

Rapid Detection of an Anthrax Biomarker by Surface-Enhanced Raman Spectroscopy

Xiaoyu Zhang,[†] Matthew A. Young,[†] Olga Lyandres,[§] and Richard P. Van Duyne^{*†}

Contribution from the Departments of Chemistry and Biomedical Engineering,
Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3113

Received October 20, 2004; E-mail: vanduyne@chem.northwestern.edu

Abstract: A rapid detection protocol suitable for use by first-responders to detect anthrax spores using a low-cost, battery-powered, portable Raman spectrometer has been developed. *Bacillus subtilis* spores, harmless simulants for *Bacillus anthracis*, were studied using surface-enhanced Raman spectroscopy (SERS) on silver film over nanosphere (AgFON) substrates. Calcium dipicolinate (CaDPA), a biomarker for bacillus spores, was efficiently extracted by sonication in nitric acid and rapidly detected by SERS. AgFON surfaces optimized for 750 nm laser excitation have been fabricated and characterized by UV–vis diffuse reflectance spectroscopy and SERS. The SERS signal from extracted CaDPA was measured over the spore concentration range of 10^{-14} – 10^{-12} M to determine the saturation binding capacity of the AgFON surface and to calculate the adsorption constant ($K_{\text{spore}} = 1.7 \times 10^{13} \text{ M}^{-1}$). At present, an 11 min procedure is capable of achieving a limit of detection (LOD) of $\sim 2.6 \times 10^3$ spores, below the anthrax infectious dose of 10^4 spores. The data presented herein also demonstrate that the shelf life of prefabricated AgFON substrates can be as long as 40 days prior to use. Finally, these sensing capabilities have been successfully transitioned from a laboratory spectrometer to a field-portable instrument. Using this technology, 10^4 bacillus spores were detected with a 5 s data acquisition period on a 1 month old AgFON substrate. The speed and sensitivity of this SERS sensor indicate that this technology can be used as a viable option for the field analysis of potentially harmful environmental samples.

Introduction

The rapid and accurate identification of bioagents is a vital task for first-responders in order to facilitate timely and appropriate actions in the event of a biological attack. *Bacillus anthracis*, a spore-forming bacterium and a dangerous pathogen for the disease anthrax, is an important example. *B. anthracis* bacteria exist in two different forms: rod-shaped organisms and spores. Rod-shaped organisms grow and divide in a nutrient-rich environment. When the food supply is depleted, the organisms turn into spores that can survive for decades. Structurally, a spore consists of a central core cell surrounded by various protective layers. Calcium dipicolinate (CaDPA) exists in these protective layers and accounts for $\sim 10\%$ of the spore's dry weight;¹ therefore, it is a useful biomarker for bacillus spores.²

Among the potential biological warfare agent candidates, *B. anthracis* spores are of particular concern. First, they are highly resistant to environmental stress and are relatively easily produced into weapon-grade material outside the laboratory. Second, anthrax is an infectious disease, requiring medical attention within 24–48 h of initial inhalation of more than

10^4 *B. anthracis* spores.³ However, the diagnosis of anthrax is not immediate because it takes 1–60 days for anthrax symptoms to appear in humans.⁴ Therefore, the rapid detection of *B. anthracis* spores in the environment prior to infection is an extremely important goal for human safety.

In the last two decades, various biological and chemical techniques have been developed to detect bacillus spores. Two important biological methods are the polymerase chain reaction (PCR)^{5–7} and immunoassays.^{8,9} PCR, a primer-mediated enzymatic DNA amplification method, requires expensive reagents, molecular fluorophores, and considerable sample processing prior to analysis. The limit of detection (LOD) based on PCR detection of bacterial *pagA* gene is $\sim 10^3$ spores in 3 h.⁷ Immunoassays, which rely on the interaction between antibodies and *B. anthracis* cell surface antigens, can detect 10^5 spores in 15 min.⁹ However, in immunoassays, it is necessary to employ specific antibodies for the desired agents and to individually

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adjust the mobile-phase conditions for their capture, elution, and separation.

Recently, relatively rapid chemical methods for the detection of bacillus spores have been developed. For instance, photoluminescence detection based on the formation of terbium (Tb(III)) dipicolinate was found to have a LOD of 10^3 *B. subtilis* colony-forming-units·mL⁻¹ in 5–7 min.¹⁰ This method compares the enhanced luminescence of the terbium dipicolinate complex to Tb(III) alone. However, an increase in the luminescence intensity can also occur by the complexation of Tb(III) with aromatic compounds other than dipicolinic acid.¹¹ Due to the frequency of false positives and its limited ability for target analyte identification, alternative approaches with improved selectivity would be a welcome addition to the arsenal of anthrax detection methods.

