Hindawi Publishing Corporation
International Journal of Spectroscopy
Volume 2012, Article ID 176851, 6 pages
doi:10.1155/2012/176851

Research Article

Detection of *Bacillus anthracis* Spores Using Peptide Functionalized SERS-Active Substrates

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Received 22 February 2012; Accepted 27 August 2012

Academic Editor: Kevin Spencer

The need for portable technologies that can rapidly identify biological warfare agents (BWAs) in the field remains an international priority as expressed at the 2011 Biological Weapons Convention. In recent years, the ability of surface-enhanced Raman spectroscopy (SERS) to rapidly detect various BWAs at very low concentrations has been demonstrated. However, in the specific case of *Bacillus anthracis*, differentiation at the species level is required since other bacilli are common in the environment, representing potential false-positive responses. To overcome this limitation, we describe the use of a peptide attached to the SERS-active metal that selectively binds *Bacillus anthracis*-Sterne as the target analyte. Using this approach, 10^9 *B. anthracis*-Sterne spores/mL produced an intense dipicolinic acid spectrum upon the addition of acetic acid, while the same concentration and treatment of *B. cereus* and *B. subtilis* did not.

1. Introduction

Since the distribution of *Bacillus anthracis* spores through the US Postal System, there has been a persistent fear that biological warfare agents (BWAs) will be used by terrorist against our warfighters abroad and our civilians at home. The extreme lethality, speed of infection, low cost, and ability to aerosolize bioagents, such as *B. anthracis,* make this scenario attractive to terrorists and a serious concern. This concern was emphasized by the Secretary of State Hillary Rodham Clinton at the December 2011 Biological Weapons Convention held in Geneva, who 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]."

Our inability to rapidly detect BWAs was revealed during the Fall of 2001 as the nation became anxious not knowing the extent of the anthrax attack. This anxiety was largely due to the several days required to grow spores in culture to sufficient numbers for detection. It became apparent that emergency and medical responders need portable technologies that can determine what has been contaminated by BWAs and who has been infected with sufficient speed and sensitivity to minimize fatalities. Since that time, there has been considerable effort to develop such technologies, such as polymerase chain reaction (PCR) systems [2] and immunoassay kits [3, 4]. While PCR systems are capable of identifying *B. anthracis* spores in ca. 2 hours and have been installed in over 300 regional US Postal Offices [5], each system is limited in the number of samples that it can process, and the systems are not portable. In contrast, immunoassays are very portable, but are unable to detect a lethal dose which has been estimated at ca. 10,000 spores (100 ng) by the Center for Disease Control [6], and, consequently, the false-positive identification rate remains unacceptably high [7]. In addition to PCR and immunoassays, other technologies are being developed that focus on speed, sensitivity, and portability. These are largely spectroscopic technologies, which include fluorescence [8], luminescence [9], mass spectrometry [10], infrared [11], and Raman spectroscopy (RS) [12, 13].

Prior to the 2001 attacks, we began investigating the capabilities of Raman and surface-enhanced Raman spectroscopy (SERS) to detect BWAs, specifically *B. anthracis*. RS has many of the desired attributes for this application. First, the rich molecular information of RS can be used to differentiate

bacterial spores from common substances, such as hoax materials [14]. This can be accomplished in microseconds using spectral search and match routines. Second, RS, combined with chemometrics, allows bacterial identification at the genus level [11, 15]. Third, RS does not require sample preparation, as the excitation laser is simply focused on the sample. Fourth, spectral acquisition is fast, requiring a minute or less. Lastly, portable RS systems have been developed in the past decade making it suitable for field measurements. In fact the ability of Raman spectroscopy to measure *Bacillus* spores on mail sorting equipment has been demonstrated [12]. While sensitivity of the analyzer was sufficient to detect visible quantities of spores, it was unable to detect invisible quantities on contaminated surfaces that could result in infection. Recognizing this limitation we also have been investigating surface-enhanced Raman spectroscopy to detect trace quantities of spores, since it is capable of increasing Raman signal intensities by as much as 6 orders of magnitude [16]. These efforts focused on the detection of dipicolinic acid (DPA) as an anthrax biomarker [17] as it represents approximately 10% of the spore weight as calcium dipicolinate (CaDPA) [18, 19]. In the past few years we developed a method capable of detecting a few hundred spores on a surface, well below the infectious dose, within 5 minutes using a portable Raman analyzer and a SERS-active capillary to collect the sample [20]. Key to successful measurements was the use of acetic acid to digest the spores and release DPA, which occurs within 1 minute at room temperature [21, 22]. The value of this method is predicated on the fact that only two common spore-forming bacteria, *Bacillus* and *Clostridium*, contain CaDPA. Nevertheless, there still exists a chance that this method could result in false alarms since several common outdoor bacilli, such as *B. cereus*, *B. subtilis*, and *B. megaterium*, could also produce a DPA SERS signal. In an effort to provide differentiation at the species level we have been investigating the ability of functionalizing silver particles with species-specific molecular recognition elements, such as antibodies, aptamers, and small peptides, to bind target BWAs. Here we present, to our knowledge, the first report of a bacterium, *B. anthracis*-Sterne, selectively bound to a peptide and detected by surface-enhanced Raman spectroscopy.

