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Fluorescence Assay Based on Aptamer-Quantum Dot Binding to *Bacillus thuringiensis* Spores

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Abstract A novel assay was developed for the detection of *Bacillus thuringiensis* (BT) spores. The assay is based on the fluorescence observed after binding an aptamer-quantum dot conjugate to BT spores. The *in vitro* selection and amplification technique called SELEX (Systematic Evolution of Ligands by EXponential enrichment) was used in order to identify the DNA aptamer sequence specific for BT. The 60 base aptamer was then coupled to fluorescent zinc sulfide-capped, cadmium selenide quantum dots (QD). The assay is semi-quantitative, specific and can detect BT at concentrations of about 1,000 colony forming units/ml.

Keywords *Bacillus thuringiensis* · Aptamer · Quantum dots · SELEX · Fluorescence

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Introduction

Since the seminal work of Alivisatos, there has been tremendous progress in using semiconductor nanocrystals (quantum dots or QD) as fluorescent labels for biological samples [1–4]. For this particular assay system which uses QD for the detection of a *Bacillus*, the zinc-sulfide capped, cadmium selenide QD which fluoresce at 655 nm have an advantage over organic fluorophores in the range of wavelengths that may be employed for excitation and a narrow emission spectrum. With an appropriate choice of excitation wavelength, the background fluorescence of the bacteria is attenuated and because of the narrow emission spectrum, there is no overlap of fluorescence intensity with that produced from the bacteria. In addition, no photo bleaching of the QD has been observed as can occur with organic fluorophores [4–8]. Because of the narrow emission spectrum, QD may be used for multiplexed optical coding [3, 7, 8]. Finally the limit of detection for our QD assay (1000 CFU) is lower than that reported for a fluorophore-based assay for *Bacillus anthracis* in which about 6,000 *Bacillus* spores were detected [9].

SELEX (Systematic Evolution of Ligands by EXponential enrichment) [10] has made possible the isolation of oligonucleotide sequences with the capacity to recognize virtually any class of target molecule with high affinity and specificity. These oligonucleotide sequences, referred to as aptamers [11], are beginning to emerge as a class of molecules that rival antibodies in both therapeutic and diagnostic applications including biological [12–15] and bacterial detection [16, 17]. Unlike antibodies, aptamers can be denatured (via heat or chemicals such as urea) and renatured repeatedly without loss of function [18, 19].

We now report the preparation of a class of QD attached to an aptamer sequence (hereafter, abbreviated as aptamer-QD) which is specific for *Bacillus thuringiensis* (BT, kurstaki

strain) spores. BT is an endospore forming bacterium which produces intracellular protein crystals that are toxic to a large number of insect larvae [20–23]. *B. thuringiensis* is closely related to *B. cereus*, a food poisoning agent [21, 24] and *B. anthracis*, the causative agent of anthrax [20, 25]. Although BT is harmful to humans only at very high doses (approximately $>10^{11}$ CFU/ml) [26, 27], the method developed for binding of aptamer-QD to these spores can be applied to a wide range of harmful biological agents such as *B. anthracis* and *B. cereus*.

Experimental

Determination of the aptamer sequence and secondary structure specific for BT

The aptamer specific for BT was obtained using SELEX according to the whole spore method of Bruno and Kiel, 1999 [17], except that the template and primers were more similar to Bruno and Kiel's later work [28]. Aptamers were cloned into chemically competent *E. coli* using a TOPO TA kit from Invitrogen (Carlsbad, CA). Plasmids were extracted and purified using Qiagen miniprep spin columns. DNA sequencing was performed using M13 Primers on an Applied Biosystems ABI 377 system at Brooks-City Base, TX. A sixty base 5'-thiol modified oligonucleotide (5'-HS-(CH₂)₆-CAT CCG TCA CAC CTG CTC TGG CCA CTA ACA TGG GGA CCA GGT GGT GTT GGC TCC CGT ATC-3') based on the aptamer sequence was then purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Free web-based "Vienna RNA" software (<http://www.tbi.univie.ac.at/~ivo/RNA/>) was used to obtain the secondary structure. The software used DNA parameters at room temperature.

