Detection of a Surrogate Biological Threat Agent (*Bacillus globigii*) with a Portable Surface Plasmon Resonance Biosensor

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ABSTRACT

New methods and technology are needed to detect biological agents that threaten the health of humans and domestic animals. The bacterium *Bacillus anthracis*, causal agent of anthrax, has been used as a biological warfare agent. Here, we extend the work of Chinowksy et al. (2007) to the detection of a surrogate of *B. anthracis, B. globigii* (also known as *B. atrophaeus, B. subtilis* var. *niger, B. subtilis* var. *subtilis*) in a mixed sample containing two different species of *Bacillus* using a portable surface plasmon resonance (SPR) biosensor (SPIRIT 4.0, Seattle Sensor Systems). Two methods (direct capture and antibody injection) were used to determine the limit of detection for spores of *B. globigii* and to detect spores of *B. globigii* in a mixed sample containing at least one other *Bacillus* spp. Spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) with direct capture at a minimum concentration of 10^7 spores/mL, and with antibody injection at a concentration of 10^5 spores/mL. Spores of *B. globigii* were also detected when mixed with *B. pumilus* spores in the same sample at equal concentrations (10^7 spores/mL) using antibody injection. An SPR method using synthetic miRNA was adapted to the portable SPR unit (SPIRIT), and preliminary experiments suggested that the target sequence could be detected. SPR methods using nucleic acids have an exciting future in the detection of biological agents, such as *B. anthracis.* With the availability of portable instrumentation to accurately detect biological warfare agents such as *B. anthracis*, emergency

responders can implement emergency protocols in a timely fashion, limiting the amount of people and domestic animals exposed.

Dedicated to

my family and REB

for their love and support

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Attributions

Described here are the affiliations and contributions of mentors and fellow graduate students to the development and success of the chapters detailed in this thesis.

David G. Schmale, III, Department of Plant Pathology, Physiology, and Weed Science

Dr. Schmale served as the chair of the committee team, corresponding author for Chapter 2, provided direction and guidance to all described work, in addition to valuable editing advice.

Hope Gruszewski, Department of Plant Pathology, Physiology and Weed Science

Ms. Gruszewski was listed as a co-author for Chapter 2, provided her expertise in Surface Plasmon Resonance training and assisted with numerous experiments.

Craig Powers, Department of Civil and Environmental Engineering

Mr. Powers was essential in the maintenance of the Seattle Sensor SPR unit and assisted with fluidic system repairs. Mr. Powers also lended his talents to remedy SPR software issues.

Jacob Barney, Department of Plant Pathology Physiology, and Weed Science

Dr. Barney served as a committee member and provided editing advice, support, and statistical assistance as experiments developed.

Boris A. Vinatzer, Department of Plant Pathology, Physiology, and Weed Science

Dr. Vinatzer also served as a committee member and provided his guidance, support, and editing advice.

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Chapter 1. Introduction

Biodetection is the sensing of biological and or chemical substances in a particular sample or area (7). It can be applied to a variety of different fields such as food safety, environmental monitoring, and military applications (5, 23, 25). Early and specific detection of biological warfare agents are critical aspects of defense in the event of a bioterrorist attack (23, 25). The ideal biodetection system needs to be rapid, sensitive, and specific to a biological threat in addition to being portable and easy to use (6). There are many different types of biodetection that involve electrochemical, potentiometric, piezoelectric, and optical methods (6, 29). Each of these strategies has their benefits and limitations such as size, limits of detection, specificity, and how quickly detection occurs. One such method of detection, Surface Plasmon Resonance (SPR), has been identified as an optical technique that may identify biological and chemical substances rapidly, specifically, and label-free (29, 37).

SPR biosensors have been used to detect a variety of biological and chemical substances in the laboratory (7, 37). SPR uses a light-scattering effect in which polarized light passes through a prism and reflects off the backside of a metallic surface at a range of angles larger than the critical angle (45° in relation to the metallic surface) (2, 7) (**Figure 1**). At a particular angle relative to the gold surface known as the resonance angle, the intensity of the polarized light is absorbed by electrons in the gold surface to generate what is known as a surface plasmon wave that travels along the gold surface (7). This resulting plasmon is observed by a dip in the intensity level of refracted light when collected into the detector. This dip in intensity can be used to convey information about substances that come into contact with the sensor surface.

Seattle Sensor Systems has developed a unique SPR device that is small, portable and very durable (**Figure 2**). Enclosed in a Pelican hard case, it weighs roughly 1.4 kgs and is the size of a lunchbox. It is designed to harbor 4 different sensors, each with 3 different channels allowing for multiple readings on the same sensor. The 3 channels of each sensor can then be averaged together to display a single, uniform response. This SPR device utilizes a complex fluidics system with a peristaltic pump to pull or flow an injected, liquid sample across the surface of all four of the sensors. Responses are viewed in real-time, graphically; using a custom program designed using MATLAB on a dedicated laptop computer.

Chinowsky et al. (7) highlighted the potential of SPR to detect a number of agents, including the bacterium *Bacillus globigii* (a mimic of *B. anthracis*). The genus *Bacillus* contains spore-forming bacteria that can be readily found in soil and water (9, 12). *Bacillus* spp. are gram positive, rod-shaped bacteria that are able to form spores when they enter adverse or stressful conditions (27). Once the bacteria have entered their spore phase, they become resistant to many harsh conditions including ultraviolet radiation, heat, chemicals, and ionizing radiation (26). Of particular interest is the bacterium *Bacillus anthracis*, a pathogenic bacterium discovered by Robert Koch to be the causative agent of anthrax, which afflicts both humans and animals alike in various parts of the world (4, 18, 22). In addition to causing naturally occurring outbreaks of anthrax, this bacterium has been characterized as a biological warfare agent to be used against humans (9, 20, 36). One example of *B. anthracis* used as a biological warfare agent was the anthrax attacks on America in the Fall of 2001. *B. anthracis* spores were made into a powdered form and distributed through the US Postal Service in which 22 people were infected by either cutaneous or inhalation anthrax (24). There are three general types of anthrax infections; cutaneous (entering through an open wound), ingestion, and inhalation (inhalation being the most

lethal) (9). In the event of inhalation, spores are taken up by alveolar macrophages. From there, they germinate and lyse the phagolysosomal compartment, releasing vegetative cells that circulate through the body, infecting the blood and resulting in death by the combination of lethal toxin and edema toxin produced by the vegetative cells (8, 26, 27). Even before the 2001 anthrax attacks in the United States, *B. anthracis* was considered to be a biological weapon (24, 33). Because *B. anthracis* has been considered as a biological warfare weapon for an extended period of time, and is still considered to be a threat to biosecurity (9, 38), it is important to be able to characterize a biodetection system using *B. anthracis* as a potential bacterium for a bioterrorist attack.

A related bacterium, *Bacillus globigii* (also known as *B. atrophaeus, B. subtilis* var. *niger, B. subtilis* var. *subtilis*), has been used extensively by the military as a biological warfare spore simulant for a variety of different biological warfare agents for over 60 years, including *B. anthracis* (10, 35). Strains of *B. globigii* are nonpathogenic to humans and also present distinct colony morphologies when grown on agar plates (10). Other studies have used *B. thuringiensis*, a close relative of *B. anthracis*, as a simulant (3, 13). Different factors must be considered when selecting appropriate surrogates for pathogenic microorganisms, such as phylogenetic relationships, morphology, and ease of access (30). Chinowsky et al. (7) demonstrated successful detection of *B. globigii* spores as well as a number of other pathogens and toxins using an older version of a portable SPR biosensor developed by Seattle Sensor Systems. Here, we extend the work of Chinowsky et al. (7) by detecting spores of *B. globigii* in a mixed sample containing two species of *Bacillus*.

One of the many different receptors that can be immobilized on the gold surface of an SPR sensor is an antibody. Antibodies immobilized on the surface of a sensor increase the

specificity and affinity for a particular pathogen that comes in contact with the sensor by binding to recognition sites of a pathogen (e.g., on the surface of a spore). Antibodies function by identifying a number of antigens (polyclonal antibodies) or a single, specific antigen (monoclonal antibodies). Although antibodies used in SPR have been shown to be able to detect a variety of different biological substances (7, 17), antibodies have not been used in SPR to differentiate between *Bacillus* spp. One of the major challenges of detecting *B. anthracis* spores is the frequency of generating false positives. This is because the spore structures that serve as antigens, such as the spore coat and exosporium, are very similar among species (11, 15, 19, 36). Further, *B. anthracis* has a very short phylogenetic distance to other *Bacillus* spp., particularly within the *B. cereus* group (14, 40). Therefore, it is important to determine the ability of antibodies to differentially detect spores of different *Bacillus* spp.

Another receptor that can be immobilized on the surface of a sensor and a potential detection target for SPR biodetection is a nucleic acid. One advantage that nucleic acids might have over antibodies is that they allow for nucleotide specific binding that may increase the specificity to detect a pathogen (32). Further, nucleic acids are abundant within all cells, allowing for numerous biomarker sites within a particular target (31). Hana Sipova of the Jiri Homola group (Institute of Photonics and Electronics, Czech Republic) recently published a new technique that suggests highly specific and lowered limits of detection via the detection of microRNA (miRNA) (32). miRNA are short, ribonucleic acid molecules that regulate a variety of biological functions by interacting with mRNA (32). Sipova et al. (32) were particularly interested in this technique because miRNA have been implicated in a number of different deficiencies within the human body. Binding to the miRNA sequence is achieved through the use of complementary DNA oligonucleotides immobilized on the sensor surface. These immobilized

DNA oligonucleotides will bind to the miRNA in a sequence specific manner. Further, they use an antibody that recognizes a DNA*RNA hybrid complex to receive a stronger detection signal.

miRNA are typically only found in eukaryotic cells (16). However, bacteria contain several hundred copies of small RNA (sRNA), which are very similar to miRNA (16, 34). Although miRNA and sRNA do differ slightly in biogenesis, the functional equivalence between the two is uncanny (16). sRNAs are normally untranslated sequences roughly 50 to 250 nucleotides in length (16). sRNA have been implicated in the pathogenicity of many types of bacteria including *Mycobacterium* species, *Listeria* species, and *Pseudomonas* species to name a few (1, 21, 39). These sRNA sequences play varying roles in biofilm formation, quorum sensing and virulence (16). There is the potential for sRNA targets to be used in SPR to detect spores of *B. globigii*. This method might also be applied to a larger RNA molecule such as mRNA. Zezza et al. (41) describes the detection of a much larger DNA molecule (570 bp) with a small, 25-nucleotide probe. mRNA molecules specific to encoding virulence proteins within *B. anthracis* (such as the previously discussed lethal and edema factors) may be potential targets. This would allow for detection of virulent strains of *B. anthracis* that can be useful for a variety of different applications including biological warfare and soil studies.

In conclusion, biodetection is a very useful tool that can be applied to a number of different fields of study. A new method of detection known as SPR has recently been characterized as a rapid and specific technique that could revolutionize the biodetection industry. *Bacillus anthracis*, the causative agent of anthrax, poses a threat to human and animal health as both a ubiquitous soil pathogen as well as a potential biological warfare agent. New methods, such as antibody and nucleic acid detection, are needed to be able to differentially detect a pathogen such as *B. anthracis* from other closely related *Bacillus* spp. This will allow for an

earlier implementation of emergency protocols that may limit exposure of individuals to such a pathogen.

Research Objectives

1. Generate a *Bacillus* spore library.

2. Screen antibodies for specificity against *B. globigii* spores and other related *Bacillus* spp.

3. Identify and compare the limit of detection (LOD) for direct capture and antibody amplification to Chinowsky et al (7).

