Electrochemical Immunoassay Detection of *Bacillus anthracis* Protective antigen

Zoraida P. Aguilar*, Padhma Y. Narasimhan, Chamika Wansapura, Andrea Henrichs, Julie Aday, Ysmael Aguilar, and Leo Estorninos

Vegrandis, LLC

700 W. Research Ctr. Blvd., ENRC 1208, Fayetteville, AR 72701
Tel: (479) 527-6591
Fax: (479) 571-8814
Email: zoraida.aguilar@vegrandis.com

Abstract

A self-contained microelectrochemical immunoassay detection of *Bacillus anthracis* protective antigen (PA) has been developed. A microcavity array (VEG4x1™) of 50-µm diameter cavities with self-contained microelectrodes were used for the detection of as low as 2.5 pg of *B. anthracis* Protective Antigen in a less than 12 min total assay time from capture to signal generation. The microelectrochemical sandwich enzyme-linked immunosensor involved covalent immobilization of the capture antibody on the free end of mercaptoundecanol self-assembled monolayers at the bottom Au-microdisk electrode that also serves as the bottom of the microcavities. Signal generation involved alkaline phosphatase hydrolysis of the enzyme substrate *p*-aminophenyl phosphate to *p*-aminophenol that is detected with the wall middle layer gold as the detecting electrode and the top layer gold as the pseudoreference/auxiliary electrodes. The microelectrodes in the cavity exhibited background signals lower than signal for the lowest concentration detected that was 2.5 pg.

Introduction

Anthrax is one of the most potent bacterial diseases caused by a naturally occurring organism and is relatively easy to contract (1). The spores have a half-life of 100 years and are resistant to desiccation, solvents, extreme pH, temperature, pressure, UV and IR (2). Anthrax infections occur via any of three routes: cutaneous, intestinal, or inhalation. Cutaneous anthrax results in a sore with a black center that results from contact with an infected animal, its hide, or its carcass. Left untreated, 20% of cases progress to systemic infections that are usually fatal. Intestinal anthrax that results from eating contaminated meat is extremely rare but can also be fatal. The most deadly form of the disease is inhalation anthrax which occurs when aerosolized spores are inhaled (5) causing a rapidly progressing, systemic infection with a 90 to 100% fatality rate (2, 3, 4). Inhalational anthrax has an infective dose of 8,000 to 10,000 spores (5).

*Bacillus anthracis* is a spore-forming bacterium that can cause illness and death in exposed animals and humans (3). There are three forms of anthrax: cutaneous, gastrointestinal, and inhalational (26). Exposure to aerosolized *B. anthracis* spores may cause inhalational anthrax, the most deadly form of the disease. The anthrax-laced letters that were sent in the wake of the 11 September 2001 terrorist attacks on the World Trade
Center and the Pentagon have made tragically clear the urgency of developing effective prophylactic and therapeutic treatments for this infection. A total of 11 confirmed cases of inhalational anthrax and 8 cases of cutaneous anthrax were reported during that incident. Five Americans died of inhalational anthrax despite aggressive antibiotic treatment (3).

In general, when anthrax spores are inhaled, larger spores lodge in the upper respiratory tract while spores between 1 to 5 micron can penetrate the alveoli in the lungs. The immune system responds destroying some of the spores. Spores that are not destroyed germinate releasing the three toxins lethal toxin (LT), edema factor (EF), and the protective antigen (PA) which is the central component of the anthrax toxins (6) that enter the blood stream resulting in hemorrhage and tissue decay (7). PA (83 kDa) initially binds to cell surface receptors (8) followed by cleavage of PA by cell-associated furin-like proteases, releasing a 20-kDa fragment (9, 10) to produce the activated form, PA63 (63 kDa). The next steps results to binding of LF (or EF) to PA63 (11,12). The PA63-LF (or PA63-EF) complexes are internalized and within the acidic environment of the endosomes, LF and EF are translocated into the target cell cytoplasm (13, 14) where they exert their toxic effects (15, 16). PA by itself has no known deleterious effects but the presence of the anthrax toxins are required for massive bacteremia, since the toxins exert strong antiphagocytic effects that appear to favor the growth and spread of vegetative bacilli (17). Antibiotic treatment of inhalational anthrax victims is effective if started shortly after exposure but may be less effective if delayed even by hours (18).

