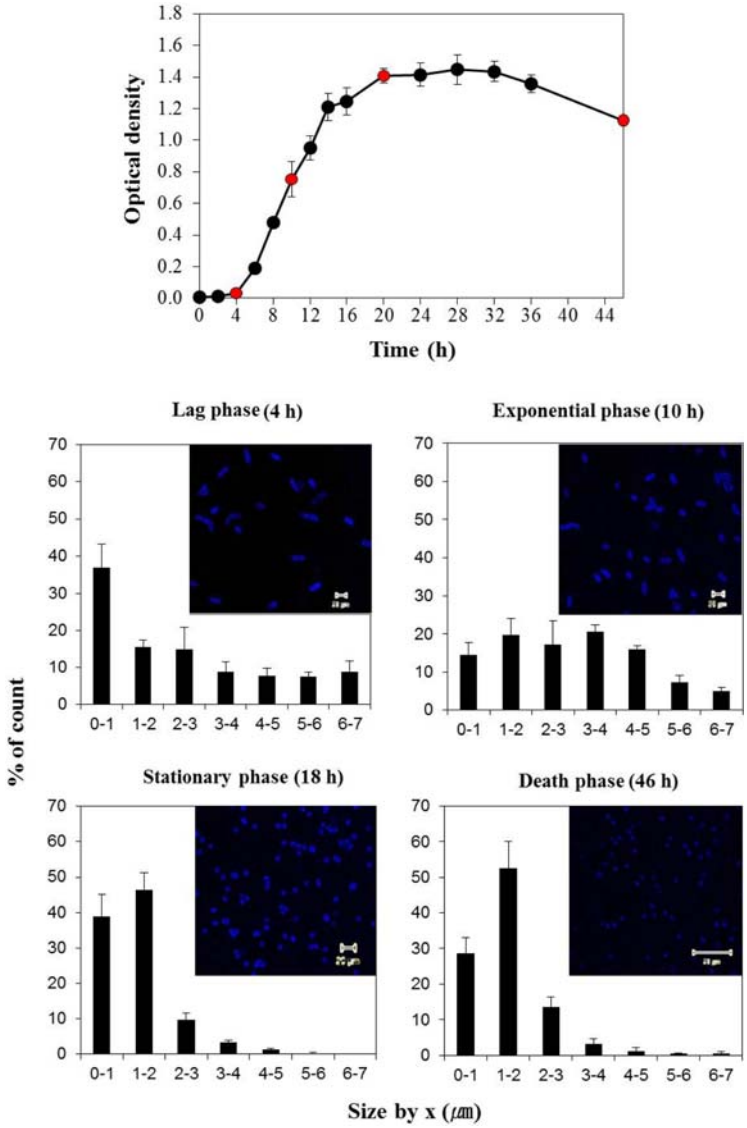

Fig. 2 (A) *P. aeruginosa* cell growth curve (B) cell size distribution according to different cell growth stages, lag, exponential, stationary, and death. Microscopic images of *P. aeruginosa* stained with DAPI were observed.

quorum sensing molecules in stationary phase can cause biofilm formation and virulence of bacteria (Navarro Liorens et al., 2010). Therefore, cell culture in the stationary phase was used in all experiments.

Selection of probes. To confirm the conjugation of QD-aptamer, fluorescence property was evaluated. The result of fluorescence intensity was measured at 488 nm excitation wavelength. The emission wavelengths of FITC and QD were 520 and 650 nm, respectively. This result shows that conjugation of QDs and aptamers were successful (Fig. 3A). The velocity of migration is determined by charge-to-mass ratio. An increase of charge-to-mass ratio by conjugation of aptamers caused faster migration velocity compared to QDs (Fig. 3B).

