

Detection of *Bacillus* Spores by Surface-Enhanced Raman Spectroscopy

Stuart Farquharson¹, Chetan Shende¹, Alan Gift² and Frank Inscore¹

¹*Real-Time Analyzers,*

²*University of Nebraska, Omaha, USA*

1. Introduction

On September 18 and October 9, 2001, two sets of letters containing *Bacillus anthracis* spores passed through the United States Postal Service's Trenton, NJ, Processing and Distribution Center (Jernigan et al., 2001). The first set was destined for Florida and New York, while the second set was destined for Washington, DC. The infection of 22 people by these spores resulted in 5 deaths: a media employee in Florida, two postal workers in DC, a hospital worker in New York, and a retired woman in Connecticut (Inglesby et al., 2002). This bioterrorism event closely followed the September 11, 2001 attack on the Pentagon building and the World Trade Center towers, which added to the nation's concern about terrorism within US borders. There was additional anxiety associated with this second attack, in that the extent of spore distribution along the east coast was unknown, and it took a long time to determine who was infected. This was even true for the letter that was mailed to Senator Tom Daschle at the Hart Senate Office (HSO) Building in DC. While the powder that fell from the letter was immediately suspected as *B. anthracis*, due to the previous week's news from Florida and New York, and collection of nasal swabs from HSO personnel was initiated within 9 hours (Hsu et al., 2002), it still took several additional days to determine who was exposed and infected (Jernigan et al., 2001).

This delay was due to the fact that the spores must be germinated and grown in culture media to sufficient cell numbers so that they can be measured by standard methods. Presumptive *B. anthracis* was based on shape (1 to 1.5 by 3 to 5 μm rods), lack of motility, lack of a hemolysis on a sheep blood agar plate, susceptibility to β -lactam antibiotics and to γ -phage lysis, and staining for gram-positive bacteria (Center for Disease Control and Prevention, CDC, 2001). The time consuming component of this analysis is the culture growth of cell colonies. Simply put, the fewer the initial number of spores, the less likely a sample will produce detectable colonies. Only samples collected from surfaces or individuals that had a high probability of being contaminated produced colonies that were evident in 24 hours. Furthermore, tests were initially limited to individuals within the vicinity of where the letter was opened, which proved insufficient, as three postal workers at the Brentwood, DC, Processing and Distribution Center became infected, two fatally (Jernigan et al., 2001; Sanderson et al., 2002). Upon notification of their hospitalization, the CDC initiated collection of several hundred environmental (mostly surface) samples from

associated facilities and several thousand nasal swab samples from visitors to these facilities, including first responders, and their employees. According to the team at the National Institutes of Health, who processed nearly 4000 samples, current methods of culture growth and analysis were “extremely time-consuming and labor-intensive” (Kiratisin et al., 2002).

From these bioterrorist attacks, it became clear that considerably faster methods of analysis are required. This would expedite assessment of the extent of attack, including the path of such letters from destination back to origination. More importantly, it would minimize fatalities, since it was learned that if exposure is detected within the first few days, the majority of victims can be treated successfully using Ciprofloxacin, doxycycline and/or penicillin G procaine (Bell et al., 2002).

At the time of the attack two rapid methods were used, immunoassay kits and polymerase chain reaction (PCR) analyzers. Immunoassay methods use competitive binding of the bioagent (as an antigen) and its labeled conjugate for a limited number of antibodies. These methods can be relatively fast (<20 minutes) with modest sensitivity. The latter was not an issue for the Senator Daschle letter, which contained billions of spores (Kennedy, 2001). However, as yet no well-defined anthrax antigen has been identified (Bell et al., 2001; Kellogg, 2010), and as a result, the false-positive rates remain unacceptably high. Consequently, immunoassay development has shifted to detecting the *B. anthracis* proteins involved in infection. This includes the protective antigen and lethal factor (Bell et al., 2002; Mabry et al., 2006; Tang et al., 2009). But these immunoassays require several days after the onset of infection for these proteins to reach detectable concentrations, even with the use of time-resolved fluorescence detection, which may not provide sufficient time to substantially improve the odds of successful treatment (Tang et al., 2006).

During the 2001 bioterrorism event NIH employed Applied Biosystems (Foster City, CA) to use PCR to sequence the 16S rRNA *B. anthracis* gene (Kiratisin et al., 2002). However, the specificity of this gene for *B. anthracis* was in doubt as Bacilli are highly homologous to the extent that *B. anthracis*, *B. cereus* and *B. thuringiensis* may belong to one species (Helgason et al., 2000, Sacchi et al., 2002). Since the attacks, more definitive *B. anthracis* gene sequences have been identified. Specifically, the genes within the toxin encoding pXO1 plasmid and the capsule-encoding pXO2 plasmid are being targeted for analysis. The development of “real-time” PCR systems that combine the use of primers to separate these organism-specific nucleic acid sequences and polymerases to amplify the sequences (Bell et al., 2001 & 2002; Thayer, 2003), resulted in the installation of such systems at some 300 regional US Postal offices through 2006 at a cost of \$600 million (Shane, 2004; Leingang, 2004).

While, PCR and immunoassays continue to be developed, other methods are also being developed that rapidly assess the extent of contamination in the event of an attack, such as fluorescence, luminescence, mass spectrometry, and Raman spectroscopy (Nudelman et al., 2000; Pellegrino et al., 2002; Hathout et al., 2003; Farquharson et al., 2004, respectively). These methods focus on portability and sensitivity, rather than on specificity.

Most of these methods have been focusing on the detection of calcium dipicolinate (CaDPA) or its derivatives as a *B. anthracis* signature since it has been reported that CaDPA represents 10 to 15% by weight of these spores (Fig. 1; Janssen et al., 1958; Murrell et al., 1969; Hindle & Hall, 1999; Ragkousi et al., 2003; Liu et al., 2004; Phillips & Strauch, 2002). This is a valid approach, first because only 13 genera of spore-forming bacteria contain CaDPA (Berkeley

& Ali, 1994), but only *Bacillium* and *Clostridium* are common (and of interest; Phillips & Strauch, 2002), and second, the most widespread, potentially interfering spores, such as pollen and mold spores, do not. Relatively fast methods have been developed to chemically extract the acid of CaDPA, dipicolinic acid (DPA; Pellegrino et al., 2002), and then to detect it directly by mass spectrometry (Beverly et al., 1996; Hathout et al., 2003), fluorescence (Nudelman et al., 2000), or indirectly by luminescence (Pellegrino et al., 2002; Rosen et al., 1997). Although mass spectrometry provides a relatively high degree of discrimination and sensitivity, it still requires significant time due to sample preparation. Hot dodecylamine (DDA) has been used to extract DPA and form a highly luminescent complex with terbium (Pellegrino et al., 2002). Although measurements have been performed in as little as five minutes, it was found that as many as three concentration-dependent complexes can form, each with different lifetimes. This, coupled with the fact that the Tb^{3+} cation produces the same luminescence spectrum, makes determinations of low spore concentrations problematic. Furthermore, the combination of heat and the DDA surfactant severely degrade the spore, generating cell debris. This requires sample cleanup and in this particular case, $AlCl_3$ has to be added to remove phosphates that would interfere with the photoluminescent measurement.

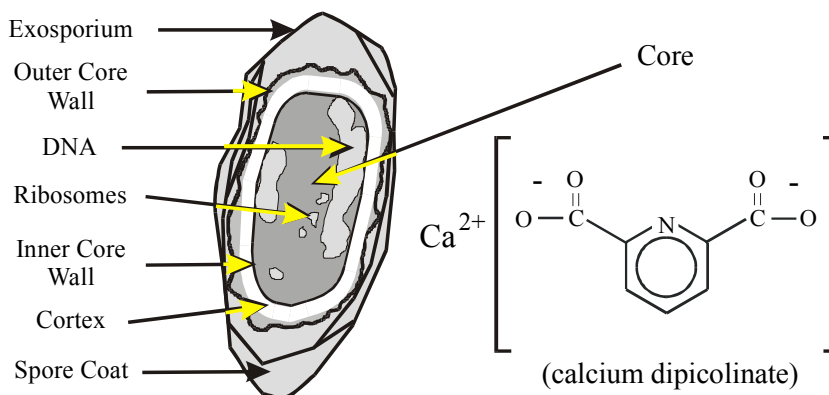


Fig. 1. Illustration of a *Bacillus* spore with major components indicated, and chemical structure of calcium dipicolinate. Deprotonated dipicolinic acid is shown within the brackets.

An alternative method, Raman spectroscopy (RS), is attractive in that very small samples can be measured without preparation. The sample need only be placed at the focal spot of the excitation laser and measured. Moreover, the rich molecular information provided by Raman spectroscopy usually allows unequivocal identification of chemicals and biologicals. As early as 1974 the Raman spectrum of *Bacillus megaterium* was measured and shown to be dominated by CaDPA (Woodruff et al., 1974). However, the spectrum was collected using pure spores and took hours to acquire. Since that time, considerable improvements in Raman instrumentation have led to laboratory measurements of single *Bacillus* spores and field measurements of spores captured from a mail sorting system (Esposito et al., 2003; Farquharson et al., 2004). Unfortunately, the single spore measurements required the use of a microscope system and time consuming efforts to locate the spores, while the field measurements required milligrams of sample.

Two approaches are widely used to improve the sensitivity of Raman spectroscopy; resonance Raman spectroscopy and surface-enhanced Raman spectroscopy (SERS). The former method involves laser excitation at or near the wavelength of a molecular electronic absorption to substantially increase the interactions between the radiation and molecular states, and was used more than a decade ago to analyze *Bacillus* spores (Ghiamati, 1992). The value of this technique is limited by the extremely low energy conversion of ultraviolet lasers, which require substantial power supplies and thus confine measurements to laboratory settings.

SERS involves the absorption of incident laser photons within nanoscale metal structures, generating surface plasmons, which couple with nearby molecules (the analyte) and thereby enhance the efficiency of Raman scattering by six orders of magnitude or more (Jeanmaire & Van Duyne, 1977; Weaver et al., 1985). More than a decade ago, we began investigating the potential of SERS to measure *B. anthracis* ssp. spores, beginning with the first measurement of the biomarker dipicolinic acid (Farquharson, 1999). In this chapter we summarize our efforts to develop a field portable analyzer that uses a simple digesting agent to extract DPA from *B. anthracis* spores for detection by SERS.