Compared to photoluminescence, vibrational spectroscopy possesses highly specific chemical information content and, therefore, is capable of uniquely identifying target analytes. Both Fourier transform infrared (FT-IR)¹² and Raman^{13,14} spectroscopies have demonstrated the ability to discriminate among different bacterial spores. However, the implementation of near-infrared (NIR) and mid-infrared spectroscopies has fundamental limitations due to the competitive absorption of water and inherent spectral congestion. In contrast, Raman spectroscopy is well-suited to applications in aqueous environments because of the small Raman scattering cross section of water.¹⁵ Recently, for example, single bacterial spores have been detected using micro-Raman spectroscopy.¹⁴ As a consequence of different individual sporulations, however, the micro-Raman spectra vary significantly from one spore to another. Additionally, the applications that require complex instrumentation used in this approach restrict its applicability in field-portable measurements. The detection of bacillus spores by normal Raman spectroscopy (NRS) has also been demonstrated;¹⁶ however, NRS suffers from low sensitivity, so that long data acquisition times (5–13 min) and high laser powers (400 mW) are required. In comparison, surface-enhanced Raman spectroscopy (SERS) yields more intense Raman signals at much lower laser excitation power. SERS produces very large enhancements in the effective Raman cross section of species spatially confined within the electromagnetic fields generated by excitation of the localized surface plasmon resonance (LSPR) of nanostructured noble metal surfaces.¹⁷ The SERS signals of ensemble-averaged molecules show enhancements up to 8 orders of magnitude over NR signals.¹⁸ Furthermore, the low power required by SERS allows the development of a compact, field-portable detection system.

In the present paper, we describe a procedure for the rapid

extraction of CaDPA from *B. subtilis* spores, simulants for *B. anthracis* spores, followed by SERS detection on reproducible,¹⁹ stable^{20,21} silver film over nanosphere (AgFON) substrates. Our previous studies have shown that when the localized surface plasmon resonance (LSPR) maximum of a AgFON substrate closely matches the laser excitation wavelength, the maximum SERS signal intensity results.^{18,22} In this study, AgFON surfaces were fabricated using 600 nm spheres in order to optimize SERS intensity for 750 nm laser excitation. We demonstrate a LOD of $\sim 2.6 \times 10^3$ spores with a data acquisition period of 1 min and a laser power of 50 mW. To place these results in context, it should be noted that previous published SERS studies of anthrax detection via the CaDPA biomarker were 200 times less sensitive and required 3 times more laser power.²³ Similarly, previous published NRS studies were 200 000 times less sensitive and required 8 times more laser power.¹⁶ The data presented herein also demonstrate that AgFON substrates provide stable SERS spectra for at least 40 days. Finally, a portable SERS device successfully produces a SERS spectrum from 10^4 spores in 5 s using a 1 month old prefabricated AgFON. This is, to our knowledge, the first reported result that utilizes a compact vibrational spectrometer for the detection of bacillus spores.

Experimental Section

Materials. All of the chemicals used were of reagent grade or better. Ag (99.99%) was purchased from D. F. Goldsmith (Evanston, IL). Glass substrates were 18 mm diameter, No. 2 cover slips from Fisher Scientific (Pittsburgh, PA). Pretreatment of substrates required H₂SO₄, H₂O₂, and NH₄OH, all of which were purchased from Fisher Scientific (Fairlawn, NJ). Surfactant-free white carboxyl-functionalized polystyrene latex nanospheres with diameters of 390, 510, 600, and 720 nm were obtained from Duke Scientific Corporation (Palo Alto, CA) and Interfacial Dynamics Corporation (Portland, OR). Tungsten vapor deposition boats were purchased from R. D. Mathis (Long Beach, CA). Nitric acid 70% (Fisher Scientific), dipicolinic acid (2,6-pyridinedicarboxylic acid, DPA), calcium hydroxide, and benzenethiol (Aldrich Chemical Co., Milwaukee, WI) were used as purchased. Water (18.2 MΩ/cm) was obtained from an ultrafilter system (Milli-Q, Millipore, Marlborough, MA). Calcium dipicolinate (CaDPA) was prepared from DPA and calcium hydroxide according to the method of Beiley and co-workers.¹

Spore Samples. *B. subtilis* was purchased from the American Type Culture Collection (Manassas, VA). Spore cultures were cultivated by spreading the vegetative cells on sterile nutrient agar plates (Fisher Scientific), followed by incubating at 30 °C for 6 days. The cultures were washed from the plates using sterile water and centrifuged at 12 000g for 10 min. The centrifuging procedure was repeated five times. The lyophilized spores were kept at 2–4 °C prior to use. Approximately 1 g of sample was determined to contain 5.6×10^{10} spores by optical microscopic measurements (data not shown). The spore suspension was made by dissolving spores in 0.02 M HNO₃ solution and by sonicating for 10 min.