The use of an anti-toxin antibody could be an important standalone therapy against antibiotic-resistant strains of anthrax. The central role of PA in the pathophysiology of anthrax makes it an excellent therapeutic target. Reports have indicated that vaccination with the PA-based human anthrax vaccine (19) or purified PA (20) results in the development of a protective immune response. Protective passive immunization with polyclonal antibodies against toxin proteins, particularly PA, has been demonstrated from challenge with *B. anthracis* spores (21, 22). More importantly, antibody titers against PA correlate with protective immunity against spore challenge (22-25).

Anthrax diagnosis can be performed through serologic detection based on microbial culture, PCR, or immunochemistry. However, microbial culture is time consuming requiring about 6-24 hours to get a result (27). PCR is very reliable but it requires expensive instrumentation and reagents (28). Multiplexing PCR leads to more non-specific products due to inappropriate primer pairing making the identification of the amplicons difficult to impossible (29). Existing rapid ELISA takes more than 3 h to complete and the traditional ELISA is a monoplex technology allowing only for the detection of one analyte per assay (30) taking days to screen exposures to all possible biowarfare agents using this technique.

The CDC has developed a quantitative ELISA for the serologic detection of anthrax using the anti-protective antigen specific immunoglobulin G (anti-PA IgG) in human serum (30, 31) using fluorescence detection with a minimum detection concentration at 3.0 µg/mL with a reliable detection limit (RDL) of 0.09 µg/mL at a dynamic range of 0.06 to 1.6 µg/mL. We previously reported a microelectrochemical


serologic assay for antibodies against *B. anthracis* as a measure of exposure (32). The assay was used to detect as low as 10 pg of antibodies against anthrax.

As a confirmatory test to the serologic diagnosis for anthrax infection, we report the detection of *B. anthracis* PA in a 3 min capture using the VEG4x1™ microcavity array chip. The assay was performed using a three antibody system in a 12 min total assay time from capture to signal generation detecting as low as 2.5 pg of PA.

**Experimental Section**

**Chemicals and materials**

The protective antigen (PA 83 is 83,000 daltons and PA 63 is 63,000 daltons), mouse anti-PA, and the goat anti-PA-IgG (or anti-PA IgG) were obtained from List Biologica (Campbell, CA). The donkey anti-goat IgG (or 2Ab-AP) was obtained from Jackson Immunolaboratories (West Grove, PA). Tris(hydroxymethyl) aminomethane (Tris), alkaline phosphatase, 1-ethyl-3-(3-dimethylamino)-propyl carbodiimide hydrochloride (EDC), lithium chloride anhydrous, sodium azide, sodium citrate, tween 20, *p*-nitrophenyl phosphate (PNPP), and *p*-aminophenol (PAP) hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin fraction powder (BSA) was from Equitech Bio Inc. (Kerrville, TX). Dried Skim milk was from EMD (Gibbstown, NJ). Potassium chloride was obtained from Aldrich (Milwaukee, WI). The *para*-aminophenylphosphate (PAPP) was synthesized, purified, and characterized in-house as previously described (32). Aqueous solutions were prepared using high-purity deionized (DI) water from Barnstead Nanopure Diamond. A Canadian maple leaf Au coin (99.99%) was purchased from USA Gold (Denver, Co) and a Cr plated W rod (Kurt J. Lesker Company) served as sources for metal deposition. Si wafers were obtained from Silicon Quest International. Polyimide (PI-2723, HD Microsystems, Du Pont) was used according to DuPont specifications. All other chemicals and materials were from VWR. All chemicals were reagent grade and used as received.