Determination of colony forming units

Bacillus thuringiensis (BT, kurstaki strain) spores were provided by the U.S. Air Force Research Laboratory at Brooks City-Base, TX, while the *Bacillus globigii* (BG; *Bacillus subtilis* var. niger) spores were obtained from the U.S. Army Dugway Proving Ground, UT. Ludox density gradient centrifugation of the BT spores was used in order to remove the protein crystals associated with the BT according to a previously published procedure [29]. The weight of the purified BT spores was determined by weighing a tube containing only 1 ml of PBS; this weight was subtracted from the weight of purified BT spores in 1 ml of PBS.

Tryptic soy agar (TSA) was obtained from Fluka (St. Louis, MO) and four percent TSA plates were prepared. Stock solutions of 1 mg/ml of BT or BG spores were prepared

in 1X PBS buffer (1.0 M phosphate buffered saline, pH 7.4) obtained from Fisher (Houston, TX).

Five TSA spread plates were spotted with 100 μ l of 1:1,000 diluted BT or BG spores, five plates with 100 μ l of 1:10,000 diluted BT or BG spores, and two plates with 100 μ l of PBS buffer. The plates were incubated at 37°C overnight and the colony counts were obtained and reported as CFU.

Preparation of aptamer-QD

A QDot 655 antibody conjugation kit containing the protocol and all necessary reagents for coupling thiols to zinc sulfide capped, cadmium selenide quantum dots (QD) which fluoresce at 655 nm was purchased from Invitrogen (Carlsbad, CA).

Briefly, to activate the QD, 13.9 μ l of 10 mM SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) were added to 125 μ l of QD and left for one hour at room temperature. A solution of 5' thiol modified aptamer was prepared at 1 mg/ml in 300 μ l of 1X PBS. To reduce the aptamer, 6.1 μ l of 1 M dithiothreitol (DTT) was added to the solution and left for 30 min at room temperature. Both the solution of activated QD and the solution of reduced aptamer were passed separately through a desalting NAP-5 column (Sephadex G-25), and 500 μ l of each were collected. The QD and reduced aptamers were then reacted at room temperature for one hour. To quench the conjugation reaction, 10.1 μ l of β -mercaptoethanol (final concentration of 100 μ M) was added to the mixture and left at room temperature for 30 min. The reaction product was concentrated using a 0.5 ml concentrator (at 7000 rpm, 3000 \times g, for 15 min), then added to a Superdex 200 column and eluted with PBS. A total of 250 μ l of column-purified, aptamer-QD were collected.

Ten μ l of aptamer-QD was dissolved in 1 ml of RO-purified water. A UV-2100 Spectrophotometer (Unico, San Diego, CA) was used to measure the absorbance at 260 nm. The actual concentration of aptamer-QD was determined by comparison with the initial aptamer concentration (1.08 mg/ml). The percent aptamer conjugated to the QD was determined to be 98%.

Binding of aptamer-QD to bacillus spores

Thirty μ l of aptamer-QD were placed in a tube and diluted with 600 μ l of PBS. In a typical reaction, 100 μ l of the diluted aptamer-QD in PBS was added to 900 μ l of 10⁷, 10⁶, 10⁵, 10⁴, 10³ CFU of BT spores. The mixture was vortexed briefly (speed 8) on a VSM -3 Vortex Mixer (Shelton Scientific, Peosta, IA) and left to react at room temperature for 20 min. Essentially, the same procedure was repeated for a series of controls: aptamer-QD with no spores, unconjugated QD

with BT spores, aptamer-QD bound to purified BT spores, and aptamer-QD bound to BG spores.

Prior to collection of the spores, 13 mm Durapore membrane filters (PVDF, hydrophilic, 0.45 μm , Millipore, Billerica, MA) were washed twice with PBS. The spores exposed to either the aptamer-QD or unconjugated QD were then collected on the filter and washed three times with 1 ml of 1 PBS. Washed spores were collected in 1 ml of PBS. All experiments were performed in duplicate.