The approach and ultimately the success of these efforts not only depend on the instrumentation, but also on the specific terrorist scenario being addressed. This has significant implications for the choice of sampling. For example, detecting a plume of spores released from an airplane is very different than detecting spores in envelopes passing through a mail sorting machine. Here the focus is the detection of spores on surfaces to assess the extent of an attack. For this application specificity at the *Bacillus* spp. level is sufficient, i.e. it is not critical to differentiate between the various *Bacillus* spp. At present there are no guidelines defining the required sensitivity. However, an extensive number of surface samples were collected from the Brentwood, DC, mail Processing and Distribution Center and their analysis can be used as a guide to estimate sensitivity requirements (Sanderson et al., 2004). This analysis determined that the highest concentrations of spores, not surprisingly, were in the immediate vicinity of delivery barcode sorter machine number 17, which processed both letters. Analysis of dust above, within 30 meters, and 30-60 meters of this machine recorded average values of 310, 67, and 10 CFU/in². Since the last average value included measurements that detected "zero" spores, it lacks the certainty of the other values. Consequently, and somewhat subjectively, we have chosen the middle value, 67 CFU/in² (10 spores/cm²), as a minimum requirement for measurement sensitivity. This value should not be construed as a definition of lethality.

Additional measurement requirements include the ease of sampling and speed of analysis. Based on the 2001 attacks, we consider a minimum requirement of 500 measurements per 24 hours as reasonable. Of course more than one analyzer could be used to accomplish this, but the fewer the analyzers, the lower the cost and the number of operators. If one analyzer is used, then the required measurement time would be less than 3 minutes. This would include the time to collect, deliver, measure, and analyze the sample. This suggests that sampling should involve a method or device to rapidly collect the sample (e.g. a wet swab or vacuum system) and deliver it to the measurement compartment of the analyzer. It also suggests that the analyzer should be portable to minimize or eliminate sample delivery time.

With these criteria in mind, we have developed a three-step method to detect dipicolinic acid extracted from surface spores by surface-enhanced Raman spectroscopy. The first step employs acetic acid to break apart the spores and release CaDPA into solution as DPA. The second step employs single-use, disposable, sol-gel filled capillaries to separate the DPA from other cell components and simultaneously deliver it to the SER-active metal particles. The third step employs a portable Raman analyzer to measure the SER spectrum and to identify and quantify the spores, if present. Development of this three-step method and measurements of *Bacillus* ssp. spores on surfaces are presented.

2. Experimental

Dipicolinic acid (2,6-pyridinedicarboxylic acid), acetic acid (glacial, 99.7%), dodecylamine (DDA), 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 (Ghiamati, 1992). *Bacillus cereus*, *B. subtilis*, and *B. megaterium* were grown on nutrient agar plates at 30°C for 7 days (Ghiamati, 1992). The vegetative cells were placed into distilled water and lysed by osmotic pressure. The resultant spores were collected by scraping them into distilled water and pelleting them by centrifugation at 12,100 x g for 10 minutes. The spore pellet was re-suspended in distilled water and lyophilized, and scraped into glass vials for Raman spectral measurements. Approximately 1 gram each, determined to be 99% pure by microscopic observation, was produced for this study. The density of the spores varied from 0.06 to 0.11 g/mL, indicating a high amount of entrained air.

Calibrated samples of *B. cereus* spores were prepared by weighing 1-5 mg specks, dispersing them in 10 mL water using sonication and vortexing. Sonication was accomplished using an Ultrasonik 300 (Neytech, Burlington, NJ), while vortexing was accomplished using a Vortex-Genie 1 (Scientific Industries, Bohemia, NY). The spores per volume water were calibrated by using a Brite-line, phase-contrast hemocytometer counting grid (AO Scientific Instruments, Buffalo, NY) and imaged using an Olympus BX 51 microscope (Olympus America, Center Valley, PA) fit in-house with a Sony Cyber-shot 2.1 digital camera.

An initial stock solution of 20 mg of DPA in 20 mL HPLC grade water (Fischer Scientific, Fair Lawn, NJ) was prepared for the pH study. The pH of this solution was 2.45 as verified using a pH electrode (Corning 314 pH/Temperature Plus, Corning, NY) that had been calibrated with pH 4.00, 7.00, and 10.00 buffer solutions (Fischer Scientific). For all pH measurements a single 2-mL glass vial coated with silver-doped sol-gel was used (*Simple SERS Sample Vial*, Real-Time Analyzers, Inc. (RTA), Middletown, CT). The vial was never moved from the sample holder to ensure that the same portion of silver-doped sol-gel was examined. Two pH series were performed. First, 2 mL of the stock solution was added to the vial and measured. Then the 2 mL solution was returned to the stock solution and made basic using 0.1 M KOH. Prior to re-addition of the solution to the SER-active vial, the vial was first rinsed three times with distilled water, then twice with the new solution prior to SERS measurement. This procedure was followed to obtain spectra at pH 3.55, 4.33, 4.87, 5.59, 10.69 and 11.66. Next the solution was brought to a pH of 2.00 by adding 0.1 M HNO_3 , and the spectrum was recorded. Again KOH was added drop wise to make the solution more basic. Spectra were obtained at pHs of 3.83, 5.10, 7.35 and 8.22. Next HNO_3 was added

drop wise so that spectra could be obtained at pHs of 2.19, 1.71, 1.35 and 1.17. Throughout this process, no more than 20 drops of acid or base were added, and therefore the concentration was diluted by no more than 10%.

For concentration measurements, a second stock solution of DPA was prepared as above and used to prepare all lower concentration samples by serial dilution using HPLC grade water. Initial spore experiments employed 78 °C, 50 mM dodecylamine to digest the spores and release dipicolinic acid for measurement. Pre-weighed spore particles were placed on a glass plate for these measurements. Final spore measurements employed room temperature acetic acid to digest the spores. Pre-weighed spores were used to prepare a stock solution for calibration using a counting grid (see below), from which a known number of spores were dried on a glass plate. For both digesting chemicals, after 1 min exposure, the degraded spore sample was drawn into a SER-active capillary (*Simple SERS Sample Capillary*, RTA) for measurement.

The SER-active vials were prepared according to published procedures (Farquharson & Maksymiuk; 2003), using a silver amine precursor to provide the metal dopant and an alkoxide precursor to provide the sol-gel matrix. The silver amine precursor consisted of a 5/1 v/v ratio of 1N AgNO₃ to 28% NH₃OH, while the alkoxide precursor consisted of a 2/1 v/v ratio of methanol to tetramethyl orthosilicate (TMOS). The alkoxide and silver amine precursors were mixed in an 8/1 v/v ratio, then 140 μL were introduced into 2 mL glass vials, which were then spin-coated. After sol-gel formation, the incorporated silver ions were reduced with 0.03M NaBH₄. The SER-active capillaries were prepared in a similar manner with the following modifications. The alkoxide precursor employed a combination of methyltrimethoxysilane (MTMS) and TMOS in a v/v ratio of 6/1, which was mixed with the amine precursor in a v/v ratio of 1/1. Approximately 15 μL of the mixed precursors were then drawn into a 1-mm diameter glass capillary coating a 15-mm length. After sol-gel formation, the incorporated silver ions were again reduced with dilute sodium borohydride.

All Raman spectroscopy measurements were performed using 785 or 1064 nm laser excitation and Fourier transform Raman spectrometers (RTA, model *RamanID-785* and -*1064*; Farquharson et al., 1999). For pure Na₂DPA, CaDPA, and the spore samples 1064 nm excitation was used, for pure DPA and DPA solutions both 785 and 1064 nm laser excitation were used, while for all DPA SERS measurements, solutions or extractions, 785 nm laser excitation was used. 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, respectively, Spectran, Avon, CT).

For 1064 nm excitation, a 24 mm diameter f/0.7 aspheric lens focused the beam to a 600 μm spot on the sample and to collect the scattered radiation back along the same axis. An f/2 achromat was used to collimate laser beam exiting the source fiber optic, while a 4 mm prism was used to direct the beam through an f/0.7 aspheric lens that focused the beam to a 600 micron spot on the sample. The scattered radiation was collected back along the same optical axis, while a second f/2 lens focused the beam into the collection fiber optic. A short pass filter was placed in the excitation beam path to block the silicon Raman scattering generated in the source fiber from reflecting off sampling optics and reaching the detector. A long pass filter was placed in the collection beam path to block the sample Rayleigh scattering from reaching the detector. For 785 nm excitation, a similar optic probe was used,

except a dichroic filter was used to reflect the laser light to sample and pass the Raman scattered radiation to the collection fiber. In this case the beam was focused to a 300 μm spot on the sample. Also, appropriate short and long pass filters were used for this wavelength. All spectra presented were collected using 8 cm^{-1} resolution.

In the case of Raman spectral measurements of spores, the samples were placed on a glass slide with the probe aimed downward. In the case of SER-active vials or capillaries, the samples were mounting horizontally on an XY positioning stage (Conix Research, Springfield, OR), so that the probe aimed upwards and the focal point of the aspheric lens was just inside the vial or capillary.

For the SERS concentration and extraction measurements, nine spectra were recorded along the length of the capillary with 1 mm spacing. As a practical approach to minimizing the variability associated with the SER activity as a function of sample position, the three high and three low intensity spectra were discarded, while the three median spectra were averaged and reported. Relative standard deviations for all concentrations are reported as percent standard deviation in Table 2. SER spectra were collected in 1-min or less as indicated in figure captions.

3. Results and discussion

The present application begins with a Raman spectral analysis of *Bacillus* spores with regards to contributions from calcium dipicolinate. The primary CaDPA peaks occur at 659, 821, 1014, 1391, 1446, 1573, 3062, and 3080 cm^{-1} in the spore spectrum (Fig. 2), and can be 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, an asymmetric OCO stretch, and the CH symmetric and asymmetric stretches, respectively (Table 1, Farquharson et al., 2004).

