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Monitoring DPA Release from a Single Germinating *Bacillus subtilis* Endospore via Surface-Enhanced Raman Scattering Microscopy

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During the past decade there has been a great deal of emphasis placed on developing reliable and rapid methods for the detection of *Bacillus anthracis*, the causative agent of anthrax. The Center for Disease Control estimates the inhalation LD_{50} of *B. anthracis* to be on the order of 100 ng, or about 10^4 spores.[1] Methods such as staining, immunoassays, polymerase chain reaction (PCR),[2] electrophoretic, and chromatographic techniques[3] are all quite capable of detecting *B. anthracis*, and in some cases differentiating *Bacillus* species, well below the human LD_{50}. However, since these methods generally take on the order of hours to complete, appropriate interventions are delayed.

Spectroscopic techniques such as photoluminescence, FT-IR, Raman, and CARS[4] have also successfully been used to identify *Bacillus* endospores through the detection of the acidic and/or calcium-chelated dipicolinate ion (DPA). Advantageously, DPA represents ca. 10% of the total spore weight and is not found in other common spores such as pollen or mold. Extraction of DPA using dodecylamine[5] or nitric acid[6] can be followed by direct measurement using vibrational spectroscopy or photoluminescence of terbium after complexation with DPA. Raman spectroscopy can be used for the direct detection of DPA as it exists inside the spore. These measurements were first reported in 1974 for *B. megaterium*[4c] and were recently used to detect relatively small amounts of *B. cerus* on a mail sorting system.[1] Raman microspectroscopy has been used to detect DPA contained in a single *Bacillus* endospore.[7] Several orders of magnitude of signal enhancement as well as reduction of the excitation power and spectral collection time can be obtained with surface enhanced Raman scattering (SERS) spectroscopy.[5,6,8]

A recent report from this laboratory detailed the first study of the DPA release kinetics of *B. subtilis* (an anthrax stimulant) using SERS spectroscopy.[9] Germination was initiated by the natural germinant L-alanine and was monitored by the increase in DPA signal over time. The DPA release kinetics were measured as a function of L-alanine concentration and temperature and averaged over several hundred spores. Understanding the kinetics of DPA release can assist in determining a mechanistic picture of how L-alanine and other germinants initiate the transformation of endospores to vegetative cells. This level of biochemical understanding is an important step for developing new *B. anthracis* therapeutics.

In this communication, we extend this method for monitoring the DPA release from a single germinating *B. subtilis* endospore. High *S*/*N* ratio SERS spectra were obtained with excitation power as low as 3 mW and 1 min spectral collection times. Previous reports measured DPA localized within a single spore.[10] The current method detects only the signal of DPA that is released during the beginning of the germination process owing to the extreme surface localization of the SERS phenomenon. Hence, this method is selective for actively germinating spores.

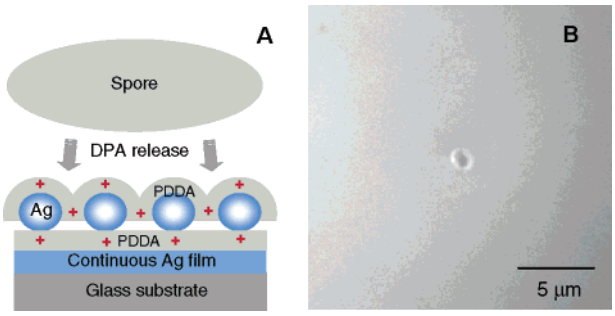

Figure 1. (A) Schematic of the SERS substrates with an adsorbed spore and (B) a brightfield image of a single *B. subtilis* endospore on a SERS-active substrate at 100× magnification taken in reflection mode.

B. subtilis endospores and SERS-active substrates were prepared as previously reported.[8a,9] The sandwich-type SERS substrates were fabricated by assembling 100 nm Ag particles on poly(diallyldimethylammonium chloride) (PDDA) modified silver mirror film (Figure 1A). A typical surface density of the particles was 12−15 μm^{-2}. The assembled particles were further modified with PDDA rendering a positive surface charge and providing adsorption of the negatively charged spores to the substrate. The substrates were exposed overnight to a dilute spore suspension to obtain low surface coverage of the spores. The average spacing between spores from which the SERS was measured was 30 μm ensuring that the collected spectra originated from a single endospore without the interference from DPA released by neighboring spores. Also, no DPA signal was detected from the substrate alone ca. 10 μm away from a germinating spore further indicating that the SERS signal was localized to the proximity of the spore. This localization resulted from trapping negatively charged DPA molecules by the layers of the positively charged PDDA polymer.