AgFON Substrate Fabrication. Glass substrates were pretreated in two steps. (1) Piranha etch (**CAUTION: piranha solution should be handled with great care**), 3:1 H₂SO₄/30% H₂O₂ at 80 °C for 1 h, was

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used to clean the substrate, and (2) base treatment, 5:1:1 H₂O/NH₄OH/30% H₂O₂ with sonication for 1 h was used to render the surface hydrophilic. Approximately 2 μ L of the nanosphere suspension (4% solids) was drop coated onto each substrate and allowed to dry in ambient conditions. The metal films were deposited in a modified Consolidated Vacuum Corporation vapor deposition system with a base pressure of 10⁻⁶ Torr.²⁴ The deposition rates for each film (10 \AA /s) were measured using a Leybold Inficon XTM/2 quartz crystal microbalance (QCM) (East Syracuse, NY). AgFON substrates were stored in the dark at room temperature prior to use.

UV-Vis Diffuse Reflectance Spectroscopy. Measurements were carried out using an Ocean Optics (Dunedin, FL) SD2000 spectrometer coupled to a reflection probe (Ocean Optics) and a halogen lamp (Model F-O-Lite H, World Precision Instruments, Sarasota, FL). The reflection probe consists of a tight bundle of 13 optical fibers (12 illumination fibers around a collection fiber) with a usable wavelength range of 400–900 nm. All reflectance spectra were collected against a mirrorlike Ag film over glass substrate as a reference.

SERS Apparatus. A battery-powered Raman spectrometer (model InSpector Raman, diode laser excitation wavelength $\lambda_{\text{ex}} = 785$ nm) was purchased from DeltaNu (Laramie, WY),²⁵ which was used to demonstrate the feasibility of a field-portable device for spore detection. The remaining data were acquired using a macro-Raman system. This system consists of an interference filter (Coherent, Santa Clara, CA), a 1 in. holographic edge filter (Physical Optics Corporation, Torrance, CA), a single-grating monochromator with the entrance slit set at 100 μ m (model VM-505, Acton Research Corporation, Acton, MA), a liquid-N₂-cooled CCD detector (Model Spec-10:400B, Roper Scientific, Trenton, NJ), and a data acquisition system (Photometrics, Tucson, AZ). A titanium-sapphire laser (CW Ti:Sa, Model 3900, Spectra Physics, Mountain View, CA) pumped by a solid-state diode laser (Model Millennia Vs, Spectra Physics) was used to generate λ_{ex} of 750 nm. All of the measurements were performed in ambient conditions.

Results and Discussion

Optimization of SERS Substrates for Near-Infrared (NIR)

Laser Excitation. One goal of this work is to demonstrate the feasibility of using SERS for rapid detection of the anthrax biomarker, CaDPA, using a low-cost, battery-powered, and portable Raman spectrometer. Typically, such spectrometers use an NIR diode laser as the excitation source. One popular diode laser excitation wavelength is 785 nm. To mimic a 785 nm diode laser, we have used a CW Ti:Sa laser tuned to 750 nm as the laser excitation source. It should also be noted that NIR excitation reduces the native fluorescence background from microorganisms.

Previously, an important correlation between nanoparticle structure, as reported by the spectral position of the LSPR relative to the laser excitation wavelength, and the SERS intensity was demonstrated.^{18,22} The maximum SERS intensity is obtained from a AgFON surface when the laser excitation wavelength coincides with the LSPR maximum. Since AgFONs are not optically transparent, the reflectivity minimum was used to locate the LSPR maximum.

AgFON substrates for SERS measurements using 750 nm laser excitation were optimized by first measuring the dependence of the LSPR spectral position on nanosphere diameter. Figure 1 shows the UV-vis diffuse reflectance spectra of AgFON substrates with nanospheres having diameters of 390, 510, and 600 nm. A AgFON sample was also fabricated using

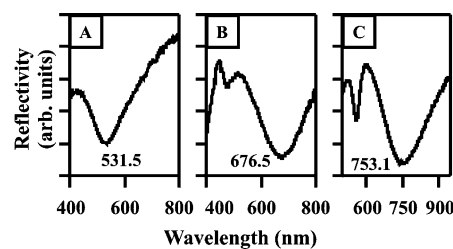


Figure 1. UV-vis diffuse reflectance spectra of different AgFON substrates in air. (A) $D = 390$ nm, $d_m = 200$ nm; (B) $D = 510$ nm, $d_m = 200$ nm; and (C) $D = 600$ nm, $d_m = 200$ nm.