4. Detect *B. globigii* spores within a mixed sample containing at least one other *Bacillus* spp. spore.

5. Adapt Sipova miRNA detection protocol to portable Seattle Sensor Systems SPR unit.

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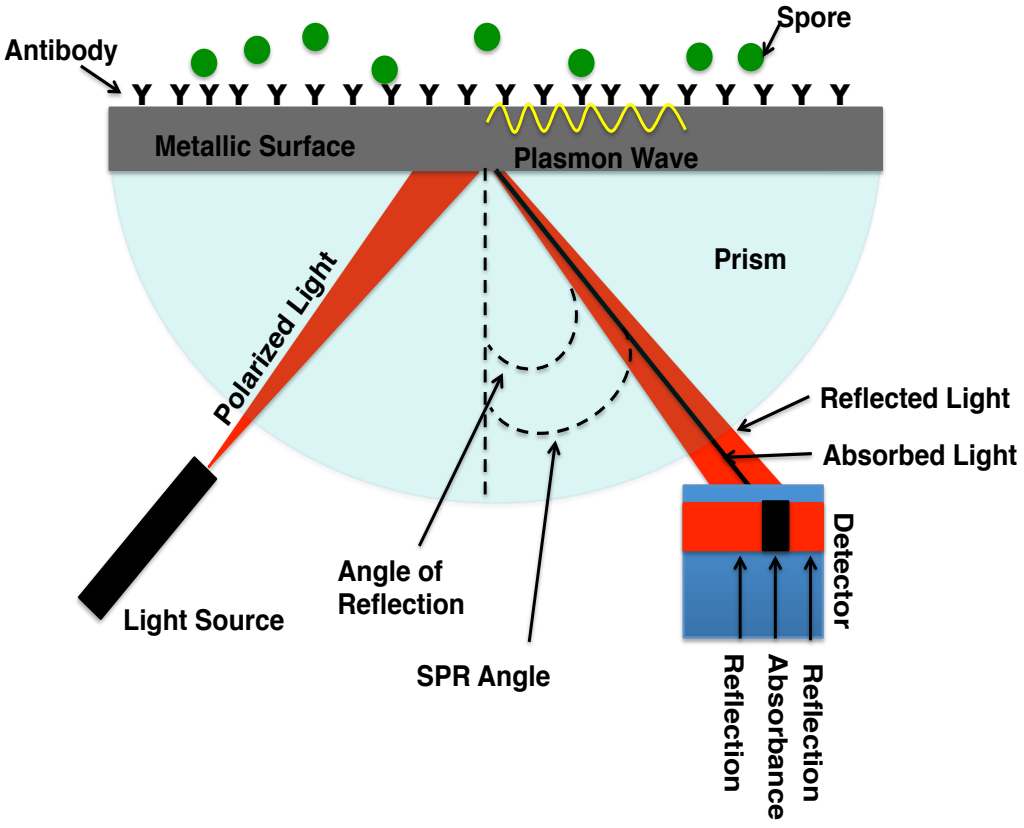

Figure 1. Surface Plasmon Resonance (SPR) Kretschmann Configuration. Each sensor consists of a polarized light source, prism, metallic surface (usually gold) and a detector. Light passes through the prism, reflects off the backside of the metallic surface and into the detector. When polarized light makes contact with the metallic surface, a plasmon wave is generated on the surface of the metal. This is observed by a loss in intensity as recorded by the detector as a dark band. Receptors are immobilized on the surface of the metallic plate to make sensors specific to a target analyte. When the target analyte (spores) interacts with the immobilized receptor (antibodies), the position of the dark band shifts and can be observed graphically. (Adapted from 27).

Figure 2. Seattle Sensor Systems Portable SPR Unit. The unit has a tube for buffer (A), a syringe for injection of a sample (B), 4 sensors (C), a peristaltic pump (D), and a waste container (E). The buffer (A) is pulled by a peristaltic pump (D) across the sensor surfaces and into a waste container (E). A syringe (B) is used to inject samples into an injection loop before the sample enters the flow cell. The flow cell is where the sample interacts with the sensor surfaces. Results are viewed on a laptop computer in real-time.

Chapter 2: Detection of a Surrogate Biological Threat Agent *Bacillus globigii* with a Portable Surface Plasmon Resonance Biosensor

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ABSTRACT

Bacillus anthracis **is a spore-forming bacterium that can cause anthrax in humans and animals. New methods and technology are needed to quickly and accurately detect potential biological warfare agents, such as *B. anthracis*. Here, we extend the work of Chinowksy et al. (2007) to the detection of a simulant of *B. anthracis* (*B. globigii*) in a mixed sample containing two different species of *Bacillus* using a portable surface plasmon resonance (SPR) biosensor (SPIRIT 4.0, Seattle Sensor Systems). Direct capture and antibody injection were used to determine the limit of detection for spores of *B. globigii,* and to detect spores of *B. globigii* in a mixed sample containing at least one other *Bacillus* spp. Spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) with direct capture at a concentration of 10^7 spores/mL, and with antibody injection at a concentration of 10^5 spores/mL. Spores of *B. globigii* were also detected when mixed with *B. pumilus* spores in the same sample at equal concentrations (10^7 spores/mL) using antibody injection, highlighting the potential for SPR to detect a target bacterium in a mixed sample of closely related species. With the availability of portable instrumentation to accurately detect biological warfare agents such as *B. anthracis*, emergency responders can implement protocols in a timely fashion, limiting the amount of people and/or domestic animals exposed.**

Keywords: anthrax, *B. anthracis*, differentiated, mixed sample, spore, SPR,

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INTRODUCTION

Early and specific detection of biological warfare agents are critical aspects of defense in the event of a bioterrorist attack (18, 20). Ideal detection systems need to be rapid, sensitive,

portable, and specific to a biological threat (3). These systems include a variety of different methods of detection (3, 20, 23): electrochemical; potentiometric; piezoelectric; and optical that have benefits and limitations including cost, portability, specificity to the agent of interest, limit of detection, and time to detection. Optical methods offer the advantage of a label-free and real-time detection of a target pathogen. One such optical method based on Surface Plasmon Resonance (SPR) has recently been miniaturized (4), and may be used to detect biological and chemical agents with a high level of specificity (18, 23, 29).

SPR uses the light-scattering effect generated when polarized light passes through a prism and reflects off the backside of a metallic surface at a range of angles larger than the critical angle (4). At a particular angle relative to the gold surface (the resonance angle), the intensity of the polarized light is absorbed in a surface plasmon that travels along the gold surface (4). The resulting plasmon is observed by a reduction in intensity at the detector. This dip in intensity can be used to convey information about the sensor surface.

Seattle Sensor Systems has developed a unique, portable SPR biosensor that can be used in manned or unmanned biodetection operations (19). The device is enclosed in a Pelican hard case and contains four different sensors, each with three different channels (for multiple readings on the same sensor). This device uses a fluidics system with a peristaltic pump to flow a liquid sample across the sensor surface. Responses are viewed in real-time using a custom program on a laptop computer.

The biological warfare agent *Bacillus anthracis* is a gram positive, rod-shaped, spore-forming bacterium that can cause the disease anthrax in humans and animals (2, 15, 17, 21, 22, 31). This bacterium has been used as a biological warfare agent (28), and is still considered to be a threat to biosecurity (33). Consequently, new methods and technology are needed to accurately

detect *B. anthracis* to allow for implication of anthrax prevention in a variety of settings, such as in the anthrax attacks of 2001.

A number of simulants (surrogates or mimics) for *B. anthracis* have been used to develop and test new biosensor technology. *Bacillus globigii*, a non-pathogen with distinct colony morphology on agar plates, has been used as a simulant in military operations for over 60 years (7, 29). Other studies have used *B. thuringiensis*, a close relative of *B. anthracis*, as a simulant (1, 12). Different factors must be considered when selecting appropriate surrogates for pathogenic microorganisms, such as phylogenetic relationships, morphology, and ease of access (24). *B. globigii* was selected as a simulant because (1) it is a non-pathogen of humans and domestic animals and (2) it does not require any special handling (4, 7).

Antibodies are common receptors that are immobilized on sensor surfaces for detection in SPR because of their specificity for target antigens and robustness (23). Further, there are a variety of different antibody-based methods that may be used in order to detect *Bacillus* spp. with SPR. In this scenario, antibodies are immobilized on the gold surface of a sensor and allow for specific detection of a particular target by recognizing and binding of antigens unique to the target. Antibodies function by recognizing a range of antigens (polyclonal antibodies) or a single, specific antigen (monoclonal antibodies). Although antibodies used in SPR have been shown to be able to detect a variety of different biological substances such as (4, 14), they have not yet been used in SPR to differentiate between *Bacillus* spp.

To address this knowledge gap, we extended the work of Chinowsky et al. (4) to further characterize a new, portable method to rapidly and accurately detect a simulant of *B. anthracis* (*B. globigii*) in a mixed sample using SPR. The specific objectives of our work were to: (1) screen antibodies for their specificity against spores of *B. globigii* and other related species; (2)

compare the limit of detection (LOD) for spores of *B. globigii* with direct capture and antibody injection SPR methods to that of Chinowsky et al. (4); and (3) detect spores of *B. globigii* in a mixed sample containing at least one other *Bacillus* spp. With the availability of portable instrumentation to accurately detect biological warfare agents such as *B. anthracis*, emergency responders can implement emergency protocols in a timely fashion, limiting the amount of people and/or domestic animals exposed.

MATERIALS AND METHODS

Generation and purification of spores of *Bacillus* spp. Spores of *Bacillus* spp. were generated following a nutrient depletion protocol developed by Harrold *et al.* (11). Briefly, plates of 3.0% trypticase soy broth (TSB) (Cat. No. 211768, Becton Dickinson, Sparks, MD) and 0.5% yeast extract (Cat. No. Y1625-250G, Sigma Aldrich, St. Louis, MO) were inoculated with *Bacillus globigii, B. circulans, B. polymyxa, B. thuringiensis* (ATCC13367)*, Lysinibacillus sphaericus* (ATCC33722)*,* and *B. albolactis,* obtained from Katherine Rodgers of the Virginia Tech Biological Sciences Department and incubated overnight at 37 °C. Resulting colonies were transferred to 3.5 mL of 3.0% TSB and 0.5% yeast extract liquid media and incubated at 37 °C overnight. The resulting suspensions were transferred to 500 mL of 0.3% TSB on a shaking incubator (Excella E24, New Brunswick Scientific, Enfield, CT) at 37 °C for 6 days.

Spores were isolated from the liquid cultures through a series of washing steps, based in part on the hydrophobic tendencies of spores (11). First, 1 L of the spore suspension was centrifuged at 6,000 g for 15 minutes. The supernatant was discarded, and the pellet at the bottom of the centrifuge tube was washed 3 times by vortexing with 30 mL of 0.1 M NaCl solution. To reform the pellet again, the solution was centrifuged at 10,000 g for 5 minutes and

the supernatant was discarded. After the washing steps, the pellet containing the spores and cell debris was resuspended in 5 mL of diH_2O water. To isolate the spores from the cell debris, a solution containing 11 mL 50% PEG-4000 (Cat. No. H273-61, Mallinckrodt Chemicals, Phillipsburg, NJ), 17 mL 3M potassium phosphate consisting of 1.76 M K_2HPO_4 (Cat. No. P-3786, Sigma, St. Louis, MO) and 1.24 M KH_2PO_4 (Cat. No. 3246, JT Baker, Center Valley, PA), and 22 mL diH_2O water was added to the pellets and vigorously shaken by hand for 15 min. The resulting suspensions were centrifuged for 2 min at 100 g, and 12-15 mL of the top layer containing the purified spores was collected. Purified spores were visualized following the staining procedure of Harrold *et al*. (11). Aliquots of 10 µL containing purified spores suspension were transferred to microscope slides, flooded with 5.0% malachite green (Cat. No. 211079, MP Biomedicals, Solon, OH), and heated over a beaker containing boiling water for 5 min. After 5 min, the malachite green was washed off with diH_2O for 30 seconds. A counter stain of 0.6% safranin (Cat. No. ICN15204025, Fisher Scientific, Hanover Park, IL) was added to visualize vegetative cells. Slides were flooded with this stain for 30 seconds and then washed with diH_2O. Microscope slides were then visualized under a light microscope, and images of spores were captured with a digital camera (e.g., Supplemental Figure 1).