The spores were imaged in reflection geometry because of the opaque nature of the SERS substrate (Figure 1B). Before the SERS measurement, 10 μL of a 150 mM L-alanine solution was dropped onto the substrate with adsorbed spores and sealed using a coverslip and vacuum grease. Individual spores were located, and spectral collection was initiated within 1−2 min after the L-alanine addition. Imaging and spectral acquisition was done using a 100×/1.3 N.A. oil immersion objective on an Olympus inverted microscope equipped with a Raman spectrograph and liquid nitrogen cooled CCD camera. The 647.1 nm line from a Kr^+ laser was used for excitation. To characterize the germination kinetics, a series of SERS spectra were collected at 1.5 min intervals over 30 min time period. Each spectrum was accumulated during a 60 s interval followed by a 30 s waiting period between the scans. All spectra measured from the same spore were normalized to a broad band of PDDA[9] at 794 cm^{-1}. This band served as an internal standard for the quantitative comparison of SERS spectra acquired at

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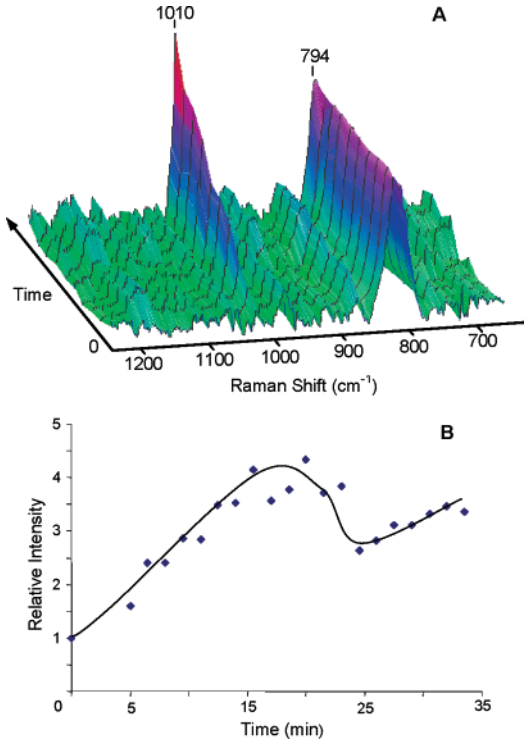

Figure 2. (A) SERS spectra of a single *B. subtilis* endospore following addition of 100 mM L-alanine as a function of time. The total time on the time axis is 20 min. Part B shows the normalized intensity of the peak at 1010 cm^{-1} as a function of time.

different time intervals as well as provided high reproducibility between individual SERS substrates.

A three-dimensional plot containing SERS spectra measured from a single germinating endospore at different time intervals following addition of L-alanine is shown in Figure 2A. The peak at 1010 cm^{-1} is characteristic of the breathing mode of the pyridyl ring[5] in the DPA molecule. It does not overlap with any other vibrational modes in the system, and therefore it is a convenient marker for monitoring the germination process.[9] A weak intensity at 1010 cm^{-1} was already detected at zero time (before the addition of L-alanine) and attributed to the normal Raman scattering of the DPA inside the spore. This weak intensity remained unchanged without the presence of germinant. After the addition of the germinant, the peak intensity increased indicating the ongoing germination of the spore. In the process of germination, DPA molecules were released and captured by the positively charged PDDA that was in the space between the adjacent silver nanoparticles as well as in the space between the silver nanoparticles and the silver mirror film. The local electromagnetic field in these places is enhanced as compared to the incident field because of the plasmon coupling among the particles themselves and between the particles and the conduction electrons in the silver film. This enhanced local field led to the observed SERS phenomenon. The surface area on the substrate from which the SERS signal was collected was determined by the near diffraction-limited laser spot and was estimated to be about 1 μm^2.

A typical kinetics for DPA release during the germination of a single spore is plotted in Figure 2B. The intensity of the peak at 1010 cm^{-1} increased steadily for the first ~15 min after the addition of L-alanine. A noticeable decline of the intensity was observed at later times followed by an additional increase of the signal. Even though the decline was characteristic for all studied spores, its magnitude varied significantly between individual spores owing to the spore heterogeneity.[9] The first 15 min were attributed to the germination step during which progressively more DPA was released. The beginning of the decline indicated the completion of this step after which the release of DPA was diminished. The signal decline was due to diffusion that reduced the local DPA concentration in the area probed by the laser beam. The exact nature of the additional increase of the signal at longer times is currently unclear. It could reflect additional DPA release from endospores at later stages of germination or the kinetics of DPA adsorption on SERS substrate. After ca. 35 min, the DPA signal reached a constant value and no further kinetics were observed.

In conclusion, this work reports the first direct spectroscopic monitoring of DPA release from a single germinating spore of *B. subtilis*. The SERS microspectroscopy described here enables the measurement of the kinetics without ensemble-averaging. Such information will aid the fundamental understanding of the germination process of endospores from various species that contain DPA including *B. anthracis*. From an analytical perspective, this method is proof-of-principle for the SERS detection limit at the single spore level. This represents a 100- to 1000-fold improvement over previously reported detection limits for SERS-based measurements of DPA in endospores.

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