Anal. Chem. **2009,** *81,* 7561–7570

Identification and Quantitation of *Bacillus globigii* Using Metal Enhanced Electrochemical Detection and Capillary Biosensor

Samuel K. Mwilu, Austin O. Aluoch, Seth Miller, Paula Wong, and Omowunmi A. Sadik*

Department of Chemistry, Center for Advanced Sensors & Environmental Monitoring (CASE), State University of New York-Binghamton, P.O. Box 6000, Binghamton, New York 13902

Alim A. Fatah

Office of Law Enforcement Standards, National Institute of Standards and Technology, Technology Services, 100 Bureau Drive, Mail Stop: 2000, Gaithersburg, Maryland 20899-8102

Richard D. Arcilesi

Battelle Memorial Institute, 505 King Avenue, Columbus, Ohio 43201

Presented herein are two detection strategies for the identification and quantification of *Bacillus globigii,* a spore forming nonpathogenic simulant of *Bacillus anthracis.* The first strategy involves a label-free, metal-enhanced electrochemical immunosensor for the quantitative detection of *Bacillus globigii* (*atrophaeus*). The immunosensor comprises of *antibacillus globigii (BG)* antibody self-assembled onto a gold quartz crystal electrode via cystamine bond. A solid-phase monolayer of silver underpotentially deposited onto the cystamine modified-Au-electrode surface is used as the redox probe. The monolayer was also generated by adsorbing silver nanoparticles on the gold electrode. When the antibody-modified electrode is exposed to BG spores, the antibody−antigen (Ab-Ag) complex formed insulated the electrode surface toward the silver redox probe. The variation of redox current was found to be proportional to the concentration of the BG spores between 1×10^2–3.5×10^4 spores/mL. A detection limit of 602 spores/mL was obtained, which is well-below the infectious dose of anthrax spores at 2.5×10^5 spores/mL. The second approach involves the use of ultrasensitive portable capillary biosensor (UPAC) to detect the spores. The capillary is an enclosed system that acts as the flow cell, the waveguide, and the solid support for immobilized bimolecular probes. An evanescent excitation generates a signal from an antigen−antibody−fluorophore complex, which propagates along the capillary and is guided to the detector. A limit of detection of 112 spores/mL was reported using the UPAC sensor. Both methods showed lower detection limits compared to the conventional ELISA. The effect of potential interferants tested using *Bacillus pumilus* confirmed the selectivity for the analyte. This work should allow the first responders to rapidly detect and quantify *Bacillus globigii* spores at concentrations that are well-below the infectious dose.

There is an increasing need to develop tools that can be used not only to detect but to also effectively combat pathogens and biochemical warfare agents (BWA). Current efforts for monitoring bioagents include immunological, nucleic acid and tissue-based detection techniques, stand-alone detectors, and purely instrumental methods.[1] Immunoassay-based detection with antibodies is perhaps the only technology that has been successfully employed for detection of bacterial cells, spores, viruses, and toxins alike.[1] Antibodies specific to virtually any compound can be produced so long as the compound of interest is able to trigger an immunological response. Most immunological techniques target surface antigens. This obviates the need for cell lysis or antigen purification prior to performing the assay.

The spores of *B. anthracis* have been widely recognized as BWA because of their ability to cause mortality in humans and their long life under unfavorable conditions. *B. anthracis* spores have a long shelf life and can survive for 2 years in pond water and 40 years or more in soil.[2] The spores are also highly heat resistant and can only be inactivated by boiling in water for 25 min[3] or by dry heat at 140 °C for 3 h.[2] Due to the low rate of inactivation when used as an aerosol, anthrax spores have been weaponized for aerosol biowarfare applications and food contamination.[4] Airborne spores remain infectious until they fall to the ground, where most of them are inactivated by sunlight. If inhaled

* Corresponding author. Fax: (607) 777-4478. E-mail: osadik@binghamton.edu.

(1) Fatah, A. A.; Richard D. Arcilesi, J.; Chekol, T.; Lattin, C. H.; Sadik, O. A.; Aluoch, A. *Guide for the Selection of Biological Agent Detection Equipment for Emergency First Responders*, 2nd ed.; Department of Homeland Security, Ed.; 2007, pp Guide 101−106.
(2) Watson, A.; Keir, D. *Epidemiol. Infect.* **1994**, *113*, 479–490.
(3) Burrows, W. D.; Renner, S. E. *Environ. Health Perspect.* **1999**, *107*, 975–984.
(4) Christopher, G. W.; Cieslak, T. J.; Pavlin, J. A.; Eitzen, E. M. *JAMA* **1997**, *278*, 412–417.

10.1021/ac900834e CCC: $40.75
Published on Web 08/18/2009

though, the spores produce diseases with high mortality and morbidity if antibiotic treatment is not administered quickly. BWA aerosols are usually invisible, odor- and taste-free, and difficult to detect after initial release.[5] Currently, clinical and strategic countermeasures have been developed including vaccines and antibiotics to treat more than 10 million people infected with anthrax.[6] Nevertheless, experts agree that an effective initial response will not only have to be robust and well rehearsed but also timely since there is only a short window to provide prophylaxis or implement effective countermeasures.[6−8] The key challenge is to actually determine when or whether an attack has occurred.[9] Usually, the initial symptoms after BWAs infection are difficult to distinguish from symptoms due to infections from more benign biological agents. Moreover, an incubation period lasting up to several days is often required before a victim displays serious symptoms characteristic of exposure to pathogens.[5] The need for molecular techniques which can identify chemical markers from known biological agents is therefore critical for early detection of pathogens and bioagents.

Traditional methods for detecting bacteria are complex and involve prolonged steps such as pre-enrichment, biochemical screening, and serological confirmation steps.[10,11] Using these methods, enumerating coliform bacteria (colony counts) is often slow requiring up to 72 h to obtain and confirm results. Furthermore, the results of such tests are often difficult to interpret[12] rendering the whole procedure impractical for onsite application where rapid detection is extremely important. Some modern techniques such as polymerase chain reaction (PCR) have been successfully used to detect pathogens with detection limits in the femto or even attogram range.[13] Hartley and Baeumner demonstrated the exquisite sensitivity of PCR by detecting DNA from a single anthrax spore.[14] Even though the PCR technique is extremely sensitive, it requires pure samples and hours of processing and expertise in molecular biology.[15−17] These involve spore/microorganism lysis to make the DNA available for amplification and removal of contaminants such as RNase or DNase enzymes and other proteins, among other purification steps. Biosensors have the ability to meet the requirements of decentralized biodefense applications as compared to the traditional assays. We hereby present two novel biosensor approaches that can assist first responders to rapidly detect and quantify BG spores.