Spores previously isolated (from the top layer) were diluted with diH_2O to a final a volume of 50 mL and centrifuged at 6,000 g for 30 min. The resulting pellet was washed four times with 5 mL of 0.1 M NaCl, resuspended by shaking, and centrifuged at 10,000 g for 5 min. After the fourth wash, the pellet was vortexed once more and resuspended in 5 mL of PBS (Cat. No. 14190-136, Life Technologies, Grand Island, NY). Viability of the resulting suspension was assessed by distributing aliquots of 20 µL of the final spore suspension on plates of TSB, and colonies were observed the next day.

Spore suspensions were quantified using a Neubauer haemocytometer (Cat. No. 1492, Hausser Scientific, Buffalo, NY). Ten-fold dilutions of lab-generated spores and commercial spore suspensions of *B. globigii* were prepared at 10^7, 10^6, 10^5, 10^4, 10^3 spores/mL.

SPR unit and sensor configuration. A Seattle Sensors SPIRIT portable SPR unit was used for all of the experiments. The unit had 4 sensors, two peristaltic pumps, and buffer and waste tubes. The running buffer used in all experiments was 0.1% Tween-20 (BP337-100, Fisher Scientific, Hanover Park, IL) PBST and was pulled by a peristaltic pump through the fluidics system, across the sensor surfaces, and into a waste container. A syringe was used to inject samples into an injection loop before it entered the flow cell. The flow cell is where the sample interacted with the sensor surfaces. Results were viewed in a SPR32bit custom program generated in MATLAB (acquired from Scott Soelberg) and through a Graphical User Interface in real-time.

Four SPR SPREETA sensors (Cat. No. SS01A, SensiQ, Oklahoma City, OK) were used on the SPR device. Three sensors were coated with a commercially available anti-*B. globigii* antibody (Cat. No. TC-7008-001, Tetracore, Rockville, MD). The fourth sensor was coated with a commercial anti-ovalbumin antibody (Cat. No. TC-7003-001, Tetracore, Rockville, MD) to serve as a reference sensor and an indicator of any potential non-specific binding. Gold sensor surfaces were coated with 20 µL of the antibody solution in PBS at a concentration of 3.75 mg/mL and incubated overnight at room temperature in a plastic box containing a small amount of water to prevent dehydration (hereafter referred to as a humidity box). After incubation, excess antibody solution was recovered by pipetting and stored for later use. After coating, sensors were either inserted into the SPR unit for immediate use or stored for later use with 20 µL of PBS within a humidity box at 4 °C. Detection of the specific agent was determined at the

end of the direct capture step and at the end of the amplification step as a change in Refractive Index Units (RIU) between the detection and reference sensors, consisent with the methods of Chinowksy et al. (4). Sensors were ordered with detection sensors (sensors coated with anti-*B. globigii*) as the first to interact with the sample and the reference sensor as last to see the sample for SPR Experiment 1, 2 and 3 (Table 1). To confirm that the reference sensor was not exhibiting the lowest RIU response because of a positional effect (last sensor in the array), the reference sensor was shifted to be the first to interact with the sample in SPR experiment 4: LOD with Antibody Injection.

Dot Blots. Dot blots were performed to observe the potential interaction of spores of different *Bacillus* species with commercial anti-*B. globigii* antibodies. A 0.45 μm nitrocellulose membrane (Cat. No. 162-0115, Bio Rad, Hercules, CA) was used to capture a spore suspension, and then interact with a primary antibody (anti-*B. globigii*). Binding of spores to anti-*B. globigii* was observed through the addition of a secondary fluorescent antibody (Cat. No. 31345, Thermo Scientific, Rockford, IL) that binds directly to the primary antibody (anti-*B. globigii*). The fluorescence was observed through the application of Lumi-Phos WB substrate (Cat. No. 34150, Thermo Scientific, Rockford, IL) followed by exposure using a ChemiDoc XRS+ (Bio Rad, Hercules, CA) gel doc system.

Aliquots of 20 μL of PBS spore suspensions (10^7 spores/mL) of lab-generated *B. globigii, B. pumilus* (ATCC27142), and *Lysinibacillus sphaericus* (ATCC33722), 20 μL of ovalbumin (Cat. No. 825051, Fisher Scientific, Hanover Park, IL) and PBS (negative controls) were pipetted onto the nitrocellulose membrane and left to dry for 20-30 minutes. The membrane was then blocked using TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20) with 3.5% milk for 1

hour at room temperature. After blocking, the membrane was incubated with the 1° antibody solution (2 μL anti-*B. globigii* in TBST with 3.5% milk) for 1 hour. After incubation, it was washed 3 times for 15 minutes using TBST with 3.5% milk. The membrane was then incubated with the 2° antibody solution (2 μL of anti-Rabbit fluorescent antibody in TBST with 3.5% milk) for 1 hour. After the second incubation, the membrane was washed with TBST 3 times for 15 minutes, followed by a final wash with TBS (10 mM Tris, pH 8, 150 mM NaCl) for 15 minutes. After the final washing step, Lumi-Phos WB substrate was added with a minimum of 0.125 mL per cm^2 of membrane and incubated for 3 minutes. The chemiluminesence was then observed using ChemiDoc XRS+ system.

SPR Experiment 1: Direct Capture Method Injections of *B. globigii* proceeded from 10^7 to 10^3 spores/mL sequentially, using 50 mM NaOH (Cat. No. S3201, Fisher Scientific, Fairlawn, NJ) as a regeneration buffer to remove bound spores from the immobilized antibodies on the sensor surface. Injections were conducted as independent 1 mL aliquots.

SPR Experiment 2: Antibody Injection Method. New sensors were coated as described previously, but with the addition of a blocking step. First, sensors were coated with 20 μL of anti-*B. globigii* antibodies and incubated overnight at room temperature in a humidity box. Second, antibodies were recovered for later use and rinsed with PBST by insertion into the SPR unit and flowing at a rate of 20 μL/min for 10 min. Third, sensors were removed from the SPR unit and blocked with 20 μL of PBSTB (PBS with 0.05% tween-20, 2% BSA) and incubated for 1 hour at room temperature within the humidity box. PBSTB was then recovered, disposed of

and the sensor rinsed once more with PBST by insertion into the SPR unit, flowing at a rate of 20 μL/min.

Antibody injection samples were prepared at a concentration of 10 μg/mL and rotovaped using the CentriVap Concentrator to evaporate trace reagents that may interfere with the SPR signal. Antibody samples were injected after the entire spore sample passed completely through the flow channel. This point was chosen to maximize the amount of binding of spores to antibodies, and can be observed graphically in real time as the point when the RIU signal begins to drop (e.g., **Figure 2**). Antibody samples of 1 mL were injected in the same fashion to that of the spore sample and allowed to run completely through the flow channel at a rate of 30 μL/min. After the antibody solution passed through the flow channel, as evident by another dip at the end of the curve (e.g., **Figure 4**), 1 mL of regeneration buffer at 50 mM was injected to release both antibodies and captured spores. After 2-3 minutes of flowing regeneration buffer, 1 mL of PBST was injected to return the system to the original running buffer.

SPR Experiment 3: Detection of target in mixed spore samples. Samples of both *B. pumilus* and *B. globigii* were rotovaped using a CentriVap Concentrator (Labconco, Kansas City, MO) from 1 mL original volume to 0.5 mL at room temperature. The 0.5 mL suspensions were then combined to make a total volume of 1 mL with 10^7 spores/mL of both *B. globigii* and *B. pumilus* within a single sample. Samples were then vortexed to homogenize the solution and then directly injected into the SPR device with a sterile needle and flowed across the sensors surfaces at a rate of 30 μL/min for both the direct capture and antibody injection method. However, the latter method added an additional injected antibody. Once the spore sample had completely passed through the flow channel and had the most interaction with all sensor surfaces, an

antibody solution of anti-*B. globigii* at a concentration of 10 μg/mL was injected and flowed across the sensor surface at a rate of 30 μL/min. The regeneration process of the sensors was the same for both direct capture and antibody injection methods. Once all injections had been completed, the regeneration buffer (50 mM NaOH) was injected and flowed at 30 μL/min across the sensor surface to remove bound antibodies and spores for future injections. After 3 to 5 minutes of regeneration, PBST was injected to return the system to its running buffer state.

SPR Experiment 4: LOD with Antibody Injection. Serial dilutions of *B. globigii* were generated from concentrations of 10^7 – 10^4 spores/mL using PBS as the buffer solution. Samples with a volume of 1 mL were prepared and injected in sequential order from 10^7-10^4 spores/mL and the differential between detection and reference sensors was recorded for each sensor. The mean response of the detection sensors was generated and subtracted from the reference sensor for each serial dilution injection.

RESULTS

Generation and purification of spores of *Bacillus* spp. Spores were successfully produced and quantified for *B. globigii*, *L. sphaericus*, *B. albolactis*, *B. thuringiensis*, *B. circulans*, and *B. polymyxa*. Figure 1 of the supplemental figures shows an image of *B. globigii* spores that were successfully generated in the lab, stained with malachite green.

Dot Blots. Results from the dot blots showed that lab-generated spores of *B. globigii* interacted with anti-*B. globigii* antibodies at concentrations of 10^7 and 10^5 spores/mL (**Figure 1**). Spores of *L. sphaericus* and *B. pumilus* did not interact with anti-*B. globigii* antibodies at these same

concentrations (**Figure 1**). Ovalbumin and PBS were used as negative controls to show any non-specific binding and illustrate residual background issues.

SPR Experiment 1: Direct Capture Method. Lab-generated spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) at 10^7 spores/mL with direct capture using the SPR unit (**Figure 2**). Spores were not detected with this SPR method at concentrations lower than 10^7 spores/mL (data not shown). However, there was not a statistically significant difference between detection and reference based on 4 sequential, replicate injections ($P = 0.51$). This might be explained by sensor attrition of the signal over multiple injections (**Figure 3**), at least in part. Figure 2 illustrates an injection of 10^7 spores/mL of *B. globigii* on a new sensor with freshly coated antibodies. After 21 minutes, there was a mean response of 123.39 RIUs for sensors coated with anti-*B. globigii*, compared to the reference sensor response of 108.13 RIUs (**Figure 2**). The time of 1476 seconds was selected because it corresponds to the time at which the maximum amount of spores was expected to interact with the antibodies on the sensors surface and was consistent with the end of detection observed in Chinowsky et al. (4). Further, as sensors were exposed to additional injections in sequence, the antibodies would become less sensitive to incoming injections, making detection more challenging to observe.