Fluorescence spectra

A Cary Eclipse Fluorescence Spectrophotometer (Varian, Palo Alto, CA) was used to obtain the fluorescence spectra. Solutions were excited at 400 nm and the fluorescence read between 430 and 700 nm. Excitation and emission slits were set to 10 nm and the spectra were run with the detector voltage set at 600 V.

Results

Determination of BT and BG colony forming units

The CFU for BT and BG spores was determined as follows: the 1:1000 dilution of spores was overgrown, therefore only the colonies grown at a 1:10,000 dilution of spores were counted. The average was taken and multiplied by the dilution factor to determine the CFU. The number of CFU for BT was determined to be 1.4×10^7 CFU and the number of CFU for BG was determined to be 1.8×10^8 CFU.

Fluorescence spectra

Figure 1 illustrates the intrinsic fluorescence of the BT spores. BT spores do not fluoresce at 655 nm which is the fluorescent wavelength of the QD, but they do exhibit fluorescent intensity at 455 nm. BG spores also, did not exhibit fluorescence intensity at 655 nm (data not shown).

Unconjugated QD were tested against BT spores to determine if there is any non-specific binding between QD and BT spores. The spectra obtained are shown in Fig. 2. Even at the highest concentrations of BT spores, we observed a minimal amount of fluorescence at 655 nm which could be attributed to non-specific binding.

The spectra obtained for aptamer-QD bound to BT are shown in Fig. 3. The fluorescence intensity varies with BT in the range 10^3 to 10^6 CFU, thus demonstrating that aptamer bound to QD can still recognize BT spores. The QD have a nominal size of 20 nm which is about the size of a large protein or antibody. In a previous report Zhao and coworkers published scanning electron micrographs of much larger 60 nm silica nanoparticles attached to an antibody bound to E. Coli [30].

Since BT spores contain crystals that may interfere with the binding between aptamer-QD and BT spores, the BT spores were isolated from the crystals using density gradient centrifugation. Purified spores were then reacted with the aptamer-QD and the results are shown in Fig. 4. From Fig. 4 it can be seen that there is an enhanced fluorescence intensity associated with the purified BT spores. Although the removal of the crystals yields a more intense signal and better consistency in reproducing the results from reaction to reaction,