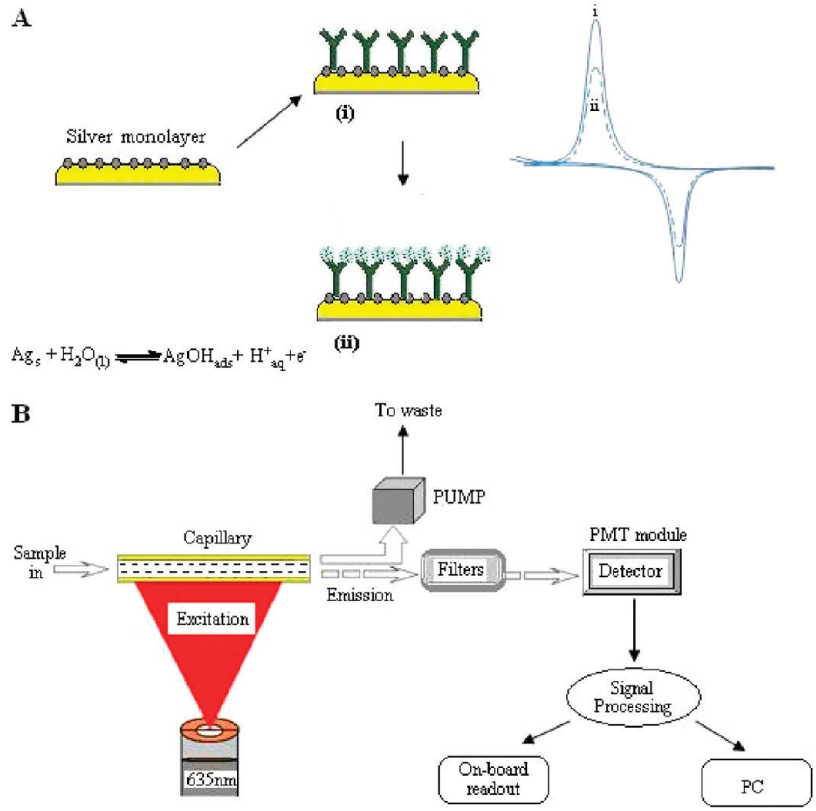
Biosensor Principles and Concepts. In designing the BG biosensor, our guiding principle is to overcome some of the challenges encountered in the development of analytical detection for monitoring BWA. BWAs are infectious at very low doses. For inhalation anthrax, the infectious dose is estimated to be 6000 spores or 2.0×10^5spores/mL.[2] Therefore BWA detection systems need to exhibit high sensitivity to be able to detect at least 2000 spores forming bacteria. Another challenge is the complex and rapidly changing environmental background that requires these detection systems to exhibit a high degree of specificity. The biosensor system must be able to identify the target analyte while rejecting or at best minimizing the detection of other nonpathogenic biological background. Finally, it must have a short response time and simple sample preparation requirements to find applications as potential biodefense systems[1] for military and first responder applications.

In metal-enhanced electrochemical detection (MED), a solid-phase monolayer of silver is deposited onto gold electrode using underpotential deposition (UPD).[18−20] The surface is then modified with an antibody as the biorecognition element. As shown in Scheme 1A, MED relies on the oxidation/reduction of the upd silver, which significantly enhances the sensitivity of the system.[19] By oxidizing the silver monolayer, and in the presence of immobilized Ab molecules, highly reactive oxides of silver are generated *in situ* causing a change in the electronic properties of immobilized monolayer. By scanning in the reverse direction, the current is measured which is attributed to the reduction of the oxide layers. If an antigen is introduced into the medium, the molecular recognition occurring is evidenced by a corresponding change in the redox properties of the silver monolayer. Changes in the redox currents are dependent on the concentrations of the antigen. The concentration-depended signals are attributed to interfacial charge transfer barrier and related "site-blocking effects" of the nonelectroactive BG antigen.

UPAC is an integrating optical sensing glass capillary platform (38 mm in length and 0.8 mm inside diameter) on which antibodies are covalently attached to the inner wall. The principle of the integrating waveguide biosensor is based on the excitation of an optical waveguide at a 90° angle and a subsequent collection of the emitted fluorescence from one end of the waveguide[21,22] (Scheme 1B). The emitted light is collected on a photomultiplier tube (PMT) or a photodiode. The waveguide/flow channel can be a silica capillary tube or a polymer. In addition to being highly sensitive, UPAC uses the selectivity of the immobilized antibody on a "plug and play" basis for molecular recognition of various bioaffinity reagents including proteins, nucleic acids, cells, and bacteria.[25,26] The configuration took advantage of the waveguide properties of the capillary to integrate

(5) Ivnitski, D.; O'Neil, D. J.; Gattuso, A.; Schlicht, R.; Calidonna, M.; Fisher, R. *Biotechniques* **2003**, *35*, 862–869.
(6) Khan, A. S.; Morse, S.; Lillibridge, S. *The Lancet* **2000**, *356*, 1179–1182.
(7) Kaufmann, A. F.; Meltzer, M. I.; Schmid, G. P. *Emerging Infect. Dis.* **1997**, *3*, 83–94.
(8) Meselson, M.; Guillemin, J.; Hugh-Jones, M.; Langmuir, A.; Popova, I.; Shelokov, A.; Yampolskaya, O. *Science* **1994**, *266*, 1202–1208.
(9) Gooding, J. J. *Anal. Chim. Acta* **2006**, *559*, 137–151.
(10) Hobson, N. S.; Tothill, I.; Turner, A. P. F. *Biosens. Bioelectron.* **1996**, *11*, 455–477.
(11) Tietjen, M.; Fung, D. Y. C. *Crit. Rev. Microbiol.* **1995**, *21*, 53–83.
(12) Ivnitski, D.; Abdel-Hamid, I.; Atanasov, P.; Wilkins, E. *Biosens. Bioelectron.* **1999**, *14*, 599–624.
(13) Higgins, J. A.; Ibrahim, M. S.; Knauert, F. K.; Ludwig, G. V.; Kijek, T. M.; Ezzell, J. W.; Courtney, B. C.; Henchal, E. A. *Ann. N.Y. Acad. Sci.* **1999**, *894*, 130–148.
(14) Hartley, H. A.; Baeumner, A. J. *Anal. Bioanal. Chem.* **2003**, *376*, 319–327.
(15) Meng, J.; Doyle, M. P. *Microbes Infect.* **2002**, *4*, 395–397.
(16) Meng, J.; Zhao, S.; Doyle, M. P.; Mitchell, S. E.; Kresovich, S. *Int. J. Food Microbiol.* **1996**, *32*, 103–113.
(17) Sperveslage, J.; Stackebrandt, E.; Lembke, F. W.; Koch, C. *J. Microbiol. Methods* **1996**, *26*, 219–224.
(18) Aluoch, A. O.; Sadik, O. A.; Bedi, G. *Anal. Biochem.* **2005**, *340*, 136.
(19) K'Owino, I. O.; Agarwal, R.; Sadik, O. A. *Langmuir* **2003**, *19*, 4344–4350.
(20) K'Owino, I. O.; Mwilu, S. K.; Sadik, O. A. *Anal. Biochem.* **2007**, *369*, 8–17.
(21) Breimer, M. A.; Gelfand, Y.; Sadik, O. A. *Biosens. Bioelectron.* **2003**, *18*, 1135–1147.
(22) Ligler, F. S.; Breimer, M.; Golden, J. P.; Nivens, D. A.; Dodson, J. P.; Green, T. M.; Haders, D. P.; Sadik, O. A. *Anal. Chem.* **2002**, *74*, 713–719.
(23) In *Pierce Biotechnology Inc.* http://www.piercenet.com/files/1776gj4.pdf.
(24) Green, N. M. *Biochem. J.* **1965**, *94*, 23C–24C.
(25) In *Pierce Biotechnology Inc.* http://www.piercenet.com/haba/habacalc.cfm.
(26) Wang, W.; Efrima, S.; Regev, O. *Langmuir* **1998**, *14*, 602–610.

Scheme 1. (A) Principle of MED Using the Ag/Ag$^+$ Redox Couple[a] and (B) UPAC Design[b]



[a] The Ab-Ag complex causes insulation of electrode surface from the redox probe which is reflected in the reduction of peak current. [b] The capillary serves as support and waveguide for integrating fluorescent signal following the Ab-Ag recognition.

the signal over an increased surface area without simultaneously increasing the background noise from the detector. The capillary provides multifunctionality—serving as the sensor template, waveguide, and microfluidics—and the sensor surface is never exposed to the outer environment. The anti-BG antibody is immobilized onto the entire inner surface of silanized glass capillaries using a bifunctional cross-linker (GMBS). A fluorescent reagent provides the recognition signal after Ab-Ag binding, and a photomultiplier (PMT) fitted at the end is used to collect the integrated fluorescence. Analysis of different analyte concentrations is attained by using multiple capillaries each coated with anti-BG antibody.