At each ratio of bacteria number to aptamer number, FITC-labeled and QD-aptamers were injected in different volumes. From the results, only FITC-labeled aptamers showed fluorescence intensity (129.1±24.3 a.u.). QDs are widely used in place of traditional fluorescent dyes due to their advantages including photostability and unique size-dependent fluorescent properties. However, sensitivity of QDs can be decreased when high concentrations of proteins are presented and conjugated with magnetic beads for the capture and detection of bacteria (Zhao et al., 2009; Gilmartin and O'Kennedy, 2012). In addition, physical deformation of QD can occur when it is near the surface of bacteria (Dwarakanath et al., 2004) and their large size could affect the folding structure of aptamer, which would be the reason leading preferentially to the low binding affinity of QD-aptamer probe.

Optimum binding time and incubation speed. The effects of reaction time and incubation speed for FITC-labeled aptamers and bacteria were investigated. The optimum reaction time was 30 min and showed high fluorescence intensity of 99.6±1.1 a.u..

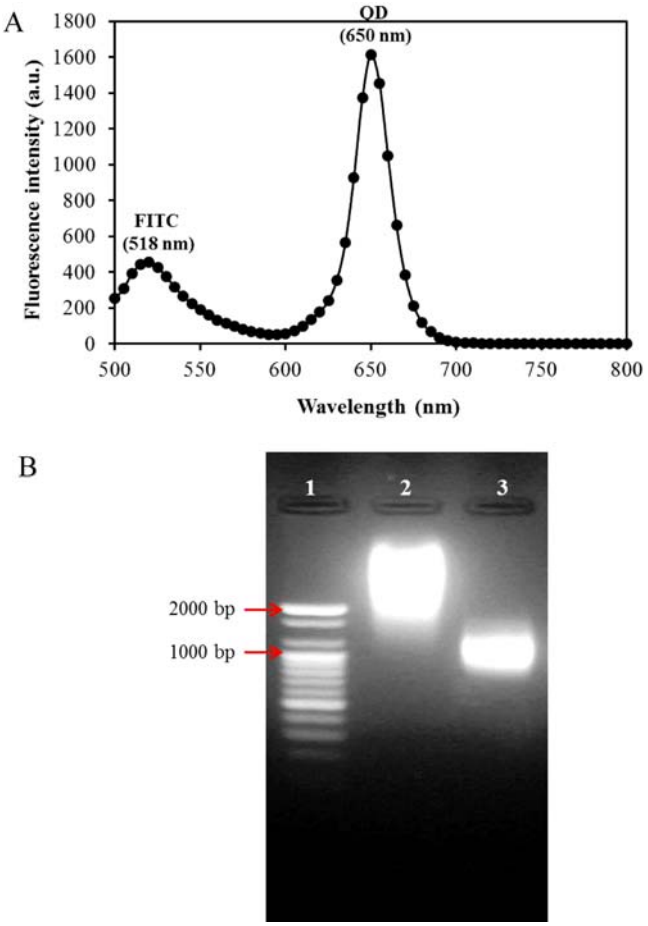

Fig. 3 (A) Confirmation of QD and aptamer conjugation. FITC (λ_{em}, 518 nm) and QD (λ_{em}, 650 nm) fluorescence were detected (B) Gel electrophoresis of QD and QD-aptamer complex (lane 1, Marker; lane 2, QD; lane 3, QD-labeled aptamer)

