J. Microbiol. Biotechnol. (2006), 16(11), 1713–1719

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Rapid and Accurate Detection of *Bacillus anthracis* Spores Using Peptide-Quantum Dot Conjugates

PARK, TAE JUNG[1], JONG PIL PARK[1], GWI-MOON SEO[3], YOUNG GYU CHAI[3], AND SANG YUP LEE[1,2]*

[1]*Metabolic and Biomolecular Engineering National Research Laboratory, (BK21 Program), BioProcess Engineering Research Center, and Center for Ultramicrochemical Process Systems, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea*
[2]*Department of BioSystems and Bioinformatics Research Center, Center for Systems and Synthetic Biotechnology, and Institute for the BioCentury, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea*
[3]*Division of Molecular and Life Sciences, Hanyang University, Ansan 426-791, Korea*

Received: April 9, 2006
Accepted: July 12, 2006

Abstract A method for the simple, rapid, specific, and accurate detection of *Bacillus anthracis* spores was developed by employing specific capture peptides conjugated with fluorescent quantum dots (QDs). It was possible to distinguish *B. anthracis* spores from the spores of *B. thuringiensis* and *B. cereus* using these peptide-QD conjugates by flow cytometric and confocal laser scanning microscopic analyses. For more convenient high-throughput detection of *B. anthracis* spores, spectrofluorometric analysis of spore-peptide-QD conjugates was performed. *B. anthracis* spores could be detected in less than 1 h using this method. In order to avoid any minor yet false-positive signal caused by the presence of *B. thuringiensis* spores, the B-Negative peptide, which can only bind to *B. thuringiensis*, conjugated with another type of QD that fluoresces at different wavelength was also developed. In the presence of mixed *B. anthracis* and *B. thuringiensis* spores, the BABA peptide conjugated with QD525 and the B-Negative peptide conjugated with QD585 were able to bind to the former and the latter, specifically and respectively, thus allowing the clear detection of *B. anthracis* spores against *B. thuringiensis* spores by using two QD-labeling systems. This capture peptide-conjugated QD system should be useful for the detection of *B. anthracis* spores.

Key words: *Bacillus anthracis*, quantum dot, capture peptide, peptide-conjugated nanoparticle, anthrax detection

*Corresponding author
Phone: 82-42-869-3930; Fax: 82-42-869-8800;
E-mail: leesy@kaist.ac.kr

Bacillus anthracis is a Gram-positive, aerobic, and spore-forming bacterium that causes anthrax, a lethal disease of human and animals [5, 14, 19, 22, 23]. It is one of the biggest threats to many countries because of its potential use in bioterrorism, which had actually occurred in the United States of America in the fall of 2001 [3, 16, 22]. Once exposed to internal tissues, the spores germinate for vegetative cell growth and produce toxins, often resulting in the death of the host within several days. *B. anthracis* spores are highly resistant to normally destructive environmental factors to living cells, such as heat, toxic chemicals, desiccation, and physical damages. These characteristics make them suitable for a potential biological warfare. Therefore, the rapid and accurate detection of *B. anthracis* spores in the environment prior to infection is extremely important for human safety and national security.

Various biological and chemical techniques have been developed to detect *Bacillus* spores. So far, complex, cumbersome, and time-consuming lab-based assays that require spore germination and outgrowth of vegetative cells have been used [2, 5]. Other methods include polymerase chain reaction (PCR) [3, 5, 7, 21] and immunoassays [1, 6, 8, 12, 19]. PCR, a primer-mediated enzymatic DNA amplification method, requires considerable effort in sample processing prior to analysis. For the analysis part itself, the PCR-based detection of the *pagA* and *lef* genes encoding the protective antigen toxin in *B. anthracis* may take 2 to 5 h or longer [1, 6, 13, 22]. Immunoassays, which rely on the interaction between antibodies and *B. anthracis* cell surface antigens, can detect 10^3 spores in 12 h [3, 10, 17, 18, 21]. Although this direct spore detection system is

relatively fast, the current antibody-based detection method suffers from the lack of accuracy and limited sensitivity, which result in unacceptably high levels of both false-positive and false-negative responses [3, 13]. Therefore, a better detection system needs to be developed.