MATERIALS AND METHODS

Reagents and Stock Solutions. HEPES (N-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) hemisodium salt (99%), silver nitrate (99.999%), cystaminedichloride(2,2′-diaminodiethyldisulfide), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), sodium azide (NaN_3), tween 20 (polyoxyethylene sorbitan monolaurate), diethanolamine buffer pH 9.8, Tris-base (Tris-hydroxymethylaminomethane), trizma-HCL (99%), sodium borohydride ($NaBH_4$), silver nitrate ($AgNO_3$), phosphoric acid (H_3PO_4), and paranitrophenol (PNPP) tablets were purchased from Sigma-Aldrich company, Milwaukee, WI, USA. PNPP solution was prepared by dissolving two tablets in 10 mL to make 0.1 M in 0.5 M diethanolamine (DEA) buffer (pH 9.8) containing magnesium chloride and bovine serum albumin (BSA). Sodium hydroxide (NaOH (98.7%), sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$), sodium hydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_20$), sodium chloride (NaCl), sodium phosphate heptahydrate ($NaH_2PO_4 \cdot 7H_2O$), and sodium hydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) were purchased from J.T. Baker, Phillipsburg, NJ. Sulfo-NHS-LC-Biotin kit (Cat No. 21435) was purchased from Pierce Biotechnology Inc., Rockford, IL. The kit contained sulfo-NHS-LC-Biotin, D-Salt dextran desalting columns, HABA (2-(4′-hydroxyazobenzenebenzoic acid)), and avidin. Both goat and rabbit anti-*Bacillus globigii* IgG (TC-7014-0002) were purchased from Tetracore Inc., Rockville MD, while *Bacillus globigii* (*B. atrophaeus*) spore suspension (ATCC 9372; SUN-06) was purchased from NAMSA, Northwood, OH.

All stock solutions were prepared using Nanopure water with resistivity of 18MΩ or better. To avoid contamination all glassware, pipettes, pipet tips, equipments, and bench tops were disinfected with ethanol before and after performing the experiments. A stock solution of 0.01 HEPES was prepared by dissolving the 2.383 g of HEPES reagent in water, and the pH was adjusted to 7.2 with sodium hydroxide. PBS (phosphate buffer saline) consisted of 0.045 M NaH_2HPO_4, 0.06 M Na_2HPO_4, 0.1 M NaCl, and 0.004 M NaN_3 in Nanopure water. PBST consisted of PBS with 0.05% (v/v) Tween 20. PBSTB consisted of PBST with 1% BSA (w/v). All the phosphate buffers were adjusted to pH 7.2 with concentrated NaOH. TBST buffer was prepared by 0.045 M Trizma-HCl, 0.0055 M Tris-base, 0.1 M NaCl, 0.004 M NaN_3, and 0.05% (v/v) in Nanopure water. TBSTB consisted of TBST with 1% BSA. All Tris buffers were adjusted to pH 8.0 with concentrated HCl or NaOH. Carbonate buffer was made by titrating 320 mL of 0.1 M Na_2CO_3 containing 0.004 M NaN_3 with 0.1 M $NaHCO_3$ to pH 9.6.

Antibody Biotinylation. It was necessary to biotinylate the rabbit anti-BG IgG in order to eventually couple it to avidin-alkaline phosphatase (AV-ALP). Sulfo-NHS-LC-Biotin was used as the biotinylating reagent. According to procedures outlined on Pierce Technology,[23] the extent of labeling depends on the size and distribution of amino groups on the proteins and the amount of reagent used per protein concentration. One mg/mL of solution of rabbit anti-BG IgG required a 20-fold excess of sulfo-NHS-LC-biotin. For 1 mL of a 1 mg/mL rabbit anti-BG IgG (150,000 MW), ~13.5 μL biotin solution was used. Briefly 10 mM Sulfo-NHS-LC-Biotin was prepared by dissolving 2.2 mg in 400 μL ultrapure water (**Note**: Sulfo-NHS-LC-Biotin is moisture sensitive and should be stored at −20 °C in a desiccant. To avoid moisture condensation, the vial should be equilibrated to room temperature before opening. The NHS-ester moiety readily hydrolyzes and becomes nonreactive, thus the biotin solution should not be prepared in stock and any unused reconstituted reagent should be discarded). Biotinylation was achieved by mixing 1 mL of rabbit anti-BG (1 mg/mL) in PBS buffer with 13.5 μL of the biotin reagent, and the mixture was incubated for 1 h. The excess biotin was removed using protein desalting column Zeba (Pierce Biotechnology, Rockford, IL), which was prepared by breaking off the bottom plug and placing the column into a 15 mL centrifuge tube. The column was centrifuged at 4500 rpm to remove the storage buffer. It was then equilibrated by adding 2.0 mL of PBS buffer before injecting the protein-biotin mixture onto the column and centrifuged. This step was repeated three times, and the antibody-biotin mixture was loaded at the center of the resin bed. The column was placed in a fresh 15 mL tube and centrifuged for 2 min. The biotinylation was confirmed by the avidin-HABA assay.[24]

Avidin-HABA Assay. The HABA assay was used to quantify the amount of biotin label incorporated into the rabbit anti-BG antibody. The HABA/avidin solution was prepared by mixing 10 mg of avidin with 600 μL of the HABA solution in 19.4 mL of PBS. 900 μL of this solution was transferred into a 1 cm UV−vis cuvette, and absorbance was measured at 500 nm. 100 μL of the biotinylated antibody was added into the cuvette and mixed well. The absorbance of this solution was also measured at 500 nm. This was repeated to get a constant reading for at least 15 s. The HABA calculator[25] from Pierce Biotechnology Inc. was used to evaluate concentration of biotin per molecule of rabbit anti-BG.

UPAC Protocol. *Capillary Cleaning and Silanization.* Glass capillary tubing (with flame polished ends) of 1 mm (o.d.), 0.78 mm (i.d.), and 100 mm length obtained from Warner Instruments Inc. (Hamden, CT) was used. A 1/32 inner diameter-inch Tygon tubing was utilized for all solution delivery and waste lines. All capillary preparations were carried out at room temperature and ambient atmosphere. To facilitate application of reagents to only the interiors of the capillaries, the capillaries were attached to the tips of 1 cc Norm Ject syringes (ideal since these do not react with toluene) using 1 cm lengths of 1/32″ ID Tygon tubing affixed to Luer adapters. Simultaneous treatment of capillaries was accomplished by attaching capillaries in series using 1 cm lengths of Tygon tubing. The capillary ends were inserted in the tubing and held by the friction between the capillary and the tubing. A 2 cm length of Tygon tubing was attached to the end of the capillary distal to the syringe allowing the end of the capillary to be dipped into treatment solutions without contacting the outer surface of the capillary. Using this series assembly, solutions were flushed through the capillary with ease by drawing on the syringe plunger. Care was taken to exclude air pockets within the capillaries.

Capillaries were cleaned by incubating with 1:1 methanol hydrochloric acid solution for 30 min and dried thoroughly by purging the interiors with a gentle flow of nitrogen gas and exhaustive rinsing with nanopure water. This was then followed by incubation with concentrated sulfuric acid for 30 min before being blow-dried with nitrogen gas. The capillaries were subsequently incubated for 1 hour with 2% mercaptopropyltrimethoxysilane (Sigma-Aldrich) in anhydrous toluene. The silanized capillaries were washed with toluene and dried thoroughly with a gentle flow of nitrogen gas. The capillaries were stored in an airtight tube containing a drying agent. This type of storage is suitable for keeping the capillaries viable up to a year.

Antibody Immobilization. The silanized capillaries were cut into 3.8 cm length and attached in series using bits of silicon Tygon tubing. 3.13 mg of N-(maleimidobutyrylocy)succinimide (GMBS) from Pierce chemicals was dissolved in 50 μL of dimethyl sulfoxide (DMSO) and then diluted in a 10 mL volumetric flask using anhydrous ethanol. Using a fresh plastic syringe (1 cm^3) the GMBS solution was drawn three times through the capillary dismal, and on the last draw the capillary ends were clamped and incubated for 30 min at room temperature. This was later rinsed with anhydrous ethanol and blow-dried with nitrogen. Goat anti-BG was used as the antibody, while BG spores were used as the antigen in a sandwich type assay. 20 μg/mL of the goat anti-BG antibody in PBS buffer was drawn and incubated in the capillary distal for 1 h. The capillary surface was then blocked with bovine serum albumin (BSA) to prevent nonspecific adsorption by incubating with PBSTB buffer for 15 min. Different concentrations of the antigen were prepared (25−1000 ng/mL). The antigens were incubated in the capillary attached in triplicates for 15 min and measurements taken thereafter using a Science Workshop Interface equipped with DataStudio software from PASCO Company (Roseville, CA). Assay blanks were subjected to all the steps except addition of the antigen.