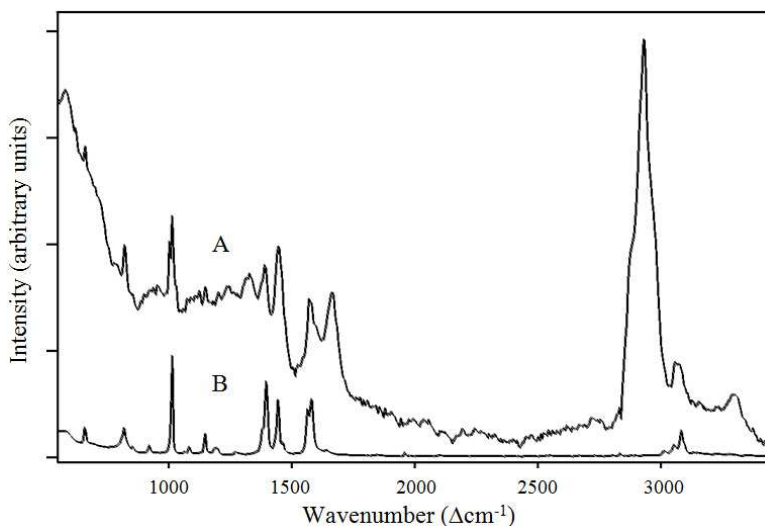


Fig. 2. RS of A) *Bacillus cereus* spores and B) calcium dipicolinate. Spectral conditions: 500 mW of 1064 nm at the sample, 5 min acquisition time, 8 cm^{-1} resolution.

The remaining peaks can be assigned to protein modes associated with the peptidoglycan cell wall, such as amino acids and peptide linkages (amide modes; Woodruff et al., 1974; Ghiamati, 1992; Grasselli et al., 1981; Bandekar, 1992; Austin et al., 1993). The former include peaks with little intensity at 821, 855, 900 cm^{-1} , which are assigned to several CC bending modes, as well as the phenylalanine modes that appear at 1003 and 1598 cm^{-1} . The latter include the amide I peak at 1666 cm^{-1} , which is primarily a C=O stretch, and amide III combination peaks at 937, 1241, and 1318 cm^{-1} , which are various CC and CN stretching combinations (peak positions are given for *B. cereus*). 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.

Next, the amount of CaDPA available in a spore that could be measured as DPA was considered. Although it is often stated that *Bacillus* spp. spores contain 10-15% calcium dipicolinate by weight (Janssen et al., 1958; Liu et al., 2004), this value has been reported as low as 1% (Halverson, 1961). Since this amount will be used to calculate the number of spores measured, it is important to have as accurate a number as possible. For this reason, the Raman spectra of *Bacillus subtilis*, *B. megaterium*, and *B. cereus* were acquired (Fig. 3). In fact it was found that the most obvious differences between the spectra for the three *Bacillus* species are the CaDPA peaks. In particular, the 1014 cm^{-1} peak noticeably changes intensity, especially when compared to the neighboring phenylalanine peak at 1003 cm^{-1} . If it can be assumed that the composition of these *Bacilli* is very similar, then it may be assumed that the relative phenylalanine concentration is nearly constant and its Raman peak can be used as an internal intensity standard. (The amide I peak at 1666 cm^{-1} could also be used.) Using the ratio of the CaDPA and phenylalanine peak heights suggests then that the salt concentrations for *B. megaterium* and *B. cereus* are 1.85 and 2.05 times that of *B. subtilis*. In the latter case, a recent study using resonance Raman spectroscopy of the same sample

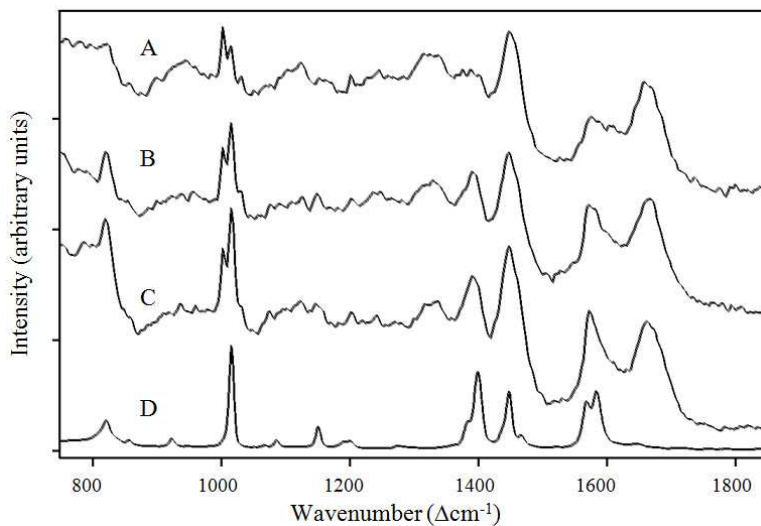


Fig. 3. RS of A) *B. subtilis*, B) *B. megaterium*, C) *B. cereus*, and D) CaDPA. Spectral conditions as in Fig. 2.

concluded that the CaDPA peak intensity corresponded to 6-7 weight percent (Nelson et al., 2004). This suggests that the CaDPA weight percent for the *B. megaterium* and *B. cereus* spore samples are 11-13 and 12.5-14.5 wt%, respectively, or in the case of DPA, 9-11 and 10-12 wt% (based on MW). It should be noted that the differences between these *Bacillus* spp. does not imply that the CaDPA concentrations are species specific. It is more likely that experimental conditions during the original growth of the bacteria, such as time, temperature, and available nutrients, influenced the extent of sporulation. Consequently, any calculations of the number of spores based on DPA content should assume a range of at least 5-13 weight percent of the spores. As a practical matter $10 \pm 5\%$ will be used here.

For comparison purposes, the spectra of CaDPA and DPA are shown in Fig. 4, along with Na_2DPA , while the observed spectral peaks with vibrational mode assignments are listed in Table 1. The assignments for both CaDPA and DPA, based on literature (Carmona 1980; Hameka et al., 1996), were used to assign the peaks observed for Na_2DPA . Both DPA and Na_2DPA contain unique peaks with significant intensity at 760 and 1730 cm^{-1} , respectively. Since neither peak is observed in the spectrum of CaDPA, it can be concluded that this sample does not contain either chemical as an impurity.

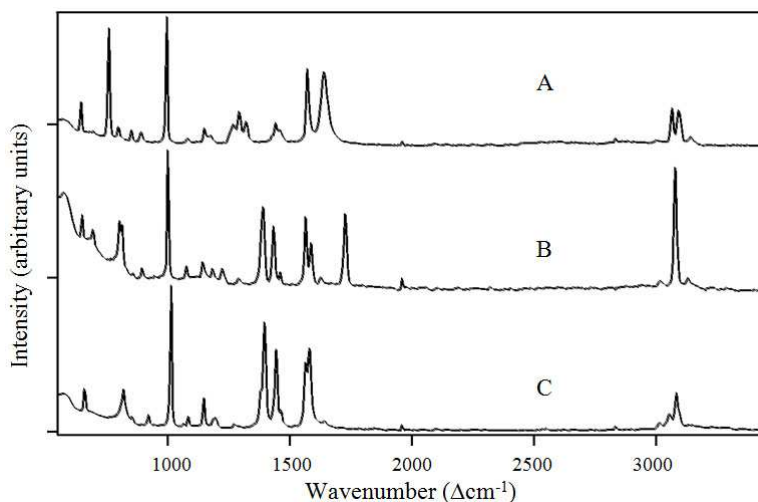


Fig. 4. RS of A) dipicolinic acid B) disodium dipicolinate, and C) calcium dipicolinate. Spectral conditions as in Fig. 2.

Next, dipicolinic acid was analyzed by SERS. The assignment of SERS peaks to vibrational modes is less straightforward than for RS peaks due to the metal-to-molecule surface interactions that shift and enhance various vibrational modes to different extents. Furthermore, it is usually found that RS spectra of analytes in solution more closely match the SER spectra than in the solid-state. However, it is usually beneficial to acquire and examine both when making assignments. Since DPA dissolves in water only sparingly, 1N KOH was used to dissolve 80 mg/mL. The RS spectrum of the solution phase is largely the same as the solid phase except for some minor changes in peak frequencies, intensities, and widths (Table 1). Notably, the 760 cm^{-1} peak in the solid phase is completely absent in the solution phase, while a new peak at 1386 cm^{-1} appears in the solution phase. The former

<i>B. Cereus</i>	CaDPA	Na ₂ DPA	DPA solid	DPA solution	SERS	Tentative Assignments
	403	413			405	
	433		425		458	CC ring bend ^a
	478	499	489			C-CO ₂ str ^a
		575	573		567	
659	661	650	646	652	657	CC ring bend ^a
		696				
			760		(795)	HO-C=O in-plane def ^b
		805	801			
821	820	814		822	812	CC str ^d , CaDPA CH out-of-plane def ^b
855	857	856	853		858	CC str ^d
900		897				CC str ^d
925	923					CaDPA
937						CC str + amide III ^d
1003						phe sym ring str. ^{c,d}
1014	1015	1003	997	1001	1006	sym ring breath ^e
					1029	
1077	1086	1079	1085	1087		trigonal ring breathing ^b
1150	1150	1147	1153	1154	1157	CH bend ^a
	1199	1185	1179	1191	1184	
		1227			1230	
1241						amide III (b) ^{c,d}
	1274	1256	1271			CC str ^b
		1293	1296		1285	
1318						CH ₂ bend, amide III ^d
			1324			C=O str
1377sh	1383					CH bend ^a
1391	1398	1393		1386	1381	OCO sym str ^c
1446	1447	1437	1445	1438	(1426)	CH ₂ bend, ^d CaDPA ring CH bend ^a or CC str ^b
	1466	1464	1461		1466	ring CC str
1573	1568	1569	1575	1572	1567	OCO asym str
1583	1583	1589			(1590)	CC ring str
1598sh						phe sym ring str. ^{c,d}
	1643	1634	1643			carboxylate ^e
1666						amide I ^{c,d}
		1704/30				C=O str (doublet)
2879sh						CH ₃ sym str ^f
2934						CH ₂ antisym str ^f
2968sh						CH ₃ antisym str ^f
	3019	3021				
3062	3060		3070			aromatic CH sym str ^f
3080	3088	3084	3098			CH antisym str ^f
		3137	3150			CH str ^f
3302						amide NH str ^{c,d}

Table 1. Tentative Raman vibrational mode assignments for dipicolinates. a is from Hameka et al., 1996; b is from Carmona, 1980; c is from Woodruff et al., 1974; d is from Bendaker, 1992, e is from Ghiamati et al., 1992; f is from Grasselli et al., 1981 and Austin et al., 1993.

peak is likely associated with carboxylic acid groups (e.g. HO-C=O deformation), while the latter peak is likely associated with deprotonated carboxylic acid groups (e.g. O-C-O stretch). The latter assignment is consistent with a sample pH of 10 due to the 1N KOH. The former assignment is supported by the fact that the peak does not disappear when DPA is dissolved in the aprotic solvents dimethylsulfoxide or *N,N*-dimethylformamide.