Williams *et al.* [23] developed several peptide ligands that can bind specifically to *B. anthracis* spores. Based on this excellent development, we devised a rapid and simple method for detecting *B. anthracis* spores by using specific-binding peptides-conjugated quantum dots (QDs). QDs have ideal characteristics for dense spectral multiplexing, a narrow emission range, and a long lifetime, and have the potential to simplify the multiplexed analysis using different QDs [9, 10]. Specific binding of three capture peptides conjugated with QDs to two attenuated *B. anthracis* strains, *B. anthracis* ΔSterne ($pXO1^-$, $pXO2^-$) and *B. anthracis* Sterne 34F2 ($pXO1^+$, $pXO2^-$), was examined by using fluorescence confocal microscopy and flow cytometry. Since the two species, *B. cereus* and *B. thuringiensis*, are the most similar strains to *B. anthracis* based on their genome sequences, they were used as control strains to be compared during the detection of *B. anthracis* [15]. For more convenient and high-throughput detection of *B. anthracis* spores, a spectrofluorometric assay system was also developed.

MATERIALS AND METHODS

Materials and Strains

QD525 and QD585 streptavidin conjugates (10–15 nm in diameter, 1 mM solution) were purchased from Quantum Dot Corp. (Hayward, CA, U.S.A.). The *Bacillus* strains used in this study are listed in Table 1. The experiments with *B. anthracis* ΔSterne ($pXO1^-$, $pXO2^-$) and *B. anthracis* Sterne *34F2* ($pXO1^+$, $pXO2^-$) were carried out at Hanyang University (Prof. Y.G. Chai's laboratory) to avoid transport problems. The capture peptides were chemically synthesized and purified by high-performance liquid chromatography according to the manufacturer's procedure (Peptron, Daejeon, Korea). The N-termini of the capture peptides were modified with biotin for their conjugation to streptavidin-QDs.

Cultivation of Cells and Purification of Spores

B. anthracis strains were cultured in brain heart infusion (BHI, BD Biosciences, Sparks, MD, U.S.A.) medium at 30°C for 5 days. *B. subtilis* DB104 and *B. thuringiensis* strains were cultivated at 37°C and 30°C, respectively, and 250 rpm for 3 days in CDSM sporulation medium [11]. Spores mixed with vegetative cells were harvested from 50 ml of the culture by centrifugation (10,000 ×*g*, 10 min) and were resuspended in 0.2 ml of 20% (w/v) urografin (Sigma, St. Louis, MO, U.S.A.). This suspension was gently layered over 1 ml of 50% (w/v) urografin in a 1.5 ml microcentrifuge tube, and then centrifuged (16,000 ×*g*, 10 min) at 4°C. The collected pellets containing only free spores were stored at −20°C.

Flow Cytometric Analysis

The purified spores were washed and subsequently resuspended in phosphate-buffered saline (PBS, pH 7.5) solution. The biotin-conjugated peptides (5 mg/ml) were incubated with streptavidin-conjugated QD525 at 30°C for 1 h and washed with PBS solution three times to remove unbound peptides. Then, spores were mixed with peptide-QD525 conjugates in PBS solution and incubated at 30°C for 30 min to examine binding affinities. Unbound spores and free peptides were removed by washing with PBS solution three times. Then, spores were collected by centrifugation (10,000 ×*g*, 10 min) at 4°C. Spores with bound peptide-QD525 conjugates were resuspended in PBS solution, and fluorescence was measured by fluorescence-activated cell sorting (FACS) using FACSCalibur instrument and CellQuest Pro software (BD Bioscience, Palo Alto, CA, U.S.A.). All samples were analyzed for the relative fluorescence intensities by FL1 green and FL2 orange fluorescence detectors, having 530±15 and 585±21 nm bandpass filters, respectively.

Fluorescence Imaging

For fluorescence imaging, the samples were mounted on poly-L-lysine-treated slide glasses (Cel & Associates, Inc., Pearland, TX, U.S.A.) for immobilization of spores and examined by using an LMS 510 confocal laser scanning

Table 1. *Bacillus* strains used in this study.

Bacillus strains	Relevant characteristics	Reference or source
B. subtilis DB104	*nprE18 aprEΔ3*	[24]
B. thuringiensis 4Q7	Plasmidless mutant of *B. thuringiensis* var. *israelensis*	BGSC[a]
B. thuringiensis HD1	Wild-type isolate, ATCC 33679	ATCC[b]
B. cereus 1092	KCTC 1092	KCTC[c]
B. anthracis ΔSterne	($pXO1^-$, $pXO2^-$)	Prof. Chai[d]
B. anthracis Sterne *34F2*	($pXO1^+$, $pXO2^-$)	Prof. Chai[d]

[a]*Bacillus* Genetic Stock Center, Columbus, OH, U.S.A.
[b]American Type Culture Collection, Manassas, VA, U.S.A.
[c]Korean Collection for Type Cultures, Daejeon, Korea
[d]Prof. Y.G. Chai's laboratory, Hanyang University, Ansan, Korea

Table 2. Capture peptides used in this study.