Enzyme-Linked Immunosorbent Assay (ELISA). Unless otherwise stated the ELISA vinyl microtiter plate wells (Costar) were filled with 100 μL of the reagents during incubation. Ten μg/mL of goat anti-BG prepared in 0.1 M carbonate buffer pH 9.6 was immobilized onto the microtiter plate as the capture antibody and incubated overnight at 4 °C. The plate was then rinsed several times with PBST buffer pH 7.2 and tapped dry. It was subsequently incubated overnight with blocking buffer (1 mg/mL BSA in PBS) at 4 °C. Again the plate was washed with PBST buffer and tapped dry. The plate was coated with *Bacillus globigii* spore standards ($2 \times 10^5 - 10 \times 10^5$ spores/mL) prepared in PBSTB buffer pH 7.2 and incubated overnight at 4 °C. The plate was again washed with PBST buffer and incubated with the detecting antibody, rabbit anti-BG-biotin (10 mg/mL), for 2 h at room temperature. The plate was washed with TBST buffer and tapped dry after which avidin-alkaline phosphatase prepared in TBSTB buffer (1:20000) was added. Two h later PNPP solution was added, and the color was allowed to develop (usually 30 min to 2 h). Absorbance readings were obtained using a Synergy HT Multi-Mode Microplate Reader plate reader (Biotech, VM, USA) with a fixed wavelength at 405 nm with blank correction.

Cross Reactivity. The effect of other potential interferences such as proteins or spores of the *Bacillus* family was tested using ELISA. The microtiter plate was prepared as previously described and incubated with two different concentrations of *Bacillus pumilus.* The absorbance readings obtained for *B. pumilus* spores were compared with varying concentrations of *B. globigii*. The assay control was subjected to all the steps but for the analyte.

Electrochemical Procedures. All electrochemical measurements were performed in a conventional three-electrode cell using Princeton Applied Research (formally EG&G) potentiostat (Tennessee) equipped with WinEchem data acquisition software. QA-A9M-Au(M)-50 gold resonators (9 MHz) (Area = 0.196 cm^2) purchased from Advanced Measurement Technology Inc. (Oak Ridge, Tennessee) were used as the working electrodes. Saturated silver electrode (Ag/AgCl-sat'd) and platinum wire were used as the reference and counter electrodes, respectively. A well-type electrochemical cell, made from Teflon with a capacity of 300 μL, was used for the electrochemical experiment. Solutions were purged with nitrogen for 5 min before electrochemical measurements were taken. All microgravimetric quartz crystal microbalance (QCM) experiments were performed using QCA 917-quartz crystal analyzer (Princeton Applied Research). Unless otherwise stated the QCM measurements were performed in open circuit.

Synthesis of Oleate Stabilized Silver Nanoparticles. Silver nanoparticles were synthesized according to a procedure adopted from Wang et al.[26] In this method, nanosized hydrophobic, oleate stabilized silver organosols in hexane are obtained. Briefly, 25 mL of 1×10^{-3} M $AgNO_3$ was added to 25 mL of 4×10^{-3} M $NaBH_4$ containing 2.5×10^{-4} M sodium oleate (surfactant) with vigorous stirring at 0 °C. A brown-yellowish colloidal solution stabilized by sodium oleate was obtained. To the 25 mL of this solution, 0.2 mL of 0.1 M H_3PO_4 (inducer) and 25 mL of hexane were added. A phase transfer is rapidly induced, and the aqueous phase becomes colorless. The final pH of the aqueous phase was controlled in the range of 4.0−5.0. The hydrophobic colloid is stable, and the nanoparticles retain their integrity even after the solvent is evaporated and the dried deposit resuspended in other solvents.[26]

Adsorption of Silver Nanoparticles on the Electrode Surface. The gold quartz crystal was cleaned in freshly prepared Piranha solution (H_2O_2−H_2SO_4, 1:3). It was then rinsed with water and finally with ethanol. The electrode surface was blow-dried with a stream of nitrogen. 200 μL of the colloid solution in hexane was added to the Teflon well that houses the gold quartz crystal electrode. This was left exposed under the hood for 45 min to evaporate the hexane. The silver nanoparticle modified gold electrode was characterized by transmission electron microscopy (TEM) and UV−vis spectroscopy.

Underpotential Deposition of Silver. The gold quartz crystal was cleaned in Piranha solution (H_2O_2:H_2SO_4, 1:3), rinsed with water and ethanol, and finally blow-dried with ethanol. Silver was electrochemically deposited on the gold surface using 1 mM silver nitrate solution prepared in 0.01 M acetate buffer, pH 5.3. Chronoamperometry was used to deposit the silver monolayer on the quartz crystal. The following potentiostat settings were applied: initial potential 503 mV, potential step 1 of 303 mV (49.90s), and potential step 2 of 503 mV (10.11s).[19] UPD under these conditions forms a thin film monolayer of monodispersed silver.

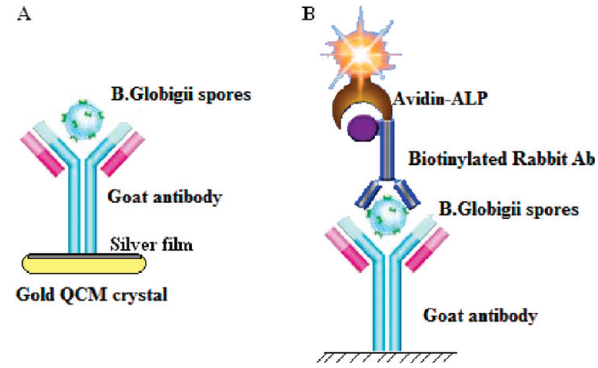


Figure 1. (A) MED immunoassay format for the detection of BG spores. A silver monolayer is underpotentially deposited (UPD) onto a gold electrode. Goat anti-BG antibody is attached to the electrode via cystamine chemistry. The redox properties of the silver layer are measured with varying concentration of BG spores. A decrease in i_p is observed before and after Ab-Ag binding. (B) Sandwich immunoassay format for the UPAC assay.

Electrode Modification. The silver−gold electrode was soaked in 0.02 M cystamine prepared in (Nanopure water) under room temperature conditions for 2 h. The electrode was then rinsed with copious amounts of water to remove physically adsorbed cystamine. The cystamine-modified electrode was soaked for 3 h in a solution consisting of 10 μg/mL of goat anti-B *globigii* and 10 mM EDC (coupling reagent) prepared in 0.01 M HEPES buffer (pH 7.2). The electrode was rinsed with the HEPES buffer and characterized by cyclic and differential pulse voltammetry using the HEPES buffer as electrolyte.

RESULTS AND DISCUSSION

The objectives of this work are 2-fold: The first was to develop an electrochemical immunoassay for *Bacillus globigii* spores, a nonpathogenic stimulant of *B. anthracis* spores. The immunoassay was made by either self-assembly of silver nanoparticles or underpotential deposition of a thin film of silver onto a gold quartz crystal, attaching the antibody via cystamine chemistry and finally exposing the probe to *Bacillus globigii* spores, as depicted in Figure 1(A). The decrease in peak current resulting from oxidation of the silver film was directly proportional to the concentration of antigen (spores) in the sample. The strategy for direct signaling using the reactivity of silver ions was recently developed in our laboratory for detection of DNA interaction.[19,20] Biospecific interaction of the antibody with the antigen is indicated using the current change from the redox reaction at the silver monolayer resulting in insulation of the electrode surface.[18,27]

The second objective was to detect the BG spores using an ultrasensitive portable capillary (UPAC). The protocol for the capillary fluorescence ELISA (enzyme linked immunosorbent assay) is presented in Figure 1(B). The UPAC sensor utilizes the wave guiding properties of the capillary to provide signal enhancement and hence a superior detection strategy when compared to conventional ELISA. The portability aspect of the UPAC sensor also offers an attractive attribute for detection of bioagents.