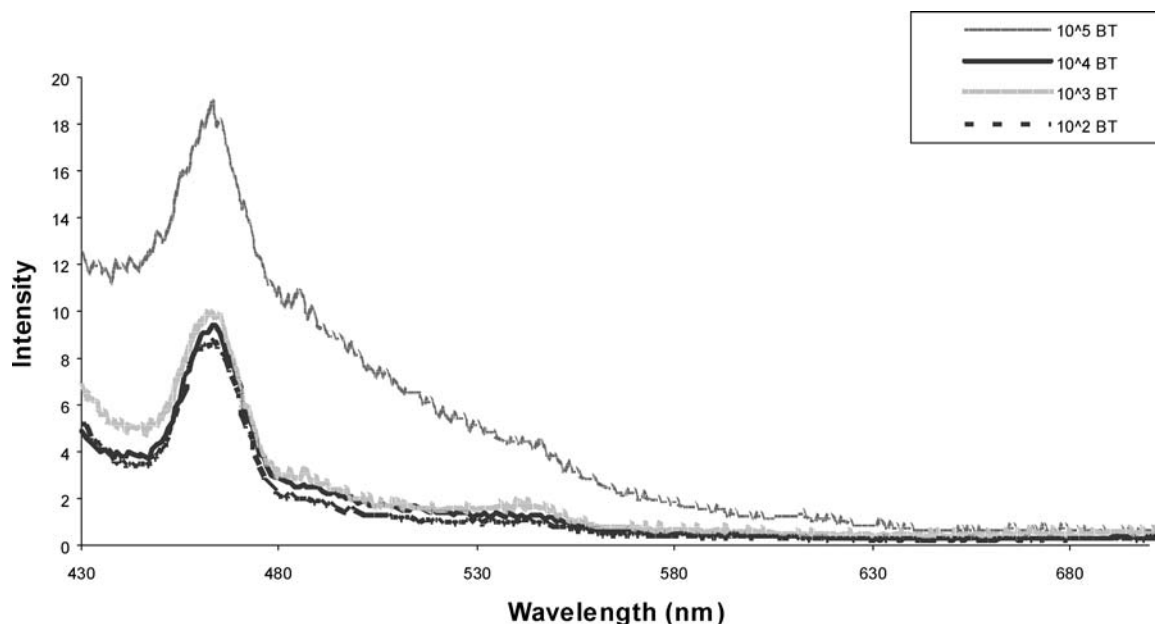
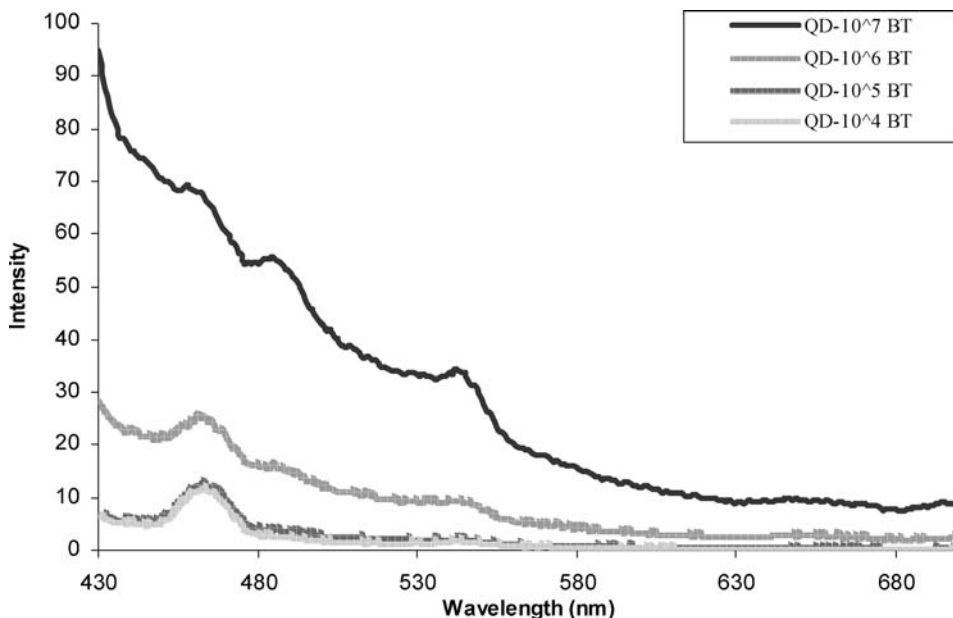


Fig. 1 Fluorescence Spectra of BT spores. Solid line represents fluorescence spectra of 10^4 CFU of BT; other dilutions are shown in the legend

Fig. 2 Fluorescence Spectra of unconjugated QD reacted with BT spores. Solid line is the spectra of unconjugated QD reacted with 10^7 CFU of BT spores. Other dilutions are shown in the legend area



this would be impractical and an unnecessary step in trying to assess the presence of BT spores in the environment.

To determine the specificity, aptamer-QD was reacted with BG spores. From the results in Fig. 5 it can be

seen that aptamer-QD also bind to BG spores. However the intensity at 655 nm at a given value of CFU is lower for the BG spores when compared with the BT spores.