Capture peptide	Sequence (N-terminus to C-terminus)	Reference
BA-1	Biotin-ATYPLPIRGGGC	[23]
BABA	Biotin-ATYPLATYPLC	[23]
B-New	Biotin-ATYPLATYPLPIRGGGC	This study
B-Negative	Biotin-SLLPGLPGGGC	[23]

microscope (Carl Zeiss, Jena, Germany). Samples were excited by 488 nm argon and 543 nm HeNe laser, respectively, and the images were filtered with longpass 505 and 575 nm filters. All images were generated from 4–5 serial images made by automatic optical sectioning.

Fluorescence Assays

Fluorescence assays were carried out by using Spectrofluorometer (Model VICTOR^2, PerkinElmer, Shelton, CT, U.S.A.) and Wallac 1420 Workstation software (PerkinElmer). The fluorescence data were saved in Microsoft Excel and the capture images in Wallac 1420 Manager. To detect fluorescence signals of the QD525, the fluorescence was read after excitation (485±7 nm bandpass laser filter) and emission (535±12.5 nm bandpass filter) on a VICTOR^2 plate reader. For the detection of QD585 signals, 550±4 nm bandpass filter for excitation and 579±12.5 nm bandpass filter for emission were used. All peptides (50 ml of 5 mg/ml) were incubated with streptavidin-conjugated QDs (4 μl of 1 μM) at 30°C for 1 h and washed with PBS buffer three times to remove unbound peptides. For the examination of the binding affinities among the peptides and various spores, peptide-QD conjugates were incubated with *Bacillus* spores (ca. 2×10^7 CFU/ml) at 30°C for 30 min. To each well of a 384-well black flat-bottomed plate was added 25 μl of spore-peptide-QD complex solution in PBS buffer containing 1% (v/v) Tween 20. The output of the plate reader is generated under the sensitivity setting of high scale ($0-10^6$) in relative fluorescence units (RFU).

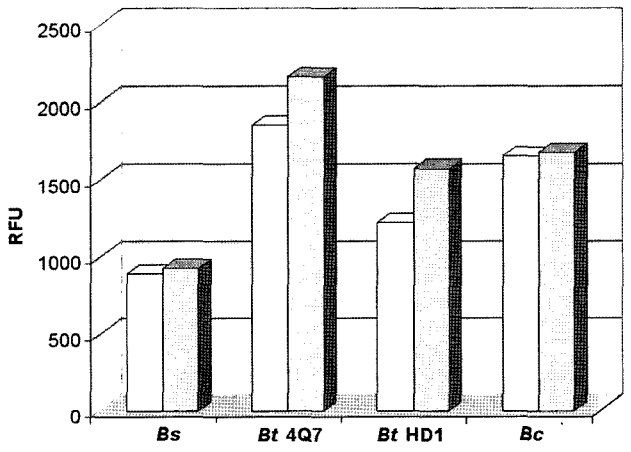

Fig. 1. Fluorescence intensity of various *Bacillus* spores after binding to the BA1 (white bar) and BABA (grey bar) peptides conjugated with QD585.
Fluorescence intensities obtained for the spores in the *B. cereus* group were higher than that for *B. subtilis* spores by 1.5–2.3 folds. Abbreviations: *Bs*, *B. subtilis*; *Bt* 4Q7, *B. thuringiensis* 4Q7; *Bt* HD1, *B. thuringiensis* HD1; *Bc*, *B. cereus*. Fluorescence is reported in RFU, relative fluorescence unit, under the sensitivity setting of high scale ($0-10^6$).

Results and Discussion

Analysis of Peptide Binding to *Bacillus* Spores

To develop a method for the rapid and simple detection of *B. anthracis* spores, three capture peptides developed by Williams *et al.* [23] and a newly designed peptide were used (Table 2). The capture peptides were modified with biotin at their N-termini for the attachment to the streptavidin-conjugated QDs.

First, we used the spores of *B. cereus* and *B. thuringiensis*, which are genetically very similar to *B. anthracis* [15], and *B. subtilis* as model strains. This was because we wanted to reduce the risk of carrying out experiments with *B. anthracis*, even though they are attenuated harmless strains. The biotin-