The SER spectrum of 1 g/L DPA in water is more like the solution than solid phase as shown in Fig. 5. The quality of this SER spectrum is considerably better than the first reported SER spectrum of dipicolinic acid obtained on a silver electrode in an electrolytic cell (Farquharson et al., 1999). In fact, not only are most of the peaks of the solution phase RS spectrum observed in the SER spectrum, but peaks shift no more than 10 cm^{-1} and change little in relative intensity. These similarities suggest a weak molecule to silver surface interaction. The RS to SERS shifts of the major peaks are: 652 to 657 cm^{-1} , 822 to 812 cm^{-1} , 1001 to 1006 cm^{-1} , 1386 to 1381 cm^{-1} , 1438 to 1426 cm^{-1} , 1572 to 1567 cm^{-1} . The SERS peaks are assigned according to CaDPA above and literature as follows (Ghiamati et al., 1992; Woodruff et al., 1974): the 1006 cm^{-1} peak is assigned to the symmetric ring stretch, the 1381 cm^{-1} peak to the symmetric O-C-O stretch, the 1426 cm^{-1} peak to the symmetric ring C-H bend, and the 1567 cm^{-1} peak to the asymmetric O-C-O stretch. The greatest difference between the RS and SER spectra is the appearance of a new peak in the latter spectrum at 795 cm^{-1} (see below).

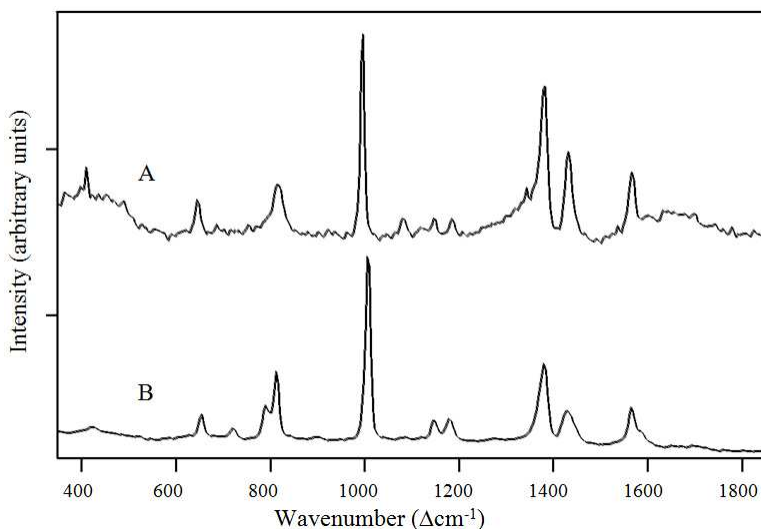
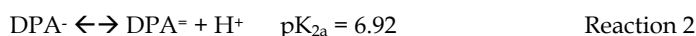
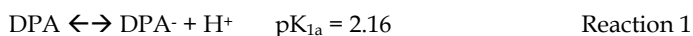


Fig. 5. A) RS of 80 mg DPA in 1 mL 1N KOH in a glass capillary. B) SERS of 1 mg DPA in 1 mL water in a silver-doped sol-gel filled glass capillary. Spectral conditions: A) 450 mW of 785 nm, 5 min acquisition time and B) 150 mW of 785 nm, 1-minute acquisition time; both 8 cm^{-1} resolution.

Next the pH dependence of both the measurement and analyte was considered. This could be significant if an acid or a base is used to digest spores and extract the CaDPA. It is widely known that the pH of the solution can have an effect on the SER signal (Laserna et al., 1988; Dou et al., 1999), particularly in the case of metal colloids where pH affects the extent of

aggregation (Laserna et al., 1988), which in turn affects the plasmon field and the Raman signal enhancement. Other SER-active media are more tolerable to pH changes, such as metal coated spheres and posts, or silver-doped sol-gels, as used here. Although these sol-gels may not be affected by pH, the analyte is a diprotic acid and the neutral and ionic forms of DPA, DPA^- , or $\text{DPA}^{=}$, must be considered. These species may interact with the silver quite differently and consequently influence the amount that each vibrational mode is enhanced. For example, it might be expected that $\text{DPA}^{=}$ will interact more strongly with electropositive silver increasing the chemical component of the SERS mechanism. Furthermore, added enhancement might be expected for the vibrational modes of the deprotonated carboxylic acid groups that participate in this interaction, or for modes that are favorably aligned perpendicular to the surface due to this interaction.

The relative concentrations of DPA, DPA^- , and $\text{DPA}^{=}$ can be determined at any pH as long as the pK_{a} s are known and the initial concentration. According to Lange's Handbook of Chemistry, the pK_{a} s are 2.16 and 6.92 (Dean 1979), and the deprotonation reactions are:



The relative concentrations can then be determined by expressing $[\text{DPA}]$ and $[\text{DPA}^{=}]$ in terms of $[\text{DPA}^-]$ using Reactions 1 and 2, and summing all three to equal the total starting concentration, here 1 g/L, viz:

$$[\text{DPA}] + [\text{DPA}^-] + [\text{DPA}^{=}] = 1 \text{ g/L} \quad (1)$$

substituting from Reactions 1 and 2:

$$([\text{H}^+][\text{DPA}^-])/K_{1\text{a}} + [\text{DPA}^-] + (K_{2\text{a}}[\text{DPA}^-])/[\text{H}^+] = 1 \text{ g/L} \quad (2)$$

rearranging:

$$[\text{DPA}^-] = 1 \text{ g/L} / (1 + [\text{H}^+]/K_{1\text{a}} + K_{2\text{a}}/[\text{H}^+]) \quad (3)$$

As shown in Fig. 6, at pH less than $\text{pK}_{1\text{a}}$ DPA dominates, at pH between the pK_{a} s DPA^- dominates, and above $\text{pK}_{2\text{a}}$ $\text{DPA}^{=}$ dominates.

Fig. 7 shows SER spectra of DPA for pH 4.87, 5.59, 7.35, 8.22, 10.69, and 11.66 with spectra of the 800 cm^{-1} region for pH 1.35, 1.71, 2.19 and 3.83 (inset). Overall there is only a modest decrease in intensity for most of the peaks as a function of pH. For example, the 1006 cm^{-1} peak assigned to the pyridine ring stretching mode decreases by $\sim 7\%$ from pH 2 to 11. The greatest changes observed, yet still modest, are in the peak intensities at 795, 812, 1567, and 1590 cm^{-1} between pH 1.3 and 5.5. These peaks change intensity as pairs. The 795 cm^{-1} peak loses intensity as the pH becomes basic, while the 812 cm^{-1} peak gains a little intensity. Similarly, the 1567 cm^{-1} peak loses intensity as the pH becomes basic, while the 1590 cm^{-1} peak gains intensity. The intensities of the former pair are plotted as a function of pH in Fig. 6. The peak heights were divided by the peak height of the 1006 cm^{-1} peak at each pH and then scaled with the lowest value set to 0 and the highest to 1 g/L. As can be seen the 795 cm^{-1} peak tracks the DPA concentration, while the 812 cm^{-1} peak tracks the DPA^- concentration. The former peak is likely associated with carboxylic acid groups, just as in the case of the 760 cm^{-1} peak in the solid phase RS spectrum of DPA.

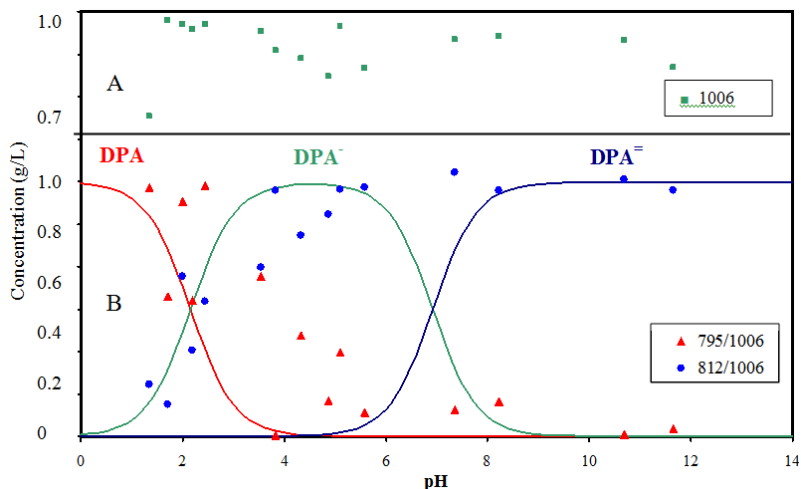


Fig. 6. DPA and its anion concentrations as a function of pH (lines). A) The 1006 cm⁻¹ peak intensity is shown as measured, but scaled to a 0 to 1 g/L concentration range. B) The 795 and 812 cm⁻¹ peak intensities are normalized to the 1006 cm⁻¹ peak intensity and then scaled. These two peaks appear to represent DPA and DPA⁻, respectively, but both with DPA⁻ character.