**Buffer Solutions**

The buffer solutions used in this study were as follows: (a) dilution buffer, DB: 0.1 M PBS, pH 7.4, b) rinsing buffer, RB: 0.01 M PBS with 0.05% Tween 20, c) blocking buffer (BB): 0.1 M PBS, pH 7.4 with 5% dried skim milk, 1% Tween 20, 1% Triton 100, and 0.05% NaN₃, d) 4 mM mercaptooctadecane (MOD) in acetate TBSA that consists of 0.1 M acetate buffer, pH 5.5 with 0.2 % BSA, and 0.02 % Tween, e) 0.1 M Tris Buffer, pH 9.0.

**Microcavity Chip VEG 4X1™**

The VEG4X1™ microcavity array chips (called Figure 1A) containing 50-µm diameter cavities in an 4 x 1 array were fabricated using previously published methods with a slight change in size (33, 34, 35). The VEG 4X1™ are 1 cm x 1.2 cm with contact pads that are 5 mm long and 1 mm wide located on one end of the chips. The cavities are 2 mm apart and are located 2 mm from the opposite end (Figure 1A).

**Electrode Quality Control**

After dicing, the chips were soaked for 3 to 5 days in cleaning solutions. The chips were washed thoroughly with DI water and soaked in DI water before the next step. Initial quality control procedure on all three microelectrodes in each of the 50-µm
diameter cavities consisted of sonication followed by testing for shorting and continuity of contact between the contact pads and the connectors. The electrochemical quality control consisted of electrochemical signal generation using cyclic voltammetry (CV) in a two-electrode setup in which one of the cavity electrodes served as the working electrode while the top/rim electrode served as counter and pseudoreference electrodes. The CV tests were performed using 10 µL of 4 mM K₃Fe(CN)₆ in 0.1 M KCl spread across all 4 microcavities on a chip. The microelectrodes were also tested with 4 mM PAP in 0.1 M Tris, pH 9.0 because the signal for PAP was later used to demonstrate the self-contained immunoassay detection of the *B. anthracis* PA. Microelectrodes that fouled during the immunoassay were excluded in the analysis. To eliminate electrode variability in sensitivity, only electrodes that gave similar signals (within less than 5% variation from 36 nA) when calibrated with 4 mM PAP and 4 mM K₃Fe(CN)₆ were used in all the studies.

Functionalization with the Capture Antibody, Mouse Anti-PA IgG (1⁰Ab)

Non-specific adsorption of proteins on the microcavity walls during the immunoassay was prevented with an established passivation process (33) that involved immersion in acetate TBSA with 4 mM mercaptoundecanol (MOD) for 4 h or more. This passivation process also protected the TNB, and top-layer Au from fouling during the immunoassay. To activate the RMD for immobilization of the capture antibody, 1⁰Ab, the passivation layer was selectively removed by electrochemical desorption at +0.6 V at a scan rate of 10 V/s, in 0.1 M KCl (32, 33). After cleaning the bottom electrode, the microcavity chips were soaked in 5 mL of 4 mM MUOL in ethanol for 12 h to create self-assembled monolayers (SAMs) of mercaptoundecanol (MUOL) (33). The microcavity chips were rinsed with Ar-purged ethanol three times, washed with DI water three times, and dried by careful tapping on lint-free paper. Coupling of the capture probe to the free ends of the SAMs was performed in 25 µg/mL (unless otherwise indicated) solution of ⁰Ab in the presence of 0.2 M EDC (18). The capture probe was placed across all four microcavities in the array at a volume of 10 µL, incubated at room temperature (RT) in a sealed Petri dish for 30 min and subsequently washed two times with 100 µL of washing buffer (WB). The chips were subsequently soaked in 200 µL of blocking buffer (BB) for 1 min. The series of washing, blocking, and rinsing steps were performed after exposure of each chip to the various components of the assay to prevent non-specific adsorption. Maintaining a humid environment in a covered Petri dish minimized evaporation during incubation of the immunoassay components.

Capture of the Analyte, *B. anthracis* PA (PA)

Each 1⁰Ab-immobilized microcavity was exposed to 200 nL solution of PA that was incubated for 10 min (unless otherwise stated) in a humid environment to allow the formation of mouse-1⁰Ab + PA. The resulting mouse-1⁰Ab + PA microcavity was washed, blocked, and rinsed thoroughly to remove all excess and non-specifically adsorbed analyte.