SPR Experiment 2: Antibody Injection Method. An injection of anti-*B. globigii* into the SPR unit further increased the RIUs above the reference sensor (**Figure 4**). Figure 4 shows an injection of *B. globigii* spores with an injection of anti-*B. globigii*. Figure 5 illustrates an injection of *B. pumulis* spores followed by an injection of anti-*B. globigii*. The time from injection to the assessment of detection with spores of *B. globigii* and *B. pumilus* was estimated

to be 24 minutes following the Chinowsky et al. protocol. The mean response of the anti-*B. globigii* sensors with an injection of 10^7 spores/mL of *B. globigii* was 137.57 RIUs, compared to 126.57 RIUs for the reference sensor and that anti-*B. globigii* sensors were significantly greater than anti-ovalbumin sensors over 2 replicates ($P = 0.03$). For a single injection of spores of *B. pumilus*, sensors 4, 3 and 2 had signal responses of 137.6 ± 7.3, 136.03 ± 17.5, and 135.1 ± 8.1 respectively, while sensor 1 had a signal response of 132.97 ± 5.1 (**Table 1**). These results show that the injection of an antibody post injection of spores of *B. pumilus* did not increase the difference between detection and reference sensors (**Figure 5**). Though these results were based on a single injection (**Table 1**), the non-detection of spores of *B. pumilus* was also supported by the dot blot results that showed spores of *B. pumilus* were not detected with sensors coated with anti-*B. globigii* (**Figure 1**).

SPR Experiment 3: Detection of target in mixed spore samples. Spores of *B. globigii* were not detected when mixed with *B. pumilus* at equal concentrations at a concentration of 10^7 spores/mL using direct capture (data not shown), but were detected in a mixed sample at equal concentrations using antibody injection (**Figure 6**). For one particular sample using the direct capture method, detection sensors averaged a signal response of 572 RIUs as compared to the reference sensor response of 577 RIUs. This could be in response to the elevated concentration of *Bacillus* spores in the injected sample. Experiments were repeated 4 times and showed similar results with detection and reference signal responses of ~600 RIUs. All detection sensor responses were below the reference sensor, indicating no specific detection of *B. globigii* from *B. pumilus* using the direct capture method. Thus, the sensors are responding to the increased number of spores as indicated by the elevated RIU responses, but were unable to differentiate

and detect *B. globigii* from *B. pumilus*. However, antibody injection was able to detect *B. globigii* when mixed with *Bacillus* spp. in the same sample. When anti-*B. globigii* was injected, there was a sharp drop in RIU signal, rather than an increased or amplified signal observed in pure *B. globigii* injections (**Figure 4**). However, the response leveled off and detection sensors diverged from reference sensors at the 100 second time mark. In Figure 6, detection sensors produced an averaged signal response of 140.18 RIUs while the reference sensor response was 123.3 RIUs at the 730 second time mark. Results showed that anti-*B. globigii* sensors were significantly greater than anti-DON sensors over 2 replicates ($P < 0.01$). The overall time it took to achieve detection beginning with the spore sample injection was 27 minutes. These RIU signal responses and the detection/reference sensor differential are similar to that of an injected sample of pure *B. globigii* at 10^7 spores/mL followed by antibody amplification (**Figure 4**).

SPR Experiment 4, LOD with Antibody Injection

The injection of anti-*B. globigii* after sample injection lowered the LOD from 10^7 to 10^5 spores/mL (**Figure 7)**. Figure 7 shows the dose response of spores of *B. globigii* from 10^7-10^4 spores/mL using antibody injection. Figure 7 illustrates the difference between the detection and reference sensors as the concentration of spores also decreases. Further, there is also a maximum difference between detection and reference sensors, as evidenced by the logarithmic trendline (**Figure 7**). Using the trend equation, $y = 17.48(1-e^{((-1.04 \times 10E-05)\times x)})$, our LOD for an RIU difference of 10 was 8.16×10^4 spores/mL and for an RIU difference of 5, our LOD was 3.24×10^4 spores/mL (**Figure 7**).

DISCUSSION

Surface Plasmon Resonance (SPR) has recently been miniaturized, and may be used to detect biological and chemical agents with a high level of specificity (4, 23, 32). New, portable SPR methods need to be developed and tested for the rapid and accurate detection of biological threat agents. Here, we show that a portable SPR biosensor can be used to detect spores of *B. globigii*, a surrogate for *B. anthracis* (4, 7, 29) using commercial antibodies at a concentration of 10^5 spores/mL. Chinowsky et al. (4) identified their LOD concentration to be 9.0×10^4 CFU/mL. Their LOD, however, was based on CFUs which represents viable (culturable) spores in their suspension. It is likely that some of the spores in their suspension were not viable, and since their SPR method could not distinguish between dead and living spores, their reported LOD likely underestimates the true number of spores in suspension. This work could extend to the rapid, onsite detection of other biological threat agents in a variety of emergency response scenarios.

Commercially produced antibodies were able to differentially detect *B. globigii* from *B. pumilus.* Dot blots were inoculated with *B. globigii* and other *Bacillus* spp. and showed that anti-*B. globigii* specifically binds to *B. globigii* and not to *B. pumilus*. The dot blot results were extended to SPR; sensors coated with anti-*B. globigii* showed strong responses to spores of *B. globigii* and not *B. pumilus* using direct capture and antibody injection. This differential response is noteworthy, since many *Bacillus* spp. have similar proteins on the surface of their spores (8, 16) which could present a challenge in the accurate detection of the target (34). Previous work has shown that monoclonal antibodies have been characterized to detect *B. anthracis* from several other *Bacillus* spp. with separate injections using SPR and subtractive inhibition assays (32). However, this technique has not been challenged with mixed sample injections containing more than one *Bacillus* spp., nor applied to direct capture or antibody injection methods. Some studies have attempted to detect targets in complex samples/matrices

from environmental samples using SPR (10, 26). Little work has been accomplished in the differential detection of a target *Bacillus* spp. within a mixed sample of other *Bacillus* spp.

Spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) with direct capture at a concentration of 10^7 spores/mL. It is important to note that previously exposed sensors were not as sensitive as freshly coated sensors in detecting spores of *B. globigii* (**Figure 3**), thus this observed LOD might vary if the sensors had been previously exposed to spores. The LOD of 10^7 spores/mL is high compared to other biodetection methods (3). However, these results are in agreement with previous literature, indicating that larger molecules (in our case higher concentrations) are required for direct detection to be successful (23). SPR using direct capture was unable to differentiate a mixed sample of spores of *B. globigii* and *B. pumilus* at a concentration of 10^7 spores/mL. Thus, additional methods (such as antibody injection, discussed next) were warranted to further decrease the LOD and detect the target in a mixed sample.

When antibodies were injected at the phase in which spores had achieved maximum contact with immobilized antibodies, spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) at a concentration of 10^5 spores/mL. We identified a logarithmic trend between sample concentration and the difference in RIUs between the detection and reference sensors. This might be explained by a saturating effect with how many spores can be captured as well as how many injected antibodies can bind to captured spores on the sensors. Using the log trendline equation, we can extrapolate 2 different LOD with RIU differences of 10 and 5. With a minimum RIU difference of 10, our LOD was 8.16×10^4 spores/mL. With a minimum RIU difference of 5, our LOD was 3.24×10^4 spores/mL.

The logic behind injecting an antibody stems from the generation of what is known as a 'sandwich assay' with the constituents of immobilized antibody, spore, and injected antibody (4, 10). The addition of the injected antibody, and the resulting formation of the 'sandwich,' increases the molecular weight of the binding complex, further increasing the RIU signal (23). The use of the sandwich assay reduced the LOD by 100 fold. The 'sandwich assay' or antibody injection method is a technique that has been used to strengthen the detection signal and provide more specific signal responses for many different biodetection methods (3, 4, 10).

The work described here provides evidence that SPR might be suitable for the detection of *B. anthracis* spores in a proposed biolocial attack. In a real world scenario, the infectious dose for *B. anthracis* in a building is dependent on factors such as room size, amount of air circulation, and pulmonary ventilation (6). The mathematical model developed by Fennelly et al. (6) suggests that if 10,000 people are exposed to between 77,500 – 275,000 *B. anthracis* spores for 1 hour in a moderate-size room (61 m^3), 2-5% of exposed people will contract inhalation anthrax, depending on the pulmonary ventilation rate. This model suggests that if all spores within this hypothetical room could be collected and suspended into a 1 mL sample, detection of *B. anthracis* spores would be achieved for the upper 89% of this spore range (100,000-275,000). Previous work has identified the use of SPR in conjunction with bioaerosols (19, 30). However, it is possible to become infected with *B. anthracis* with as few as 100 spores and possibly even 1-3 (9). This is an obvious limitation of the technology described here, and likely an unrealistic expectation with antibody methods in the current SPR configuration that we used.

There is the possibility of pushing the technology to detect an even lower concentration of spores, which would allow for less collection time and potentially reduce exposure of people to a potential biological attack with *B. anthracis*. Additional experiments using a higher

concentration of injected antibodies may be able to lower the LOD into the lethal dose range for *B. anthracis*, but this was not tested in our experiments. With a higher concentration of injected antibodies, saturation of the captured spores will be more likely to occur, which may further increase the RIU signal of detection sensors (4). Nevertheless, there would be some tradeoffs between how effective high concentrations of antibody injection would be at decreasing the LOD and the overall cost of purchased antibodies. Eventually, captured spores would be saturated with antibodies, leading to an excess or waste of injected antibodies at higher concentrations. Previous work has also used direct capture and antibody injection to detect concentrations of *B. globigii* spores at 9.0×10^4 CFU/mL and detailed the use of subtractive inhibition assays in SPR to obtain low a LOD of 10^4 CFU/mL (4, 32). We identified our LOD in spores/mL, which is a true number of the target in suspension. However, this also has limitations because both viable and non-viable spores are detected; some of the spores may not be able to cause disease. Thus, our LODs are consistent with previous literature (4, 32).

Spores of *B. globigii* were also detected when mixed in a sample with *B. pumilus* at equal concentrations (10^7 spores/mL) using antibody injection. The injection of the antibody post-mixed sample injection produced a sharp drop in RIUs, eventually leading to divergence between detection and reference sensor signals (**Figure 6**). One possible explanation for this sharp drop might be explained by the removal of spores that are interacting with the sensor surface and that are not necessarily captured; the injection of the antibody produced a sandwich that pushed all non-captured spores out of the system and amplified the detection sensor signals by specifically binding to captured *B. globigii* spores. Though SPR has been used to identify targets in environmental samples (10, 26), additional work is needed to determine the limitations of this

method in complex matrices containing other potential contaminants that might interfere with the antibodies.

Future SPR work might leverage assays using nucleotides and immunomagnetic beads (27), which could ultimately enhance specificity and lower limits of detection. One promising approach might be the use of small RNAs (sRNAs) or micro RNAs (miRNAs) that exist in high numbers in individual cells (e.g., 13, 26). Immobilized oligonucleotides have the potential to be more advantageous than immobilized antibodies because binding would be sequence specific and there are multiple copies of sRNA within a single spore (25, 26). sRNA detection has the potential for more oligonucleotide receptors to be bound with a lower spore concentration as compared to antibody receptors. This approach, however, may create some additional challenges, such as optimization of spore lysis and subsequent harvesting of sRNA prior to degradation by enzymes such as RNases.

CONCLUSIONS

Methods using a new, portable SPR biosensor were extended from the work of Chinowsky et al, (4) to detect a simulant of *B. anthracis* (*B. globigii*) in a mixed sample. Two methods (direct capture and antibody injection) were used to determine the limit of detection (LOD) for spores of *B. globigii* and to detect spores of *B. globigii* in a mixed sample containing at least one other *Bacillus* spp. Spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) with direct capture at a concentration of 10^7 spores/mL, and with antibody injection at a concentration of 10^5 spores/mL and are consistent with previous literature (4, 32). Spores of *B. globigii* were differentiated from *B. pumilus* in a mixed sample at equal concentrations (10^7 spores/mL) using antibody injection. With the availability of portable

instrumentation to accurately detect biological warfare agents such as *B. anthracis*, emergency responders can implement emergency protocols in a timely fashion, limiting the amount of people and/or domestic animals exposed. Future work could integrate nucleotide-based assays (such as sRNAs) to improve specificity and decrease the LOD.