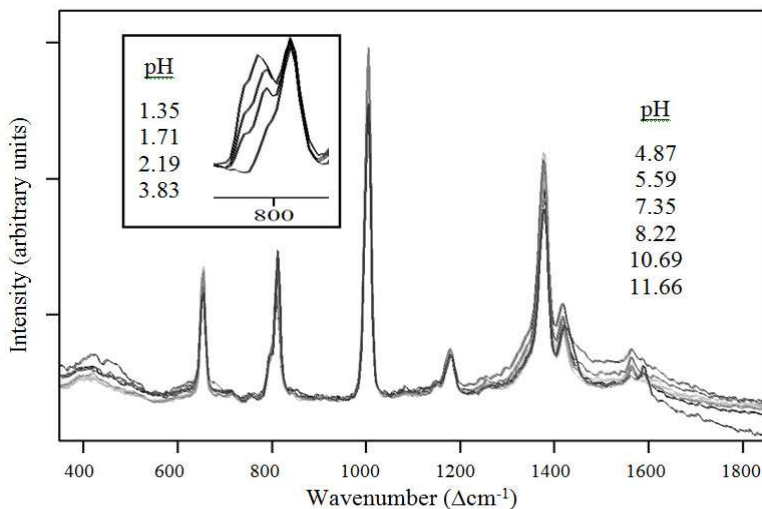


Fig. 7. SERS of 1 mg/mL dipicolinic acid as a function of pH. The spectral intensities have been normalized to the 812 cm⁻¹ peak. Inset: Expanded view of low wavenumber region. Spectral conditions: 100 mW of 785 nm, 44 sec acquisition time, 8 cm⁻¹ resolution.

However, a 35 cm⁻¹ shift is somewhat inconsistent with a weak analyte-to-surface interaction. It is also apparent in Fig. 6 that the concentrations of DPA and DPA⁻ based on the 795 and 812 cm⁻¹ peak intensities are shifted to the basic side of the predicted curves. This shift may

be due to the silver surface influencing the carboxylic acid dissociation energy. Or the peaks may contain contributions from the DPA^- species. Although clarifying this point will require further measurements, the most important conclusions from this data is that the SER intensity for most of the prominent DPA peaks change little as a function of pH, and that the silver-doped sol-gels do not appear to influence the measurement to any significance.

Next, the response of the SER intensity for DPA as a function of concentration was examined. A preliminary calibration curve was prepared by measuring 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 mg/L samples. Fig. 8 shows SER spectra for 100, 1, and 0.01 mg/L samples measured using 100 mW of 785 nm and 1-min acquisition time. It can be seen that even at 10 $\mu\text{g/L}$ the signal-to-noise ratio is quite good. The SER intensity was taken as the peak height at 1006 cm^{-1} minus the value at 950 cm^{-1} as the baseline. For each concentration, a different capillary was used. Spectra were measured at nine points along the length of each capillary and the median values are plotted in Fig. 9. It is obvious that the response is not linear, in that the peak heights change from 0.2 to 1.5, while the concentration changes over 4 orders of magnitude. This Langmuir isotherm response is typical for SERS substrates where signal intensity is a function of available silver surface area (Mullen & Carron, 1994).

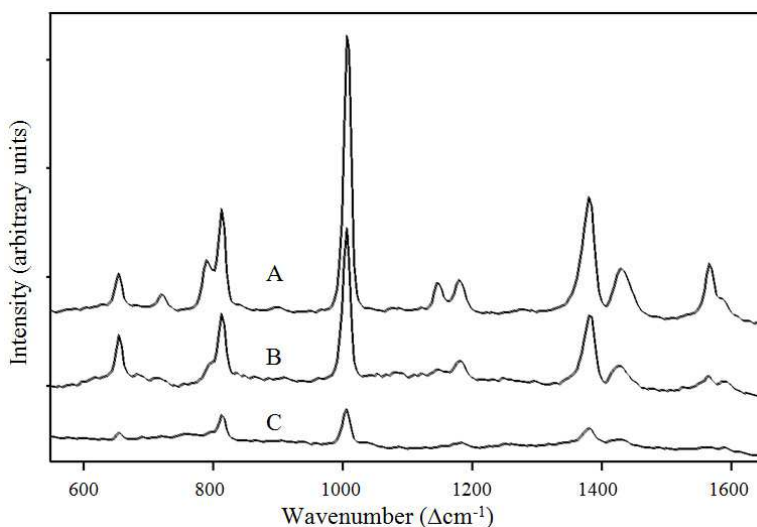


Fig. 8. SERS of DPA in water at A) 100, B) 1, and C) 0.01 mg/L (100 pg in 10 μL sample) using the SER-active capillaries, 100 mW of 785 nm and 1 min acquisition time.

All of these values were also used to estimate limits of detection (LOD), defined as the concentration that produces a signal three times as intense as the baseline noise. The signal was taken as the height of the 1006 cm^{-1} peak, while the noise was the relative standard deviation of baseline noise measured between 50 and 150 cm^{-1} . The LODs are for 1-min measurements using 100 mW of 785 nm laser excitation and 8 cm^{-1} resolution. As Table 2 indicates, the lower the measured concentration, in general, the lower the predicted LOD. Note that the lowest concentration sample, 10 $\mu\text{g/L}$ (0.01 mg/L), suggests that 0.7 $\mu\text{g/L}$ can

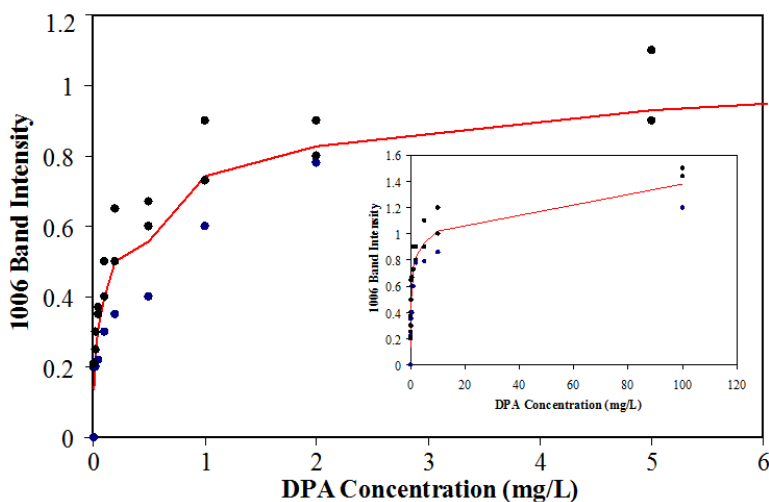


Fig. 9. Plot of SER intensity for 1006 cm^{-1} peak of DPA as a function of concentration using 100 mW of 785 nm and 1-min acquisition time. Line connects average value at each concentration. Inset includes 10 and 100 mg/L data.

be measured (S/N equaled 33 for the 1006 cm^{-1} peak). This is consistent with the fact that attempted measurements of 1 $\mu\text{g/L}$ samples did yield spectra, but not in every case. It is also worth noting that only 10 μL samples were used to generate the spectra, or in the case of the 10 $\mu\text{g/L}$ sample, the actual sample was 100 pg DPA in 10 μL water.

Conc mg/L	spores in 0.1 mL	Signal (ave)	Std Dev	RSD (%)	Noise	S/N	LOD factor	LOD mg/L	LOD spores/0.1 mL
0.01	1229	0.14	0.12	86.68	0.0033	41.4	13.8	7.24E-04	89
0.02	2457	0.25	0.05	20.00	0.0042	59.5	19.8	1.01E-03	124
0.05	6143	0.31	0.08	25.99	0.0043	72.9	24.3	2.06E-03	253
0.1	12,285	0.40	0.10	25.00	0.0047	85.1	28.4	3.53E-03	433
0.2	24,570	0.50	0.15	30.00	0.005	100.0	33.3	6.00E-03	737
0.5	61,425	0.56	0.14	25.17	0.006	92.8	30.9	1.62E-02	1986
1	122,850	0.74	0.15	20.24	0.0067	110.9	37.0	2.70E-02	3322
2	245,700	0.83	0.06	7.78	0.008	103.3	34.4	5.81E-02	7133
5	614,251	0.93	0.16	16.90	0.0067	138.8	46.3	1.08E-01	13276
10	1,228,501	1.02	0.17	16.75	0.0096	106.3	35.4	2.82E-01	34687
100	12,285,012	1.38	0.16	11.50	0.0122	113.1	37.7	2.65E+00	325820
~1*	100,000	0.70	0.14	20.11	0.0055	126.7	42.2	2.37E-02	2368
~0.22**	22,000	0.45	0.10	23.20	0.0159	28.3	9.4	2.33E-02	2332

* Approximate concentrations for dodecylamine surface measurements, see below.

** Approximate concentrations for acetic acid surface measurements, see below.

Table 2. Estimated limits of detection in terms of mg DPA per L water and corresponding spores per 0.1mL DDA.

Finally, an enhancement factor (EF) for DPA can be estimated by comparing the measurement conditions and signal intensities for the 10 $\mu\text{g/L}$ SERS and 80 g/L RS. The

spectra are plotted on the same scale in Fig. 10. The 1006 cm^{-1} peak heights are nearly identical at 0.20 and 0.173 (arbitrary units), while the laser power at the sample and collection time were somewhat different at 150 and 450 mW, and 1 and 5 minutes for the SERS and RS, respectively. In both cases, 1-mm capillaries were used to hold the samples, and the same sample optics were used. Taking the concentration into account yields an estimated enhancement factor of 2.4×10^7 . It is difficult to determine the precise number of molecules in the field of view for the sol-gel, and this number may represent better than average enhancement, i.e. better than 10^6 , or it may reflect the ability of the sol-gel to concentrate the sample. In either case, the measurement of $10\text{ }\mu\text{g/L}$ suggests that 10 ng of spores in a $100\text{ }\mu\text{L}$ solution of a digesting chemical can be measured; assuming all of the CaDPA is made available as DPA (10%). Recent estimates suggest that this mass corresponds to 1000 spores (Inglesby et al., 2002).

