

# Species Selective Measurement of 10 *B. anthracis*-Sterne Spores within 10 Minutes by Surface-Enhanced Raman Spectroscopy

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## Abstract

The use of biological warfare agents by terrorists remains a global concern. While there has been substantial effort since the 2001 distribution of *Bacillus anthracis* spores through the US Postal System to develop analyzers to detect this and other biological agents, the analyzers lack sensitivity, lack specificity (produce high false-positive rates), are too slow, or cannot be fielded. For the past decade we have been investigating the ability of surface-enhanced Raman spectroscopy (SERS) to overcome these limitations. Recently, we developed an assay by functionalizing silver nanoparticles with various peptides to selectively bind *B. anthracis*, and then adding acetic acid and silver colloids to release and detect, respectively, dipicolinic acid as a biomarker by SERS. Here we describe the successful measurement of *B. anthracis*-Sterne spores with a 10- to 20-fold selectivity over other *Bacillus* species at 10<sup>5</sup> spores/mL, using the peptide functionalized SERS assay with a sensitivity capable of detecting 10 spores in a 10<sup>3</sup> spores/mL sample in 6.5 minutes. This measurement represents 6 orders-of-magnitude improvement over our previous peptide based SERS assay measurements.

**Keywords:** *Bacillus anthracis*; Anthrax; Biological warfare agents; Surface-enhanced Raman spectroscopy

## Introduction

During the most recent 2011 Biological Weapons Convention held in Geneva, US Secretary of State Hillary Rodham Clinton stated “Unfortunately, the ability of terrorists to develop and use these weapons is growing. Terrorist groups have made it known they want to acquire these weapons [1].” Consequently the need for a portable technology that can rapidly identify biological warfare agents with high specificity (no false-positive responses) and sensitivity (e.g. 10<sup>4</sup> spores for *B. anthracis* [1]) in the field remains. During the past decade, various techniques have been developed to detect and identify biological agents. Notable techniques include mass spectrometry [2], fluorescence [3], luminescence [4], infrared [5,6], and Raman spectroscopy [7], as well as the polymerase chain reaction (PCR) [8]. While all of these techniques have been employed for biological agent detection to some extent (e.g. identification of anthrax on mail sorting equipment by PCR), none of them satisfy all of the requirements of speed, sensitivity, selectivity, and field ruggedness, especially the latter as required by military personnel.

The most successful field techniques are based on immunoassays, such as enzyme-linked immunosorbent assays (ELISA) [9,10]. In an effort to improve upon this technique, a number of other antigen-antibody based binding event reporter methods have been investigated. These techniques include cantilever [11,12], electrochemical [13], magnetic [14], piezoelectric [15], and surface plasmon resonance (SPR) based devices [16]. While these techniques are relatively fast, inexpensive, and easy to use, they suffer from high false-positive and false-negative rates due to a lack of specificity and sensitivity [17]. In the specific case of *B. anthracis*, other *Bacillus* spores, such as *B. cereus*, are common in the environment, and they share the same surface antigens used for antibody binding [18], increasing the false-positive rate.

Recognizing the limitations of all the techniques mentioned above, we have been developing a surface-enhanced Raman spectroscopy (SERS) based assay for the detection of trace quantities of biological

agents, since it is capable of amplifying Raman signal intensities by 6 orders of magnitude or more [19]. We demonstrated the potential of SERS to detect *Bacillus* spores by performing the first measurements of dipicolinic acid (DPA) as a biomarker more than a decade ago [20]. DPA is a suitable biomarker, as it represents approximately 10% of the spore mass in the form of calcium dipicolinate (CaDPA) [21]. In the past few years, we developed and patented a method to extract DPA using acetic acid [22]. This method allowed detecting ~200 spores within 3 minutes [23,24], which is well below the estimated infectious dose of 10<sup>4</sup> spores for *B. anthracis* [2]. Although the use of DPA as a biomarker is a logical approach, the chances of false-positive identification are significant, since both *Bacillus* and *Clostridium* bacteria contain CaDPA in their protective outer layers. To overcome this limitation, we have been investigating the possibility of functionalizing silver nanoparticles with peptides that specifically bind *B. anthracis*, so that these spores can be selectively measured by SERS. Recently, several peptides have been identified that demonstrate high selectivity towards *B. anthracis* versus other *Bacillus* [25,26]. Peptides offer several advantages over the traditional antibody-antigen binding assays, in that they can be easily synthesized, functionalized, and covalently attached to sensor surfaces. Furthermore, they are more tolerant to heat and moisture, which extends their usable lifetime making them suitable for field use.