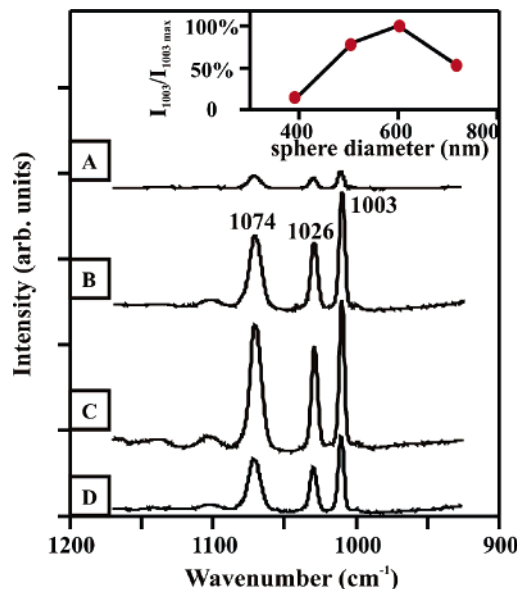


Figure 2. SERS spectra of 20 μ L, 1 mM benzenethiol in ethanol on different AgFON substrates. (A) $D = 390$ nm, $d_m = 200$ nm; (B) $D = 510$ nm, $d_m = 200$ nm; (C) $D = 600$ nm, $d_m = 200$ nm; and (D) $D = 720$ nm, $d_m = 200$ nm. The inset shows the variation of the benzenethiol SERS intensity ratio ($I_{1003}/I_{1003,\text{max}}$) with sphere sizes. $I_{1003,\text{max}}$ is taken from spectrum 2C. For all spectra, $\lambda_{\text{ex}} = 750$ nm, $P_{\text{ex}} = 3$ mW, acquisition time = 1 min.

720 nm diameter spheres; however, the spectrum is not shown because the reflectance minimum is shifted beyond the red limit (~ 900 nm) of the CCD detector. In Figure 1, the reflectance spectrum of AgFON substrate C (nanosphere diameter, $D = 600$ nm, and mass thickness of Ag film, $d_m = 200$ nm) shows a reflectivity minimum near 753 nm, attributable to the excitation of the LSPR of the silver film. This substrate is expected to show the largest intensity for 750 nm laser excitation. To further confirm this expectation, SERS spectra of 1 mM benzenethiol in 20 μ L of ethanol on the AgFON substrates with $D = 390, 510, 600,$ and 720 nm (Figure 2) were measured. The largest SERS enhancement of benzenethiol was, in fact, observed from the AgFON with $D = 600$ nm and $d_m = 200$ nm (Figure 2C). Therefore, this AgFON substrate was chosen as optimal for the bacillus spore detection experiments which follow.

Extraction of CaDPA from Spores. CaDPA was extracted from spores by sonicating in 0.02 M HNO₃ solution for 10 min. This concentration of the HNO₃ solution was selected because of its capability of extraction and its benign effect on the AgFON SERS activity. The sonication procedure was performed because no SERS signal of CaDPA was observed from the spore solution prior to sonication (data not shown). To test the efficiency of

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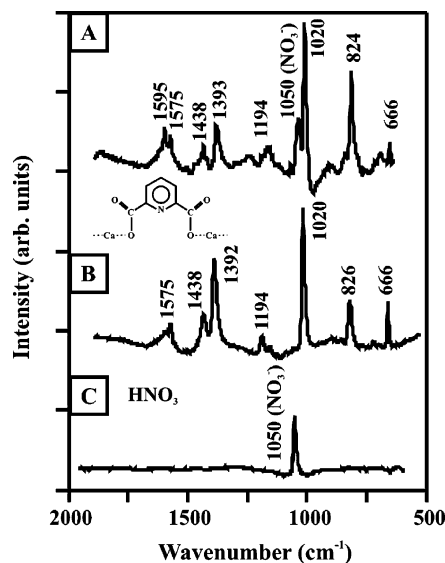


Figure 3. (A) SERS spectrum of 3.1×10^{-13} M spore suspension (3.7×10^4 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3) on a AgFON substrate. (B) SERS spectrum of 5.0×10^{-4} M CaDPA. (C) SERS spectrum of $0.2 \mu\text{L}$ of 0.02 M HNO_3 ; $\lambda_{\text{ex}} = 750 \text{ nm}$, $P_{\text{ex}} = 50 \text{ mW}$, acquisition time = 1 min, $D = 600 \text{ nm}$, $d_{\text{m}} = 200 \text{ nm}$.