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Experiment	SPR Run	Sensor 4	Sensor 3	Sensor 2	Sensor 1	Δ RIU (avg.)
Direct Capture	**Run 1**	125.1	125.4	119.7	108.1	15.3
Bg spores	**Run 2**	122.0	127.6	142.2	128.9	1.7
	Run 3	117.1	107.3	99.1	110.1	-2.2
	Run 4	108.4	110.9	116.0	111.0	0.7
Antibody Injection	**Run 1**	136.8	134.9	141.0	126.6	11.0
Bg spores	**Run 2**	114.0	128.3	128.0	108.3	15.2
Antibody Injection	**Run 1**	137.6	136.0	135.1	133.0	3.3
Bp spores						
Antibody Injection	**Run 1**	142.9	139.7	137.9	123.3	16.9
Mixed Spores	**Run 2**	130.7	123.1	125.7	114.9	11.6

Table 1. Raw RIU Data for *B. globigii*, *B. pumilus*, and Mixed Sample Detection with Antibody Receptors. For the experiment Direct Capture *B. globigii* (Bg) spores, sensor 4, 3, and 2 were coated with anti-Bg. Sensor 1 was coated with anti-ovalbumin (Ova). There was no significant difference between detection and reference sensors over the 4 sequential replicates ($P = 0.51$). For Antibody Injection of Bg spores, sensors 4, 3, and 2 were coated with anti-Bg and sensor 1 was coated with anti-DON. We noted that in this case, there was a significant difference between detection and reference sensors over the 2 replicates ($P = 0.03$). For Antibody Injection *B. pumilus* (Bp), sensors 4, 3, and 2 were coated with anti-Bg and sensor 1 was coated with anti-DON. For Antibody Injection Mixed Spores, sensors 4, 3, and 2 were coated with anti-Bg and sensor 1 was coated with anti-DON. There was a significant difference between detection and reference sensors ($P < 0.01$).

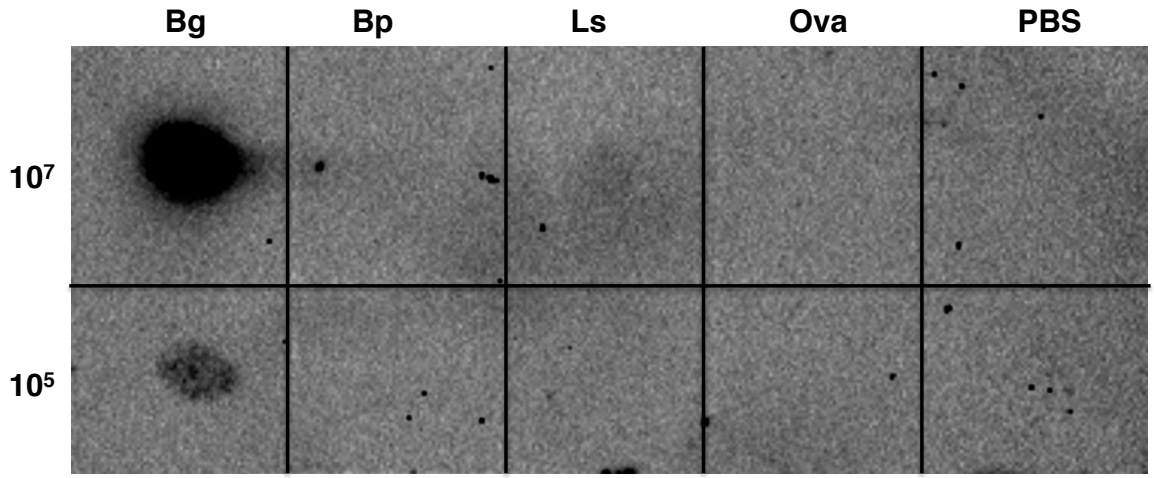

Figure 1. Anti-*B. globigii* dot blot. A dot blot showing the potential interaction among commercial antibodies of *B. globigii* and spores of *B. globigii* (Bg), spores of *B. pumilus* (Bp), spores of *L. sphaericus* (Ls), Ovalbumin (Ova), and PBS buffer. The membrane was dotted from left to right with lab-generated *B. globigii*, commercial *B. pumilus*, lab-generated *L. sphaericus*, Ovalbumin and PBS. Ovalbumin and PBS were used as negative controls to indicate non-specific binding and residual background interference. The top row was dotted at a concentration of 10^7 spores/mL and the bottom row was dotted at concentration of 10^5 spores/mL. Anti-*B. globigii* demonstrated a strong interaction with lab-generated *B. globigii* at both 10^7 and 10^5 spores/mL. Spores of *B. pumilus* and *L. sphaericus* did not interact with anti-*B. globigii*.

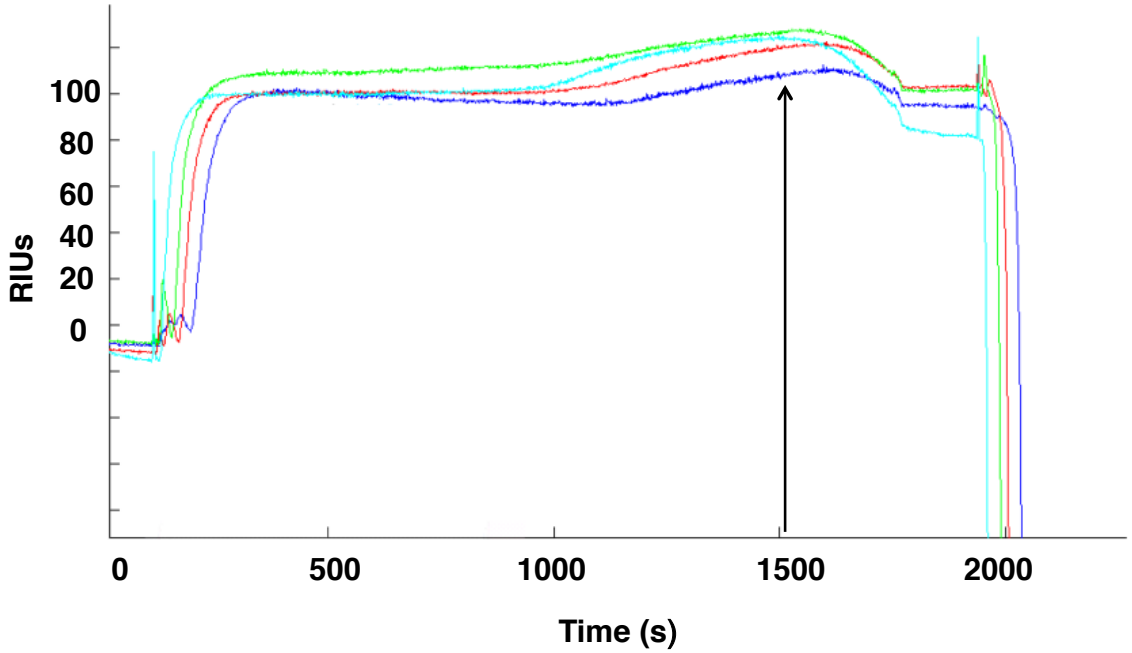

Figure 2. Direct capture detection of *B. globigii* spores. A sensorgram showing the reaction of sensors coated with physisorbed antibodies to Bg and Bg spores at a concentration of 10^7 spores/mL. Sensors coated with anti-*B. globigii* are represented by (4) light blue (middle top), (3) green (top), and (2) red (middle bottom) lines shown above. Anti-Ovalbumin, the reference sensor, is represented as the (1) dark blue line. The initial upshift indicates the injection point of the sample, followed by a leveling off where the rate of binding and unbinding of sample to immobilized antibodies reaches an equilibrium stage. The sharp drop in signal is the point where regeneration buffer (50 mM NaOH) is injected to remove bound spores from the immobilized antibody to allow for multiple injections. The signal point was chosen to be at the time stamp just before the first dissociation event (time = 1476 s) and indicated by the arrow. This point was chosen because it allows for the maximum amount of binding of spores to the immobilized antibodies, generating the strongest signals for all sensors. Sensor data reflected at this point were 125.07 ± 3.09 for sensor 4 (light blue), 125.4 ± 6.58 for sensor 3 (green), 119.7 ± 11.63 for sensor 2 (red) and 108.13 ± 13.92 for sensor 1 (dark blue). Spores of *B. globigii* spores were detected using this method, since all detection sensors are >10 RIUs above the reference sensor (4).

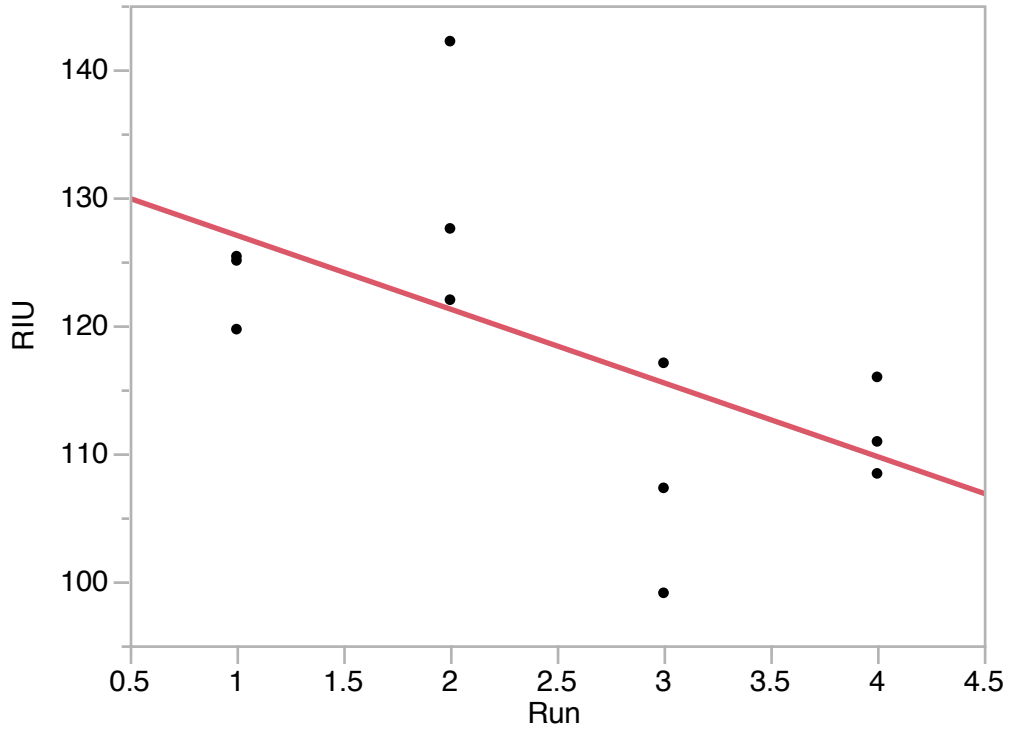

Figure 3. Direct capture method sensor attrition. This graph shows that over a series of 4 injections, there is a a significant decrease in RIU response ($P = 0.04$). The linear trend equation corresponds to RIU = 132.79667 - 5.7606667*Run with an R^2 value of 0.35.