2. Experimental

2.1. Materials. Acetic acid (glacial, 99.7%), cysteine, dipicolinic acid (2,6-pyridinedicarboxylic acid), and all chemicals used to produce the silver-doped sol-gels were obtained at their purest commercially available grade from Sigma-Aldrich (Milwaukee, WI) and used as received. Calcium dipicolinate was prepared from disodium dipicolinate (Na_2DPA), which was prepared from DPA according to previous publications [23]. *B. anthracis*-Sterne, *B. cereus*, and *B. subtilis* were obtained from American Type Culture Collection (Manassas, Virginia) and grown on nutrient agar plates at 30°C for 24 hours and then stored until used. For each spore sample, 10 nutrient agar plates were inoculated and incubated at 30°C for 4-5 days. Following incubation, growths on the plates were harvested with 50 mL of sterile distilled water using a sterile hockey stick. The harvested sample was centrifuged for 5 min at 10,000 rpm in a Sorval SS-34 Rotor (Thermo Fisher, Waltham, MA). The pellet was washed four times by resuspending it in 25 mL of sterile distilled water and repeating the centrifugation. The final pellet was resuspended to a total volume of 10 mL in sterile distilled water. This sample was diluted by a factor of 100 in distilled water. A portion of this sample was placed in a hemocytometer to perform a direct spore count. The counts for *B. anthracis*-Sterne, *B. cereus*, and *B. subtilis* were 5.7×10^9, 1.7×10^{10}, and 6.1×10^9 spores/mL, respectively. In all cases, the 10^{-2} diluted samples were further diluted to 10^{-7} in sterile distilled water, and in each case a 0.1 mL sample was plated in triplicate on nutrient agar plates that were incubated overnight at 30°C. The viability counts for *B. anthracis*-Sterne, *B. cereus*, and *B. subtilis* were 2.1×10^{10}, 9.3×10^9, and 6×10^9 CFUs/mL, respectively. Three mL aliquots of the above endospore suspension were placed into three lyophilization vials and stored overnight at −80°C. The frozen samples were dried under vacuum for 12–15 hr, sealed with butyl rubber stoppers, and screw-capped.

2.2. SERS-Active Capillaries. The SERS-active capillaries (Simple SERS Sample Capillary, RTA) were prepared according to published procedures [24, 25], 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 1 N $AgNO_3$/28% NH_3OH/CH_3OH, while the alkoxide precursor consisted of methyltrimethoxysilane. The SERS capillaries were prepared by drawing 20 μL of the silver-doped sol-gels into 10 cm long, 1-mm diameter glass capillaries to produce ca. 1 cm plugs. The plugs were allowed to gel and cure, after which the incorporated silver ions were reduced with dilute $NaBH_4$.

The SERS capillaries were functionalized with a peptide synthesized to specifically bind *B. anthracis*-Sterne spores (not vegetative cells) by drawing a 5 mg/mL peptide/water solution into the SERS capillaries and allowing the peptide to attach to the silver overnight. The peptide was synthesized with a cysteine residue at the C-terminus so that it could form a covalent bond through the thiol group to the silver. Cysteine also serves as a spacer, in that the peptide is displaced from the silver surface by about 2–10 angstroms, providing space for the spores to bind. The functionalized sol-gel was then washed with a solvent to remove unbound peptide.