this extraction method, a 3.1×10^{-13} M spore suspension (3.7×10^4 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3) was deposited onto a AgFON substrate ($D = 600 \text{ nm}$, $d_{\text{m}} = 200 \text{ nm}$). The sample was allowed to evaporate for less than 1 min. A high signal-to-noise ratio (S/N) SERS spectrum was obtained in 1 min (Figure 3A). For comparison, a parallel SERS experiment was conducted using 5.0×10^{-4} M CaDPA (Figure 3B). The SERS spectrum of *B. subtilis* spores is dominated by bands associated with CaDPA, in agreement with the previous Raman studies on bacillus spores.^{16,23} The SERS spectra in Figure 3, however, display noticeable differences at 1595 cm^{-1} , which are from the acid form of dipicolinate.²⁶ The peak at 1050 cm^{-1} in Figure 3A is from the symmetrical stretching vibration of NO_3^- .²⁷ Because of its prominence, this peak is used as an internal standard to reduce the sample-to-sample deviations.

Temporal Stability of AgFON Substrates. An ideal detection system should run unattended for long periods of time, require infrequent maintenance, and operate at low cost. Previous work has demonstrated that bare AgFON surfaces display extremely stable SERS activity when challenged by negative potentials in electrochemical experiments²⁰ and high temperatures in ultrahigh vacuum experiments.²⁸ In this work, the temporal stability of AgFON substrates was studied over a period of 40 days. SERS spectra of 4.7×10^{-14} M spores (5.6×10^3 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3), well below the anthrax infectious dose of 10^4 spores, were captured on AgFON substrates of different ages (Figure 4). The intensity ratios between the strongest CaDPA peak at 1020 cm^{-1} and the NO_3^- peak at 1050 cm^{-1} (I_{1020}/I_{1050}) were measured to quantitatively compare the AgFON substrates of different ages (shown in Figure 4 inset). Both the CaDPA spectral band positions and intensity patterns remained constant over the course of 40 days,

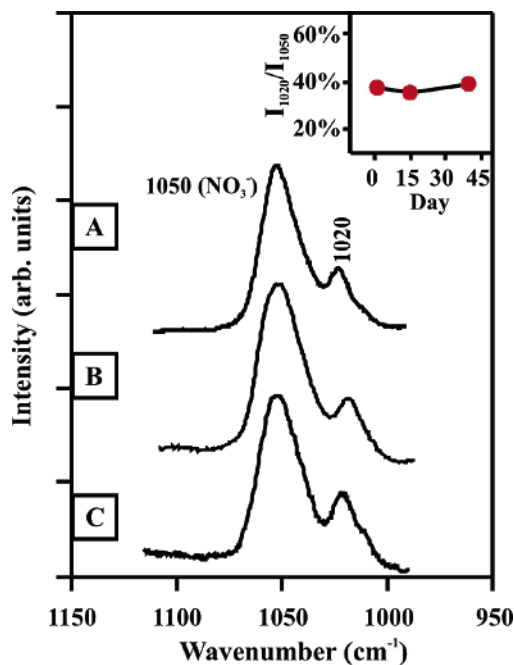


Figure 4. SERS spectra demonstrate the long-term stability of AgFON substrates, monitored for 1–40 days. SERS spectra of 4.7×10^{-14} M spore suspension (5.6×10^3 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3) on AgFON substrates. (A) A 1 day old AgFON, (B) a 15 day old AgFON, and (C) a 40 day old AgFON. The inset shows the intensity ratio (I_{1020}/I_{1050}) variation with time; $\lambda_{\text{ex}} = 750 \text{ nm}$, $P_{\text{ex}} = 50 \text{ mW}$, acquisition time = 1 min, $D = 510 \text{ nm}$, and $d_{\text{m}} = 200 \text{ nm}$.

indicating the long-term stability of the AgFON as SERS substrates for potential field-sensing applications.

Adsorption Isotherm and LOD for Bacillus Spores on AgFON Substrates. The quantitative relationship between SERS signal intensity and spore concentration is demonstrated in Figure 5A. Each data point represents the average intensity at 1020 cm^{-1} from three samples, with the standard deviation shown by the error bars. At low spore concentrations, the peak intensity increases linearly with concentration (Figure 5A inset). At higher spore concentrations, the response saturates as the adsorption sites on the AgFON substrate become fully occupied. Saturation occurs when the spore concentrations exceed $\sim 2.0 \times 10^{-13} \text{ M}$ (2.4×10^4 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3).