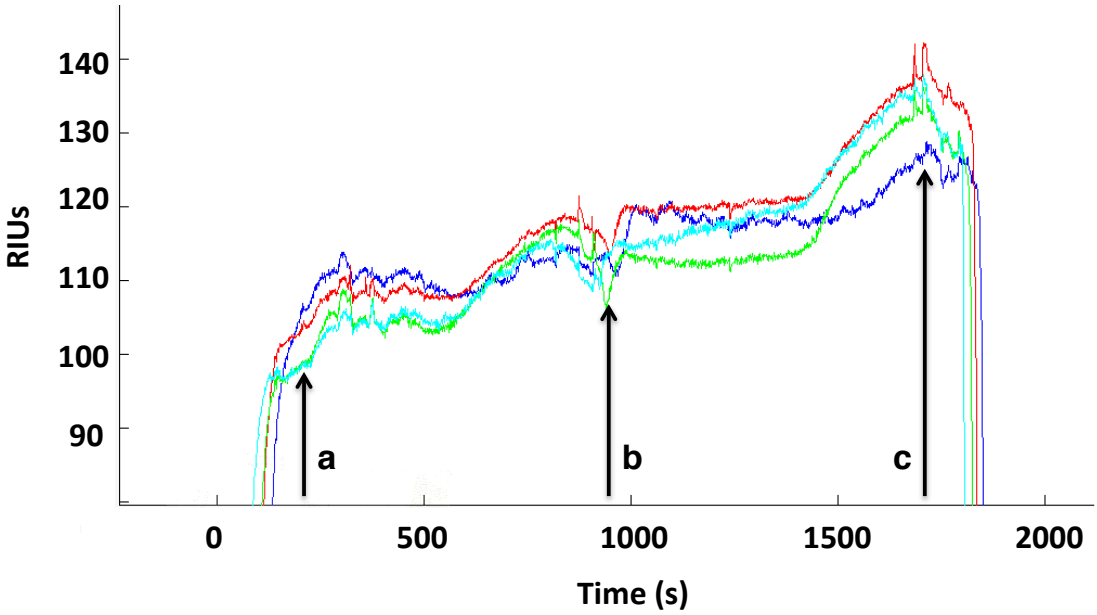

Figure 4. Antibody injection with *B. globigii* spores. A sensorgram showing an injection of spores of *B. globigii* at 10^7 spores/mL, followed by an injection of *B. globigii* antibodies (anti-Bg) at a concentration of 10 µg/mL. Arrow (a) indicates the point of spore injection, arrow (b) indicates the injection of anti-Bg, and arrow (c) indicates the detection point. Sensor 4 (light blue) is coated with anti-Bg, sensor 3 (green) was coated with anti-Bg, sensor 2 (red) was coated with anti-Bg and sensor 1 (dark blue) was coated with anti-DON. Sensor 4, 3, and 2 have signal responses of 136.8 ± 2.6, 134.9 ± 12.3, and 141.0 ± 0.9 respectively, while sensor 1 has a signal responses of 126.6 ± 6.0. These results show that the injection of an antibody post injection of a Bg spore sample increased the difference between detection and reference sensors, producing a strong detection signal.

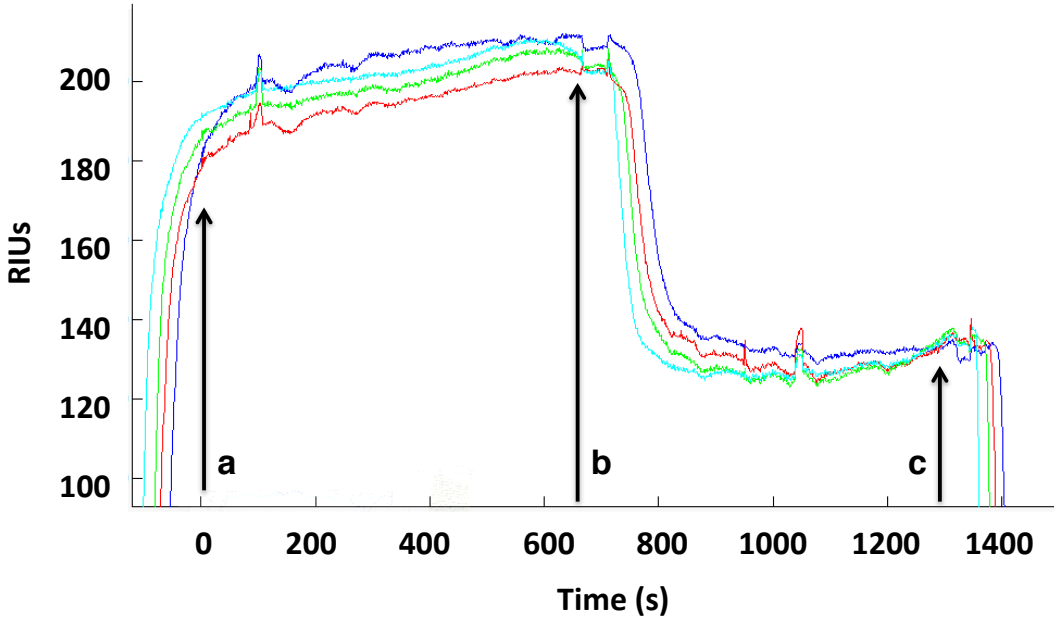

Figure 5. Antibody injection with *B. pumilus* spores. A sensorgram showing an injection of spores of *B. pumilus* 10^7 spores/mL, followed by an injection of *B. globigii* antibodies (anti-Bg) at a concentration of 10 µg/mL. Arrow (a) indicates the point of spore injection, arrow (b) indicates the injection of anti-Bg, and arrow (c) indicates the detection point. Sensor 4 (light blue) is coated with anti-Bg, sensor 3 (green) was coated with anti-Bg, sensor 2 (red) was coated with anti-Bg and sensor 1 (dark blue) was coated with anti-DON. Sensor 4, 3 and 2 have signal responses of 137.6 ± 7.3, 136.03 ± 17.5, and 135.1 ± 8.1 respectively, while sensor 1 has a signal response of 132.97 ± 5.1. These results show that the injection of an antibody post injection of a Bp spore sample did not increase the difference between detection and reference sensors.

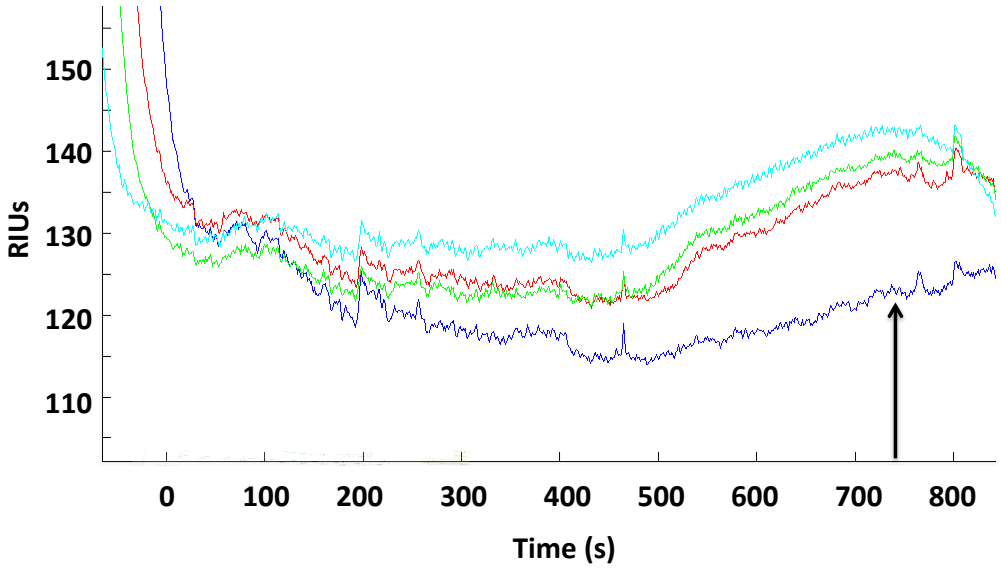

Figure 6. Differential detection of *B. globigii* in a mixed sample. Injection of mixed spore suspension *B. globigii* and *B. pumilus* at equal concentrations (10^7 spores/mL), followed by injection of anti-*B. globigii* at a concentration of 10 µg/mL. The sensorgram shows the portion of the run following the injection of anti-*B. globigii*. Sensor 4 (light blue), sensor 3 (green) and sensors 2 (red) were coated with anti-B. globigii. Sensor 1 (dark blue) was coated with anti-DON. The arrow indicates the detection point at 730 s. Sensors 4, 3, and 2 showed signal responses of 142.9 ± 4.5, 139.7 ± 12.5, and 137.9 ± 13.1. Sensor 1 showed a signal response of 123.3 ± 8.8. A divergence of detection from reference sensors was observed, indicating differential detection of *B. globigii* with in a mixed sample.

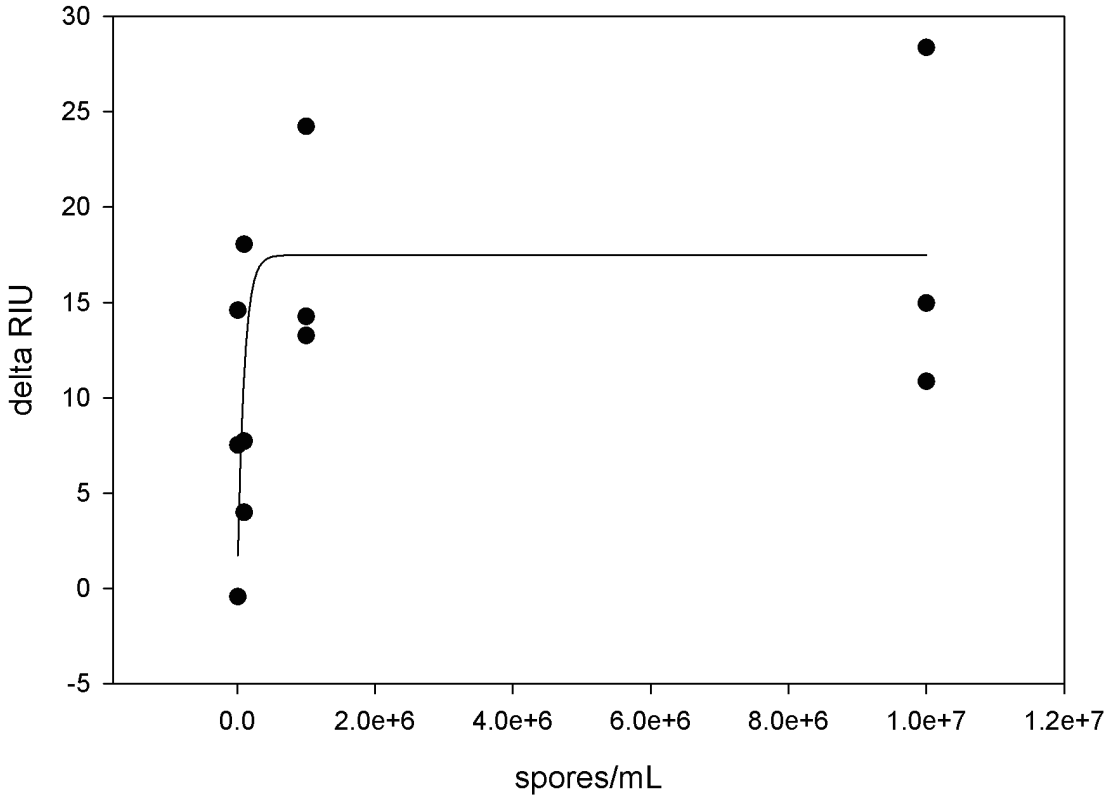

Figure 7. Antibody injection limit of detection (LOD). This graph shows that as the concentration of a spore sample decreases, the difference in response between detection and reference sensors also decreases. The injection of an antibody solution post-sample injection amplified the difference between detection and reference sensors at the original limit of detection (LOD) of 10^7 spores/mL. Further, it shows that there is likely a saturating effect, in which there is a limited amount of spores that can be bound to the immobilized antibodies. Using the trend equation, $y = 17.48(1-e^{((-1.04 \times 10E-05)\times x)})$, we can extrapolate our LOD. For an RIU difference of 10, our conservative LOD is 8.16×10^4 spores/mL. For an RIU difference of 5, our more aggressive LOD is 3.24×10^4 spores/mL.

