

focusing the 488 nm laser beam ~ 2 mm deep into the bulk of the crystals to avoid contributions from potential depletion layers. As shown in Fig. 1b, the dependence of the Raman shift on the degree of deuteration is almost perfectly linear and fits very well with $D = -2.684\text{cm}^*R + 2452.6$, where D is the degree of deuteration (in %) and R is the spectral mean of the PO_4 vibration in cm^{-1} . A linear correlation coefficient of 0.998 indicates an excellent linear dependence of the Raman peak shift with degree of deuteration. This result shows that the shift of the PO_4 peak is simply caused by the linear increase in atomic mass due to isotope substitution, which decreases the length of hydrogen-like bonds.

This excellent linear dependence allows us to map the profile of the D/H exchange layer at the surface of DKDP crystals by acquiring Raman spectra and determining the position of the PO_4 peak for various depths. This method is preferable over other methods such as determining the strength of the OD vibration directly (e.g., at 715 cm^{-1}), because the position of the most intense peak in the Raman spectrum can be measured more precisely than the intensity of some of the weakest peaks in the spectrum. This is demonstrated in Fig. 2, where depth-dependent Raman spectra (Fig. 2a) and the resulting exchange layer profiles for two DKDP crystals are shown (Fig. 2b). The spectra in Fig. 2a were obtained from a depth scan of a DKDP crystal with 75% degree of deuteration in the bulk, grown at 45°C . The spectra start out as DKDP with $\sim 30\%$ deuteration close to the surface and approach the bulk DKDP spectrum within a few micrometers of depth. The fact that the relative degree of deuteration does not extend to 0% D is due to the limited depth resolution of the Raman microprobe, which averages over $\sim 4\ \mu\text{m}$ in depth. Figure 2b depicts the resulting D/H exchange layer profiles for this and a second crystal grown at 63°C , respectively. Both crystals had the same exposure to ambient conditions and their main difference is the temperature at which they were grown. The different exchange layer profiles indicate that crystals grown at different temperatures have different proton conductivities,¹³ which leads to a difference in their rate of deuterium depletion. The parameters controlling this behavior are currently the objective of a detailed study, the results of which will be reported elsewhere.

CONCLUSION

In conclusion, we have shown that the shift of the totally symmetric PO_4 stretch mode in the Raman spectrum of DKDP crystals scales linearly with degree of deuteration. This allows us to correlate Raman peak positions to deuteration levels in these crystals. We have presented a new technique to determine D/H diffusion profiles in DKDP frequency conversion crystals based on micro-Raman spectroscopy. This technique is fast, inexpensive, and works under various environmental conditions, which will allow us to better understand and control deuterium depletion in DKDP crystals.

ACKNOWLEDGMENTS

We would like to thank M. Runkel for first discovering DKDP cracking, R. Floyd for providing DKDP crystals, and L. Chase and A. Burnham for their support and helpful discussions. This work was performed under the auspices of the U.S. Department of Energy by the University

of California, Lawrence Livermore National Laboratory under contract # W-7405-Eng-48.

1. J. J. De Yoreo, A. K. Burnham, and P. K. Whitman, *Int. Mat. Rev.* **47**, 113 (2002).
2. C. E. Barker, R. A. Sacks, B. M. Van Wonerghern, J. A. Caird, J. R. Murray, J. H. Campbell, K. Kyle, R. B. Ehrlich, and N. D. Nielsen, *Proc. SPIE-Int. Soc. Opt. Eng.* **2633**, 501 (1995).
3. T. Suratwala, paper to be published.
4. Cleveland Crystals, Inc., <http://www.clevelandcrystals.com/KDP.shtml#table>.
5. E. A. Popova, I. T. Savatinova, and I. A. Velichko, *Sov. Phys. Solid State* **12**, 1543 (1971).
6. I. P. Kaminow, R. C. C. Leite, and S. P. S. Porto, *J. Phys. Chem. Solids* **26**, 2085 (1965).
7. J. A. Subramony, B. J. Marquardt, J. W. Macklin, and B. Kahr, *Chem. Mat.* **11**, 1312 (1999).
8. H. Tanaka, M. Tokunaga, and I. Tatsuzaki, *Solid State Commun.* **49**, 153 (1984).
9. R. J. Nemes, G. M. Meyer, and J. E. Tibballs, *J. Phys. C* **15**, 59 (1982).
10. M. A. Yakshin, D. W. Kim, Y. S. Kim, Y. Y. Broslavets, O. E. Sidoryuk, and S. Goldstein, *Laser Physics* **7**, 941 (1997).
11. I. Takenaga, Y. Tominaga, S. Endo, and M. Kobayashi, *Solid State Commun.* **84**, 931 (1992).
12. C. Krenn, personal communication.
13. M. Sharon and A. K. Kalia, *J. Solid State Chem.* **21**, 171 (1977).