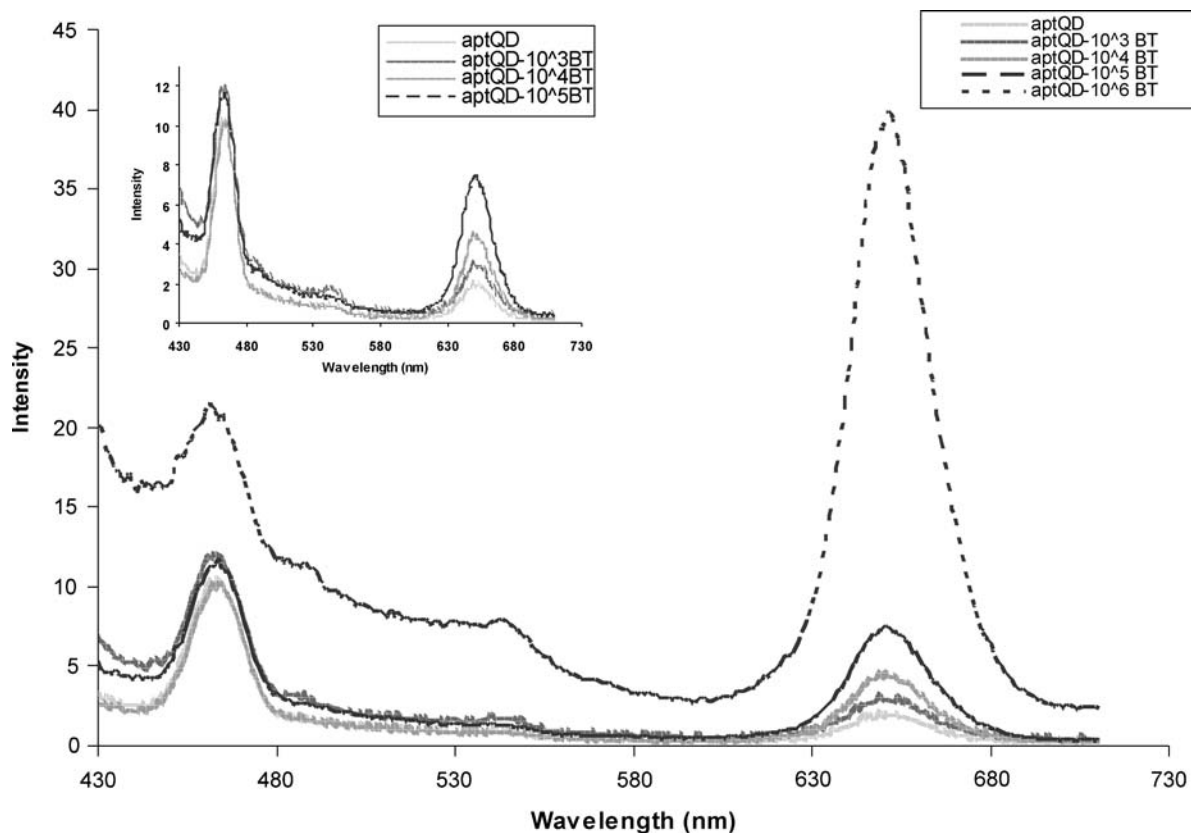


Fig. 3 Fluorescence Spectra of aptamer-QD bound to BT spores. Dashed line is the spectrum of aptamer-QD reacted with 10^6 CFU of BT (apt QD- 10^6 -BT). Other dilutions are shown in the legend area.

An inset in the upper left hand corner represents the same data with the exclusion of the data for 10^6 CFU of aptamer-QD bound to BT spores