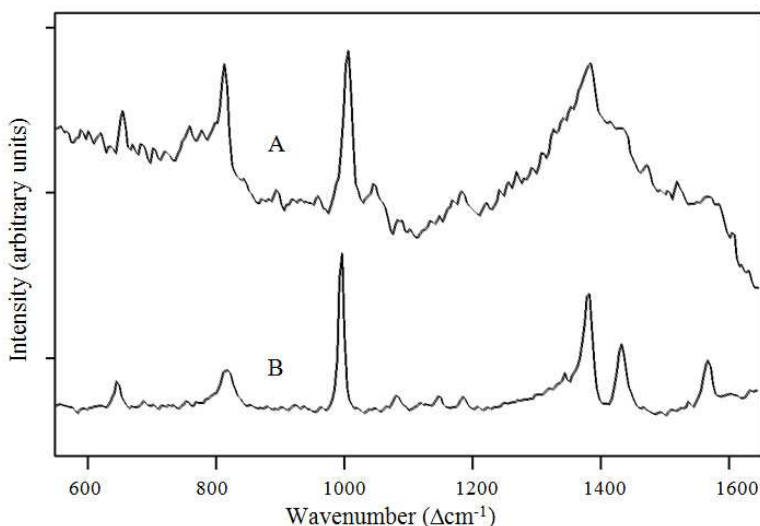


Fig. 10. A) SERS and B) RS of DPA plotted on the same scale, but offset. Conditions: A:B 10^{-5} :80 $\mu\text{g/L}$, 150:450 mW of 785 nm, and 1:5 min acquisition time. $\text{EF} = 2.4 \times 10^7$.

Next, methods were developed to rapidly extract CaDPA as DPA from *Bacillus* spores. Initially, DPA was obtained from *B. cereus* spores following the procedure of Pellegrino et al. Specifically, a 2 mg sample was placed in 2 mL of 5 mM dodecylamine in ethanol that was heated and maintained at $78\text{ }^\circ\text{C}$ for 40 minutes. Approximately $10\text{ }\mu\text{L}$ of this solution was drawn into a SER-active capillary and measured. Since SER spectra of DPA were readily observed, shorter heating periods, higher DDA concentrations and smaller spore masses, were examined. In due course experiments were performed in which $100\text{ }\mu\text{L}$ of $78\text{ }^\circ\text{C}$ 50 mM DDA in ethanol was added to $\sim 10\text{ }\mu\text{g}$ samples of spore specks (~ 1 million spores) placed on a surface. After 1 minute exposure, approximately $10\text{ }\mu\text{L}$ of the solution were drawn into a SER-active capillary and measured. Fig. 11 shows a representative spectrum for one of these capillaries using a 1-min acquisition time. The primary DPA peaks at 657 cm^{-1} , 810 cm^{-1} , 1006 cm^{-1} , 1382 cm^{-1} , and 1428 cm^{-1} are easily observed, even in the case of a 2-sec scan.

Furthermore, an attempted measurement of 50 mM DDA (without sample) did not produce a spectrum that might interfere with the measurement (Fig. 11C). The amount of DPA extracted was estimated to be between 0.5 and 5 mg/L by comparing the 0.7 signal intensity of the 1006 cm^{-1} peak to that measured for DPA in water (see Table 2). In fact this intensity is closest to that obtained for the 1 mg/L samples. This value can be used to estimate the number of spores in the 100 μL DDA sample. Assuming, as stated above, that a spore contains approximately 10% DPA by weight, and that 100 spores have a mass of ~ 1 ng, then this corresponds to 100,000 spores per 100 μL DDA or $\sim 10\%$ of the spores in the prepared particles. This low percentage could be due to incomplete degradation of the spores by DDA, interference from the spore cell debris, inefficient collection of the sample from the surface, or inefficient transfer of the DPA to the silver particles.

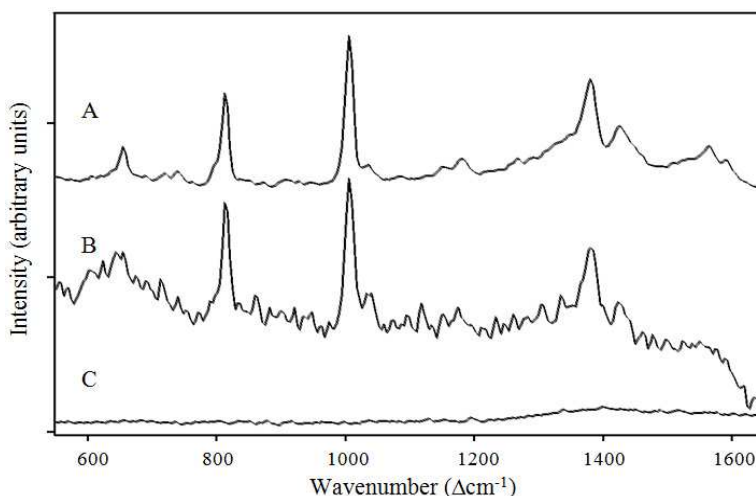


Fig. 11. SERS of DPA extracted from ~ 10 μg *B. cereus* particle using 100 μL of 50 mM hot DDA acquired in A) 1 minute and B) 2 seconds. C) Attempted SERS of 50 mM hot DDA in ethanol using silver-doped sol-gel coated glass capillary acquired in 1 minute. Spectral conditions: 150 mW of 785 nm, 8 cm^{-1} resolution.

Although, these initial measurements clearly demonstrated the potential of rapid spore analysis using SERS, the approach had two significant limitations. First, the sensitivity is insufficient, and second the use of a hot reagent severely limits its practical use in the field. To overcome these limitations we investigated numerous acids, bases and solvents (e.g. nitric acid, potassium hydroxide, phenol, etc.), separately and as mixtures that could digest spores rapidly at room temperature, and make available all or nearly all of the DPA for SERS detection.

Eventually, it was found that glacial acetic acid had the appropriate properties. However, reactivity of acetic acid with the sol-gel and competition with DPA for the silver surface was a concern, so a series of DPA concentration samples were prepared and measured. Fig. 12 shows a SER spectrum of 100 pg DPA in 10 μL acetic acid (10 ppb) and a SER spectrum of pure acetic acid drawn into SER-active capillaries.

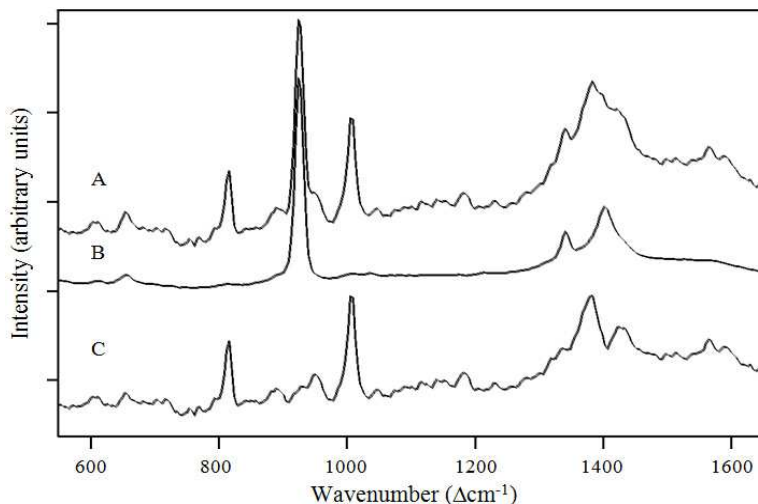


Fig. 12. A) SERS of DPA in acetic acid at 10 $\text{pg}/\mu\text{L}$, B) SERS of acetic acid, and C) difference spectrum of A-B). Spectral conditions: 85 mW of 785 nm, 1 min acquisition time, 8 cm^{-1} resolution.

Acetic acid produced several peaks, notably at 925, 1340, and 1405 cm^{-1} . Subtraction of the acetic acid spectrum reveals a standard SER spectrum of DPA (Fig. 12C). The SER spectral intensity of DPA in acetic acid as a function of concentration is very similar to that previously measured in water. In fact the 1006 cm^{-1} peak intensity, once corrected for the difference in laser power is nearly identical for the same concentration in water (compare 0.13 in acetic acid versus 0.14 in water, Table 2). Fig. 13 shows SER spectra of four orders of magnitude of DPA

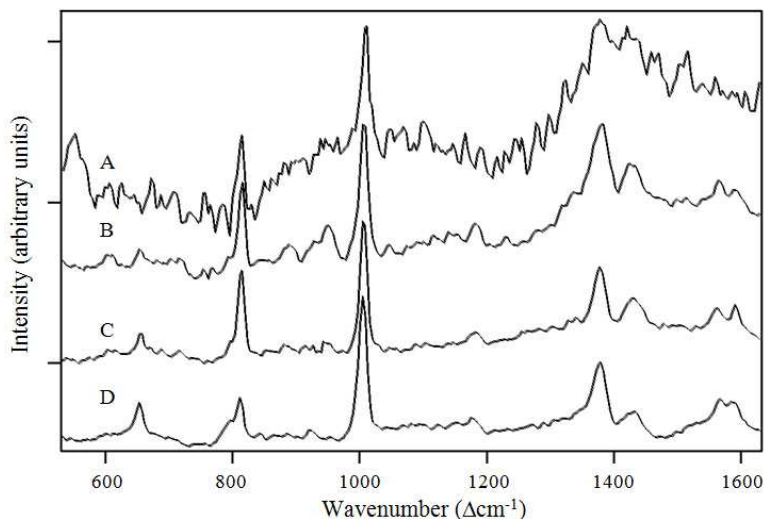


Fig. 13. SERS of DPA in acetic acid at concentrations of A) 1, B) 10, C) 100, and D) 1000 $\text{pg}/\mu\text{L}$. Spectral conditions: 85 mW of 785 nm, 1 min acquisition time, 8 cm^{-1} resolution.

concentration in acetic acid, in which the dominant 1006 cm^{-1} peak intensity is held constant and the base line noise is observed to increase with decreasing concentration.