Rapid Dipicolinic Acid Extraction from *Bacillus* Spores Detected by Surface-Enhanced Raman Spectroscopy

STUART FARQUHARSON* ALAN D. GIFT, PAUL MAKSYMIAK, and FRANK E. INSCORE

Real-Time Analyzers, Inc., East Hartford, Connecticut 06108

Index Headings: **Dipicolinic acid; *Bacillus* spores; Anthrax; Surface-enhanced Raman spectroscopy.**

INTRODUCTION

The anxiety caused by the distribution of anthrax endospores through the U.S. postal system in October 2001 was exacerbated by the long time required for positive identification of the *Bacillus anthracis* spores and the unknown extent of their distribution. Since that time, many methods capable of rapid field analysis have been investigated to augment or replace the laboratory method of growing microorganisms in culture media, which takes days to perform.^{1,2} Prominent among these approaches are polymerase chain reactions (PCR),³ immunoassays, and detection of calcium dipicolinate as a biochemical signature. PCR employs primers to separate organism-

Received 10 October 2003; accepted 14 November 2003.
* Author to whom correspondence should be sent.

specific nucleic acid sequences (e.g., capsular protein encoding gene for *Bacillus anthracis*),⁴ and polymerases to amplify the segment until it is detectable. Recently, amplification times have been substantially reduced, and complete analysis can now be performed in an hour or less. Immunoassay methods are also being developed that use competitive binding of the bioagent (as an antigen) and its labeled conjugate for a limited number of antibodies. Although analyses can be performed in under 30 minutes, a well-defined anthrax antigen has not yet been identified,⁵⁻⁷ and consequently, the false-positive rate is unacceptably high.⁸

A number of other methods are being developed with a focus on the detection of calcium dipicolinate (CaDPA) and its derivatives as a *B. anthracis* signature. This is so because only spore-forming bacteria contain CaDPA and the most common potentially interfering spores, such as pollen and mold spores, do not. Relatively fast methods have been developed to chemically extract CaDPA and then detect it directly by fluorescence⁹ or indirectly by luminescence.^{10,11} In the latter case, hot dodecylamine (DDA) has been used to extract dipicolinic acid (DPA), and terbium has been utilized to form a highly luminescent DPA complex.¹¹ 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 Tb³⁺ cation produces the same luminescence spectrum, makes determinations of low spore concentrations problematic.

It has been long known that Raman spectra of *Bacilli* spores are dominated by bands associated with CaDPA¹² and that these spectra may provide a suitable anthrax signature at the genus level.¹³ Since that time considerable improvements in Raman instrumentation have led to laboratory measurements of single *Bacilli* spores¹⁴ and to field measurements of spores captured from a mail-sorting system.¹⁵ However, the single spore measurements required complex instrumentation that is not rugged, while the field measurements required milligrams of sample. Furthermore, the Raman spectra of both measurements contained fluorescence contributions that would increase uncertainty in quantification.

In related research, we demonstrated that nanogram quantities of DPA could be detected by fluorescence-free, surface-enhanced Raman spectroscopy (SERS).¹⁶ We also demonstrated that microliter volumes of chemicals can be detected by SERS using metal-doped sol-gel-packed glass capillaries.¹⁷ Towards the goal of developing a rapid, field, SERS-based, anthrax spore detector, we have combined our previous research, and we now report that DPA can be extracted from a 10 μg *B. cereus* spore sample using DDA in 1 minute and can be detected by SERS in an additional 1 minute.