Previously, we reported the ability of this approach to detect 10<sup>9</sup> *B. anthracis*-Sterne spores per mL in less than 20 minutes [27].

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Received January 20, 2016; Accepted February 27, 2016; Published March 03, 2016

**Citation:** Shende C, Huang H, Sperry J, Farquharson S (2016) Species Selective Measurement of 10 *B. anthracis*-Sterne Spores within 10 Minutes by Surface-Enhanced Raman Spectroscopy. J Anal Bioanal Tech 7: 302. doi:[10.4172/2155-9872.1000302](https://doi.org/10.4172/2155-9872.1000302)

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Unfortunately, the addition of the peptide likely dampened the plasmon field responsible for the SER effect. Here we describe the addition of a second SER-active material to the assay in the form of a silver colloid to measure 10 *B. anthracis*-Sterne spores in a  $10^3$  spores/mL sample within 10 minutes, representing an improvement in sensitivity of 6 orders-of-magnitude!

## Materials and Methods

### Materials

All chemicals, reagents, and solvents, including those used to prepare the SER-active sol-gels, were purchased and used as received from Sigma-Aldrich (Milwaukee WI). All *Bacillus* samples were obtained from the American Type Culture Collection (Manassas, VA) and prepared by Professor Jay Sperry (University of Rhode Island) [28]. Stock solutions were serially diluted to produce the measured concentrations. Concentrations were verified by direct count of spores in  $4 \times 10^{-3}$   $\mu\text{L}$  triplicate samples using a light microscope [25]. The concentrations of these samples were determined to be  $5.7 \times 10^9$ ,  $1.7 \times 10^{10}$ ,  $5.5 \times 10^9$ , and  $6.1 \times 10^9$  spores/mL for *B. anthracis*-Sterne, *B. cereus*, *B. megaterium* and *B. subtilis*, respectively. The ATYPLPIR peptide [26] used in this study was custom synthesized by New England Peptide (Gardner, MA). Glass capillaries, tubing, syringes and syringe ports were obtained from VWR Scientific (Arlington Heights, IL).

### SER-active sol-gel capillaries

SER-active capillaries (*Simple SERS Sample Capillaries*, RTA) were prepared according to published procedures [28,29] by mixing a silver amine precursor and an alkoxide precursor at 1:1 v/v. The silver amine precursor consisted of a 1:1:2 v/v/v ratio of 1N  $\text{AgNO}_3$ /28%  $\text{NH}_4\text{OH}$ / $\text{CH}_3\text{OH}$ , while the alkoxide precursor consisted of methyltrimethoxysilane. The SERS capillaries were prepared by drawing 20  $\mu\text{L}$  of the silver-doped sol-gels into 10 cm long, 0.8 mm inner diameter glass capillaries to produce ~1 cm long sol-gel segments. The segments were allowed to gel and cure for 12 hours, after which the incorporated silver ions were reduced with dilute  $\text{NaBH}_4$ . Silver colloids were prepared from  $\text{AgNO}_3$  and  $\text{NaBH}_4$  according to literature [30] with modifications.