Antibody Biotinylation. The HABA assay is typically used to quantify biotin incorporated onto proteins.[24] HABA (4′-hydroxyazobenzene-2-carboxylic acid) is a dye with unique spectral

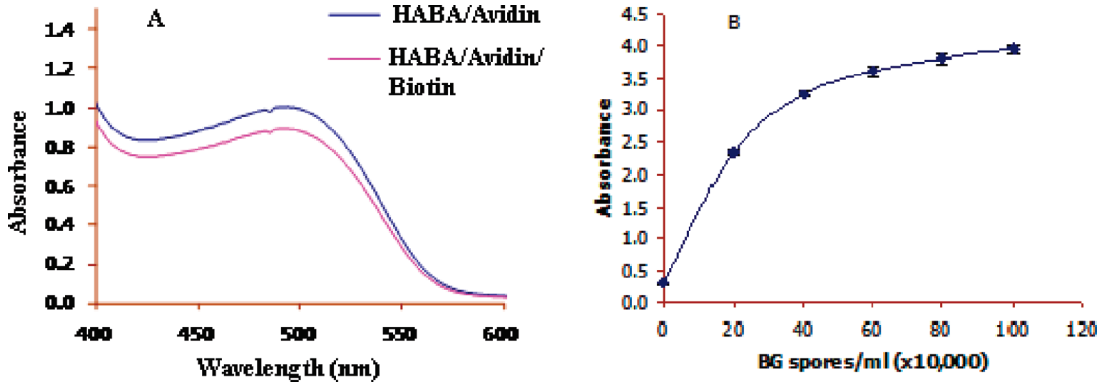


Figure 2. (A) UV−vis absorption spectra of HABA\avidin solution and HABA\avidin\biotin in PBS buffer pH 7.2. (B) A calibration plot for BG spores obtained using standard ELISA with a limit of detection of 4269 spores/mL ($n = 8$).

properties when bound to avidin which enables quantification of biotin in solution. Avidin-free HABA has an absorption peak at 348 nm, while the HABA/avidin complex has strong absorption at 500 nm. Since the affinity between HABA and avidin is relatively weak ($K_D = 5.8 \times 10^{-6}$ M) compared to the affinity between biotin and avidin ($K_D = 1 \times 10^{-15}$ M), biotin can easily replace HABA from the HABA/avidin complex, resulting in a decrease of absorption at 500 nm. The absorption of the avidin-HABA complex at 500 nm (A500) decreases proportionally with increased concentration of biotin as the HABA dye is displaced from avidin.

The absorbance measurements obtained were A_{500}H\A = 0.99 and A_{500}H\A\B = 0.88 which according to the HABA calculator corresponded to the biotin ratio of 0.56 mol of biotin per mole of rabbit anti-BG. Although this value is lower than that predicted for the procedure, it is sufficient for antibody attachment, and fewer biotin per antibody minimizes the chance of biotin's being located in the antibody-binding site.[28] Figure 2(A) shows a decrease in absorbance at 500 nm of the HABA-avidin complex when biotinylated rabbit anti-BG antibody was introduced to the solution.

Enzyme Linked Immunosorbent Assay (ELISA). A quantitative ELISA was designed to meet two objectives. First, to establish the molecular recognition between goat anti-BG IgG and *Bacillus globigii* spores and second, to compare the results with those obtained using MED and UPAC sensors. Biotinylation of rabbit anti-BG IgG was performed as described in the Experimental Section. The experimental controls were subjected to all the steps except incubation with BG spores. The controls showed significantly low signals compared to the samples. The observed signal for the control may be due to rare but probable cross-binding between the immobilized antibody and the detector antibody. However, the background signal can be minimized by using more dilute avidin-ALP solution which reduces the overall signal intensity.

A calibration plot for BG spores Figure 2(B) showed a saturation point of 4×10^5 spores/mL. Beyond the saturation point, there was no significant change in intensity when the concentration of spores was increased. This resulted in a detection limit of 4269 spores/mL. Spore aggregation due to processing, storage, and aging may affect protein binding onto

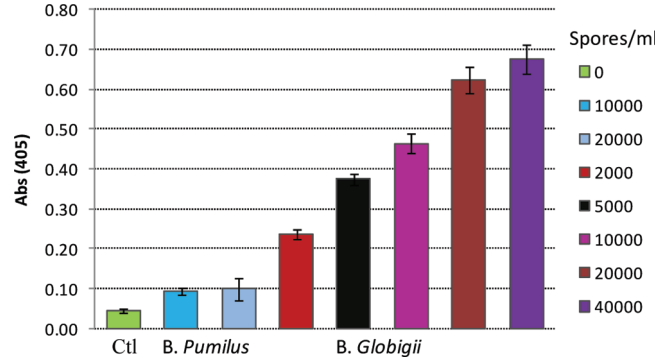


Figure 3. Effect of *Bacillus pumilus*, a close subgroup of Bacillus determined using ELISA. No significant change in the response when up to 20000 spores/mL of BP were used as the analyte. Different concentrations of BG spores show the specific response of the antibody to BG spores. $n = 5$.

the spores and hence the overall signal. In this regard, ELISA was selected to establish molecular recognition between the antibody and the analyte prior to testing the MED immunosensor. ELISA results confirmed the molecular recognition between the BG spores and the anti-BG antibodies.

Cross-Reactivity Studies. The effect of potential interferants was tested using ELISA and a subgroup of Bacillus as the analyte. *Bacillus pumilus* showed an insignificant response at concentrations five and ten times higher than the minimum concentration of BG spores, Figure 3.

MED Biosensor Design. Electrochemical sensor platforms have been developed for the detection of bacteriophage MS2,[28] microcystins,[20] and *E. coli*.[29,30] The features of electrochemical biosensors (high sensitivity, amenability to miniaturization, low cost and minimal power requirements) make them attractive for designing portable devices, but improvements are needed for more sensitive miniaturized assays. Most of the electrochemical systems rely on the use of solution based mediators to monitor biorecognition processes. The use of such mediators leads to sluggish electron transfer kinetics thereby compromising the sensor sensitivity.[31]

(27) Aluoch, A. O.; Amrute, K.; Sadik, O. A. *Sens. Lett.* **2005**, *3*, 161–163.
(28) Thomas, J. H.; Kim, S. K.; Hesketh, P. J.; Halsall, H. B.; Heineman, W. R. *Anal. Chem.* **2004**, *76*, 2700–2707.
(29) Andreescu, S.; Sadik, O. A.; McGee, D. W. *Anal. Chem.* **2004**, *76*, 2321–2330.
(30) Liu, R. H.; Yang, J. N.; Lenigk, R.; Bonanno, J.; Grodzinski, P. *Anal. Chem.* **2004**, *76*, 1824–1831.
(31) Becka, A. M.; Miller, C. J. *J. Phys. Chem.* **1993**, *97*, 6233–6239.