To be practical for long-term health and safety monitoring, a SERS-based detection system has to be capable of detecting less than the life-threatening dose of a pathogen in real or near-real time. In this study, the LOD is defined as the concentration of spores for which the strongest SERS signal of CaDPA at 1020 cm^{-1} is equal to 3 times the background SERS signal within a 1 min acquisition period. The background signal refers to the SERS intensity from a sample with a spore concentration equal to 0, which is calculated to be the intercept of the low concentration end of the spore adsorption isotherm (Figure 5A). Although lower detection limits can be achieved using longer acquisition times, these parameters are reasonable for high throughput, real-time, and on-site analysis of potentially harmful species. The LOD for *B. subtilis* spores, evaluated by extrapolation of the linear concentration range of the adsorption isotherms (Figure 5A inset), is found to be $2.1 \times 10^{-14} \text{ M}$ (2.6×10^3 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3). Furthermore, when a similar spore concentration ($2.1 \times 10^{-14} \text{ M}$, 2.6×10^3 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3) is deposited onto a AgFON surface, a

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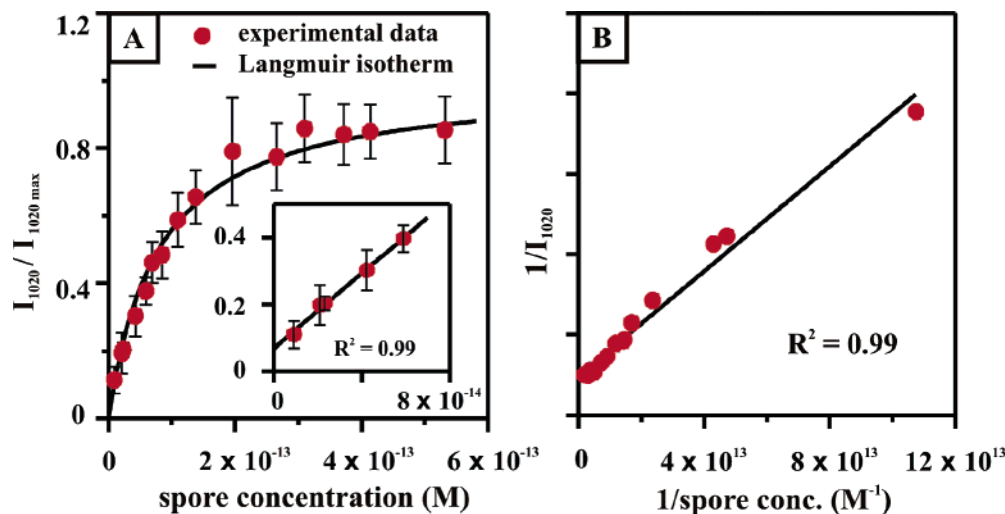


Figure 5. (A) Adsorption isotherm for *B. subtilis* spore suspension onto a AgFON substrate. I_{1020} was taken from SERS spectra that correspond to varying spore concentrations in 0.2 μL of 0.02 M HNO_3 on AgFON substrates; $\lambda_{\text{ex}} = 750$ nm, $P_{\text{ex}} = 50$ mW, acquisition time = 1 min, $D = 600$ nm, and $d_m = 200$ nm. A Langmuir curve was generated using eq 1 with $K_{\text{spore}} = 1.3 \times 10^{13} \text{ M}^{-1}$. The inset shows the linear range that is used to determine the LOD. Each data point represents the average value from three SERS spectra. Error bars show the standard deviations. (B) Adsorption data fit with the linear form of the Langmuir model (eq 2). The slope and intercept values are used to calculate the adsorption constant.

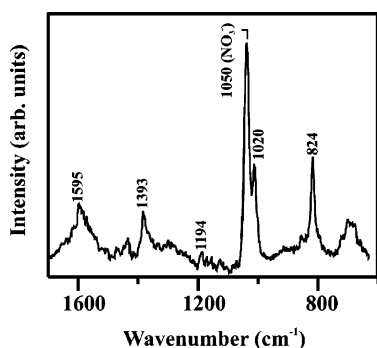


Figure 6. SERS spectrum of 2.1×10^{-14} M spore suspension (2.6×10^3 spores in 0.2 μL , 0.02 M HNO_3) on AgFON; $\lambda_{\text{ex}} = 750$ nm, $P_{\text{ex}} = 50$ mW, acquisition time = 1 min, $D = 600$ nm, and $d_m = 200$ nm.

1 min acquisition yields a SERS spectrum that clearly displays the spore Raman features at 1595, 1393, 1020, and 824 cm^{-1} (Figure 6). These data clearly demonstrate that the SERS LOD is below the anthrax infectious dose of 10^4 spores.