### Peptide functionalized SERS capillaries

A cysteine residue was attached to the C-terminus of the peptide sequence, and served to link the peptide to the SER-active metal surface. Cysteine forms a strong covalent bond with silver via the sulfur of its thiol side chain. The peptide functionalization of silver nanoparticles was carried out by adding 10  $\mu\text{L}$  of peptide solution to the SER-active sol-gel segments immobilized within the glass capillaries. After the peptide was allowed to react with the SER-active metal, water was passed through the capillary to remove any unbound peptide.

### SERS analysis

For measurements the SERS capillaries were mounted on an XY stage (Conix Research, Springfield, OR) such that the focal point of an  $f/0.7$  aspheric lens of a fiber optic probe was just inside the capillary glass wall. A software program developed in-house was used to measure 1 min spectra at 9 points spaced 1 mm apart along the length of the metal-doped sol-gel segment. A Fourier transform Raman spectrometer (Real-Time Analyzers, Middletown, CT), equipped with a 785 nm diode laser (Innovative Photonic Solutions, Monmouth Junction, NJ) and a Si-photo-avalanche detector (Perkin Elmer, Stamford, CT) was used to deliver 80 mW of power and collect spectra at  $8 \text{ cm}^{-1}$  resolution.

## Results and Discussion

Functionalization of silver nanoparticles with the *B. anthracis* specific peptide was investigated in terms of reaction times and peptide concentrations. In each case, 10  $\mu\text{L}$  of peptide was added to a previously prepared glass capillary, which was sufficient to saturate the 1 cm segment of the SER-active sol-gel. After each experiment, the SER-active sol-gel was flushed with 10 mL of HPLC grade water to remove any unbound peptide. Peptide functionalization to the silver nanoparticles was verified by observing the SERS of the peptide. Initially, 1 mg/mL peptide was allowed to react with the silver for 8 hours to ensure success. This time was decreased by factors of 2 until the SERS intensity of the peptide decreased. This occurred at binding times below 0.5 hours. As shown in Figure 1A, the SER spectrum of the peptide is very similar to cysteine, both dominated by the peak at  $657 \text{ cm}^{-1}$  due to the sulfur-silver vibrational mode, as well as cysteine peaks at 863, 1067, and  $1394 \text{ cm}^{-1}$  due to a CC stretch, CN stretch, and  $\text{CH}_2$  wag, respectively [31]. The other amino acids of the peptide do not contribute significantly to the spectrum.

In addition to the reaction time it was important to determine the peptide concentration that would provide monolayer coverage of the silver, so that chemicals or biochemicals introduced with a sample would not produce interfering SER spectral features. To establish monolayer coverage, a high concentration of DPA, 100  $\mu\text{g}/\text{mL}$ , was added to the silver nanoparticles functionalized with various amounts of peptide. Since DPA is a highly SER-active molecule, it would produce a spectrum through interaction with any silver surface uncovered by the peptide. DPA produces several characteristic SERS peaks at 659 (coincident with the dominant peptide peak), 812, 1007, and  $1381 \text{ cm}^{-1}$ , which have been previously assigned to a CC ring bend, a CH out-of-plane bend, the symmetric pyridine ring stretch, and OCO symmetric stretch, respectively [8]. It was found that the DPA spectrum was relatively intense, barely discernible, and absent at 5, 10 and 50  $\mu\text{g}/\text{mL}$  peptide, respectively (Figure 1B). Based on these measurements, peptide functionalized capillaries were prepared by reacting 100  $\mu\text{g}/\text{mL}$  peptide for 0.5 hours to ensure monolayer coverage.

Measurements of *B. anthracis*-Sterne spores, an avirulent strain of *B. anthracis*, were performed at the University of Rhode Island to determine optimum sample and reagent volumes, and reaction (binding) time for detecting *B. anthracis* by SERS. Once determined, these conditions were used to demonstrate selective binding of the *B. anthracis* peptide versus *B. cereus*, *B. megaterium*, and *B. subtilis*, as well as to determine the limit of detection for *B. anthracis*-Sterne. For each of the *Bacillus* samples, initial 1 mL wet suspensions of the spores were diluted by a factor of 300. These samples were then diluted to produce serial concentrations of  $10^5$ ,  $10^4$ ,  $10^3$  and  $10^2$  spores/mL. A starting concentration of  $10^5$  spores/mL was selected as based on 1) a previous measurement of 10 ng/mL chemical DPA using unfunctionalized SER-active capillaries [32], and 2) the fact that 10 ng DPA corresponds to  $\sim 10^5$  spores. The latter assumes that 10% of the spore mass is DPA [22], and that 1 spore has a mass of  $\sim 1 \text{ pg}$  [33,34].

