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NANO APTASENSOR FOR PROTECTIVE ANTIGEN TOXIN OF ANTHRAX

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Abstract

We demonstrate a highly sensitive nano aptasensor for anthrax toxin through the detection of its polypeptide entity, protective antigen (PA toxin) using a PA toxin ssDNA aptamer functionalized single-walled carbon nanotubes (SWNTs) device. The aptamer was developed in-house by capillary electrophoresis systematic evolution of ligands by exponential enrichment (CE-SELEX) and had a dissociation constant (K_d) of 112 nM. The aptasensor displayed a wide dynamic range spanning up to 800 nM with a detection limit of 1 nM. The sensitivity was 0.11 per nM and it was reusable six times. The aptasensor was also highly selective for PA toxin with no interference from human and bovine serum albumin, demonstrating it as a potential tool for rapid and point-of-care diagnosis for anthrax.

Keywords

Anthrax; protective antigens; toxins; aptamers; single-walled carbon nanotubes; aptasensor; SELEX

Introduction

Anthrax is a disease caused by the anthrax toxin of the spore forming Gram positive bacterium *Bacillus anthracis*. Many of the properties of *Bacillus anthracis* such as high mortality and environmental stability make them a deadly bioterrorist agent that can be used by terrorist organizations to cause wide spread casualties. The early stage of anthrax infection is often characterized by non-specific symptoms making its detection difficult. Therefore, there is a great need for prompt administration of antibiotics before a lethal infection is established. This is particularly relevant for the inhalational form of the disease. In fact, of the five fatalities in the 2001 bioterrorist attacks in the United States involving anthrax, infection was confirmed in only one individual before death¹.

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The pathogenicity of the anthrax bacteria is mainly due to the tripartite toxin it makes. The three components of the toxin are cell-binding protective antigen (PA), edema factor (EF) and lethal factor (LF). Assembly of the three toxin proteins is initiated when PA binds to a cellular receptor² and is activated by cleaving PA into two fragments: PA20 (20 kDa), PA63 (63 kDa). Receptor-bound PA63 then spontaneously self-associates to form ring-shaped, heptameric oligomers³. EF and LF bind competitively to PA63 subunits^{4, 5}, and are translocated across the membrane to the cytosol. Once within the cytosol, EF catalyzes the conversion of ATP to cAMP⁶, and LF proteolytically cleaves certain MAP kinases⁷ thus disrupting the normal functions of the cell and manifesting the disease. The protective antigen is thus an essential factor and plays an important role in the immune response. It has been demonstrated that antibodies against the PA toxin possess a neutralizing effect of the toxin as well as anti-spore activities⁸, making the PA toxin an ideal target for detection of the infection. Current detection methods include detection of spores through the calcium dipicolinate⁹ cell culture, immunological tools¹⁰, electrophysiological measurements of ion channels¹¹ and nucleic acids.

While effective these methods are not suitable for early detection¹². Therefore, a rapid, facile, sensitive and selective method of detecting anthrax toxin in body fluids would provide an invaluable tool for establishing whether anthrax is the causative agent of a biological attack leading to prompt diagnosis and targeted treatment of infected individuals.

Single-walled carbon nanotubes (SWNTs) have been extensively studied as the transducer elements of biosensors as they meet the important requirements of an efficient biosensor: excellent electrical properties and a large surface to volume ratio results in surface phenomena predominating over the chemistry and physics that happen in the bulk^{13,14}. These along with suitable bioreceptors have shown to make sensors that display high selectivity, sensitivity and real-time label-free detection capabilities. SWNTs have been successfully used to detect a variety of targets^{12–23}. Proteins (enzymes, antibodies) and more recently aptamers^{15–19} are the commonly used probes to detect the target analytes.

Aptamers are short, synthetic oligonucleotides capable of binding to wide variety of molecules with high affinity and specificity. Aptamers as probes are more advantageous compared to antibodies as 1) their synthesis does not require animal host or expensive hybridoma culture, 2) can be selected using modern combinatorial chemistry tools and 3) are more stable. For example, systematic evolution of ligands by exponential enrichment (SELEX) is the technique used to identify DNA or RNA aptamers with high affinity and specificity for different targets^{20, 21}. SELEX employs a random library of oligonucleotides from which sequences with desired characteristics of affinity and specificity are selected. A number of different SELEX processes have been proposed since its inception based on the differences in the techniques used to separate the bound and unbound random library. Capillary electrophoresis-SELEX (CE-SELEX), a variant of the conventional SELEX, uses a solution based binding and separation technique to alleviate the drawbacks²² of selection bias encountered in the conventional SELEX. The technique has been applied to different targets - Human IgE, HIV-1 Reverse Transcriptase, neuropeptide Y^{22, 23}.