SUPPLEMENTAL FIGURES

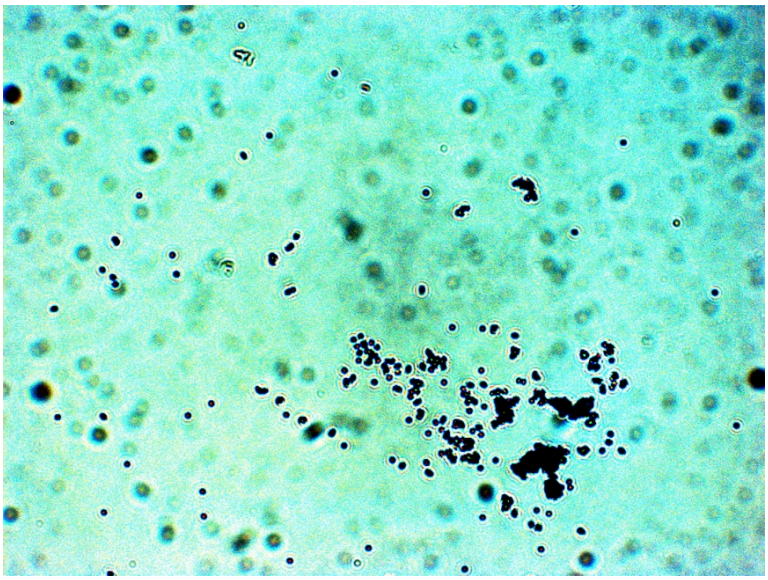

Figure 1. Lab-generated *B. globigii* spores. *B. globigii* spores have been stained with malachite green and counter-stained with safranin for differential identification between vegetative cells and spores.

Chapter 3. Detection of miRNA with SPR

Surface Plasmon Resonance (SPR) is an optical biodetection method that allows for rapid and specific detection of hazardous agents. Recently, SPR methods have been developed to target nucleic acids of specific agents. The coupling of SPR and nucleic acids has the potential to allow for an increased level of specificity and lower limits of detection when targeting a specific biological agent. An SPR method using synthetic miRNA was adapted to a portable SPR biosensor using the Krestchmann configuration, extending the work of Sipova et al. (13) that used a different SPR unit configured with a Special Diffraction Grating Structure. Preliminary experiments suggested that the target miRNA sequence (miR-122) could be detected. The results, however, were variable; the target miRNA sequence was detected by two sensors with Refractive Index Units (RIUs) of 122.13 ±15.1 and 79.5 ± 9.3, compared to a reference sensor of 68.8 ± 2.6. Though the two target sensors were > 10 RIUs above the reference sensor, additional work is needed to further assess the variability in response of sensors. SPR methods using nucleic acids have a promising future in the detection of biological agents such as *B. anthracis.*

Keywords: Biosensor, miRNA, Nucleic Acid, SPR, sRNA

INTRODUCTION

Nucleic acids play a variety of roles within all cell types, such as storage of genetic information, translation into proteins, as well as regulating cell activities (1, 7, 9, 12). Because of their abundance, nucleic acids may also be used to detect biological agents (14). Small RNAs (sRNAs) are important targets in nucleic acid detection schemes. sRNAs are short in length (50-

300 nucleotides) and play important roles within cells (13, 15, 16). Micro RNAs (miRNAs) are related to sRNAs, however, they are shorter than typical sRNA (~22 nucleotides) and have been implicated in a number of genetic disorders and can be used as biomarkers for conditions such as cancer and heart failures (14, 15). Sipova et al. (15) identified a protocol in which immobilized DNA oligonucleotides on an SPR sensor can bind to miRNA sequences resulting in detection of a target miRNA sequence (Figure 1). This was done through nucleotide specific binding between DNA and RNA sequences. The application to which Sipova et al. (15) applied this protocol was for medical diagnosis. Elevated levels of specific miRNAs have been linked to a number of health conditions such as heart failure and cancers.

As a first step toward integrating nucleic acid methods into our SPR detection scheme for biological agents, we adapted the Sipova et al. (15) to our portable SPR unit, which uses the Krestchmann configuration instead of a special diffraction grating structure configuration (Surface Plasmon Coupler and Disperser, SPRCD). The detection of nucleic acids such as sRNA may increase specificity and lower limits of detection when sensing a biological agent (15). RNA sequences are abundant within cells, allowing for multiple target sequences within a single cell, effectively increasing the odds of binding to a sequence and lowering limits of detection (15). Moreover, there may also be an increase in the specificity of binding since only a complementary sequence to that of the immobilized receptor can bind and elicit a response (15).

SPR methods using nucleic acids have a promising future in the detection of biological agents such as *B. anthracis,* where conserved spore features and a short phylogenetic distance between related species can provide challenges in distinguishing among species (3-6, 8, 10, 11). The main objective of this work was to adapt the Sipova et al. (15) for the detection of a target miRNA sequence (miR-122**)** to our portable SPR unit. If successful, this technique could be

applied to the detection of other nucleic acids that may be specific biomarkers for biological agents, such as *B. anthracis*.

MATERIALS AND METHODS

The materials and methods described below were based on the work by Sipova et al. (15). However, minor modifications were made to accommodate for the use of our portable SPR unit from Seattle Sensor Systems.

Reagents. Oligonucleotides and synthetic miRNA used in this protocol were ordered from Integrated DNA Technologies. The immobilized receptor on our detection sensor surface was a thiolated, DNA oligonucleotide with the sequence P122 SH-5'-d(CAA ACA CCA TTG TCA CAC TCC A)-3'. This DNA oligonucleotide was used to capture the complementary target miRNA sequence miR-122 5'-r(UGG AGU GUG ACA AUG GUG UUU G)-3'. The reference sensor (R1) used was coated with a different thiolated DNA oligonucleotide with the sequence R1 5'-d(TGC GTG TTT GAT TAT T)-3'-HS. A separate synthetic miRNA (miR-192) with the sequence 5'-r(CUG ACC UAU GAA UUG ACA GCC)-3' was also ordered and used as a negative control to indicate the specificity of binding. A S9.6 mouse monoclonal antibody was ordered from KeraFAST (Cat. No. ENH002, Boston, MA) to recognize the RNA-DNA hybrid complex (anti-DNA-RNA), and further amplify the signal produced by the binding of complementary RNA-DNA sequences.

Sensor Coating. Detection sensors and reference sensors were coated at the same time using their respective oligonucleotide sequence. First, 20 μL of DNA oligonucleotides was pipetted

onto the surface of SPREETA sensors (SensiQ) and incubated overnight at room temperature. Following incubation, excess oligonucleotides were recovered and stored at 4° C for later use. Sensors were then inserted into the SPR unit and washed with PBS buffer (Life Technologies) at rate of 20 μL/min for 10 to 15 min. Sensors were removed from the SPR unit and blocked with 20 μL of a 1 μM alkanethiol solution (Sigma-Aldrich) in a fume hood at room temperature for 1 hour. Next, excess blocking alkanethiol solution was recovered and disposed of and the sensor was rinsed with PBS buffer by insertion into the SPR unit at 20 μL/min for 5 min. Finally, 2mM NaOH was injected and flowed over the sensor for 5 min and then returned to normal PBS buffer.

Assay Protocol. The running buffer (PBS; $Tris_{Mg}$ [10 mM Tris-HCl, 15 mM $MgCl_2$, pH 7.4 at 25° C]) was used for all miRNA injections. Initially, running buffer was flowed across the inserted sensors for 15-20 min to generate a baseline. Target miR-122 was suspended in the running buffer at a concentration of 500 pM and injected and flowed at 20 μL/min for 10 min. Following injection of the miRNA solution, S9.6 anti-DNA-RNA solution at a concentration of 0.7 μg/mL was injected and flowed for another 5 min. To regenerate the sensor for subsequent injections, the bound antibody was first removed with a solution of 2 mM NaOH, which was injected and flowed for 2 min. Next, bound synthetic miR-122 to the immobilized P122 DNA oligonucleotide was removed by injecting 2mM of HCl flowed at 20 μL/min for 2 min. The system was then returned to the original running buffer state through injection of running buffer or flushing of the system and was ready for future injections.

RESULTS

Synthetic miRNA Injections. Synthetic miRNA (miR-122) was detected with the Seattle Sensor Systems portable SPR unit on one SPR run; Figure 2 illustrates a single injection of miR-122 with 2 detection sensors in P122 and 2 reference sensors in R1 and a plain gold sensor. The target miRNA sequence was detected by two sensors with Refractive Index Units (RIUs) of 122.13 ±15.1 and 79.5 ± 9.3, compared to the reference sensors of 68.8 ± 2.6 and 24.7 ± 3.9 at time point 2253 s. Though the two target sensors were > 10 RIUs above the reference sensor, additional work is needed to further assess the variability in response of sensors. Further, there was no significant difference between detection and reference sensors over 2 replicates (P = 0.47) Figure 3 shows the results of a negative control run; a non-target miRNA sequence (miR-192) was not detected (Figure 3). The results showed very low RIUs with P122 sensors having an average signal of 1.733 ±1.14 and 0.5 ± 1.970 RIUs, while the R1 sensor elicited a response of 7.667 ±1.266 RIUs at time point 689 s. The plain gold sensor also served as another reference sensor and responded with a signal of 0.8 ± 2.081 RIUs. The difference between all sensors is less than 10 RIUs, indicating that no detection of the non-target synthetic miRNAs. There was also no significant difference between detection and reference sensors over 2 replicates (P = 0.83). Replicates of this experiment were quite variable and do not complement the detection of miRNA.

DISCUSSION

SPR methods using nucleic acids have a promising future in the detection of biological agents, such as *B. anthracis*. Nucleic acid detection with SPR presents some potential advantages as compared to antibody detection through decreased limits of detection and increased binding specificity (14, 15). New methods of detection need to be verified and tested in order to further

advance SPR technology to reach its full potential. We adapted previously published nucleic acid SPR detection protocol to a new, portable SPR unit using commercially purchased, synthetic miRNA. This work could aid in the further characterization of SPR as a potential emergency response biodetection system for biological agents in the future.

Preliminary experiments suggested that the target miRNA sequence (miR-122) could be detected. The results, however, were variable; the target miRNA sequence was detected by two sensors with Refractive Index Units (RIUs) of 122.13 ±15.1 and 79.5 ± 9.3, compared to a reference sensor of 68.8 ± 2.6 and 24.7 ± 3.9. Though the two target sensors were > 10 RIUs above the reference sensor, additional work is needed to further assess the variability in response of sensors. However, the results from the lack of detection of our non-target sequence (miR-192) in a separate run were promising and illustrated the level of specificity to which synthetic miRNA sequences bind to one another. With further refinement and optimization of our methods, we are optimistic that we should be able to achieve consistent, repeatable responses indicating the detection of miRNA to our target sequence. If successful, we will look to apply this technique to a nucleic acid biomarker to target *Bacillus* spore spp.

CONCLUSIONS

An SPR method using synthetic miRNA was adapted from Sipova et al. 2010 (15) to a portable SPR biosensor. Preliminary experiments suggested that the target miRNA sequence (miR-122) could be detected. The results, however, were variable, and additional work is needed to further assess the variability in response of sensors. This technique could be applied to the detection of other nucleic acids that may be specific biomarkers for biological agents such as *B. anthracis.*

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Experiment	SPR Run	Sensor 4	Sensor 3	Sensor 2	Sensor 1	Δ RIU (avg.)
miRNA-122	**Run 1**	123.0	80.2	69.5	25.3	32.1
	Run 2	-38.5	-72.9	-66.0	-35.9	10.3
miRNA-192	**Run 1**	1.7	0.5	7.7	0.8	-6.6
	Run 2	11.3	16.9	11.2	9.8	2.9

Table 1. Raw data table for miRNA injections. Sensors 4 and 3 were coated with the thiolated DNA oligonucleotide sequence P122, sensor 2 was coated with a different thiolated DNA oligonucleotide sequence R1 and sensor 1 was not coated with any receptor for all experiments. There was no significant difference between detection and reference sensors for either experiment.