To determine the adsorption capacity of extracted CaDPA on a AgFON, the Langmuir adsorption isotherm was used to fit the data:^{29,30}

$$\theta = \frac{I_{1020}}{I_{1020,\text{max}}} = \frac{K_{\text{spore}} \times [\text{spore}]}{1 + K_{\text{spore}} \times [\text{spore}]} \quad (1)$$

$$\frac{1}{I_{1020}} = \frac{1}{K_{\text{spore}} \times I_{1020,\text{max}}} \times \frac{1}{[\text{spore}]} + \frac{1}{I_{1020,\text{max}}} \quad (2)$$

where θ is the coverage of CaDPA on the AgFON; $I_{1020,\text{max}}$ is the maximum SERS signal intensity at 1020 cm^{-1} when all the SERS active sites on AgFON are occupied by CaDPA; $[\text{spore}]$ is the concentration of spores (M), and K_{spore} is the adsorption constant of CaDPA extracted from spores on AgFON (M^{-1}). From eq 2, K_{spore} is calculated from the ratio between the intercept and the slope. Slope and intercept analyses of the linear

fit (Figure 5B) lead to the value of the adsorption constant, $K_{\text{spore}} = 1.7 \times 10^{13} \text{ M}^{-1}$.

Adsorption Isotherm and Extraction Efficiency of CaDPA.

Parallel studies of SERS intensities versus CaDPA concentrations indicate that the LOD is 3.1×10^{-6} M in 0.2 μL , 0.02 M HNO_3 (Figure 7A inset), and the adsorption constant for CaDPA, K_{CaDPA} , is $9.0 \times 10^3 \text{ M}^{-1}$. Under the assumption that there is no influence from the other constituents of spores on the adsorption of CaDPA from spore suspensions, the ratio between K_{spore} and K_{CaDPA} represents the extracted amount of CaDPA. Accordingly, it can be estimated that approximately 1.9×10^9 mol DPA is extracted from 1 mol spores, which corresponds to 3.0% of spore weight. Previous research found that *B. subtilis* spores contain approximately 8.9% DPA by weight.¹ Therefore, the DPA extraction efficiency of 10 min sonication in 0.02 M HNO_3 is $\sim 34\%$.

Use of Field-Portable Raman Spectrometer for Anthrax

Detection. The final goal of this project was to demonstrate the use of SERS as a field-portable screening tool by using a compact Raman spectrometer. Many field-sensing applications require the portability and flexibility not available from conventional laboratory scale spectroscopic equipment. As a first step in this direction, the Raman spectrum from 10^4 *B. subtilis* spores dosed onto a 1 month old AgFON substrate was readily acquired using a commercially available portable Raman instrument. A high S/N spectrum was achieved within 5 s (Figure 8A). The SERS peak positions and intensity pattern for the spore sample were similar to those of CaDPA recorded utilizing the same device (Figure 8B). This is the first example of using a compact, portable Raman spectrometer for the detection of bacillus spores. Coupling the portability and ease of use of this type of device with the molecular specificity and spectral sensitivity inherent to SERS, a range of possibilities are now open in the area of detecting bioagents and other chemical threats. In practical field applications of the detection method described, there might be difficulties in collecting *B. anthracis* spores out of the air and dissolving them into a small liquid volume. Most sensor modalities must face this problem.

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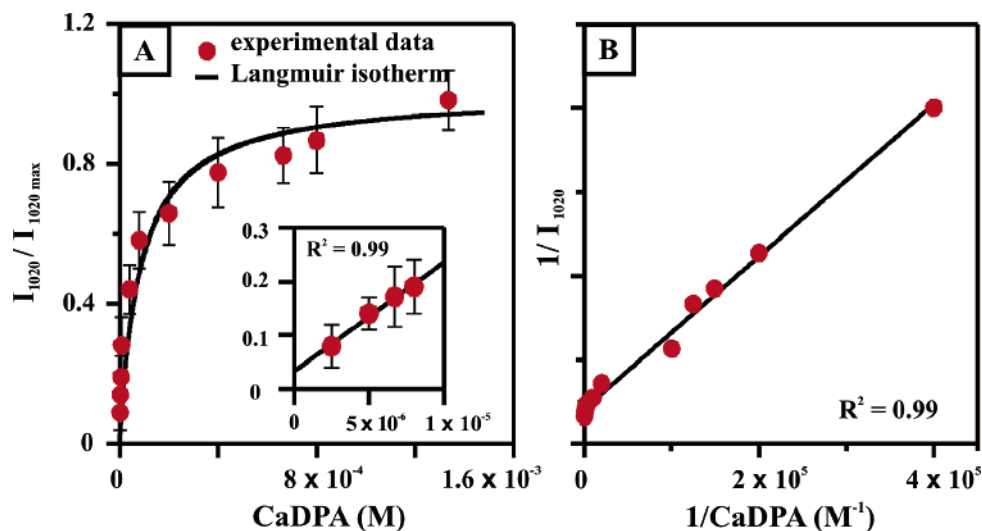