Here we report the development, characterization and evaluation of a highly sensitive and selective aptasensor for the detection polypeptide entity PA of anthrax toxin in plasma. The aptasensor consisted of ssDNA aptamers for the PA63 selected in-house using CE-SELEX from a DNA library integrated to a SWNTs-based chemiresistive transducer.

Experimental

Materials

The DNA library and Primers for PCR were acquired from Integrated DNA Technologies Inc. (Coraville, IA, USA). Streptavidin Agarose beads for single strand regeneration were obtained from Pierce (Rockford, IL, USA). Bare fused silica capillary (40 cm long by 50 μ m I.D. and 360 μ m O.D.) coated with polyvinyl alcohol was obtained from Polymicro Technologies (Phoenix, AZ, USA). PA toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA). Taq Polymerase and pGEM vector were purchased from Promega Corp. (Madison, WI, USA). CE – SELEX was performed in P/ACE MDQ Capillary Electrophoresis system (Beckman Coulter, Inc., Fullerton, CA, USA). Selection Buffer consisted of 25mM Tris, 10 mM NaCl and 1 mM $MgCl_2$, bead binding/washing buffer was 100 mM phosphate buffer and 50 mM NaCl. Single-walled carbon nanotubes with high carboxylated functionality, sold under the trade name of P3-SWNT, were purchased from Carbon Solutions, Inc. (Riverside, CA, USA). Human serum albumin and bovine serum albumin were purchased from Sigma-Aldrich (Milwaukee, WI, USA). 1-pyrenebutanoic acid succinimidyl ester (PASE) was purchased from Invitrogen (Carlsbad, CA, USA).

Methods

Aptamer Generation

Capillary electrophoresis selection: The synthetic DNA library of 40 random nucleotides flanked by 20 bases priming sites for PCR amplification in selection buffer was heated to 94 °C and cooled to room temperature followed by incubation with 20 nM of PA toxin for 20 min at room temperature. Approximately 150 nL of the above mixture was injected into the conditioned (washing/rinsing with 150 nL each of 100 mM HCl, 100 mM NaOH, double distilled H_2O and selection buffer) bare fused silica capillary of a P/ACE MDQ Capillary Electrophoresis system using 2.5 psi pressure for 13 s. An electric field of 30 kV was applied to enable the migration using normal polarity at a temperature of 25 °C and the separation of PA toxin-DNA complex and unbound DNA was monitored using a UV detector at 254 nm. All sequences migrating more than 30 s earlier than the leading edge of the peak corresponding to the unbound sequences were collected into 20 μ l of separation buffer at the capillary outlet. The exact time that the unbound sequences would reach the outlet of the capillary was calculated from the knowledge of the distance from the end of the capillary to the detector and experimentally determined migration time for the naïve library.

PCR amplification: The 20 μ l of the collected fraction containing the bound sequences were divided into four aliquots. PCR buffer was added to these fractions so that the final reaction mixture contained 1 mM of each deoxyribonucleotide triphosphate (dNTP), 1 μ M of primer 1 (5'-AGC AGC GAG GTC AGA TG-3'), 1 μ M of primer 2 (biotin/5'-TTC ACG GTA GCA CGC ATA-3'), 0.15 U/ μ l of Taq polymerase, 7.5 mM $MgCl_2$, 1 mM PCR buffer and 5 μ l of the collected fraction as template. PCR was carried out by heating the mixture to 95 °C for 5 min followed by 11 cycles of denaturation, annealing and extension for 30 s at 95 °C, 53 °C for 30 s and 72 °C for 20 s, respectively. Control PCR without any added DNA was also performed. The presence of DNA following PCR was confirmed by electrophoresis on a 2% agarose gel followed by staining with ethidium bromide.

The PCR amplified product was conjugated to streptavidin coated agarose beads, washed with binding buffer and then melted by treatment with 200 μ l 0.15M NaOH. The single stranded DNA was then concentrated using QIAEX II DNA Extraction Kit Qiagen (German Town MA).