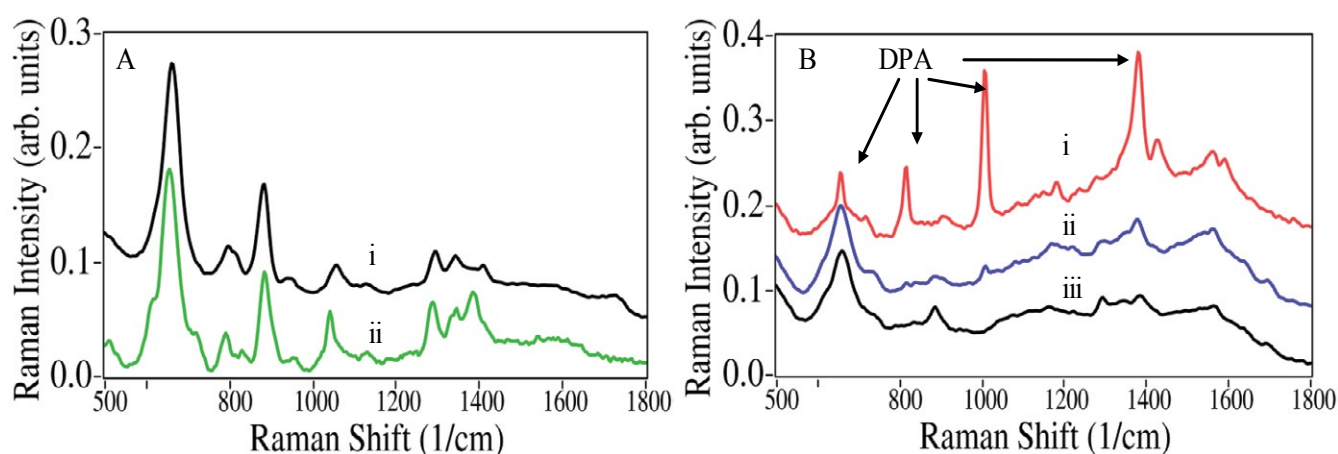
The following steps were found to repeatedly produce quality spectra for *B. anthracis*-Sterne at  $10^5$  spores/mL: 1) draw 10  $\mu\text{L}$  of spores into the peptide functionalized, SER-active capillaries; 2) allow the spores to bind to the peptides; 3) draw 50  $\mu\text{L}$  of water through the capillary to remove unbound spores; 4) draw 10  $\mu\text{L}$  of silver colloid as prepared [31] into the capillary to coat the spores; 5) draw 10  $\mu\text{L}$  acetic acid solution into the capillary to cause DPA to be released from the spores; 6) mount the capillary on the XY stage of the Raman spectrometer; and 7) measure the SERS at 9 points along the

capillary. Measurements of  $10^5$  *B. anthracis*-Sterne spores/mL using this procedure produced intense DPA spectra for binding times of 30, 15, and 5 minutes (Figure 2). Since one of the goals was to establish the minimum time to perform the measurement, the 5 min binding time was used for the *B. anthracis*-Ames samples, as it produced a sufficiently intense DPA spectrum.