2.3. Sample Preparation for Measurement. Initially, 20 μL of each bacilli sample was drawn into a functionalized SERS capillary and allowed to bind for 5, 10, and 15 minutes. After this period, 5 mL of water was drawn through the capillary to remove any unbound spores. In subsequent experiments, the samples were diluted by approximately a factor of 10 to produce 10^9 spores/mL samples in HPLC grade water, and 20 μL of acetic acid was drawn into the SERS capillaries after the water wash to effect release of DPA.

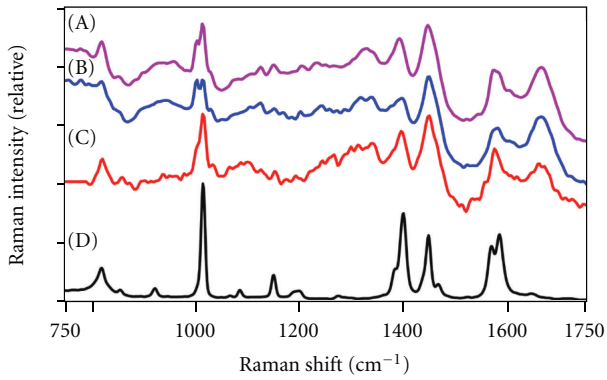

FIGURE 1: RS of (A) *B. cereus*, (B) *B. subtilis*, and (C) *B. anthracis*-Sterne spores, compared to (D) calcium dipicolinate. Spectral conditions: 500 mW of 1064 nm irradiation at the sample, (A–C) 20 min, (D) 5 min acquisition time, 8 cm^{-1} resolution.

2.4. Raman and Surface-Enhanced Raman Spectroscopy. RS measurements were typically performed using 500 mW of 1064 nm laser excitation at the sample, while SERS measurements were performed using 85 mW of 785 nm excitation at the sample (see figure captions for details). In both cases Fourier transform Raman spectrometers were used with a resolution of 8 cm^{-1} (RTA, model RamanID-785 and -1064). Fiber optics were used to deliver the excitation beam to the sample probe and the scattered radiation to the interferometer (2 m lengths of 200 and 365 μm core diameter, resp., Spectran, Avon, CT). For the RS measurements the samples were placed in sealed glass vials, mounted horizontally above the probe, and measured. For SERS measurements the capillaries were mounted horizontally on an *XY* plate reader (Conix Research, Springfield, OR), and in-house software was used to measure five points spaced along the length of the metal-doped sol-gel plug. The five-point relative standard deviation was typically 20% [22], and the reported spectra are the average of these five 1 min measurements. The spot size for both RS and SERS was approximately 300 microns in diameter.

3. Results and Discussion

The fingerprint region of the Raman spectra of bacilli is dominated by calcium dipicolinate peaks at 821, 1014, 1391, 1446, and 1573 cm^{-1}, which have been assigned to a ring CC bend, an out-of-plane CH bend, the symmetric pyridine ring stretch, a symmetric OCO stretch, a symmetric ring CH bend, and an asymmetric OCO stretch, respectively, (Figure 1) [12]. The remaining dominant spore peaks have been assigned to protein modes associated with the peptidoglycan cell wall, such as amino acids and peptide linkages [26–29]. The 1003 and 1598 cm^{-1} peaks are assigned to phenylalanine modes, while the 1318 and 1666 cm^{-1} peaks are assigned to amide III combination modes and the amide I mode, which is primarily a C=O stretch. In several cases, protein and CaDPA vibrational modes occur at or close to the same frequency, such as the 821 and 1446 cm^{-1} peaks. In fact the only obvious difference between the RS of the three bacilli is the relative intensity of the CaDPA pyridine ring stretching mode at 1014 cm^{-1}, especially when compared to the intensity of the phenylalanine ring stretch at 1003 cm^{-1}. While it is tempting to use this difference to identify the *Bacillus* species, it more likely reflects variations in the growth media and growth conditions. Indeed others have shown that dramatic spectra differences occur when different growth media and growth conditions are used [30], and only if identical growth conditions are used would identification of the bacilli at the species level be possible [15].