Completing the Sandwich Immunoassay Assembly with the Secondary Antibody, goat anti-*B. anthracis* PA IgG, (2⁰Ab)

The mouse-1⁰Ab + PA modified microcavities were subsequently exposed to 500 nL of 10 µg/mL 2⁰Ab (unless otherwise specified) for 10 min (or less) to form the sandwich consisting of mouse-1⁰Ab + PA + goat-2⁰Ab. The modified microcavity chips were washed, blocked, and rinsed thoroughly as in the previous section.
Immobilization of the Enzyme Labeled anti-2°Ab IgG (3°Ab~AP)

The mouse-1°Ab + PA + goat-2°Ab modified microcavities were subsequently exposed to 500 nL of 50 µg/mL anti-goat-3°Ab~AP for 10 min (or less) to complete the sandwich enzyme-linked immunoassay assembly. The anti-goat-3°Ab~AP was used to generate the electroactive enzymatic product that is used to report the concentration of the analyte captured in the microcavity. The enzyme label serves to amplify the signal by continuously generating the enzymatic product during incubation of the enzyme substrate. Over a controlled enzyme substrate incubation period, the concentration of the enzymatically generated electroactive species is directly related to the concentration of the analyte.

Enzymatic Generation of Electroactive Species, PAPR

After completion of the mouse-1°Ab + PA + goat-2°Ab + anti-goat-3°Ab~AP assembly, the chips were rinsed with DI water. The passivation on the top layer Au and middle layer electrodes were electrochemically desorbed by fast scan rates at +0.65 V. After cleaning, the middle layer electrode in each cavity was used to generate current from 4 mM PAP using a two-electrode configuration at a scan rate of 50 mV/s. The 4 mM PAP was used to generate the expected current when all the 4 mM PAPP is enzymatically converted to PAP. Enzymatic generation of PAP was achieved using 4 mM PAP in 0.1 M Tris, pH 9.0 (33) that was purged with Ar and kept from light to avoid oxidation (32, 33). A 200 nL drop of PAPP was placed on top of the modified microcavity, covered, and scanned immediately every 30 s over a period of 5 min. The enzyme conjugated to 3°Ab~AP hydrolyzed the PAPP to PAPR. The concentration of PAPR (that is directly related to the current is proportional to the concentration of the analyte present in the assay) was detected electrochemically.

Electrochemical Measurements

All electrochemical measurements were generated using a CH Instruments electrochemical workstation (model 1030) 8-channel multipotentiostat with a Faraday cage (CH Instruments, Inc, Austin, TX). The microcavity chip was kept in a humid environment to prevent evaporation during all electrochemical measurements.

The self-contained microelectrochemical cell setup inside the 50-µm diameter cavities involved the middle-layer Au as working electrode and the top layer Au as pseudoreference/auxiliary electrode (see the schematic in Figure 1B). The signals of all the cavities in the chips used in the assays were normalized to the 4.0 mM PAP signal of a selected microcavity. The activity of the complete (all components of the sandwich assay) and incomplete (lacks one or more components of the sandwich assay) assays in the modified microcavities (Figure 2) were determined by evaluating 200 nL PAPP solution that were incubated in the cavities. Working electrode potentials were kept within appropriate ranges to avoid electrochemical conversion of PAPP into PAPR (32, 33).

Effect of Capture probe (1°Ab) and Secondary probe (2°Ab) concentrations

The effect of the capture probe and secondary probe concentrations on the electrochemical signal was studied on 1 cm² gold-coated silicon wafer devices to establish the optimum concentrations leading to the highest signal following previously published procedure (32, 33). The effect of 1°Ab concentrations was studied using 0.5 to
100 µg/mL at fixed concentration of PA at 25 µg/mL, secondary probe (2⁰Ab) at 50 µg/mL, and the 3⁰Ab–AP was kept at 50 µg/mL. Using another set of devices the effect of the 2⁰Ab concentration was evaluated by using 0, 5, 10, 25, 50, and 100 µg/mL with fixed concentrations of 1⁰Ab at 25 µg/mL, PA at 50 µg/mL, and 3⁰Ab–AP at 50 µg/mL. All solutions were used at 20 µL/chip for these studies.