Figure 7. (A) Adsorption isotherm for CaDPA suspension onto a AgFON substrate. I_{1020} was taken from SERS spectra that correspond to varying CaDPA concentrations in $0.2 \mu\text{L}$ of 0.02 M HNO_3 on AgFON substrates; $\lambda_{\text{ex}} = 750 \text{ nm}$, $P_{\text{ex}} = 50 \text{ mW}$, acquisition time = 1 min, $D = 600 \text{ nm}$, and $d_m = 200 \text{ nm}$. A Langmuir curve was generated using eq 1 with $K_{\text{CaDPA}} = 9.5 \times 10^3 \text{ M}^{-1}$. The inset shows the linear range that is used to determine the LOD. Each data point represents the average value from three SERS spectra. Error bars show the standard deviations. (B) Adsorption data of CaDPA fit with the linear form of the Langmuir model (eq 2). The slope and intercept values are used to calculate the adsorption constant.

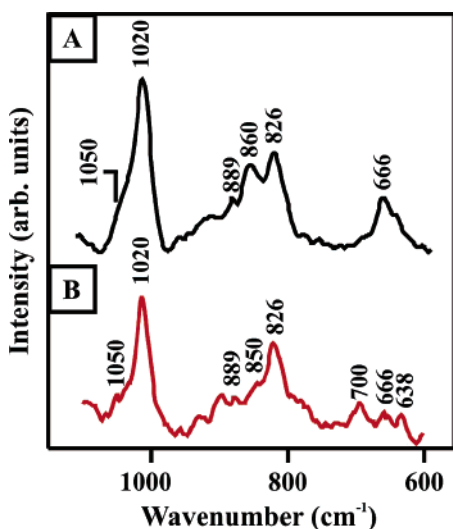


Figure 8. SERS spectra obtained by the portable Raman spectrometer. (A) SERS spectrum of $8.3 \times 10^{-14} \text{ M}$ spore suspension (1.0×10^4 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3) on 30 day old AgFON. (B) SERS spectrum of 10^{-4} M CaDPA in $0.2 \mu\text{L}$ of 0.02 M HNO_3 on 30 day old AgFON substrate; $\lambda_{\text{ex}} = 785 \text{ nm}$, $P_{\text{ex}} = 35 \text{ mW}$, acquisition time = 5 s, resolution = 15 cm^{-1} , $D = 600 \text{ nm}$, and $d_m = 200 \text{ nm}$.

Conclusions

These results represent a significant step toward the real-time detection of anthrax spores using SERS. AgFON surfaces ($D = 600 \text{ nm}$ and $d_m = 200 \text{ nm}$) were determined to be SERS intensity-optimized substrates for 750 nm laser excitation. CaDPA was rapidly extracted from *B. subtilis* spores using a 10 min sonication in 0.02 M HNO_3 , with an extraction efficiency of $\sim 34\%$. The peaks associated with CaDPA dominate the SERS spectrum of spores. The strongest peak of CaDPA at 1020 cm^{-1} was used to measure SERS intensity versus spore concentration profiles that yield an adsorption constant, $K_{\text{spore}} = 1.7 \times 10^{13} \text{ M}^{-1}$. On the basis of the linear portion of the

response curve, the LOD of *B. subtilis* spores was estimated to be $2.1 \times 10^{-14} \text{ M}$ (2.6×10^3 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3) for a 1 min data acquisition period. Furthermore, the SERS spectrum of $2.1 \times 10^{-14} \text{ M}$ spore suspension (2.6×10^3 spores in $0.2 \mu\text{L}$, 0.02 M HNO_3), well below the anthrax infectious dose of 10^4 spores, was easily measured within this acquisition time. These studies showed a lower LOD of spores and a lower incident laser power (50 mW) than any of the previously reported studies on the detection of bacillus spores based on SERS or NRS. In related experiments,²⁰ we demonstrated that the affinity between silver surfaces and dipicolinate is the key factor to lowering the LOD. Increasing the affinity binding constant by developing an appropriate capture layer on AgFON to preconcentrate CaDPA is an important future goal. The shelf life of AgFON substrates in air at room temperature is shown to exceed 40 days. Finally, preliminary integration of this novel SERS sensor with a field-portable spectrometer shows that it is possible to detect bacillus spores at the desired limit of detection ($\sim 10^4$ spores) within 5 s on a 30 day old AgFON substrate. This experiment demonstrates a necessary but not sufficient result to show the specificity of the *B. anthracis* detection based on SERS. Further confirmation awaits the SERS measurements in the presence of potential interferences.

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