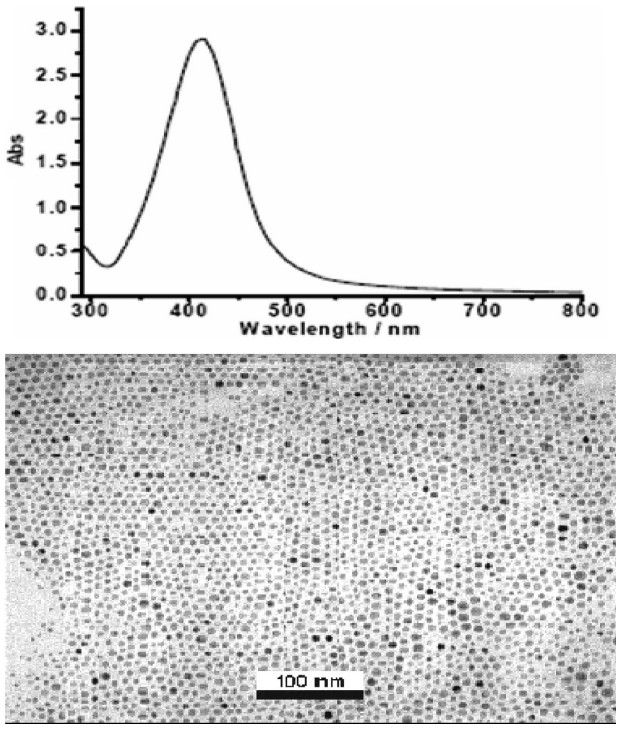


Figure 4. UV−vis spectrum and TEM picture of dried oleate stabilized silver nanoparticles employed for the MED detection.

To design the biosensor, a monolayer of BG antibodies was assembled onto the Au-electrode via cystamine chemistry.[18,27,32−34] The deposition of a thin film of silver was achieved through underpotential deposition of silver or adsorption of silver nanoparticles on the gold surface as described in the Experimental Section. Underpotential deposition is an electrochemical process whereby a single metal adlayer is electroplated onto a dissimilar metal.[35,36] The UPD phenomenon is observed when there are strong adatom-substrate interactions that are energetically more favorable than that formed during bulk electrodeposition.[35,36] Jennings et al. demonstrated the use of UPD substrates in the generation of self-assembled monolayers (SAMs) having highly organized structures compared to those that form on the parent bulk metal surface.[35,36] Indeed, alkanethiol-based SAMs on gold surfaces modified by UPD to contain a thin interlayer of silver exhibited greater stabilities toward desorption than did SAMs on native gold surfaces.[37]

Silver nanoparticles were synthesized as described in the Experimental Section and characterized by UV−vis spectroscopy and TEM, Figure 4. The oleate stabilized silver nanoparticles showed characteristic UV−vis absorption at 407 nm with a full width at half-maximum (fwhm) at 60 nm and a mean diameter of about 10 nm characteristic of monodispersed silver particles.[26,38] The MED biosensor surface was progressively characterized by scanning electron microscopy (SEM) where the bare gold electrode surface showed a relatively smooth surface with observable white strips, Figure 5A. The SEM of the electrode immobilized with silver nanoparticles was much rougher with the particles manifested as black spots, Figure 5C. This was an indication of successful adsorption of the silver particles onto the gold quartz electrode surface. These results were further confirmed by electron dispersive spectroscopy (EDS) which showed a silver peak, Figure 5D. Immobilization of cystamine on the silver nanoparticle modified gold electrode resulted in reduction of the filaments that were observed on the bare electrode. This indicated that the silver formed a covalent bond with the exposed gold surface.

In this study, silver UPD film on gold surface was utilized for self-assembly of cystamine thiol groups and subsequent coupling of *Bacillus globigii* antibody to the cystamine using EDC chemistry. Further, we found out that silver nanoparticles adsorbed onto the gold surface could play a similar role as the UPD surface and forms a thin silver film on the gold surface. Oxidizing the silver monolayer in the presence of the *Bacillus globigii* antibody resulted in reactive silver oxides accompanied by electrons producing a reversible redox signal. Incubation of the goat- anti-BG modified electrode with different concentrations of *Bacillus globigii* spores resulted in biospecific molecular recognition, forming the Ab-Ag complex. The complex formation on the electrode surface resulted in insulation of the surface toward redox probe. The extent of insulation was found to be proportional to the concentration of the BG spores, Figure 6(A).

HEPES buffer was selected as the electrolyte to avoid the interference by cations such as Cl^- that can lead to precipitation of silver ions. The formation of oxides on the submonolayer is believed to have considerable influence on the mechanisms and kinetics of anodic processes.[19,39] The results obtained show two emerging silver peaks which may be attributed to the transition of Ag(I) to Ag (II).[40] The binding and immobilization processes were confirmed by QCM measurements. The frequency change monitored in situ showed a decrease in frequency when cystamine and goat anti-BG antibodies were immobilized onto the gold quartz crystal. The frequency decrease was also observed when the electrode was exposed to 2000 BG spores/mL. The observed frequency change is consisted with Saurbrey postulation.[41] HEPES buffer used as the blank showed no significant frequency changes (ΔF) of ±5 Hz, and hence ΔFs observed were attributed to the immobilized molecules. The accompanying mass changes were evaluated and plotted indicating the corresponding cumulative adsorption densities of each successive immobilization step.[42] Figure 6(B) shows typical time courses of mass increase (ΔM) of the 9-MHz QCM, responding to the addition of HEPES buffer, cystamine, antibody, and the BG spores. The self-assembly of cystamine (MW = 260 g/mol) was confirmed from the frequency decrease (mass increase) at the QCM electrode surface corresponding to the mass change of 72 ng cm^{-2} or one monolayer. Following the immobilization of *Bacillus globigii*

(32) Sadik, O.; Ngundi, M.; Yan, F. *Biotechnol. Bioprocess Eng.* **2000**, *5*, 407–412.
(33) Katz, E.; Schlereth, D. D.; Schmidt, H.-L. *J. Electroanal. Chem.* **1994**, *367*, 59–70.
(34) Liu, Z.-M.; Yang, Y.; Wang, H.; Liu, Y.-L.; Shen, G.-L.; Yu, R.-Q. *Sens. Actuators, B* **2005**, *106*, 394–400.
(35) Chen, C. H.; Gewirth, A. A. *J. Am. Chem. Soc.* **1992**, *114*, 5439–5440.
(36) Jennings, G. K.; Laibinis, P. E. *J. Am. Chem. Soc.* **1997**, *119*, 5208–5214.
(37) Jennings, G. K.; Laibinis, P. E. *Langmuir* **1996**, *12*, 6173–6175.
(38) Petit, C.; Lixon, P.; Pileni, M. P. *J. Phys. Chem.* **1993**, *97*, 12974–12983.
(39) Chaubey, A.; Malhotra, B. D. *Biosens. Bioelectron.* **2002**, *17*, 441–456.
(40) Jerltiewicz, G. In *Interfacial Electrochemistry: Theory, Experiment, and Applications*; Wieckowski, A., Ed.; CRC Press: New York, 1999; p 966.
(41) Sauerbrey, G. *Z. Phys* **1959**, *155*, 206–222.
(42) Okahata, Y.; Niikura, K.; Sugiura, Y.; Sawada, M.; Morii, T. *Biochemistry* **1998**, *37*, 5666–5672.