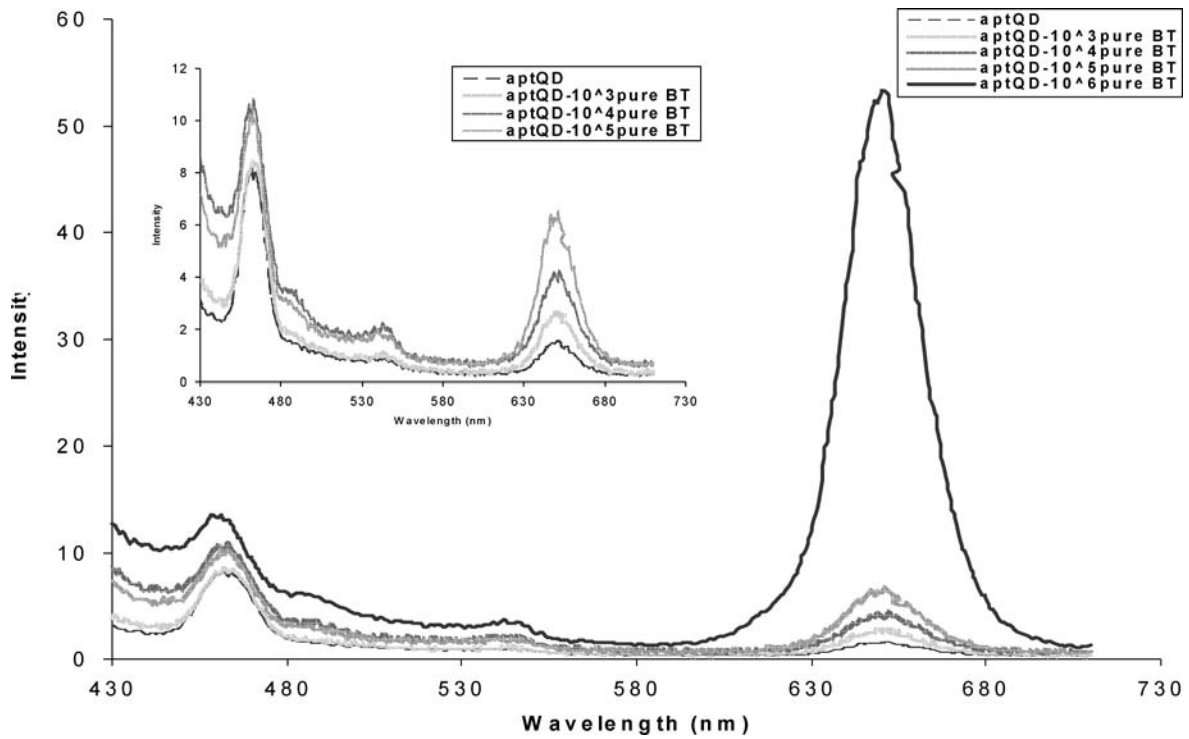


Fig. 4 Fluorescence Spectra of aptamer-QD bound to purified BT spores. Solid line is the spectrum of aptamer-QD reacted with 10^6 CFU of purified BT Spores (apt QD- 10^6 -pureBT). Other dilutions are shown

in the legend area. The inset in the upper left hand corner represents the same data with exclusion of 10^6 CFU of aptamer-QD bound to purified BT spores

Discussion

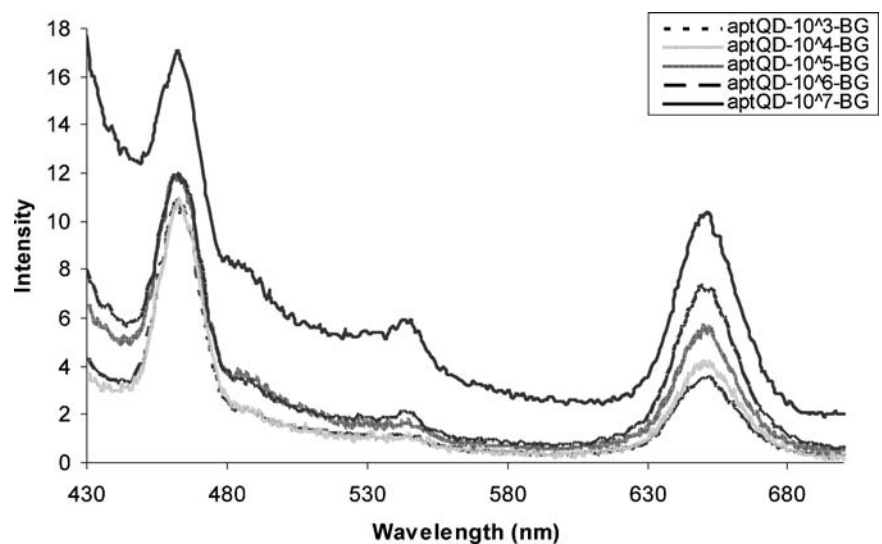
BT aptamers

Figure 6 shows the nucleotide sequence (5'-CAT CCG TCA CAC CTG CTC TGG CCA CTA ACA TGG GGA CCA GGT GGT GTT GGC TCC CGT ATC-3') and predicted stem loop structure of the aptamer specific for BT obtained from SELEX and the Vienna RNA software program respectively.