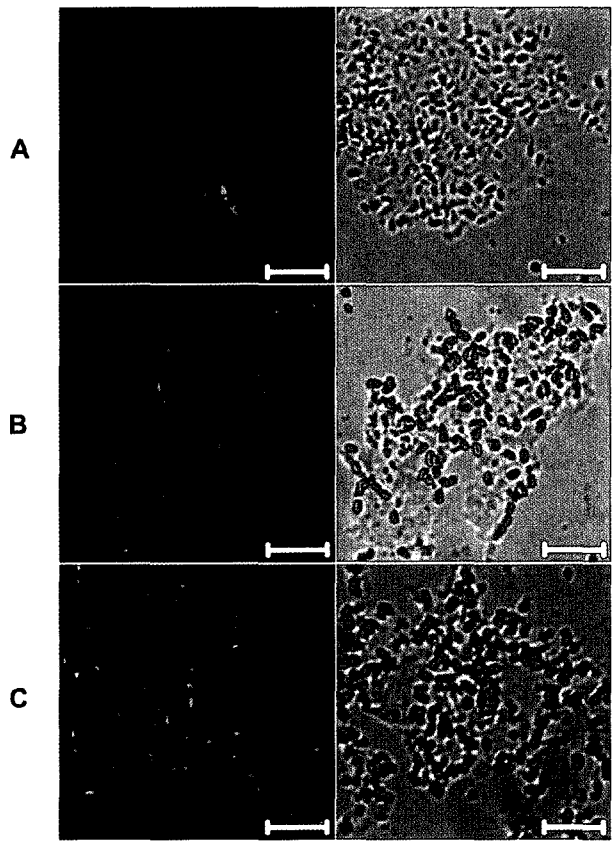

Fig. 2. Confocal (left) and optical (right) microscopic images of *B. thuringiensis* spores bound to BA1 capture peptide-conjugated QD585 after 10 min (**A**), 20 min (**B**), and 30 min (**C**) reaction.
White bars represent 10 mm.

conjugated BA1 and BABA peptides (5 mg/ml) were incubated with streptavidin-conjugated QD585 at 30°C for 1 h and washed with PBS buffer to remove unbound peptides. In order to examine the binding affinities among the peptides and various spores, BA1-QD585 and BABA-QD585 conjugates were incubated with *B. cereus*, *B. thuringiensis*, and *B. subtilis* spores (ca. 2×10^7 CFU/ml) at 30°C for 30 min. Unbound peptide-QD585 conjugates were removed by washing with PBS buffer, and spore-peptide-QD585 complexes were analyzed by spectrofluorometry. Both peptides were better able to bind to the spores of *B. cereus* and *B. thuringiensis* than to *B. subtilis* spores (Fig. 1). These results suggest that the BA1 and BABA peptides can distinguish the spores of the *B. cereus* group from *B. subtilis* spores. Considering that *B. anthracis* spores are very similar to the *B. thuringiensis* spores [4], these peptides may be used to specifically distinguish *B. anthacis* spores from other spores.

Binding of peptide-conjugated QD585 to *B. thuringiensis* spores was confirmed by confocal microscopy using 543 nm excitation and 575 nm emission filters. The biotin-conjugated BA1 peptides (50 µl of 5 mg/ml) were mixed with the streptavidin-conjugated QD585. After removing unbound QDs and free peptides by washing with PBS buffer three times, the peptide-QD585 conjugates were incubated with *B. thuringiensis* 4Q7 spores (ca. 2×10^7 CFU/ml) at 30°C up to 30 min. The spore-peptide-QD585 complexes were examined with confocal microscopy every 10 min. As can be seen from Fig. 2, the BA1 peptide-conjugated QD585 efficiently bound to *B. thuringiensis* spores within only 20–30 min.

Detection of *Bacillus anthracis* Spores among Other Strains

Based on the above results, binding of the capture peptides, BA1 and BABA, to the spores of *B. anthracis* ΔSterne ($pXO1^-$, $pXO2^-$) and Sterne ($pXO1^+$, $pXO2^-$) was examined. Additionally, a new capture peptide, B-New, was designed by fusing the BABA and BA1 peptides, possibly to enhance its binding affinity to *B. anthracis* spores. In brief, QD525 (≈4 µl) was incubated with 50 µl of three peptides (5 mg/ml) at 30°C for 1 h. Subsequently, unbound QDs and free peptides were removed by washing with PBS buffer three times. Three different peptide-QD525 conjugates were incubated with the spores of *B. cereus*, *B. thuringiensis* 4Q7, *B. anthracis* ΔSterne, and *B. anthracis* Sterne (ca. 2×10^7 CFU/ml) at 30°C for 30 min. Unbound peptide-QD525 conjugates were removed by washing with PBS buffer three times, and all spore-peptide-QD525 complexes were analyzed by FACS and confocal laser scanning microscopy. As can be seen in Fig. 3, all these capture peptides could distinguish *B. anthracis* spores from *B. thuringiensis* and *B. cereus* spores by showing higher fluorescence intensity signals. The newly designed B-New peptide did not show better binding affinity/specificity to *B. anthracis* spores