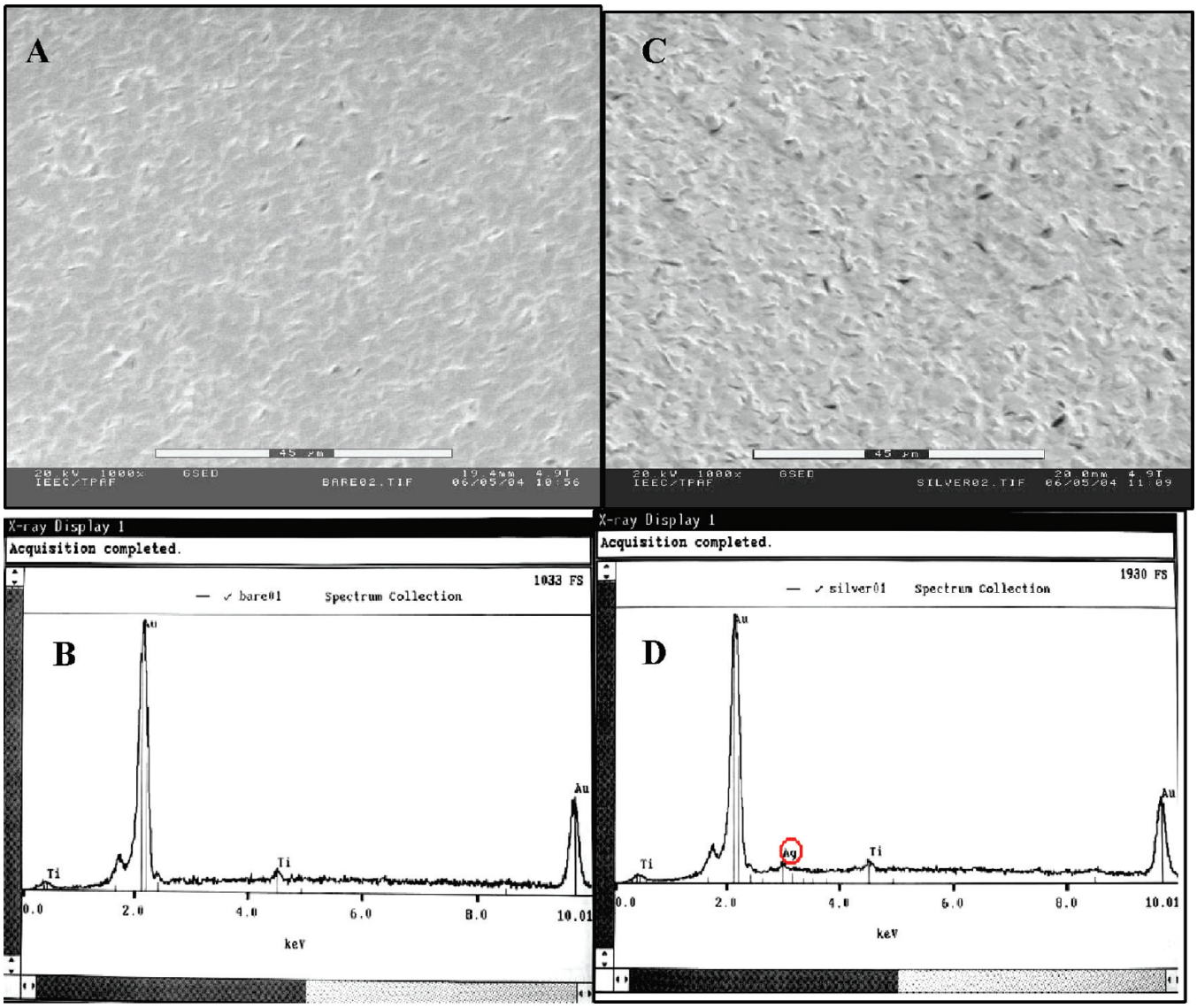


Figure 5. Scanning electron micrograph characterization (A) of bare gold quartz crystal, (B) EDS of bare gold electrode, (C) SEM of gold electrode/silver nanoparticles, and (D) EDS of gold electrode/silver nanoparticles.

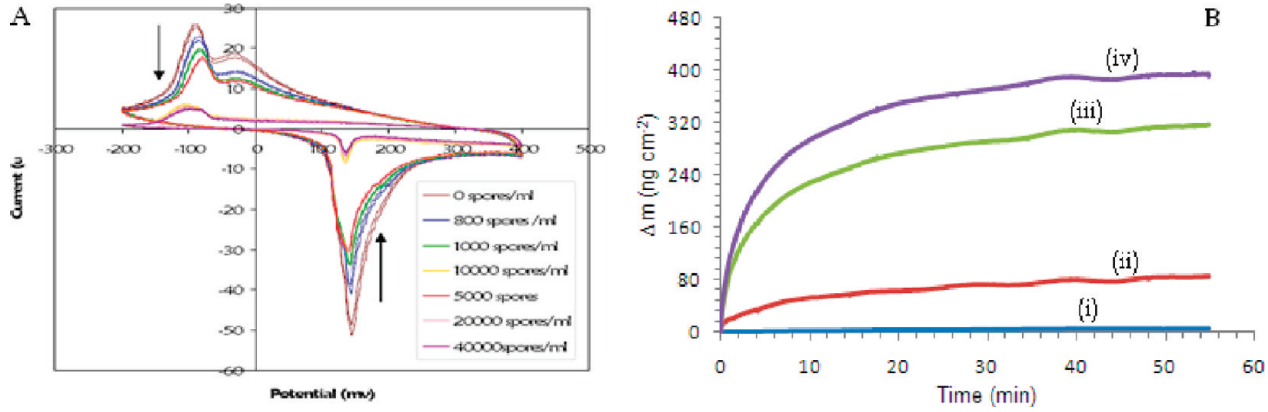


Figure 6. (A) Representative CV obtained after incubating anti-BG modified electrode with different concentrations of BG spores in HEPES buffer (pH 7.2). (B) In situ QCM measurements showing the different immobilization steps: (i) HEPES buffer, (ii) cystamine, (iii) cystamine+antibody, and (iv) cystamine+antibody+2000 spores/mL.

antibody (~150 kDa), onto the cystamine-bound QCM surface, a much larger increase in mass of 215 ng cm^{-2} was recorded. The binding between the antibody and the BG spores was further confirmed by the observed increase in mass of 68 ng cm^{-2} when the antibody-bound QCM was exposed to a solution containing 2000 spores/mL.

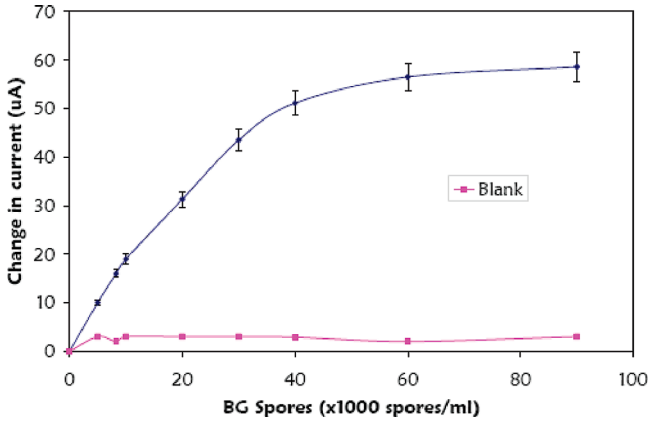


Figure 7. MED calibration curve for *Bacillus globigii* spores using cyclic voltammetry as a plot of the anodic peak current. Both cathodic and anodic peak currents decreased with increase in concentration of BG-spores. LOD=760 spores/mL (sd of blank = 0.49) and LDR=1 × 10^2-3.5 × 10^4 spores/mL.

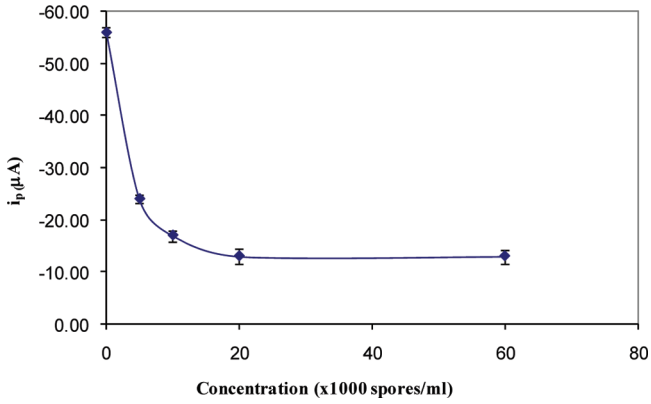


Figure 8. DPV calibration curve for BG spores using the MED concept. The insulation effects after biospecific interaction of anti-BG antibody with the spores caused a drop in peak current.

Both cathodic and anodic peak currents showed a reduction in intensity as the concentration of BG spores was increased. To further ascertain that the reduction in peak current was as a result of the antibody−antigen interactions, several cycles of CV were performed using the Au-antibody without incubation with the spores. The peak current was found to remain constant (data not shown). A plot of the change in anodic peak current versus the spore concentration showed a linear range between 100−35000 spores/mL with a detection limit of 760 spores/mL for the nanoparticle modified surface, Figure 7. A similar experiment performed using differential pulse voltammetry (DPV) gave a detection limit of 602 spores/mL (Figure 8). When the UPD surface was used, a detection limit of 854 spores/mL was recorded. The differences in detection limit between the UPD and nanoparticle modified surfaces can be explained by the higher sensitivity afforded by the nanoparticles due to increased surface area.