EXPERIMENTAL

Dipicolinic acid (2,6-pyridinedicarboxylic acid, DPA) and dodecylamine (DDA) were used as received from Sigma-Aldrich (Milwaukee, WI). Lyophilized *B. cereus* spores, prepared according to the literature,¹³ were supplied by the University of Rhode Island and used as received. Multiple particles, approximately 0.1 mm³ each,

were separated and weighed at 5 to 15 μg , representing 0.5 to 1.5 million spores. The sample masses were consistent with a previous determination of spore density at 0.081 g/mL that indicated a high degree of entrained air.

All chemicals used to prepare the silver-doped sol-gel coated capillaries were also obtained and used as received from Sigma-Aldrich. According to previously published procedures,¹⁷ two precursor solutions were prepared, mixed, and then drawn into 1-mm-diameter glass capillaries. The silver amine precursor consisted of a 5/1 v/v ratio of 1 N AgNO₃ to 28% NH₃OH, while the alkoxide precursor consisted of a 2/1 v/v ratio of methanol to tetramethyl orthosilicate. The alkoxide precursors were mixed with silver amine precursor in an 8/1 v/v ratio. Approximately 0.15 mL was drawn into the capillary, coating a 15-mm length. After sol-gel formation, the incorporated silver ions were reduced with dilute sodium borohydride, which was followed by a water wash to remove residual reducing agent.

A 100 μL drop of a 50 mM DDA solution in ethanol, pre-heated to 78 °C, was added to each of the *B. cereus* particles to digest the spore coat. After 1 minute the resultant solution was drawn into a SER-active capillary that was immediately fixed horizontally to an XY positioning stage (Conix Research, Springfield, OR) just inside the focal point of an *f*/0.7 aspheric lens. The lens focused the beam into the sample and collected the scattered radiation back along the same axis. A dichroic filter (Omega Optical, Brattleborough, VT) was used to reflect the excitation laser to the lens and pass the Raman scattered radiation collected by the lens. An *f*/2 achromat was used to collimate the laser beam exiting a 200- μm -core-diameter source fiber optic, while a second *f*/2 achromat was used to focus the scattered radiation into a 365 μm fiber optic (Spectran, Avon, CT). 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. A 785 nm diode laser (Process Instruments Inc., model 785-600, Salt Lake City, UT) was used to deliver 100 to 150 mW of power to the sample. A Fourier transform Raman spectrometer (Real-Time Analyzers, model IRA-785, East Hartford, CT) and a silicon photoavalanche detector (Perkin Elmer model C30902S, Stamford, CT) were used to acquire the SER spectra.

RESULTS AND DISCUSSION

As an initial experiment, the SER spectrum of 1 g/L of DPA in water was measured using the newly developed silver-doped sol-gel-coated capillaries (Fig. 1A). At this concentration, a high signal-to-noise ratio (S/N) is obtained in 1 min. In fact, a reasonable spectrum is obtained in the same time frame for 1 mg/L (Fig. 1B). The SER spectra are reasonably similar to the normal Raman (NR) spectrum obtained for a saturated solution of DPA in 1 N KOH (Fig. 1C), and the following band shifts are observed (NR to SER): 647 to 657 cm⁻¹, 817 to 815 cm⁻¹, 998 to 1008 cm⁻¹, 1384 to 1382 cm⁻¹, 1434 to 1428 cm⁻¹, and 1569 to 1567 cm⁻¹. Many of these bands have been previously assigned,^{12,13} such as 998 cm⁻¹ to