Results and discussion

This paper is the first time that the VEG 4X1™ (Figure 1A) arrays of microcavities with self-contained electrodes were demonstrated for 3 min analyte capture for a total assay time of 12 min. The rapid detection of B. anthracis PA at the onset of infection is very important because of the fatal consequences of the infection.

All the preliminary studies for non-specific adsorption and optimization of capture probe and secondary probe concentrations were successfully demonstrated in 1 cm² gold-coated chips that closely resembled the gold electrode at the bottom of the microcavity. Control studies in the absence of the mouse 1⁰Ab, PA, goat-2⁰Ab and analyte were performed. The results (Figure 2) indicated that the electrochemical signal from the complete assembly was significantly higher compared with signals obtained from the incomplete assemblies. These observations indicated that there was no significant non-specific adsorption of the assay components on the materials inside the cavity. Moreover, these measurements also confirmed that the signals generated from the complete assembly of capture probe + PA + goat-2⁰Ab + anti-goat-3⁰Ab–AP were due only to the captured PA. Therefore, the developed self-contained microelectrochemical signals were specifically for the capture of PA.

Optimization of the Capture Probe (1⁰Ab) and Secondary Probe (2⁰Ab–AP) Concentrations

The concentrations of the various components of the sandwich assay were optimized so that the signals were limited only by the concentration of the analyte. The signals began to plateau at 1⁰Ab concentration of 25 µg/mL (Figure 3A) when the concentration of the analyte, the secondary probes, and the enzyme-linked tertiary probes were kept constant. This indicated that the capture surface was saturated at 25 µg/mL. Hence, all succeeding experiments were performed with the optimum capture probe concentration of 25 µg/mL.

The results of the secondary probe optimization (Figure 3B) indicated that the signals began to plateau at 10 µg/mL. The increase in the signal at 25 to 100 µg/mL was an insignificant trade-off for the more than 50% additional expense in the reagent when considering the commercialization of the reported assay. For as long as the signals generated allowed linear correlations with the various concentrations studied, the use of the 10 µg/mL secondary probe served the purpose of developing a rapid, sensitive, reproducible, and inexpensive assay for the detection of B. anthracis PA. Hence, secondary probe concentration of 10 µg/mL was used in the rest of the studies.

Microelectrochemical Detection of B. anthracis PA

The optimized 1⁰Ab and 2⁰Ab concentrations were used in the assay that was transferred inside the microcavities. The sandwich immunoassay for B. anthracis PA in
photolithographically fabricated microcavities with built-in microelectrodes generated a rapid, small volume, self-contained microelectrochemical detection of antibodies against *B. anthracis* PA. The ~16 picoliter geometric volume of the ~8 µm deep cavity resulted in short time for diffusion into the capture surface at the bottom of the cavity. As a result, the self-contained nature of the assay allowed the rapid 3 min capture of *B. anthracis* PA, 3 min secondary probe immobilization, 3 min for the PAPP incubation in a 15 min total assay time.

The results given in Figure 4 indicate that it is possible to lessen the time for detection of *B. anthracis* PA. These results show that the signals from the 35 min total assay time are generally higher than the 15 min assay time. The signal for 2.5 pg *B. anthracis* PA is significantly above background even with the decreased assay time. The rapid assay may be attributed to two factors: 1) close proximity of the capture surface to the detecting electrode which is about 4 µm away; 2) minimized dilution of the enzymatically generated PAPR making it possible to record the signal even when only a few species have been formed thereby, eliminating the need for assay components incubation for 10 min incubation (3). The rapid capture, fast signal generation, and small reagent volumes are the major advantages of the self-contained microelectrochemical detection of *B. anthracis* PA.