Interactions between Silver UPD and Electrode Surface. Interfacial phenomena occurring between the upd Ag monolayer (or the Ag nanoparticles) and the underlying gold substrate could be attributed to strong chemisorption and metallic bonding. Morphology and chemical heterogeneities could play equally prominent roles in governing the interaction of the colloidal nanoparticles and the underlying gold substrate. With metallic

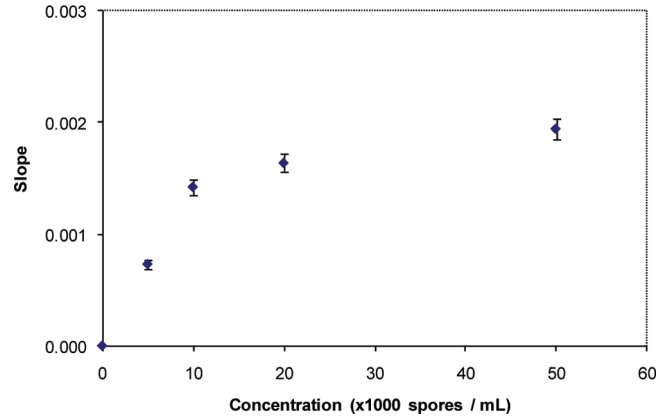


Figure 9. Calibration curve for BG spores using the UPAC sensor in single analyte capillaries. The signal slope for triplicate analyses was averaged and plotted versus the concentration of BG spores. A detection limit of 112 spores/mL was obtained.

bonding, positive cores of silver atoms are held together by a sea of electrons. These electrons are delocalized and are able to migrate through the metal lattice when an external electrical field is applied, the atoms lose their outer shell electrons to become positive ions, and a net current will result (Scheme 1A). The strength of the metallic bonding is derived primarily from the charges in the system. The larger the magnitudes of the positive charge on the metallic nuclei, the greater the strength of the metallic bonding.

This idea is strongly supported by the SEMs shown in Figure 5 revealing the continued presence of the silver islands even after several repetitive redox cycling in buffer solutions. These findings are in agreement with the work of Jennings et al.[36] in which XPS studies illustrated that silver coverage on gold was not affected by redox cycling. The persistence of the Ag peak suggests that if only van der Waals forces are involved, the Ag nanoparticles would peel off, especially during the anodic oxidation of the silver monolayer and subsequent reduction in the cathodic scan. Since fairly reversible redox peaks are obtained (Figure 6) at repetitive scanning, a stronger interaction is predicted. In that case, the stability of the antibody-modified electrode is maintained. Consequently, the observed change in the redox currents may be attributed to a multiple of fundamental microscopic processes such as the flow of charged atoms or atom agglomerates in the electrolytes, charge transfer reactions resulting from the diffusion features of the redox silver, and specific adsorption.[19] Current flow may be further affected by band structure anomalies at any grain boundaries present, especially due to the presence of additional phases provided by the avidin−biotin layers.[18−20]

Capillary Biosensor. The protocol followed in the capillary fluorescence ELISA is described in the Experimental Section. The concept of combining optical analysis with a capillary device was explored in our previous work[21,22] with results showing low detection limits and superior sensitivity compared to planar array biosensors. Capillaries can support various types of immunoassays ranging from simple sandwich types with a fluorescent tagged antibody attached to the surface, to ELISA assays where fluorescent products are enzymatically produced in solution. In binding assays, the capillary serves as a solid support for immobilizing bioreagents as well as an optical waveguide integrating the signal

Table 1. Comparison of the Analytical Characteristics of MED and UPAC Biosensors with Other Techniques Used in the Detection of *B. Globigii* or Its near Neighbor, *B. Anthracis*

technique	LOD	response time	sample preparation	references
MED immunosensor	602 spores/mL	5 min	minimal	
UPAC biosensor	112 spores/mL	30 min	minimal	
standard ELISA	4269 spores/mL	6 h	extensive	
standard PCR	250 spores/mL	12 h	extensive (PCR extraction)	43
optical leaky clad waveguide biosensor	10,000 spores/mL	40 min	autonomous	44
DOX	qualitative	30 min	minimal	45

over an increasing surface area. The complete operation and detection strategy for the capillary sensor follows an earlier model previously discussed by Ligler et al.[22] The spore concentration−response curve is based on multiple sandwich immunoassays (as described in the Experimental Section) with fresh capillaries used each time. Twenty capillaries were prepared simultaneously and incubated with goat anti-BG solution and assayed individually. The triplicate data showed good precision as indicated by the error bars in Figure 9. The linear range was observed up to 10,000 spores/mL beyond which the curve leveled off. The limit of detection for the sensor was 112 spores/mL calculated as 3 times the standard deviation of the blank. This compares well to standard PCR using BG007 primer that reportedly had an LOD of 250 spores/mL for *B.globigii* spores.[43] However PCR is not suited for rapid detection of BWA due to the amplification steps required before detection and the inherent technical skills required to perform the assay. Table 1 compares the analytical characteristics of the MED and UPAC immunosensors with other techniques used to detect *B. globigii*.

CONCLUSIONS

Novel electrochemical (MED) and fluorescence biosensors (UPAC) have been described for *Bacillus globigii* and results compared with standard ELISA. A label-free electrochemical biosensor has been developed for monitoring BG, which could be extended to other BWAs subject to the availability of appropriate antibodies. We have also developed a sandwich ELISA to confirm the molecular recognition between the goat anti-BG IgG and the BG spores and quantify the biospecific interaction for comparison with other techniques. Successful biotinylation of the goat anti-BG was also demonstrated and confirmed by avidin-HABA assay. The specificity of the goat anti-BG to the BG spores imparts appreciable sensitivity to the MED immunosensor. The response time of the MED immunosensor is about 5 min faster than conventional assays. Minimal sample preparation is required since the goat anti-BG spores are specific to antigenic proteins on the surface BG spores coating.

The MED assays have a limit of detection ~6 orders of magnitude lower than similar assays on standard ELISA platform. The CV and DPV current responses with and without the spores indicate that the concentration-depended response is due to the BG analytes. The use of silver monolayer-surface provided a unique approach for metal enhanced detection and self-assembly of antibodies onto the electrode surface. The MED immunosensor meets and exceeds the requirements of rapid, label-free biosensors for BWA detection and could serve as a benchmark for future developments of biosensors in biodefense and first responder applications. The capillary biosensor provided the best detection limit (112 spores/mL) among all the sensor platforms tested. These results were expected since the signal can be integrated over the length of the capillary via the waveguiding properties of the capillary while maintaining a constant electronic background. The response time for the UPAC sensor could take up to 30 min per single assay as compared to MED which has a response time of 5 min with minimal sample preparation. However, both formats may be used to validate each other providing a promising tool for detection and identification of potential bioterrorism agents.

(43) Kuske, C. R.; Banton, K. L.; Adorada, D. L.; Stark, P. C.; Hill, K. K.; Jackson, P. J. *Appl. Environ. Microbiol.* **1998**, *64*, 2463–2472.
(44) Zourob, M.; Mohr, S.; Brown, B. J. T.; Fielden, P. R.; McDonnell, M. B.; Goddard, N. J. *Anal. Chem.* **2005**, *77*, 232–242.
(45) Karasinski, J.; Andreescu, S.; Sadik, O. A.; Lavine, B.; Vora, M. N. *Anal. Chem.* **2005**, *77*, 7941–7949.

ACKNOWLEDGMENT

The authors acknowledge the following agencies for funding: NIST-OLED Office and Battelle via subcontract SP 0700-00-D-3180. Also acknowledged is the contribution to this work by Dr. Jason Karasinski who designed the current version of the UPAC instrument.

Received for review April 18, 2009. Accepted August 1, 2009.

AC900834E