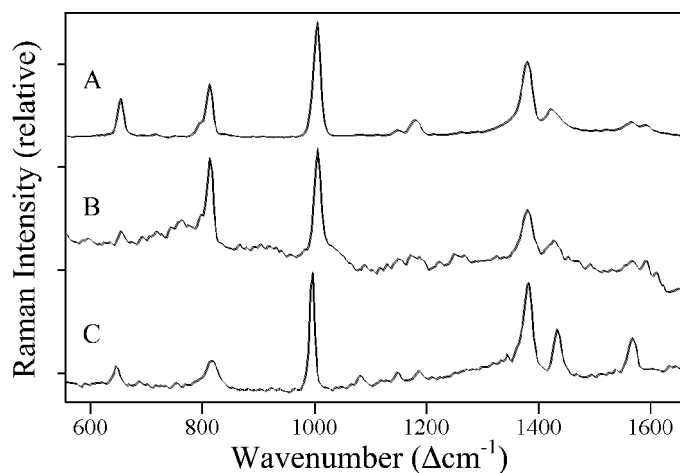


FIG. 1. SERS of DPA in water using silver-doped sol-gel-coated glass capillary for (A) 1 g/L and (B) 1 mg/L. (C) NR of saturated DPA in 1 N KOH in a glass capillary. Spectral conditions: (A) and (B), 150 mW of 785 nm, 1-min acquisition time; (C) 450 mW of 785 nm, 5-min acquisition time; both 8 cm^{-1} resolution.

the symmetric ring stretch, 1384 cm^{-1} to the O–C–O symmetric stretch, 1428 cm^{-1} to the symmetric ring C–H bend, and 1569 cm^{-1} to the asymmetric O–C–O stretch.

The first *B. cereus* samples consisted of 2 mg of spores in 2 mL of 5 mM hot DDA. The samples were maintained at 78 °C for 40 min, and while hot, approximately 10 μL was drawn into a SER-active capillary. Since spectra of DPA were obtained for these initial samples, smaller spore masses, higher DDA concentrations, and shorter heating periods were examined. In due course it was found that 10 μg of spores could be digested by 100 μL of 50 mM hot DDA in one minute and detected (Fig. 2A). In fact the signal was sufficiently intense that it can be observed in as little as two seconds (Fig. 2B). The amount of DPA that was extracted was estimated to be between 5 and 10 mg/L by comparing the signal intensity of the 1008 cm^{-1} band to that measured for DPA in water. This is consistent with previous research that found that the majority of the DPA is extracted from spores using DDA¹¹ and that *B. cereus* spores contain approximately 10% DPA by weight.¹⁸ The S/N of 127 for the 1008 cm^{-1} band in the 1-minute SER spectrum suggests a limit of detection of approximately 250 ng of *B. cereus* spores based on a S/N of 3. Finally, it should be noted that DDA did not produce a detectable SER spectrum, as shown in Fig. 2C.

CONCLUSION

We have demonstrated that by combining rapid extraction of dipicolinic acid from *Bacillus cereus* spores with chemical identification by surface-enhanced Raman spectroscopy, as little as 10 μg of spores can be detected. In fact, the entire measurement, from the time of adding hot dodecylamine to the spores to the time when the dipicolinic acid SER spectrum is acquired and analyzed, could be performed in less than two minutes. The ability of this method to distinguish between spore-forming bacteria, such as *Bacillus anthracis*, and non-DPA containing powders could help prevent costly shutdowns associated

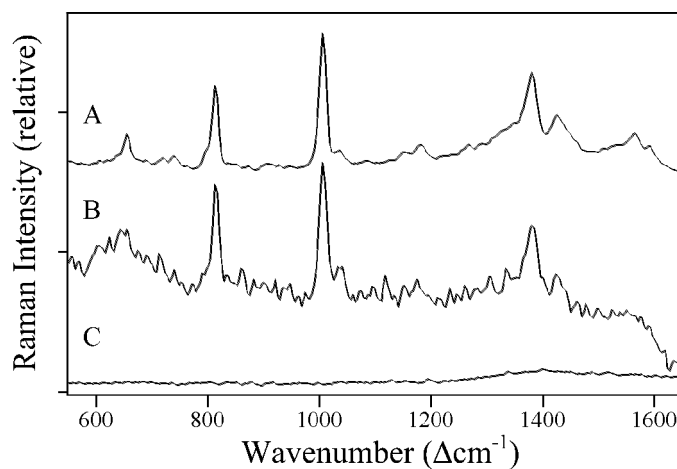


FIG. 2. SERS of DPA extracted from $\sim 10 \mu\text{g}$ *B. cereus* particle using 100 μL of 50 mM hot DDA acquired in (A) 1 minute and (B) 2 seconds. (C) Attempted SERS of 50 mM hot DDA in ethanol using silver-doped sol-gel-coated glass capillary acquired in 1 min. Spectral conditions: 150 mW of 785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

with the appearance of suspicious material or intentional mailing of common substances as an anthrax hoax. This method could also prove useful in detecting the location of anthrax endospores in mail distribution facilities if another verified attack should occur.