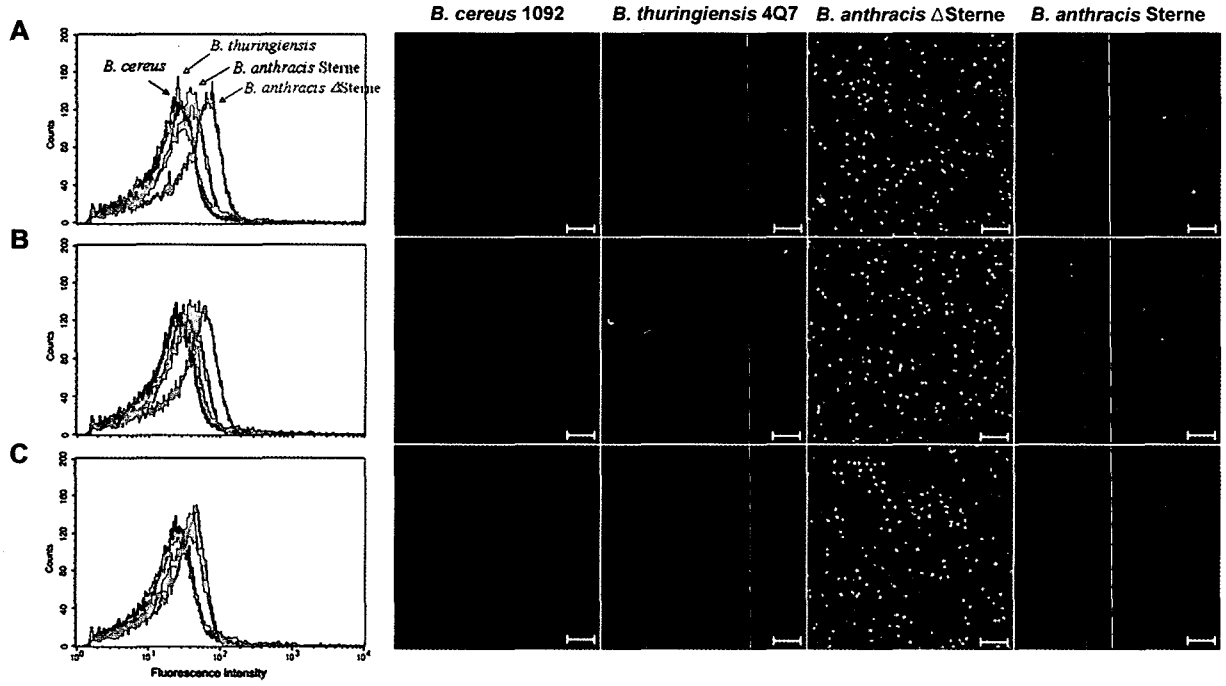

Fig. 3. Flow cytometric and confocal microscopic analyses of four types of *Bacillus* spores bound to QD-conjugated capture peptides, BABA (**A**), BA1 (**B**), and B-New (**C**).

All capture peptide-QD525 conjugates were able to bind to *B. anthracis* spores at the levels distinguishable from *B. cereus* and *B. thuringiensis*. White bars represent 10 mm.

compared with BA1 and BABA peptides. As described earlier, there was some binding of these peptides to *B. cereus* and *B. thuringiensis* spores. However, there were enough differences in fluorescence intensities for the distinguishable detection of *B. anthracis* spores.

Spectrofluorometric Detection of *B. anthracis* Spores

For more convenient high-throughput detection of *B. anthracis* spores, the fluorescence assays were carried out by spectrofluorometry. The BABA-QD525 conjugates were incubated with *B. anthracis* and *B. thuringiensis* spores (ca. 2×10^7 CFU/ml) at 30°C for 30 min with gently shaking in PBS buffer containing 1% (v/v) Tween 20. Tween 20 was included in the buffer to prevent nonspecific binding of peptide-QD conjugates to the well plate and spores. To remove unbound BABA-QD525 conjugates, these complexes were washed with PBS buffer and analyzed on a 384-well black plate by using a microplate reader (Fig. 4A). The binding capacity of BABA-QD525 conjugates to the spores was profiled by relative fluorescence intensity (Fig. 4B). As already shown in Fig. 3, we compared the capture peptides for their specificities of binding to various *Bacillus* spores. As expected, much higher fluorescence was observed in the wells containing *B. anthracis* spores-BABA peptide-QD525 complexes compared with the wells containing *B. thuringiensis* spores. This means that BABA capture peptides have a higher selectivity for *B. anthracis* spores compared with *B. thuringiensis* spores.