Cloning: A PCR amplified product was ligated to pGEM vector transformed into DH α 5 *E. coli* using electroporation. Colonies were grown on LB-agar medium supplemented with

ampicillin and X-Gal and IPTG. Transformed colonies were selected and sequenced at the University of California, Riverside Genomics Institute, Riverside, CA. The dissociation constants (K_d) for selected clones was determined by Affinity Capillary Electrophoresis (ACE) performed under the same conditions as CE-SELEX. In the ACE process the aptamers were initially labeled with a 6-carboxyfluorescein and incubated with increasing concentration of PA Toxin. The peak heights of the unbound DNA was used to calculate the K_d values. Nonlinear least-squares regression analysis was performed to determine the K_d using GraphPad Prism 4 (GraphPad Software, San Diego, CA USA).

Biosensor Fabrication

Solubilizing SWNTs and alignment: Carboxylated-SWNTs (SWNT-COOH 80–90% purity) (Carbon Solution, Inc. Riverside, CA, USA) were dispersed (1 $\mu\text{g/mL}$) in dimethyl formamide (DMF, Sigma Aldrich, MO, USA) using ultrasonic force for 60 min followed by centrifugation (10,000 RPM) to remove unsolubilized SWNTs. These processes were repeated three times with decreasing time 90, 60, and 30 min to prepare soluble SWNTs. The suspended SWNTs were aligned in the 3 micron spaced microfabricated gold electrodes by AC dielectrophoresis (DEP). In brief the procedure involves addition of 0.1 μL drop of SWNTs and applying AC voltage at a frequency of 4MHz (amplitude 0.366 V p-p) across the electrodes. The aligned SWNTs are then annealed at 300 $^{\circ}\text{C}$ for 60 minutes under reducing atmosphere (5% H_2 + 95% N_2) to minimize the contact resistance between the CNT network and the gold electrodes and to remove any DMF residues. The number of SWNTs bridging the electrode gap was controlled by adjusting the concentration of the SWNTs in the DMF solution and the droplet size.

Aptamer functionalization of SWNTs: The SWNTs are noncovalently modified using 6 mM 1-pyrenebutanoic acid, succinimidyl ester by technique developed by Chen et al.²⁴ In brief, the process involved incubating the sensor (with annealed SWNTs) with 6 mM 1-pyrenebutanoic acid, succinimidyl ester in DMF for one hour followed by thorough washing with DMF to remove excess chemical. The sensor was then rinsed with phosphate buffer (10 mM) and nanopure water. The apt 11(PA toxin aptamer) was attached onto the SWNTs by incubating with 5 μM aptamer solution in 10 mM phosphate buffer overnight at 25 $^{\circ}\text{C}$, followed by treatment with 0.1 mM ethanolamine to block excessive reactive groups for 30 min,²⁵ and finally by incubation with 0.1 % Tween 20 to prevent non-specific binding.

Sensing measurement: The devices were incubated with 5 μL of the sample for 1 min, washing the device 3 times with 10 mM phosphate buffer, 1 times with nanopure water followed by aspirating the water and measuring the I–V response in ambient air using a Keithley Source Meter (Model 236).

Results and discussion

PA toxin aptamer selection

Because of the well documented central role of PA toxin, the B component of the A–B type anthrax toxin²⁶, in manifestation of the disease, a number of immunoassays/sensors utilizing the high affinity antibodies single chain antibody fragments and affinity peptide against PA toxin have been developed^{9,27–29}. However, due to antibodies potential instability from proteolysis and the need of animal host or expensive hybridoma culture to produce, we chose to work with aptamers that are characterized by their higher stability, easy regeneration upon binding to an analyte, inexpensive *in vitro* synthesis and similar high affinity as antibodies. Previously, an RNA aptamer against PA toxin was generated by Archemix Corporation, Cambridge, MA, USA, under an U.S. Army Research Office contract 30. Since DNA sequences, with and without terminal modifications, are more resistant to nucleases than RNA sequences³¹, we chose to work with DNA aptamers. Aptamer for PA toxin was selected from