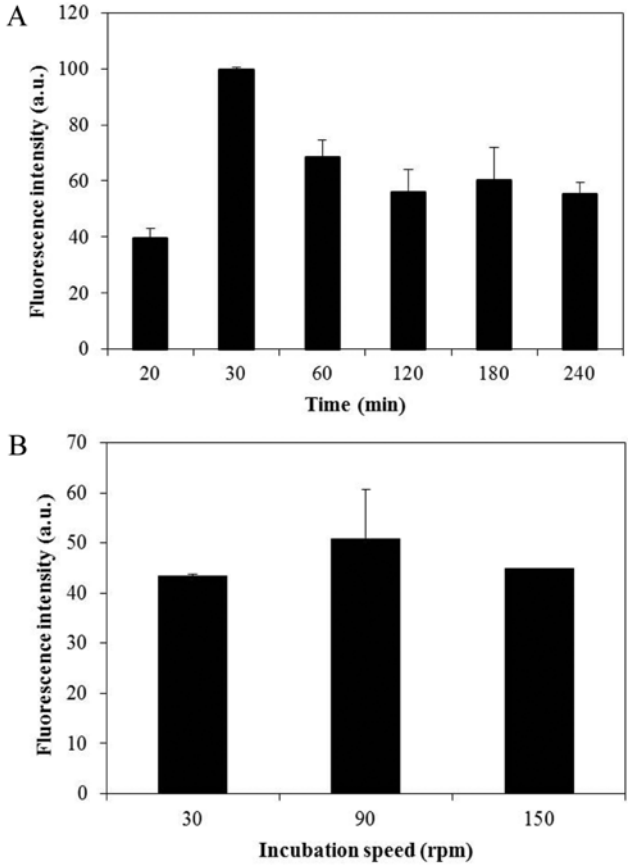

Fig. 4 Fluorescence intensity at different (A) incubation time and (B) speed. Excitation wavelength was 488 nm and emission wavelength was 518 nm.

Reaction time longer than 30 min showed lower fluorescence intensity (Fig. 4A). The developed detection biosensor was able to detect *P. aeruginosa* within minutes to hours (Table 1). However, optical biosensors that use a peptide nucleic acid probe takes 6 to 8 hours to detect *P. aeruginosa* (Esiobu et al., 2004). Thus, incubation speed did not have a significant effect on binding efficiency (Fig. 4B).

Dose response. To investigate the binding capacity of FITC-labeled aptamers in bacteria, fluorescence intensities were measured according to cell concentrations (0, 10, 100, 1000 cells/mL) (Fig. 5). LOD, LOQ, and LOL were 5.07, 5.64, and 100 cells/mL, respectively. The linear dynamic range was obtained from 5.64 to 100 cells/mL (R^2=0.9443).

P. aeruginosa is an opportunistic pathogenic bacterium with an ability to cause serious diseases (Rusin et al., 1997). Aptamers have been applied in various fields, from diagnosis to therapeutics to basic sciences, and have been applied in the detection of specific proteins, whole cells, and tissues (Joshi et al., 2009). Even though detection of bacterial cells using aptamers has many limitations due to their cell-wall structure, aptamers can bind to whole bacterial cells including *E. coli* (Akerlund et al., 1995; Zhao et al., 2009) and *Campylobacter jejuni*. However, only few studies on *P. aeruginosa*-specific aptamer have been reported (Wang et al., 2011). At the initial stage of the work in the development of aptasensor, we investigated the binding capacity of FITC-labeled aptamers with *P. aeruginosa* whole cell, and showed that these aptamers were able to rapidly (30 min) and sensitively detect *P. aeruginosa* cells (LOD, 5.07 cells/mL). *P. aeruginosa* in drinking water is often found at 3–4 cells/mL concentration (Mena and Gerba, 2009), thereby showing the potential of FITC-labeled aptamer for *P. aeruginosa* detection in drinking water.

Acknowledgments This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (2011-0006866) and in part by a grant (07SeaHeroA01-01) from Plant Technology Advancement Program funded by Ministry of Land, Transport and Maritime Affairs of the Korea Government.

