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Anal. Chem. **2006,** *78,* 1724−1729

Monitoring the Kinetics of *Bacillus subtilis* Endospore Germination via Surface-Enhanced Raman Scattering Spectroscopy

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Mirror sandwich SERS substrates (M3S) were used to monitor the kinetics of *Bacillus subtilis* endospore germination. The sandwich configuration of the substrates allows real-time observation of germination in samples that contained only several hundred endospores. The enhancement provided by the substrates is attributed to the enhanced local electromagnetic field that originates from coupling between the Ag nanoparticles and the underlying metal film as well as from coupling between the Ag nanoparticles themselves. The germination kinetics at varying concentrations of L-alanine and different temperatures were studied by monitoring the intensity and growth of the Raman peak at 1010 cm^{-1}, which is characteristic of dipicolinic acid. A total of four concentrations (50, 75, 100, and 150 mM) of L-alanine and three different temperatures (30, 37, and 55 °C) were investigated.

Over the past few years, much emphasis has been placed on the need for rapid detection and identification of *Bacillus anthracis* spores as well as other potential biological threat agents. *B. anthracis* is a Gram-positive bacteria normally found in the soil. It is the causative agent of anthrax and has been used as a biological weapon.[1] *B. anthracis* belongs to the genus *Bacillus*, one of two endospore-forming genera (*Bacillus* and *Clostridium*), and are large aerobic spore forming rod-shaped bacteria. Endospores of *B. anthracis* are resistant to adverse environmental conditions such as extreme temperature changes, drying, radiation, and toxic chemicals. When appropriate germinants are present, the spores enter an outgrowth phase and resume vegetative growth.[2] Spores can enter the body through digestion, inhalation, or skin abrasions, of which the inhalation path is the most threatening. Once exposed to internal tissues, the spores are engulfed by phagocytes where they germinate and resume vegetative growth.[3] Death usually occurs in 1−7 days after the appearance of symptoms in untreated cases of the disease. Clearly, a rapid, sensitive, and reliable method to detect *B. anthracis* endospores is crucial in responding to anthrax threats. In addition to detection methods, a comprehensive mechanistic understanding of the germination process itself is required for development of preventive and therapeutic countermeasures. Hence, methods for inhibiting or slowing down the process of germination and, consequently, prevention of infection will be developed.

Germination is breaking of the endospore dormancy phase and returning to its vegetative cell cycle and is triggered by the presence of nutrients and elevated temperatures. It involves the degradation of the endospore coat and cortex followed by the simultaneous hydration of the core and release of dipicolinic acid from within the core,[4] and as a result of these transformations, the spores appear less refractile in a light microscope. This process is followed by an outgrowth phase, in which the endospores develop into vegetative cells and begin to divide. The vegetative cells are now metabolically active and can produce new spores.[5] The process of germination involves the interaction of chemical germinants (nutrients) with specific receptors located throughout the spore. The receptors specific for germination throughout the *Bacillus* and *Clostridium* genera are the gerA operons.[6] Nutrients that trigger germination in spores include sugars, nucleosides, and single amino acids, as well as a combination of nutrients such as a mixture of asparagine, fructose, glucose, and potassium ions. L-Alanine is a common single amino acid nutrient that is recognized by the gerA operon in *B. subtilis* and is used for triggering spore germination.[7] In addition to nutrients, spores can be germinated by nonnutrient agents such as surfactants, lysozymes, and salts.[8]

The current focus of many researchers is on the development of methods for the detection and identification of *B. anthracis* with the demand shifting toward techniques that are rapid, sensitive, and cost-effective. Traditional techniques such as routine culture, staining procedures,[9] fluorescence-based assays,[10] and PCR-based methods[11] have proven to be time-consuming and expensive. The PCR approach has the advantage of being able to distinguish

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10.1021/ac052009v CCC: $33.50 © 2006 American Chemical Society
Published on Web 02/01/2006

between *B. anthracis* and other *Bacillus* species, but it is still relatively slow ($1^1/_2$−2 h) when compared to other methods of spore detection that look for characteristic chemical markers of spores.[11] Rapid methods for detecting endospores based on the presence of chemical markers include liquid chromatography,[12] gas chromatography,[13] mass spectrometry,[14] and FT-IR[15] as well as UV−visible and luminescence,[16] which are based on complexing or binding of the chemical marker. None of the above-mentioned methods provides the required sensitivity needed to detect endospores at low concentrations and they are also prone to false negatives. The most definite identification of the spores is through routine culture, in which spores are germinated on agar plates and further proliferated to form cell colonies. However, this approach is slow for rapid analysis of possible contamination incidents as it takes 24−48 h before definite identification can be confirmed.[9]