Next, experiments were performed using acetic acid to digest spore samples on a surface, collect the DPA and detect it in SER-active capillaries. As a specific example, a 2 mg sample of spores was weighed and dispersed in 10 mL of water by vortexing. The spores per volume water were calibrated by placing $10\text{ }\mu\text{L}$ on a standard hemocytometer counting grid. Each grid holds 4 nL ($0.2\times 0.2\times 0.1\text{ mm}$). Microscope images of 10 grids were recorded and 870 spores were counted, which represents $\sim 22,000$ spores per μL (Fig. 14A). The original spore solution was diluted by a factor of 10 to produce a 2200 spore per μL . From this solution, a $1\text{ }\mu\text{L}$ sample was placed on a glass slide and allowed to dry producing a 2200 spores per $\sim 0.2\text{ cm}^2$ spot (Fig. 14B). Although this represents 11,000 spores/ cm^2 , 3-orders of magnitude greater than the target sensitivity, no attempt was made to spread the spores across the surface (i.e. identical results would likely have been obtained if the sample was allowed to dry on a 10 cm^2 area). Next, $10\text{ }\mu\text{L}$ of acetic acid was added to the spore spot (Fig. 14C). After 1 minute, $1\text{ }\mu\text{L}$ of this solution was drawn into a SER-active capillary (Fig. 14D), and a 1-minute spectrum measured (Fig. 15A). The measurement represents 220 spores/ μL . The entire measurement was accomplished in less than 2.5 minutes, and at a measured pH of 2.1 the primary DPA peaks at 657 cm^{-1} , 812 cm^{-1} , 1006 cm^{-1} , 1381 cm^{-1} , and 1426 cm^{-1} are easily observed.

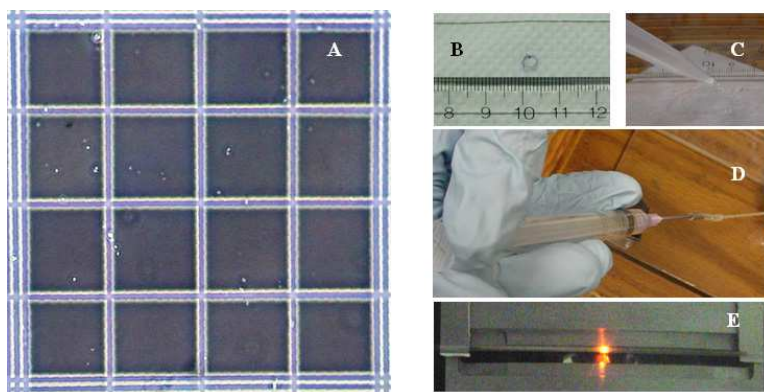


Fig. 14. A) Microscope image of initial calibration sample containing 60 spores in a 0.2×0.2 mm field of view (0.1 mm deep). B) 2200 spores were dried from $1\text{ }\mu\text{L}$ on the surface as ($\sim 0.2\text{ cm}^2$). C) $10\text{ }\mu\text{L}$ of acetic acid was added to the spores for 1 min digestion. D) $1\text{ }\mu\text{L}$ of the solution was drawn into the chemically-selective, SER-active capillary. And E) the capillary was mounted in Raman analyzer sample compartment and the spectrum collected.

The quantitative accuracy of the measurement was further verified by comparing this 220 spores/ μL spectrum to the $100\text{ pg}/\mu\text{L}$ (~ 100 spores/ μL) solution of DPA in acetic acid spectrum. This was accomplished by using the acetic acid peak at 925 cm^{-1} as an internal intensity standard and normalizing both spectra to it. It was found that the primary peak at 1006 cm^{-1} of the spore sample is \sim twice the intensity of the reference DPA sample, consistent with the 220 spore estimate. This spectral comparison also shows that the spore spectrum contains a considerable baseline offset with significant noise (background). This is

likely due to spore debris that has not been effectively excluded from the metal-doped sol-gels, and suggests that further "cleaning" the sample could improve sensitivity. The spectral background results in a modest S/N of 28, and an estimated LOD of 2330 *B. cereus* spores per 100 μL acetic acid or 1165 spores per cm^2 for the above experiment. But, as shown in Table 2, the LODs are consistently underestimated, and substantially better sensitivity should be expected.

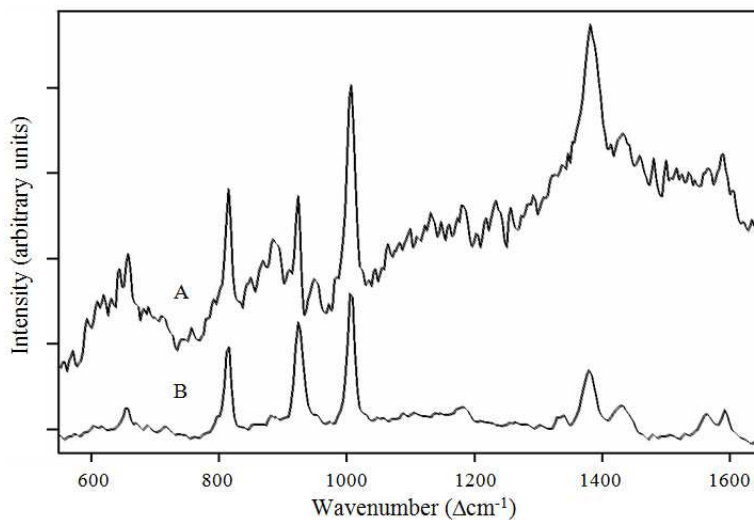


Fig. 15. SERS of A) DPA obtained from 220 *Bacillus cereus* spores using a 1-minute exposure to glacial acetic acid at room temperature and B) 100 $\text{pg}/\mu\text{L}$ solution of DPA in acetic acid for comparison. Spectral conditions: 85 mW of 785 nm, 1 min acquisition time, 8 cm^{-1} resolution.

4. Conclusions

We have demonstrated that by combining rapid extraction of dipicolinic acid from *Bacillus cereus* spores with chemical identification by surface-enhanced Raman spectroscopy, *Bacillus* spores on a surface can be identified in 2.5 minutes. This includes the time required to dispense acetic acid onto a spore contaminated surface, draw the DPA sample by syringe into a SER-active capillary, and acquire the DPA SER spectrum. Using this method, we have measured 220 spores/ μL from a 0.2 cm^2 spot containing 2200 spores (equivalent to 11,000 spores/ cm^2) in 2.5 minutes, using a simple, SER-active sampling capillary, syringe and a portable Raman analyzer. Although an estimated limit of detection based on the signal-to-noise ratio suggests that 1165 spores/ cm^2 could be measured, the LODs are consistently underestimated as shown in Table 2. In fact, 1 $\text{pg}/\mu\text{L}$ DPA in acetic acid was consistently measured. Assuming that the *B. cereus* spores contained 10 wt % DPA and each spore has a mass of 10^{-11}g , this is equivalent to 10 spores/ cm^2 for the experiment presented here. It could also be argued that the same number of spores could be spread over a 10 cm^2 region and the same amount of digesting agent would produce an equivalent SER signal. Together these arguments suggest that the present method is capable of measuring the goal of 10

spore/cm² and that SER-active capillaries could be used to measure surfaces and map distribution of anthrax endospores in mail distribution facilities or other environments should another verified attack occur. This would greatly aid in determining the extent of an attack, deciding who should be tested, and possibly tracing the origin of letters if used. Current research is aimed at extending the capability of this method to analysis of nasal swabs, and *B. anthracis* specific SERS-active materials.

5. Acknowledgements

The authors are grateful to Professor Jay Sperry of the University of Rhode Island for preparing spore samples and the National Science Foundation (DMI-0215819, DMI-0214280, DMI-0349687), the U.S. Army (DAAD13-02-C-0015, Joint Service Agent Water Monitor Program), and the Environmental Protection Agency (EP-D-06-084).

6. References

- Austin, J.C., Jordan, T., & Spiro, T.G., (1993), UVRR studies of proteins and related compounds, In *Biomolecular Spectroscopy*, Vol 21, Clark & Hester (Eds.), John Wiley & Sons, New York, NY.
- Bandekar, J., (1992), Amide modes and protein conformation, *Biochim. Biophys. Acta*, 1120, p. 123.
- Bell, C.A., Uhl, J. R., & Cockerill, F. R., (2001), Direct Detection of *Bacillus anthracis* using a Real-Time PCR Method, *ASM 101st General Meeting*, Orlando, FL, May 2001.
- Bell, C.A., Uhl, J.R., Hadfield, T.L., David, J.C., Meyer, R.F., Smith, T.F., & Cockerill, F.R. III, (2002), Detection of *Bacillus Anthracis* DNA by Light Cyclor PCR, *J. Clin. Microbiol.*, 40, p. 2897.
- Bell, D.M., Kozarsky, P.E., & Stephens, D., (2002), Clinical issues in the prophylaxis, diagnosis, and treatment of anthrax, *Emerg. Infect. Dis.*, 8, p. 222.
- Berkeley, R.C.W., & Ali, N., (1994), Classification and identification of endospore-forming bacteria, *J. Appl. Bacteriol. (Symp. Suppl.)*, 76, p. 1S.
- Beverly, M.B., Basile, F., Voorhees, K.J., & Hadfield, T.L., (1996), A rapid approach for the detection of dipicolinic acid in bacterial spores using pyrolysis /mass spectrometry, *Rapid Commun. Mass Spectrom.*, 10, p. 455.
- Carmona, P., (1980), Vibrational spectra and structure of crystalline dipicolinic acid and calcium dipicolinate trihydrate, *Spectrochim. Acta, A*, 36, p. 705.
- Centers for Disease Control & Prevention, American Society for Microbiology, & Association of Public Health Laboratories. Basic diagnostic testing protocols for level A laboratories for the presumptive identification of *Bacillus anthracis*, (2001), American Society for Microbiology, Washington, D.C. Accessed Nov 2011, Available from: <http://www.bt.cdc.gov/Agent/Anthrax/Anthraxis20010417.pdf>
- Dean, J.L., (1979), *Lange's Handbook of Chemistry*, 12th Ed., McGraw-Hill, New York, NY.
- Dou, X., Jung, Y., Cao, Z., & Ozaki, Y., (1999), Surface-Enhanced Raman Scattering of Biological Molecules on Metal Colloid II: Effects of Aggregation of Gold Colloid and Comparison of Effects of pH of Glycine Solutions between Gold and Silver Colloids, *Appl. Spectrosc.*, 53, p. 1440.