The greater limitation of Raman spectroscopy is its sensitivity. High quality spectra, such as that shown in Figure 1, require high laser powers and long acquisition times. As previously noted this can be overcome using surface-enhanced Raman spectroscopy, and several researchers have demonstrated that bacteria can be identified at the genus level [31–34]. Yet, in several cases spectra of different genera, such as *B. subtilis* and *E. coli* collected by the same research group, are virtually identical [31–33], while they are completely different between groups. Although this could be attributed to the use of gold versus silver to generate SERS, different spectra are still observed when different groups use silver [31, 32, 34]. At present there is not a consensus regarding the ability of RS or SERS to differentiate bacteria at the species level.

This uncertainty can be eliminated by functionalizing the SERS-active metal with molecular recognition elements that only bind one type of bacteria and preferably one bacterial species. Here, a peptide was synthesized to bind to the surface of *B. anthracis*-Sterne, but not other bacilli, such as *B. cereus* and *B. subtilis*. Cysteine was added to the C-terminus of the peptide so that it could form a chemical bond with the silver particles contained within the sol-gel. The SERS of the peptide functionalized silver-doped sol-gel is virtually identical to the SERS of cysteine (Figures 2(B) and 2(C)), dominated by a S-Ag stretching mode at 660 cm^{-1}, and cysteine peaks at 810, 875, 1060, 1300, 1355, and 1410 cm^{-1}. The first set of experiments involved adding various amounts of *B. anthracis*-Sterne spores to the peptide functionalized silver-doped sol-gel to see if it produced a unique spectral signature indicative of spore binding. Even after allowing the spores to bind for 15 minutes, no signature appeared, and, at best, the spores decreased the signal intensity of the peptide to a modest degree (Figure 2(A)).

Prior to the addition of acetic acid to the presumably bound *B. anthracis*-Sterne spores, the SERS for all three bacilli added to unfunctionalized silver particles was measured with the addition of acetic acid to ensure that they all produced equivalent spectra. This was indeed the case, as each *Bacillus* was dominated by the DPA peaks at 812, 1006, 1381, 1426, and 1567 cm^{-1} (Figure 3), which have the same assignments as the CaDPA peaks at similar wavenumbers (Figure 1).

In the last set of experiments, *B. anthracis*-Sterne, *B. cereus*, and *B. subtilis* samples were separately added to 3 SERS-active capillaries functionalized with the *B. anthracis*-Sterne-specific peptide, allowed to bind for 15 minutes, then washed with water to remove unbound spores, followed by

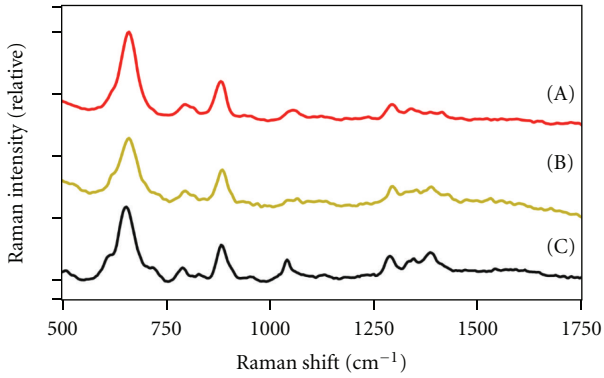

Figure 2: SERS of (A) 10^9 spores/mL *B. anthracis*-Sterne bound to the *B. anthracis*-Sterne peptide functionalized SERS-active substrate, (B) 5 mg/mL *B. anthracis*-Sterne peptide functionalized SERS-active substrate, and (C) 1 mg/mL cysteine. Spectral conditions: 80 mW of 785 nm irradiation at the sample, 1 min acquisition time, 8 cm^{-1} resolution.

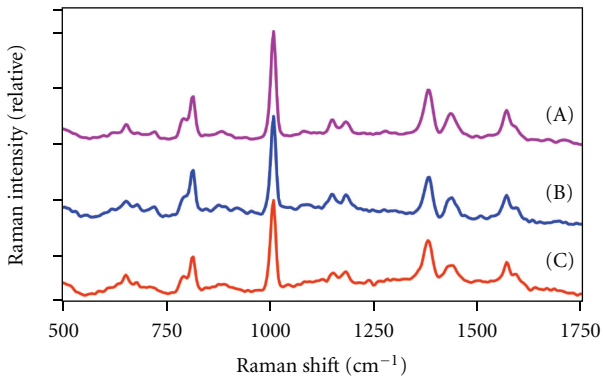

Figure 3: SERS of (A) *B. cereus*, (B) *B. subtilis*, and (C) *B. anthracis*-Sterne all at a concentration of 10^9 spores/mL treated with acetic acid. Spectral conditions as in Figure 2.

the addition of acetic acid. Only the *B. anthracis*-Sterne sample produced a DPA spectrum, while the two negative control samples produced spectra only of the peptide at reduced intensity (Figure 4), clearly demonstrating selectivity.