Since *B. thuringiensis* spores still showed weak signals to the BABA, it was necessary to develop a method for

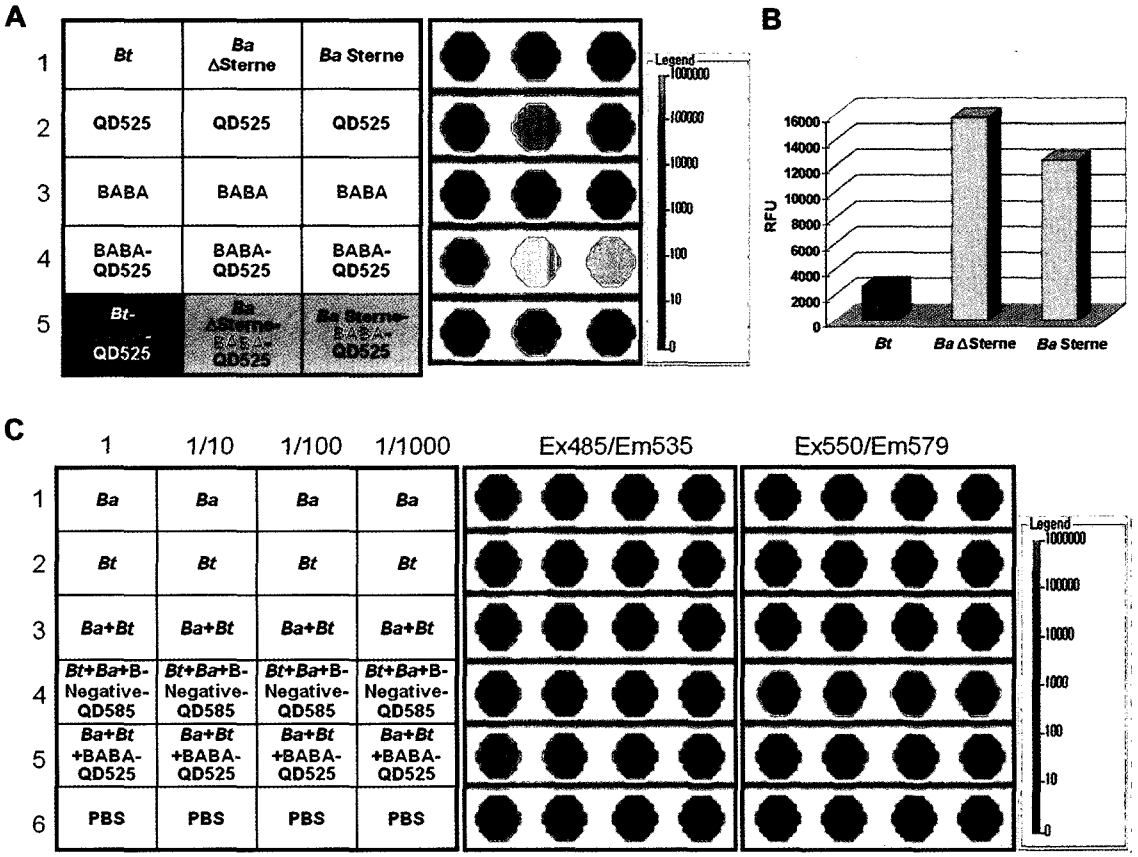

Fig. 4. High-throughput detection of *B. anthracis* (*Ba*) spores by spectrofluorometry, which can be distinguished from *B. thuringiensis* (*Bt*) 4Q7 spores.

A. Fluorescence signals obtained using BABA-QD525 complex. As the fluorescence intensity increases, the color shifts from blue to red. The rows are (from the top): 1. *Bt* 4Q7, *Ba* ΔSterne and *Ba* Sterne spores only; 2, QD525 as positive control; 3, BABA peptide only as negative control; 4, BABA-QD525 conjugates as positive control; 5, *Bt* 4Q7, *Ba* ΔSterne and *Ba* Sterne spores after binding with BABA-QD525. **B.** Fluorescence intensities after binding of BABA-QD525 complex to the spores of *Bt*, *Ba* ΔSterne, and *Ba* Sterne. Fluorescence intensities obtained with *B. anthracis* spores were 6–7 folds higher than that obtained with *B. thuringiensis* spores. Fluorescence is reported in RFU, relative fluorescence unit, under the sensitivity setting of high scale (0–10^6). **C.** Fluorescence signals obtained for *Ba* and *Bt* spores with or without binding to the BABA-QD525 and B-Negative-QD585 conjugates. The rows are (from the top): 1, *Ba* ΔSterne spores only; 2, *Bt* 4Q7 spores only; 3, *Ba* ΔSterne and *Bt* 4Q7 spores; 4, *Ba* ΔSterne and *Bt* 4Q7 spores after binding with B-Negative-QD585; 5, *Ba* ΔSterne and *Bt* 4Q7 spores after binding with BABA-QD525; 6, PBS solution as a reference. From left to right, samples were serially diluted from 1 to 1:1,000. Fluorescence intensities were read under two different conditions for QD525 and QD585 signals as indicated: Ex485, excitation (485±7 nm bandpass filter); Em535, emission (535±12.5 nm bandpass filter); Ex550, excitation (550±4 nm bandpass filter); Em579, emission (579±12.5 nm bandpass filter).