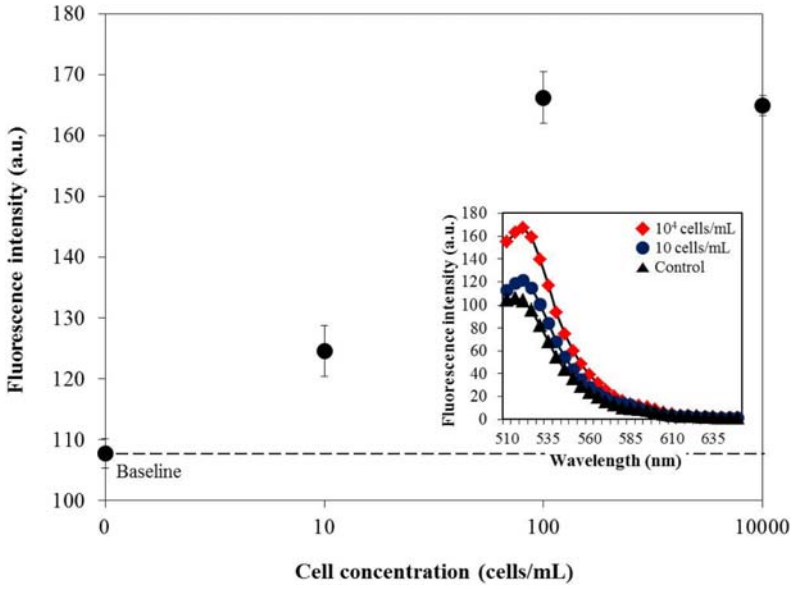

Fig. 5 Dose-response standard curve according to cell concentrations (0, 10, 100, and 1000 cells/mL). Inset image presents qualitative analysis of FITC-labeled aptamer and bacteria (fluorescence peaks of FITC were observed at 518 nm)

References

Akerlund T, Nordström K, and Bernander R (1995) Analysis of cell size and DNA content in exponentially growing and stationary-phase batch cultures of *Escherichia coli*. *J Bacteriol* **177**, 6791–7.

Cai J, Yao C, Xia J, Wang J, Chen M, Huang J et al. (2011) Rapid parallelized and quantitative analysis of five pathogenic bacteria by ITS hybridization using QCM biosensor. *Sensor Actuat B-Chem* **155**, 500–4.

Cao X, Li S, Chen L, Ding H, Xu H, Huang Y et al. (2009) Combining use of a panel of ssDNA aptamers in the detection of *Staphylococcus aureus*. *Nucleic Acids Res* **37**, 4621–8.

Carnazza S, Foti C, Gioffrè G, Felici F, and Guglielmino S (2008) Specific and selective probes for *Pseudomonas aeruginosa* from phage-displayed random peptide libraries. *Biosens Bioelectron* **23**, 1137–44.

Carpani I, Conti P, Lanteri S, Legnani PP, Leoni E, and Tonelli D (2008) Direct quantification of test bacteria in synthetic water-polluted samples by square wave voltammetry and chemometric methods. *Biosens Bioelectron* **23**, 959–64.

Doorneweerd DD, Henne WA, Reifenberger RG, and Low PS (2010) Selective capture and identification of pathogenic bacteria using an immobilized siderophore. *Langmuir* **26**, 15424–9.

Dwarakanath S, Bruno JG, Shastry A, Phillips T, John A, Kumar A et al. (2004) Quantum dot-antibody and aptamer conjugates shift fluorescence upon binding bacteria. *Biochem Bioph Res Co* **325**, 739–43.

Esiobu N, Mohammed R, Echeverry A, Green M, Bonilla T, Hartz A et al. (2004) The application of peptide nucleic acid probes for rapid detection and enumeration of eubacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa* in recreational beaches of *S. Florida*. *J Microbiol Meth* **57**, 157–62.

Gilmartin N and O'Kennedy R (2012) Nanobiotechnologies for the detection and reduction of pathogens. *Enzyme Microb Tech* **50**, 87–95.

He F and Liu S (2004) Detection of *P. aeruginosa* using nano-structured electrode-separated piezoelectric DNA biosensor. *Talanta* **62**, 271–7.

Joshi R, Janagama H, Dwivedi HP, Senthil Kumar TMA, Jaykus LA, Schefers J et al. (2009). Selection, characterization, and application of DNA aptamers for the capture and detection of *Salmonella enterica* serovars. *Mol Cell Probe* **23**, 20–8.

Kim N, Park I, and Kim D (2004) Characteristics of a label-free piezoelectric immunosensor detecting *Pseudomonas aeruginosa*. *Sensor Actuat B-Chem* **100**, 432–8.