Though it is difficult to postulate how the stem loop structure contributes to the binding of BT, it should be noted that many other aptamers with reported secondary structures also have stems, loops and bulges. These are common features of many DNA aptamers [31].

The single aptamer sequence resulted from two separate attempts at the whole spore SELEX process of Bruno and Kiel [17] followed by cloning into chemically competent *E. coli* using an Invitrogen TOPO TA cloning kit. Four clones

Fig. 5 Fluorescence Spectra of aptamer-QD reacted with BG spores. Solid line is the spectrum of aptamer-QD reacted with 10^7 CFU of BG spores (apt QD- 10^7 -BG). Other dilutions are shown in the legend area



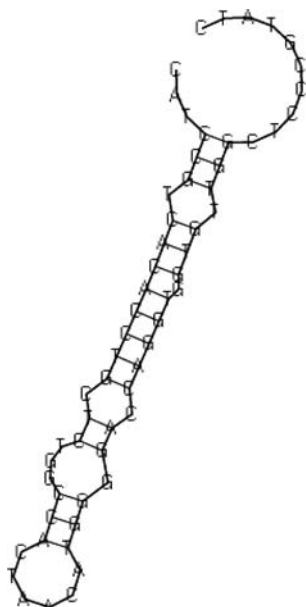


Fig. 6 Secondary loop structure of BT aptamer

were obtained after the first SELEX attempt, but no useful sequences resulted from the clones. The second SELEX attempt resulted in two clones containing identical aptamer sequences which are reported here. The fact that only one useful aptamer sequence resulted from two identical clones after two attempts at the SELEX process (each consisting of 5 rounds of selection) probably attests to the paucity of “antigenic” targets or “epitopes” for binding on the spore surface. It has been historically difficult to produce specific high affinity antibodies to bacterial spores as well, probably because the spore’s surface is relatively simple and smooth.

Fluorescence spectra

Table 1 summarizes the fluorescence data obtained for aptamer-QD binding to BT, purified BT and BG. The background fluorescence was subtracted from the absolute fluorescence intensity measured at 655 nm. Calculating the minimal detectable response as the fluorescence intensity of the control (0 CFU) plus 3 times the standard deviation of the

Table 1 Average fluorescence intensity as a function of colony forming units (CFU)

CFU	BT spores	purified BT spores	BG spores
0	1.179 (0.2)	0.955 (0.46)	1.3 (0.2)
10 ³	1.79 (0.3)	1.63 (0.08)	2.78 (0.3)
10 ⁴	3.04 (0.24)	4.607 (0.13)	3.19 (0.3)
10 ⁵	5.53 (0.43)	7.927 (0.25)	4.32 (0.32)
10 ⁶	30.3 (1.3)	67.524 (0.96)	5.97 (0.4)

Note. The standard deviation associated with the fluorescence intensity is reported within the parentheses.

fluorescence intensity at 0 CFU yields a minimum detectable signal of about 2. This in turn yields a limit of detection (LOD) between 10³ and 10⁴ CFU. The signal intensity for purified BT spores as compared to BT spores ranges from about 50% (at 10⁴ and 10⁵ CFU) to over 100% greater (at 10⁶ CFU) which is consistent with the fact that BT spores contain about 30% by weight of protein crystals. Finally, there is a marked increase in the fluorescence intensity at about 10⁵ CFU for both the BT spores and the purified BT spores when compared with the BG spores. At concentrations above 10⁵ CFU the aptamer-QD discriminates between BT and its close relative BG.

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