unambiguously distinguishing *B. anthracis* spores from *B. thuringiensis* spores. A negative-capture peptide, B-Negative, which has a specific binding affinity only to *B. thuringiensis* spores [23], was chemically synthesized. The BABA and B-Negative peptides were incubated with the streptavidin-conjugated QD525 and QD585, respectively, and were further incubated with the mixture of *B. anthracis* and *B. thuringiensis* spores at 30°C for 30 min. After washing three times, the complexes were analyzed by spectrofluorometry (Fig. 4C). Because BABA-QD525 and B-Negative-QD585 have different emission wavelengths, *B. anthracis* and *B. thuringiensis* spores could be detected by their original fluorescence signals. The *B. anthracis* spores-BABA peptide-QD525 complex was highly fluorescent at 525 nm, whereas the *B. thuringiensis*-B-Negative peptide-QD585 complex was highly fluorescent at 585 nm. Furthermore, this system allowed successful detection of *B. anthracis* spores (ca. 2×10^4 CFU/ml) diluted (up to 1:1,000 examined). These results suggest that *B. anthracis* spores can be clearly detected from the mixture of spores including the most similar *B. thuringiensis* spores by using two labeling systems employing QD525 and QD585. Consequently, more accurate and sensitive detection of *B. anthracis* spores is possible by using QDs compared with other traditional fluorophores [9, 11, 23].

In conclusion, the capture peptide-QD nanobead-based method developed in this study allows rapid, simple, and accurate detection of *B. anthracis* spores. Our fluorescence-based methods require relatively expensive materials and a laboratory equipped for analysis. However, our assays described here take less than 1 h to detect *B. anthracis* spores in the presence of other spores and microorganisms. From a practical point of view, it will be necessary to develop a sample preparation method for the detection of dilute *B. anthracis* spores present in the air, water, powder, or other environmental conditions. When a suitable sample preparation method is developed, it will be possible to employ this system successfully in the early detection of anthrax.

Acknowledgments

This work was supported by the Korean-AFOSR Nanoscience and Technology Initiative from the United States Air Force. Further supports by the KOSEF through the Center for Ultramicrochemical Process Systems and LG Chem Chair Professorship are appreciated.