a synthetic library of single stranded DNA (ssDNA) using CE-SELEX. Based on the initially established migration pattern of the library with or without PA toxin (data not shown), PA toxin-library complex was collected separately from the unbound library and PCR amplified (eleven cycles). The purified ssDNA from the PCR products was incubated with the PA toxin for the next round of SELEX. After 6 rounds of selection, the selected library was cloned and 9 clones were selected for further analysis and sequencing. As shown in Table 1, sequenced clones resulted in no consistent homology when analyzed with ClustalW, which was consistent with published results³² and is attributed to several binding motifs on the fairly large (64 kDa) PA toxin. The dissociation constant (K_d), determined by affinity capillary electrophoresis (ACE), ranged from 112 – 1140 nM. The K_d of 112 nM for the best aptamer (Apt11) was ~4-fold better compared to the PA toxin RNA aptamer (400 nM)³⁰ and in line with the K_d of other reported high affinity DNA aptamers.

SWNTs-based PA toxin aptasensor

The Apt11 PA toxin specific aptamer generated above was applied for development of the PA toxin nano aptasensor. Carboxylated SWNTs were used in our experiments to get a uniform suspension of well-separated SWNTs prior to AC dielectrophoretic alignment between microfabricated gold electrodes. Since the first use of alternating current to position carbon nanotubes³³ the process has been used extensively to align SWNTs across micro spaced electrodes. The major advantages of this technique are that it provides better control in positioning and contacting the SWNTs between metal electrodes for electronic circuits and improved sensor sensitivity and response time compared to the techniques of drop-casting,³⁴ spray-coating and catalytic growth at high temperatures or self assembly. Alignment of the SWNTs was performed at 0.3V_{P-P} and 4 MHz frequency for 5s as these parameters reproducibly gave a uniform distribution of well contacted SWNTs across the electrodes as observed under SEM and an initial resistance in the range of 20–100 K Ω after annealing at 300 °C for 1 h under 5% H₂ and 95% N₂ environment. Figure 1 shows the schematic of the steps involved in the functionalization of SWNTs with aptamers. In order to preserve the aptamer activity, the immobilization onto the SWNTs was accomplished using 1-pyrenebutanoic acid succinimidyl ester (PASE)³⁵. PASE non-covalently attaches to the SWNTs through the highly aromatic pyrenyl group by π - π bond interaction and provides a free succinimidyl ester group that can interact with the amine group at the 5' end of the PA toxin aptamer by the formation of an amide bond. The non-covalent modification of the SWNTs helps to protect their electronic properties and also provides high degree of control and specificity for immobilizing biological molecules. Immobilization of aptamers was followed by incubation with 0.1 M ethanolamine for 30 min²⁵ to neutralize/passivate excess free succinimidyl ester reactive groups and finally with 0.1% Tween20 for 30 min to block non-specific protein adsorption (NSPA) on SWNTs³⁵.

The high surface area to volume ratio and the surface carbon atoms make SWNTs extremely sensitive to perturbations/adsorption events at the surface. This was also evidenced in the present study as illustrated by the modulation of the current vs. voltage (I–V) characteristics after each event (Figure. 2). In accordance with the literature reports, aptamer immobilization onto the SWNTs decreased the device resistance (trace 3, Fig. 2) compared to the PASE-functionalized SWNTs (trace 2, Fig. 2) owing to the accumulation of negative charges from the phosphate backbone of ssDNA aptamers.^{15,33} The incubation of the aptamer-functionalized SWNTs device with 1 μ M of target analyte (PA toxin) for 1 min at room temperature dramatically increased the device resistance (trace 4, Fig. 2), attributed to positive charge accumulation of the PA toxin bound to the aptamer. The later demonstrated the potential for electrical detection of PA toxin using a simple SWNTs-based chemiresistive aptasensor.

Figure 3 shows the relationship between the SWNTs based chemiresistive aptasensor response $[(R - R_0)/R_0]$, where R is the resistance after exposure to PA toxin and R_0 is resistance after exposure to buffer] and PA toxin concentration. The resistance is calculated as the inverse of the slope of the I - V plot between -0.1 and $+0.1$ V (linear range). The dissociation constant of the PA toxin aptamer calculated from the aptasensor calibration was determined to be 225 nM. This is slightly higher than the 112 nM determined from ACE and can be explained by the fact that the aptamer is immobilized in the case of aptasensor as opposed to in the solution phase in ACE study. The aptasensor was highly sensitive, 0.11 per nM in the linear region, with a limit of detection of 1 nM. Furthermore, it had a wide dynamic range spanning up to 800 nM with a linear dynamic range up to 400 nM. The limit of detection of our aptasensor while higher than the 16 pM reported for a sandwich ELISA, the sensing required only 1 min incubation and a single recognition molecule (aptamer) as opposed to two recognition molecules, a capture single chain antibody and a secondary IgG, one hour incubation each with the capture and secondary antibodies and signal amplification through the biocatalyst horseradish peroxidase label.³⁶