Ligler FS (2009) Perspective on optical biosensors and integrated sensor systems. *Anal Chem* **81**, 519–26.

Liu C, Zeng GM, Tang L, Zhang Y, Li YP, Liu YY et al. (2011) Electrochemical detection of *Pseudomonas aeruginosa* 16S rRNA using a biosensor based on immobilized stem-loop structured probe. *Enzyme Microb Tech* **49**, 266–71.

Mena KD and Gerba CP (2009) Risk assessment of *Pseudomonas aeruginosa* in water. *Rev Environ Contam Toxicol* **201**, 71–115.

Navarro Liorens JM, Tormo A, and Martinez-Garcia E (2010) Stationary phase in gram-negative bacteria. *FEMS Microbiol Rev* **34**, 476–95.

Pang P, Huang S, Cai Q, Yao S, Zeng K, and Grimes CA (2007) Detection of *Pseudomonas aeruginosa* using a wireless magnetoelastic sensing device. *Biosens Bioelectron* **23**, 295–9.

Paoli GC (2006) Molecular approaches to immunological biosensors: phage displayed antibodies for the detection of foodborne pathogenic bacteria. In *Advances in Microbial Food Safety* (Vol. 931), pp. 41–54, ACS publications, Washington, DC, USA.

Park JS, Lee CM, and Lee KY (2007) A surface plasmon resonance biosensor for detecting *Pseudomonas aeruginosa* cells with self-assembled chitosan-alginate multilayers. *Talanta* **72**, 859–62.

Reinemann C, Stoltenburg R, and Strehlitz B (2009) Investigations on the specificity of DNA aptamers binding to ethanolamine. *Anal Chem* **81**, 3973–8.

Rodriguez-Mozaz S, Lopez de Alda MJ, and Barceló D (2006) Biosensors as useful tools for environmental analysis and monitoring. *Anal Bioanal Chem* **386**, 1025–41.

Rusin PA, Rose JB, Haas CN, and Gerba CP (1997) Risk assessment of opportunistic bacterial pathogens in drinking water. *Rev Environ Contam Toxicol* **152**, 57–83.

Sassolas A, Leca-Bouvier BD, and Blum LJ (2008) DNA biosensors and microarrays. *Chem Rev* **108**, 109–39.

Song S, Wang L, Li J, Fan C, and Zhao J (2008) Aptamer-based biosensors. *TrAC-Trend Anal Chem* **27**, 108–17.

Tennico YH, Hutanu D, Koesdjojo MT, Bartel CM, and Remcho VT (2010) On-chip aptamer-based sandwich assay for thrombin detection employing magnetic beads and quantum dots. *Anal Chem* **82**, 5591–7.

Torres-Chavolla E, and Alocilja EC (2009) Aptasensors for detection of microbial and viral pathogens. *Biosens Bioelectron* **24**, 3175–82.

Wang KY, Zeng YL, Yang XY, Li WB, and Lan XP (2011) Utility of aptamer-fluorescence in situ hybridization for rapid detection of *Pseudomonas aeruginosa*. *Eur J Clin Microbiol Infect Dis* **30**, 273–8.

Wang Y, Hammes F, Düggelin M, and Egli T (2008) Influence of size, shape,

and flexibility on bacterial passage through micropore membrane filters. *Environ Sci Technol* **42**, 6749–54.

Zhao Y, Ye M, Chao Q, Jia N, Ge Y, and Shen H (2009) Simultaneous detection of multifood-borne pathogenic bacteria based on functionalized quantum dots coupled with immunomagnetic separation in food samples. *J Agr Food Chem* **57**, 517–24.

Zhou G, Wen S, Liu Y, Li R, Zhong X, Feng L et al. (2011) Development of a DNA microarray for detection and identification of *Legionella pneumophila* and ten other pathogens in drinking water. *Int J Food Microbiol* **145**, 293–300.