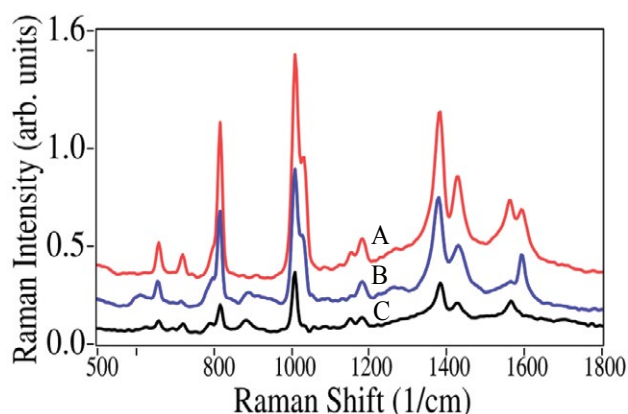
Next, the other bacilli at  $10^5$  spores/mL were measured on the *B. anthracis* specific peptide functionalized SERS capillaries. Again, measurements followed the above procedure, but in this case 15 min binding times were used to better represent the selectivity of the peptide. As shown in Figure 3A, the intensities of the  $1007\text{ cm}^{-1}$  peak for *B. cereus*, *B. megaterium* and *B. subtilis* are ~10%, 6%, and 5%, respectively, of that for *B. anthracis*-Sterne. The presence of DPA in the spectra for these bacilli is not surprising, since it is known that some non-specific binding occurs [27]. The lower discrimination against *B. cereus* has been attributed to its closer relation to *B. anthracis* [26]. It should be noted that the inability to completely flush these spores out of the porous sol-gel structure could also contribute to the lack of specificity. While this level of discrimination by itself may not suffice

for positive *B. anthracis*, the use of a second assay, such as one for *B. cereus*, could be used to rule out false positives.

Finally, *B. anthracis*-Sterne was measured at  $10^4$  and  $10^3$  spores/mL to determine the limits of detection (Figure 3B). At these lower concentrations, other weak spectral features become noticeable, such as a peak at  $930\text{ cm}^{-1}$  due to acetic acid (AA), a peak at  $1044\text{ cm}^{-1}$  due to nitrate ( $\text{NO}_3^-$ ) from the silver nitrate used to prepare the colloid, and a broad feature from  $1200$  to  $1800\text{ cm}^{-1}$  due to glass luminescence from the capillary. It is worth noting that the actual number of spores in the  $10^3$  spores/mL sample is ~10 spores, since only  $10\text{ }\mu\text{L}$  of sample was introduced into the capillary. Furthermore, the signal-to-noise ratio (S/N) is 28 using the baseline corrected  $1007\text{ cm}^{-1}$  peak height and the standard deviation noise between  $1700$  and  $1800\text{ cm}^{-1}$ . Based on a limit of detection defined as a S/N of 3, this suggests that a single spore could be detected, or 100 spores/mL assuming the SERS signal intensity is relatively linear in this range. Finally, both spectra presented in Figure 3B were acquired in 1 minute, so that the entire measurement time was ~6.5 minutes (5 min to load the sample, 0.5 min to load the reagents, and 1 min to measure the sample).