REFERENCES

1. Bell, C. A., J. R. Uhl, T. L. Hadfield, J. C. David, R. F. Meyer, T. F. Smith, and F. R. Cockerill 3rd. 2002. Detection of *Bacillus anthracis* DNA by LightCycler PCR. *J. Clin. Microbiol.* **40:** 2897–2902.
2. Cummings, R. T., S. P. Salowe, B. R. Cunningham, J. Wiltsie, Y. W. Park, L. M. Sonatore, D. Wisniewski, C. M. Douglas, J. D. Hermes, and E. M. Scolnick. 2002. A peptide-based fluorescence resonance energy transfer assay for *Bacillus anthracis* lethal factor protease. *Proc. Natl. Acad. Sci. USA* **99:** 6603–6606.
3. Hartley, H. A. and A. J. Baeumner. 2003. Biosensor for the specific detection of a single viable *B. anthracis* spore. *Anal. Bioanal. Chem.* **376:** 319–327.
4. Helgason, E., O. A. Okstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and A. Kolsto. 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* - one species on the basis of genetic evidence. *Appl. Environ. Microbiol.* **66:** 2627–2630.
5. Higgins, J. A., M. Cooper, L. Schroeder-Tucker, S. Black, D. Miller, J. S. Karns, E. Manthey, R. Breeze, and M. L. Perdue. 2003. A field investigation of *Bacillus anthracis* contamination of U.S. Department of Agriculture and other Washington, D.C., buildings during the anthrax attack of October 2001. *Appl. Environ. Microbiol.* **69:** 593–599.
6. Jackson, P. J., M. E. Hugh-Jones, D. M. Adair, G. Green, K. K. Hill, C. R. Kuske, L. M. Grinberg, F. A. Abramova, and P. Keim. 1998. PCR analysis of tissue samples from the 1979 Sverdlovsk anthrax victims: The presence of multiple *Bacillus anthracis* strains in different victims. *Proc. Natl. Acad. Sci. USA* **95:** 1224–1229.
7. Jung, S. J., H.-J. Kim, and H.-Y. Kim. 2005. Quantitative detection of *Salmonella typhimurium* contamination in milk, using real-time PCR. *J. Microbiol. Biotechnol.* **15:** 1353–1358.
8. Kwak, B.-Y., B.-J. Kwon, C.-H. Kweon, and D.-H. Shon. 2004. Detection of *Aspergillus*, *Penicillium*, and *Fusarium* species by sandwich enzyme-linked immunosorbent assay using mixed monoclonal antibodies. *J. Microbiol. Biotechnol.* **14:** 385–389.
9. Makrides, S. C., C. Gasbarro, and J. M. Bello. 2005. Bioconjugation of quantum dot luminescent probes for Western blot analysis. *Biotechniques* **39:** 501–506.
10. McBride, M. T., D. Masquelier, B. J. Hindson, A. J. Makarewicz, S. Brown, K. Burris, T. Metz, R. G. Langlois, K. W. Tsang, R. Bryan, D. A. Anderson, K. S. Venkateswaran, F. P. Milanovich, and B. W. Colston Jr. 2003. Autonomous detection of aerosolized *Bacillus anthracis* and *Yersinia pestis*. *Anal. Chem.* **75:** 5293–5299.
11. Medintz, I. L., A. R. Clapp, H. Mattoussi, E. R. Goldman, B. Fisher, and J. M. Mauro. 2003. Self-assembled nanoscale biosensors based on quantum dot FRET donors. *Nat. Mater.* **2:** 630–638.
12. Nicholson, W. L. and P. Setlow. 1990. Sporulation, germination and outgrowth, pp. 391–429. *In* C. R. Harwood, and S. M. Cutting (eds.), *Molecular Biological Methods for Bacillus*. John Wiley & Sons Ltd., Hoboken, N.J.
13. Oggioni, M., R. F. Meacci, A. Carattoli, A. Ciervo, G. Orru, A. Cassone, and G. Pozzi. 2002. Protocol for real-time PCR identification of anthrax spores from nasal swabs after broth enrichment. *J. Clin. Microbiol.* **40:** 3956–3963.
14. Öncü, S., S. Öncü, and S. Sakarya. 2003. Anthrax - an overview. *Med. Sci. Monit.* **9:** RA276–RA283.

15. Radnedge, L., P. G. Agron, K. K. Hill, P. J. Jackson, L. O. Ticknor, P. Keim, and G. L. Andersen. 2003. Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **69:** 2755–2764.
16. Reissman, D. B., E. B. Steinberg, J. M. Magri, and D. B. Jernigan. 2003. The anthrax epidemiologic tool kit: An instrument for public health preparedness. *Biosecur. Bioterror.* **1:** 111–116.
17. Rivera, V. R., G. A. Merrill, J. A. White, and M. A. Poli. 2003. An enzymatic electrochemiluminescence assay for the lethal factor of anthrax. *Anal. Biochem.* **321:** 125–130.
18. Stopa, P. J. 2000. The flow cytometry of *Bacillus anthracis* spores revisited. *Cytometry* **41:** 237–244.
19. Swartz, M. N. 2001. Recognition and management of anthrax - an update. *N. Engl. J. Med.* **345:** 1621–1626.
20. Volokhov, D., A. Pomerantsev, V. Kivovich, A. Rasooly, and V. Chizhikov. 2004. Identification of *Bacillus anthracis* by multiprobe microarray hybridization. *Diagn. Microbiol. Infect. Dis.* **49:** 163–171.
21. Wang, S. H., J. B. Zhang, Z. P. Zhang, Y. F. Zhou, R. F. Yang, J. Chen, Y. C. Guo, F. You, and X. E. Zhang. 2006. Construction of single chain variable fragment (ScFv) and biscFv-alkaline phosphatase fusion protein for detection of *Bacillus anthracis*. *Anal. Chem.* **78:** 997–1004.
22. Wang, S. H., J. K. Wen, Y. F. Zhou, Z. P. Zhang, R. F. Yang, J. B. Zhang, J. Chen, and X. E. Zhang. 2004. Identification and characterization of *Bacillus anthracis* by multiplex PCR on DNA chip. *Biosens. Bioelectron.* **20:** 807–813.
23. Williams, D. D., O. Benedek, and C. L. Turnbough Jr. 2003. Species-specific peptide ligands for the detection of *Bacillus anthracis* spores. *Appl. Environ. Microbiol.* **69:** 6288–6293.
24. Yang, M. Y., E. Ferrari, and D. J. Henner. 1984. Cloning of the neutral protease gene of *Bacillus subtilis* and the use of the cloned gene to create an *in vitro*-derived deletion mutation. *J. Bacteriol.* **160:** 15–21.