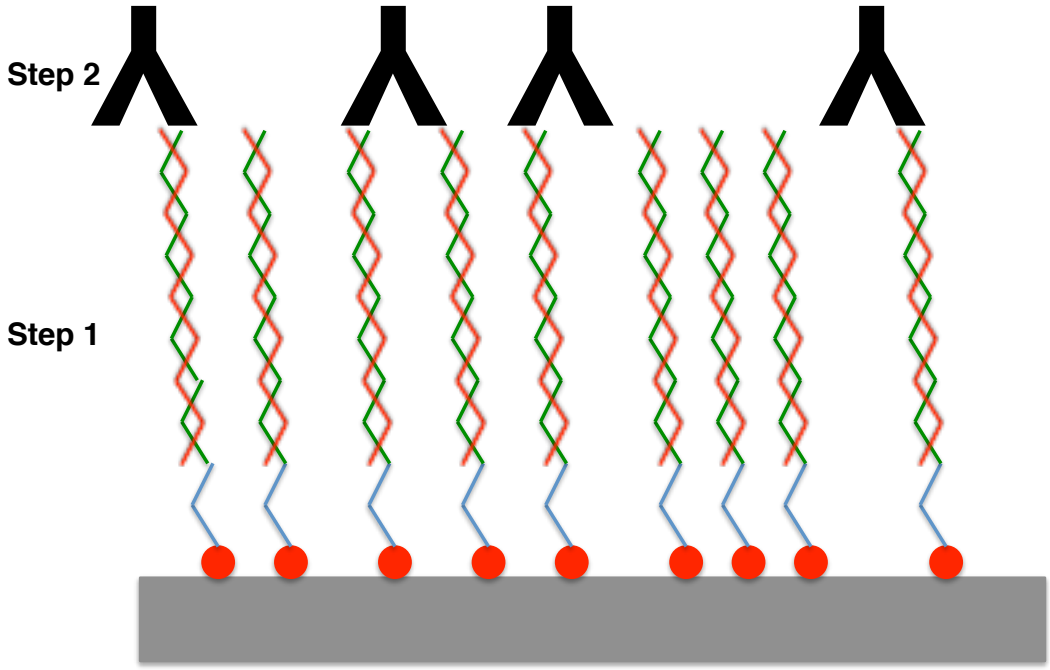

Figure 1. Surface Plasmon Resonance (SPR) Detection of Nucleic Acids. Step 1 (bottom) illustrates immobilized, thiolated DNA oligonucleotides (green) and the nucleotide specific binding to complementary miRNA (red). Step 2 (top) illustrates a monoclonal antibody that recognizes and binds the hybrid RNA-DNA complex formed to amplify the detection signal. (Adapted from 14).

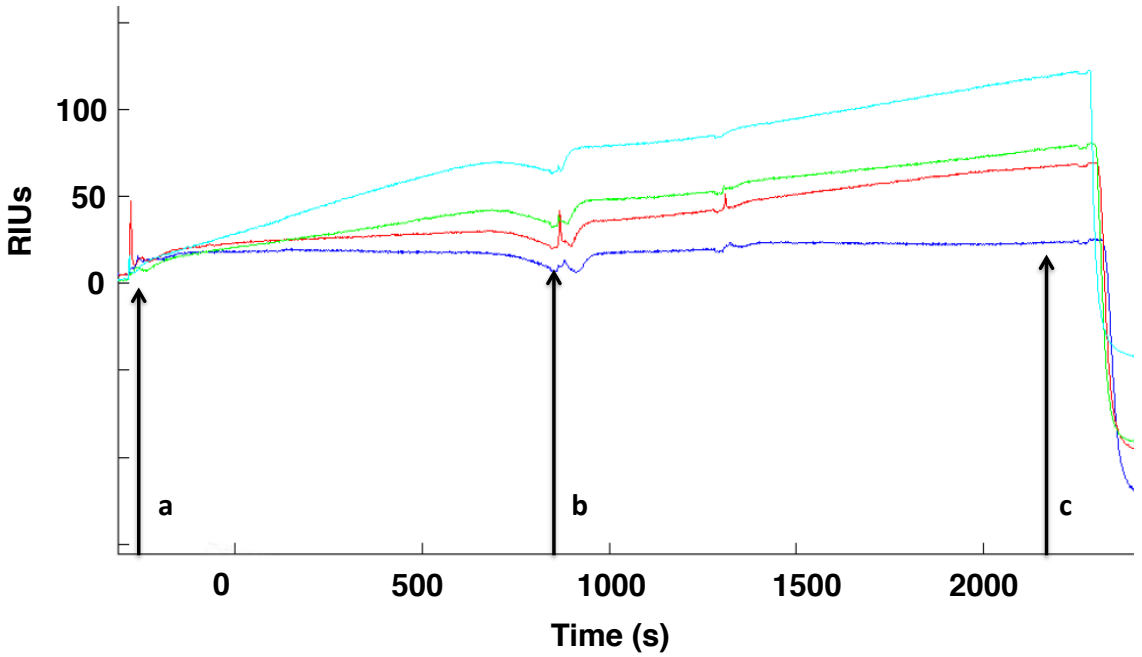

Figure 2. Detection of synthethic micro RNA (miRNA) miR-122. Sensors 4 (light blue) and 3 (green) were coated with the complementary DNA oligonucleotide sequence (P122) to miR-122. Sensor 2 (red) was coated with a different DNA oligonucleotide sequence (R1) (first control) and sensor 1 (dark blue) was not coated with any receptor (second control). Both sensors 1 and 2 were used as reference sensors to indicate any non-specific binding. Arrow (a) indicates the injection of miR-122, arrow (b) indicates injection of S9.6 Anti-DNA-RNA, and arrow (c) indicates the detection point at time 2253 s. This time point was chosen based upon the end of detection identified in Chinowsky et al. (2). Sensors 4 and 3 showed signal responses of 122.1 ±15.1 and 79.5 ± 9.3 RIUs, respectively. Sensor 2 and 1 showed signal responses of 68.8 ± 2.6 and 24.7 ± 3.9 RIUs, respectively. Though the two target sensors were > 10 RIUs above the reference sensor (and thus indicated detection), additional work is needed to further assess the variability in response of sensors.

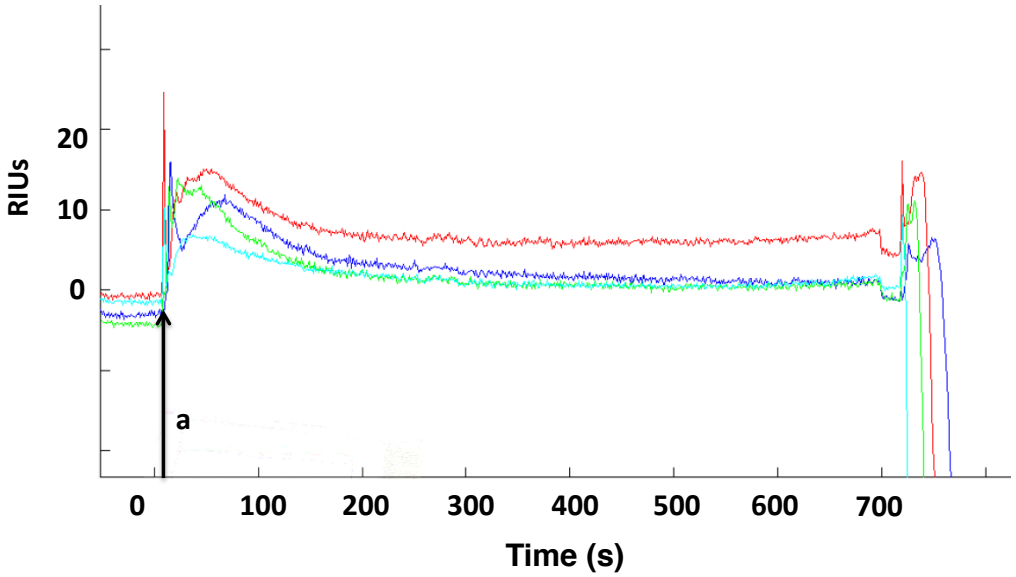

Figure 3. Control experiment for synthethic miRNA miR-192. Sensors 4 (light blue) and 3 (green) were coated with the complementary DNA oligonucleotide sequence (P122) to miR-122. Sensor 2 (red) was coated with a different DNA oligonucleotide sequence (R1) (first control) and sensor 1 (dark blue) was not coated with any receptor (second control). Both sensors 1 and 2 were used as a reference sensor to indicate any non-specific binding. Arrow (a) indicates the injection of miR-192. At time 689 s, sensors 4 and 3 showed signal responses of 1.73 ±1.14 and 0.5 ±1.97 RIUs, respectively. Sensors 2 and 1 showed signal responses of 7.67 ±1.27 and 0.8 ± 2.08 RIUs, respectively. This illustrates the lack of detection of the injected miR-192 as expected.

Chapter 4. Future Directions

The work described in this thesis highlights the potential for portable SPR instrumentation to be used to rapidly and accurately detect biological threat agents, such as *Bacillus anthracis*. Consequently, there are a number of exciting possibilities for future SPR research directions.

First, additional work is needed to optimize antibody amplification methods for the detection of *B. globigii* in pure and/or mixed sample. Previous literature uses a range of antibody amplification concentrations from 10 μg/mL to 20 μg/mL to develop the sandwich assay (1, 2). It would be ideal to maximize the potential of antibody amplification by discerning the optimal antibody injection concentration in which the best response is observed (in the context of a reasonable quantity of antibodies). Further, the antibody injection technique described here for differential detection of a target spp. within a mixed sample could be applied to detection of aerosolized spores using previously published sampling systems (3, 4, 8).

Second, additional methods could be explored to further amplify observed RIUs. One such method uses immunomagnetic beads to boost RIUs in a complex matrix (mixed samples) (7). In this configuration, antibodies would be immobilized on magnetic beads to retrieve a target pathogen from a complex, environmental sample (7). Further, this may enhance specificity and lower limits of detection by creating a much larger molecule for detection (5).

Third, approaches using nucleic acids (e.g., miRNA) should be considered to increase specificity of the protocol to the biological agent of interest (6) Nucleotide specific binding to a complementary DNA oligonucleotide coupled with a secondary antibody that recognizes DNA*RNA hybrid complexes may increase the specificity of binding and lower the limit of detection. This protocol could be adapted to a portable SPR instrument, such as the one used in

this body of work. Key to the success of this protocol will be the identification of a unique sRNA sequence for a specific *Bacillus* spp. A specific mRNA sequence may also be a target of interest, particularly one that encodes for pathogenic proteins such as the lethal or edema factor within *B. anthracis*. Vogel *et al*. (9) has also outlined methods of identifying sRNA sequences. With the identification of a unique RNA sequence for a specific *Bacillus* species and a successful protocol for the detection of miRNA using SPR, detection of RNA via SPR should be feasible. Moreover, RNA could be engineered (purchased) and/or harvested from *Bacillus* spores utilizing germination/lysis technique. Such an approach could provide an advantage over antibody-based SPR methods when detecting a target within a mixed or an environmental sample, in part because of an increased level of specificity in binding.

Finally, the protocols developed here need to be tested and validated in a variety of field experiments (e.g., natural and/or engineered emergency response scenarios). This will likely create a number of additional challenges that must be considered, such as the LOD and time to detection of a specific biological agent in a true mixed background sample. These challenges will likely engender new hypotheses and resulting experiments that must be considered in the development and deployment of portable SPR technologies in emergency response scenarios.

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