While high quality spectra of 10^9 spores/mL *B. anthracis*-Sterne were repeatedly obtained, sensitivity quickly decreased at lower concentrations, and no signal was obtained at 10^7 spores/mL. While this sensitivity is similar to other work [34], it is considerably less than previous work that included the use of acetic acid, but not the use of a binding peptide, in which 2.2×10^5 spores/mL could be measured [22]. At present this loss is partially attributed to the peptide reducing the amount of silver surface available to the DPA molecules.

In an effort to estimate an enhancement factor, a 0.55 mg/mL DPA in 1 N KOH was measured by RS (Figure 4(A)). This concentration corresponds to 55×10^9 spores assuming each spore contains 10% DPA by weight. Taking into account the relative concentrations, the signal intensity

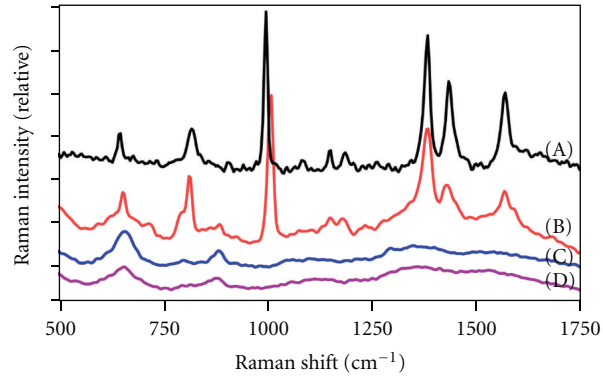

Figure 4: (A) RS of 0.55 mg/mL DPA in 1 N KOH in a plain glass capillary (intensity multiplied by 3.5 to match SERS intensity of 1000 cm^{-1} peak of (B)). Spectral conditions: 300 mW of 785 nm irradiation at the sample, 25 min acquisition time, 8 cm^{-1} resolution. SERS of (B) *B. anthracis*-Sterne, (C) *B. cereus*, and (D) *B. subtilis* all at a concentration of 10^9 spores/mL on the *B. anthracis*-Sterne peptide functionalized SERS-active substrate after a 15-min incubation period, a wash step, and the addition of acetic acid. Spectral conditions for (B)–(D) as in Figure 2.

of the symmetric stretching mode at ~1000 cm^{-1}, and the laser powers used for this measurement and the acetic acid treated peptide bound *B. anthracis*-Sterne measurement (Figure 4(B)), a modest enhancement factor of 722 is calculated.

Finally, it is worth stating that the *XY* plate reader, which held the SERS-active capillaries, was used as a matter of convenience and could easily have been replaced by a simple fixed sample holder coupled to a portable Raman analyzer so that measurements could be performed in the field. This is in contrast to previous work [31–34], in which the samples were measured using microscope systems, limiting portability.

4. Conclusion

In this work we have successfully demonstrated the use of peptide-functionalized silver particles to selectively bind a target analyte and generate surface-enhanced Raman scattering. Furthermore, it was shown that discriminate detection of spores at the species level, specifically *B. anthracis*-Sterne and not *B. cereus* and *B. subtilis*, could be performed. However, this improvement in specificity comes with a loss in sensitivity, as the inclusion of the peptide appears to reduce the available silver surface. It is also worth noting that at present the method is limited to bacteria with an extractable SERS-active signature, such as DPA. Current work is aimed at improving sensitivity by modifying the chemistry to increase the available silver surface as well as extending this analysis to other BWAs.

Authors' Contribution

All authors contributed equally to this work.

Acknowledgments

The authors are grateful to Professor Jay Sperry of the University of Rhode Island for growing and supplying the bacilli samples for the measurements presented here. They are also appreciative of funding from the National Science Foundation (DMI-0349687, IIP-0810335, and II-0956170).

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