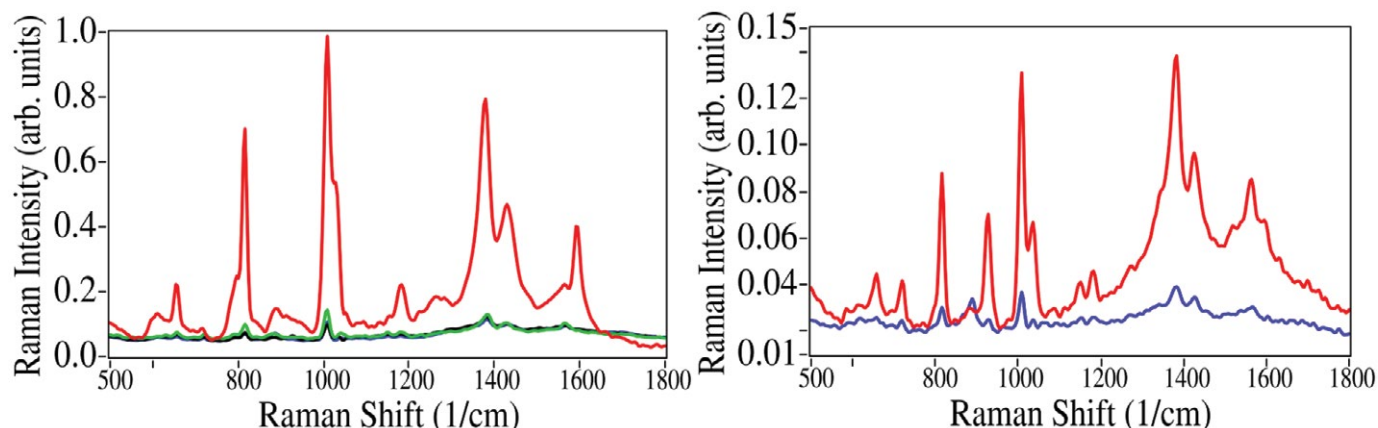


**Figure 1:** SERS of A) 1 mg/mL i) ATYPLPIRC peptide specific to *B. anthracis* functionalized in a SER-active capillary and ii) cysteine in a SER-active capillary. B) 100  $\mu\text{g/mL}$  DPA added to SER-active capillaries treated with i) 5, ii) 10, and iii) 50  $\mu\text{g/mL}$  peptide. Sample conditions: 0.5 hr peptide reaction time, DPA measured immediately. Spectral conditions: 75 mW of 785 nm laser excitation, 9 min acquisition (nine 1-min points along capillary averaged),  $8\text{ cm}^{-1}$  resolution using RTA's FT-Raman analyzer. Spectra are displayed on the same intensity scale, but offset for clarity.



**Figure 2:** SERS of  $10^5$  *B. anthracis*-Sterne spores/mL for binding times of A) 30, B) 15, and C) 5 min. Sample conditions:  $10\text{ }\mu\text{L}$  sample,  $50\text{ }\mu\text{L}$  water wash,  $10\text{ }\mu\text{L}$  silver colloid addition, and  $10\text{ }\mu\text{L}$  acetic acid addition. Spectral conditions as in Figure 1.





**Figure 3:** SERS of A) i) *B. anthracis*-Sterne, ii) *B. megaterium*, iii) *B. cereus*, and iv) *B. subtilis*, all at  $10^5$  spores/mL, conditions: 15 min binding time, 9-min spectral acquisition; and B) *Bacillus anthracis*-Sterne at i)  $10^4$  and ii)  $10^2$  spores/mL, conditions: 5 min binding time, 1-min spectral acquisition. Other conditions as in Figure 2.

## Conclusion

A method was successfully developed to selectively detect 10 *B. anthracis*-Sterne spores from a  $10^3$  spores/mL sample in 6.5 minutes. This was accomplished by functionalizing silver nanoparticles within a porous sol-gel with a *B. anthracis* specific peptide, and then adding acetic acid and silver colloids to release and detect, respectively, DPA by SERS as a biomarker. The assay provided a 10- to 20-fold discrimination against *B. cereus*, *B. megaterium* and *B. subtilis* at  $10^5$  spores/mL. Current research is aimed at developing an automated flow system coupling the output of an aerosol collector to the capillaries. The technology, once developed should prove invaluable for real-time monitoring of biological agents at potential terrorist targets.

## Acknowledgements

The authors are grateful for funding from the National Science Foundation (DMI-0349687, IIP-0810335, and I-0956170) and the Defense Advanced Research Projects Agency (N10PC2077 and D11PC20171).

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**Citation:** Shende C, Huang H, Sperry J, Farquharson S (2016) Species Selective Measurement of 10 *B. anthracis*-Sterne Spores within 10 Minutes by Surface-Enhanced Raman Spectroscopy. J Anal Bioanal Tech 7: 302. doi:[10.4172/2155-9872.1000302](https://doi.org/10.4172/2155-9872.1000302)

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