Selectivity/specificity is a critical parameter in the acceptance/utility of a sensor. The aptasensor had excellent selectivity as evidenced by an insignificant response to both Human serum albumin (dominant protein in human blood) and Bovine serum albumin (dominant protein in bovine blood) when compared to PA toxin (Figure. 4). Additionally, negative controls, i.e. SWNTs devices without PA toxin aptamer show a very small response to PA toxin, HSA and BSA.

The potential of reusing a sensor several times either with or without a regeneration step is highly desirable. We investigated regenerating the aptamer so that the sensor can be used multiple times. Incubation of the aptasensor after use with 1 μ l of 6 M guanidium hydrochloride for 15 min followed by thorough washing with 10 mM phosphate buffer restored its functionality for up to six repeated measurements (Fig. 4).

In conclusion, we synthesized ssDNA aptamers with good affinity ($K_d = 112$ nM) and selectivity for PA toxin by six rounds of CE-SELEX. Combining the aptamers with SWNTs transducer, an aptasensor for facile and rapid PA toxin detection was constructed. The 1 nM limit of detection, a wide dynamic linear range, high sensitivity and excellent selectivity make the sensor useful for direct detection in biological samples. The aptasensor also gives commercial advantage due to its simple fabrication scheme, small size and reusability. The performance of the sensor can be further improved by operating as a field-effect transistor, reducing the contamination of metallic nanotubes in the SWNT suspension and using an aptamer with lower K_d .

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Figure 1.
Schematic illustration of aptasensor fabrication steps.

Figure 2. Current versus voltage (I–V) curves of 1) unfunctionalized carboxylated SWNTs, 2) PASE modified SWNTs, 3) aptamer immobilized SWNTs, and 4) after incubation with PA toxin.

Figure 3.

Calibration plot of the PA toxin sensor. Data is average of 6 independent sensors prepared at different times and error bars represent ± 1 standard deviation. The top inset is the magnification of sensors response at lower concentrations of 0 nM to 50 nM and bottom inset depicts the linear response region of the sensor; $r^2 = 0.99$ for the line.

Figure 4. Specificity of the sensor without PA toxin aptamer (dark bars) and sensors with aptamers (striped bars) for different analytes. Analyte concentration is fixed at 100 nM and the incubation time was 1 minute. Data is the average of three independent sensors prepared at different times and error bars represent ± 1 standard deviation.

Figure 5. Regeneration of the aptasensor upon treatment with 6M Guanidium chloride. Data is the average of 6 independent sensors prepared at different times and error bars represent ± 1 standard deviation.

TABLE 1

Sequences of Clones obtained from ssDNA pool after six round of selection and CLUSTAL W (1.83) multiple sequence alignment

Clone	Sequence	K _d (nM)
C1-PA-5	TCAGACACTTTGCCAAAAACATGATACAAGTTCGCTGCC----- 40	173
C1-PA-10	----GCTTTACCGCACTTCCGATCTTTAATTTTCGAGTGTATCAT40	488
C1-PA-12	-----CATCTCGGTCGTGAACCTTACATGCATGAGTATTTGGTG 40	508
C1-PA-11	ATCACTAGTGAATTCGCG-GCCGCCTGCAGGTCGACCATAT---- 40	112
C1-PA-7	-CCCAACATCTACGGTTAGACCGGGTTACCTGAGCTGACA----- 40	526
C1-PA-6	---TTCTAGGAAATTCAAACAGGTTTGTATTTTCTAGTTGA----- 40	345
C1-PA-1	----CTATAGAGGTGCTCCAGGGCGATAAACTTATGAATATTAA---- 40	1140
C1-PA-3	----AGCTTAGTGCATATCACTCCTCGTTATAGCATGGTTATAG----- 40	661
C1-PA-9	-----AAATGATTGCTACAATACATAGAGTCATGGAGATTACATC- 40	509