Research continues to fully characterize the surface-enhanced Raman spectroscopy signal intensities as a function of sample concentration and to explore other extractants that do not require the use of elevated temperature.

ACKNOWLEDGMENTS

The authors are grateful for the support of the National Science Foundation (DMI-0296116 and DMI-0215819) and the U.S. Army (DAAD13-02-C-0015, Joint Service Agent Water Monitor program). The authors are indebted to Chetan Shende for preparing the sol-gel capillaries. The authors also thank James Gillespie, Nicholas Fell, and Augustus Fountain for providing important background information, Mark Farquharson for laboratory support, and Professor Jay Sperry of the University of Rhode Island for supplying *B. cereus* spores.

1. V. A. Pasechnik, C. C. Shone, and P. Hambleton, *Bioseparations* **3**, 267 (1993).
2. P. J. Jackson, M. E. Hugh-Jones, D. M. Adair, G. Green, K. K. Hill, C. R. Kuske, L. M. Grinberg, F. A. Abramova, and P. Keim, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1224 (1998).
3. B. R. Glick and J. J. Pasternak, *Molecular Biology: Principles and Applications of Recombinant DNA* (ASM Press, Washington, D.C., 1994).
4. C. A. Bell, J. R. Uhl, T. L. Hadfield, J. C. David, R. F. Meyer, T. F. Smith, and F. R. Cockerill, III, *J. Clin. Microbiol.* **40**, 2897 (2002).
5. D. L. Gatto-Menking, H. Yu, J. G. Bruno, M. T. Goode, M. Miller, and A. W. Zulich, *Biosens. Bioelectron.* **10**, 501 (1995).
6. J. J. Quinlan and P. M. Foegeding, *J. Rapid Methods Automation Microbiol.* **6**, 1 (1998).
7. A. A. Hindle and E. A. H. Hall, *Analyst (Cambridge, U.K.)* **124**, 1599 (1999).
8. M. S. Ascher, US Department of Health & Human Services (<http://www.hhs.gov/ophp/presentations/Ascher.doc>).
9. R. Nudelman, B. V. Bronk, and S. Efrima, *Appl. Spectrosc.* **54**, 445 (2000).
10. D. L. Rosen, C. Sharpless, and L. B. McBrown, *Anal. Chem.* **69**, 1082 (1997).

11. P. M. Pellegrino, N. F. Fell, Jr., and J. B. Gillespie, *Anal. Chim. Acta* **455**, 167 (2002).
12. W. H. Woodruff, T. G. Spiro, and C. Gilvarg, *Biochem. Biophys. Res. Commun.* **58**, 197 (1974).
13. E. Ghiamati, R. S. Manoharan, W. H. Nelson, and J. F. Sperry, *Appl. Spectrosc.* **46**, 357 (1992).
14. A. P. Esposito, C. E. Talley, T. Huser, C. W. Hollars, C. M. Schal-dach, and S. M. Lane, *Appl. Spectrosc.* **57**, 868 (2003).
15. S. Farquharson, L. Grigely, V. Khitrov, W. W. Smith, J. F. Sperry, and G. Fenerty, *J. Raman Spectrosc.*, paper accepted (2003).
16. S. Farquharson, W. W. Smith, S. Elliott, and J. F. Sperry, *SPIE-Int. Soc. Opt. Eng.* **3855**, 110 (1999).
17. S. Farquharson and P. Maksymiuk, *Appl. Spectrosc.* **57**, 479 (2003).
18. F. W. Janssen, A. J. Lund, and L. E. Anderson, *Science (Washing-ton, D.C.)* **127**, 26 (1958).