- Esposito, A.P., Talley, C.E., Huser, T., Hollars, C.W., Schaldach, C.M., & Lane, S.M., (2003), Analysis of single bacterial spores by micro-Raman spectroscopy, *Appl. Spectrosc.*, 57, p. 868.
- Farquharson, S., Smith, W.W., Elliott, S., & Sperry, J.F., (1999), Rapid biological agent identification by surface-enhanced Raman spectroscopy, *Proc. SPIE*, 3855, p. 110.
- Farquharson, S., & Maksymiuk, P., (2003), Simultaneous chemical separation and surface-enhancement Raman spectral detection using silver-doped sol-gels, *Appl. Spectrosc.*, 57, p. 479.
- Farquharson, S., Grigely, L., Khitrov, V., Smith, W.W., Sperry, J.F., & Fenerty, G., (2004), Detecting *Bacillus cereus* spores on a mail sorting system using Raman Spectroscopy, *J. Raman Spectrosc.*, 35, p. 82.
- Farquharson, S., Smith, W., Carangelo, R.C., & Brouillette, C., (1999), Industrial Raman: providing easy, immediate, cost effective chemical analysis anywhere, *Proc. SPIE*, 3859, p. 14.
- Ghiamati, E., Manoharan, R.S., Nelson, W.H., & Sperry, J.F., (1992), UV Resonance Raman spectra of *Bacillus* spores, *Appl. Spectrosc.*, 46, p. 357.
- Grasselli, J.G., Snavely, M.K., & Bulkin, B.J., (1981), *Chemical Applications of Raman Spectroscopy*, John Wiley & Sons, New York, NY, Chapter 5.
- Hameka, H.F., Jensen, J.O., Jensen, J.L., Merrow, C.N., & Vlahacos, C.P., (1996), Theoretical studies of the fluorescence of dipicolinic acid and its anion, *J. Molec. Struct. (Theory)*, 365, p. 131.
- Hathout, Y., Setlow, B., Cabrera-Martinex, R-M., Fenselau, C., & Setlow, P., (2003), Small, acid-soluble proteins as biomarkers in mass spectrometry analysis of *Bacillus* spores, *Appl. Environ. Microbiol.*, 69, p. 1100.
- Halverson, H.O., Ed., (1961), *Spores II*, American Society for Microbiology, Washington, D.C., p. 153.
- Helgason, E., Økstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I., & Kolstø, A.B., (2000), *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*-one species on the basis of genetic evidence, *Appl. Environ. Microbiol.*, 66, p. 2627.
- Hindle, A.A., Hall, E.A.H., (1999), Dipicolinic acid assay revisited and appraised for spore detection, *Analyst*, 124, p. 1599.
- Hsu, V.P., Lukacs, S.L., Handzel, T., et al., (2002), Opening a *Bacillus anthracis*-containing envelope, Capitol hill, Washington, D.C.: The Public Health Response, *Emerg. Infect. Dis.*, 8, p. 1039.
- Inglesby, T.V., Henderson, D.A., & Bartlett, J.G., (2002), Anthrax as a biological weapon: updated recommendations for management, *JAMA*, 287, p. 2236.
- Jackson, P.J., Hugh-Jones, M.E., Adair, D.M., et al., (1998), PCR analysis of tissue samples from the 1979 Sverdlovsk anthrax victims: The presence of multiple *Bacillus anthracis* strains in different victims, *Proc. Natl. Acad. Sci.*, 95, p. 1224.
- Janssen, F.W., Lund, A.J., & Anderson, L.E., (1958), Colorimetric assay for dipicolinic acid in bacterial spores, *Science*, 127, p. 26.
- Jeanmaire D.L., & Van Duyne, R.P., (1977), Surface Raman Spectroelectrochemistry, *J. Electroanal. Chem.*, 84, p. 1.
- Jernigan, J.A., Stephens, D.S., Ashford, D.A., et al., (2001), Bioterrorism-Related Inhalational Anthrax: The First 10 Cases Reported in the United States, *Emerg. Infect. Dis.*, 6, p. 933.

- Kellogg, M., (2010), Detection of biological agents used for terrorism: Are we ready?, *Clin. Chem.*, 56, p. 10.
- Kennedy, H., (2001), Daschle letter bombshell billions of anthrax spores, *New York Daily News*, October 31, 2001.
- Kiratisin, P., (2002), Large-scale screening of nasal swabs for *Bacillus anthracis*: Descriptive summary and discussion of the National Institute of Health's experience, *J. Clin. Microbio.*, 40, p. 3012.
- Laserna, J.J., Berthod, A., & Winefordner, J.D., (1988), Evaluation and optimization of experimental conditions for surface-enhanced Raman detection of analytes in flow injection analysis, *Microchem. J.*, 38, p. 25.
- Leingang, M., (2004), Post office installs anthrax detector, *The Enquirer (Cincinnati)*, Sept. 24, 2004.
- Liu, H., Bergman, N.H., Thomason, B., et al., (2004), Formation and Composition of the *Bacillus anthracis* Endospore, *J. Bacteriol.*, 186, p. 164.
- Mabry, R., Brasky, K., Geiger, R., Carrion, R. Jr., Hubbard, G. B., Leppla, S., Patterson, J. L., Georgiou, G., and Iverson, B.L.,(2006), Detection of anthrax toxin in the serum of animals infected with *Bacillus anthracis* by using engineered immunoassays, *Clin. Vaccine Immunol.* 13, p. 671.
- Mullen, K., & Carron, K., (1994), Adsorption of chlorinated ethylenes at 1-octadecanethiol-modified silver surfaces, *Anal. Chem.*, 66, p. 478.
- Murrell, W.G., Gould, G.W., & Hurst, A. Eds., (1969), *The Bacterial Spore*, Acad. Press., London, p. 215.
- Nelson, W.H., Dasari, R., Feld, M., & Sperry, J.F., (2004), Intensities of calcium dipicolinate and *Bacillus subtilis* spore Raman spectra excited with 244 nm light, *Appl. Spectrosc.*, 58, p. 1408.
- Nudelman, R., Bronk, B.V., & Efrima, S., (2000), Fluorescence Emission Derived from Dipicolinate Acid, its Sodium, and its Calcium Salts, *Appl. Spectrosc.*, 54, p. 445.
- Pellegrino, P.M., Fell Jr., N.F., & Gillespie, J.B., (2002), Enhanced spore detection using dipicolinate extraction techniques, *Anal. Chim. Acta.*, 455, p. 167.
- Phillips, Z.E., & Strauch, M.A., (2002), *Bacillus subtilis* sporulation and stationary phase gene expression, *Cell. Mol. Life Sci.*, 59, p. 392.
- Ragkousi, K., Eichenberger, P., Van Ooij, C., & Setlow, P., (2003), Identification of a New Gene Essential for Germination of *Bacillus subtilis* Spores with Ca²⁺-Dipicolinate, *J. Bacteriol.*, 185, p. 2315.
- Rosen, D.L., Sharpless, C., & McBrown, L.B., (1997), Bacterial spore detection and determination by use of terbium dipicolinate photoluminescence, *Anal. Chem.*, 69, p. 1082.
- Sacchi, C.T., Whitney, A.M., Mayer, L.W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R.S., & Popovic, T., (2002), Sequencing of 16S rRNA Gene: A Rapid Tool for Identification of *Bacillus anthracis*, *Emerg. Infect. Dis.*, 8, p. 1117.
- Sanderson, W.T., Hein, M.J., & Taylor, L., (2002), Surface Sampling Methods for *Bacillus anthracis* Spore Contamination, *Emerg. Infect. Dis.*, 8, p. 1145.
- Sanderson, W.T., Stoddard, R.R., Echt, A.S., et al., (2004), *Bacillus anthracis* contamination and inhalational anthrax in a mail processing and distribution center, *J. Appl. Microbiol.*, 96, p. 1048.
- Shane, S., (2004), Post office unveils anthrax detector, *Baltimore Sun*, July 23, 2004.

- Tang, S., Moayeri, M., Chen, Z., Harma, H., Zhao, J., Hu, H., Purcell, R.H., Leppla, S.H., and Hewlett, I.K., (2009) Detection of Anthrax Toxin by an Ultrasensitive Immunoassay Using Europium Nanoparticles, *Clin. Vacc. Immun.*, 16, p. 408.
- Thayer, A., (2003), Homeland Security: Postal Service Readies Defense - Team will install PCR-based systems to detect biohazards in mail facilities, *C&EN*, 81, p. 7.
- Weaver, M.J., Farquharson, S., & Tadayoni, M.A., (1985), Surface-enhancement factors for Raman scattering at silver electrodes: Role of adsorbate-surface interactions and electrode structure, *J. Chem. Phys.*, 82, p. 4867.
- Woodruff, W.H., Spiro, T.G., & Gilvarg, C., (1974), Raman Spectroscopy In Vivo: Evidence on the Structure of Dipicolinate in Intact Spores of *Bacillus Megaterium*, *Biochem. Biophys. Res. Commun.*, 58, p. 197.



Bioterrorism

Edited by Dr. Stephen Morse

ISBN 978-953-51-0205-2

Hard cover, 192 pages

Publisher InTech

Published online 28, March, 2012

Published in print edition March, 2012

This book consists of nine chapters, written by international authorities, discussing various aspects of bioterrorism preparedness and response. Five of the chapters are agent-specific and highlight the pathogenesis, prevention and treatment, and the potential of specific organisms (*Rickettsia* and *Yersinia pestis*) or toxins (ricin, botulinum neurotoxins, and staphylococcal enterotoxins) to be used for nefarious purposes. Four chapters discuss different aspects of detecting and responding to a bioterrorism attack. These include methods for spatio-temporal disease surveillance, international laboratory response strategies, detection of botulinum neurotoxins in food and other matrices, and the use of physical methods (ie Raman spectroscopy) to detect spores.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Stuart Farquharson, Chetan Shende, Alan Gift and Frank Inscore (2012). Detection of Bacillus Spores by Surface-Enhanced Raman Spectroscopy, *Bioterrorism*, Dr. Stephen Morse (Ed.), ISBN: 978-953-51-0205-2, InTech, Available from: <http://www.intechopen.com/books/bioterrorism/detection-of-bacillus-spores-by-surface-enhanced-raman-spectroscopy>

INTECH
open science | open minds

InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.