The self-contained microelectrochemical immunoassay detection of *B. anthracis* PA for clinical diagnosis is very promising. A fast assay would lead to less assay cost and faster turn over rate for real samples for analysis. A faster turn over rate would allow earlier treatment that can reduce fatality of the infection.

**Sensitivity and Calibration curve**

A wide range of concentrations of the analyte detected is desired for clinical diagnosis to eliminate dilution steps during sample preparation that ultimately leads to rapid assay. Using analyte capture and secondary probe immobilization time of 3 min each, 500 nL of *B. anthracis* PA from at 0, 2.5 12.5, 25, 50, 250, 500, 2500, and 5000 pg were used in the assay. The results (Figure 5A) showed that the signals began to plateau at 2500 pg which indicated that this is the maximum analyte concentration that can be detected within the linear range of the assay. The plot of current signal against the concentration of PA in duplicate analysis of 500 nL solutions of PA gave a linear curve with a least square fit given by y = 0.002 nA/pg x + 0.421 nA having an R^2 = 0.96 (y is the current in nA, c is concentration in pg) (Figure 4B). The statistical limit of detection is 1 pg (for 500 nL sample) that was established using typical equation at 99% confidence level (t is 3 and there are 16 degrees of freedom) where the slope of the calibration curve is 0.002 ± 0.421 nA/pg and the standard deviation from the blank signal is 0.18 nA (10 measurements). The lowest concentration used and detected in the assay was 5 ng/mL which contained only 2.5 pg. To our knowledge, this study is the first report on the self-contained microelectrochemical immunoassay detection of *B. anthracis* PA with the lowest absolute amount detected at 2.5 pg (30, 31). The signal at this concentration is still way above the background signal. We anticipate that detection of lower concentrations with further optimization of the assay is possible.

**Conclusions**

A small volume self-contained microelectrochemical detection of *B. anthracis* PA was developed in a VEG4x1™ array of 50-µm diameter microcavities. The assay took 12
min to complete using only 500 nL sample and reagent volumes. The lowest amount of PA detected using this assay was 2.5 pg that is, to our knowledge the lowest PA amount detected to date. The system developed was rapid with low background signal using small reagent volumes. This system can be used to diagnose as well as to confirm the serologic diagnosis of anthrax infection that are important during emergency and fatal situations.

To achieve a faster, easier, and more reliable assay, full automation of the delivery of sample and reagents is being studied in cartridges that are assembled to contain the microcavity array chip and microfluidic channels. The assay will be transferred in an array with a larger number of microcavities so that multiple analyte can be detected simultaneously.

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**Figure 1.** A) The VEG 4x1™ and the VEG 8x3™ microcavity array chips. B) The SEM of a 50-µm diameter cavity with a diagram of the sandwich-type enzyme-linked immunoassay for *B. anthracis* PA.
Figure 2. Overlay of CV data for $1^0$Ab + PA + $2^0$Ab + $3^0$Ab~AP (red line, 3.5nA), PA + $2^0$Ab + $3^0$Ab~AP (blue line), $1^0$Ab + PA + $3^0$Ab~AP (brown line), and $1^0$Ab + $2^0$Ab + $3^0$Ab~AP (green line). The signal for the complete assay is significantly higher than for any of the incomplete assemblies that almost overlap with each other.
Figure 3. A) Optimization of the capture probe using PA at 25 µg/mL, $2^0$Ab at 50 µg/mL and $3^0$Ab~AP at 50 µg/mL. Capture probe concentrations used were 0, 5, 10, 25, 50, and 100 µg/mL. B) Optimization of ($2^0$Ab) concentration using capture probe concentration of 25 µg/mL, PA at 25 µg/mL, and $3^0$Ab~AP at 50 µg/mL.
Figure 4. The results of the optimization of signal with time. The signals generated from the 20 min total assay time (left or blue bar) is generally higher than the 12 min total assay time (right or brown bar).
Figure 5. A) Dynamic range of PA concentration was studied at 0, 2.5, 12.5, 25, 50, 250, 500 and 5000 pg. B) Calibration curves for the microelectrochemical detection of *Bacillus anthracis* PA.
References