Recently, a new method for rapid detection of spores from *Bacillus* and *Clostridium* species based on surface-enhanced Raman spectroscopy (SERS) was introduced.[17] SERS is a well-recognized analytical tool capable of providing sensitive spectroscopic identification of various compounds. [18] It is often applied for trace analysis because it produces intense vibrational spectra that can be used as fingerprints of molecules. SERS was used to detect the presence of dipicolinic acid (DPA) in samples of *B. subtilis*, which is a chemical compound characteristic of endospores only and is not found in the vegetative cells. In these studies, DPA was extracted from the spores by sonication in the presence of dilute nitric acid.[17] It should be noted that the detection of DPA does not infer that *B. anthracis* endospores are present because, as stated earlier, two genera (*Bacillus* and *Clostridium*) include spore-forming species that contain DPA in their cores. However, the detection of DPA is useful for the initial screening of substances that are suspected to be *B. anthracis*.[19]

In the present work, SERS is used to detect DPA as it is released from spores during their germination. During the sporulation process, calcium dipicolinic acid is deposited in the core and represents ∼10% of the dry weight of endospores. At the onset of germination, DPA is released and can be used as a chemical signature for monitoring endospore germination of *Bacillus* species. With this approach, we emphasize the SERS technique as a new way for monitoring the germination process itself, specifically SERS ability to study the kinetics of germination. Several hundred spores were sufficient to reliably measure the kinetics of germination at different concentrations of germinant and different temperatures. Four different concentrations of L-alanine and three different temperatures were selected to demonstrate the utility of this technique with *B. subtilis* as a model for *B. anthracis*. Mirror sandwich substrates (M3S) previously reported in ref 20 were used to enhance Raman scattering from the germinating spores. We conclude that SERS is a general method for studying the germination process of other spores that contain DPA.

EXPERIMENTAL SECTION

Materials. Ag_2O (99.99%) was purchased from Alfa Aesar and hydrogen gas (99.9999%) from National Welders Supply Co. Microscope glass slides and reagent alcohol (HPLC grade) obtained from Fisher Scientific. Poly(4-vinylpyridine) 40 000 MW, poly(diallyldimethylammonium chloride) (PDDA; MW <200 000), L-alanine, and 2,6-pyridinedicarboxylic acid (dipicolinic acid) were purchased from Aldrich. *Bacillus subtilis* 168 was purchased from American Type Culture Collection (ATCC). With the exception of *B. subtilis* (strain 168), all chemicals were used as purchased with no further purification. Water with a nominal resistivity of 18 MΩ·cm was obtained from a Millipore Milli-Q system.

Sporulation of *B. subtilis* Endospores. A lyophilized stock of *B. subtilis* was aseptically inoculated into LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) and incubated in an incubator/shaker at 37 °C for >72 h to induce sporulation. Growth was monitored by both absorbance at 600 nm and phase contrast microscopy. The culture was pelleted by centrifugation. The resulting pellet was washed (resuspended and then centrifuged) 3× with a 1:1000 v/v ratio of spores to autoclaved ddH_2O to remove spent media. Spore suspensions were then heat-shocked at 72 °C for 30 min to kill any remaining vegetative cells.[21] Spore concentration was determined by counting refractile bodies (spores) using a hemocytometer and phase contrast microscopy.

Synthesis of Ag Nanoparticles. Silver nanoparticles were synthesized by a hydrogen reduction reaction previously reported by this laboratory.[22] Briefly, an aqueous solution of saturated silver oxide was reduced with hydrogen gas, and the reaction was carried out in a specially made reaction vessel. The solution was heated to 70 °C and exposed to hydrogen gas at 10 psi. Shortly after the vessel was pressurized, the solution color changed from clear to light yellow indicating the formation of silver nanoparticles from the seeds present in the vessel. The reaction was monitored using UV−visible spectroscopy. Aliquots of the reaction mixture were taken in 15-min intervals until the particles grew to an average diameter of 100 nm after which the reaction was terminated by releasing hydrogen gas.

Fabrication and Characterization of M3S Substrates. The substrates were fabricated with a procedure previously reported.[20] Microscope slides were cut into 25 × 10 mm strips and cleaned with soap and water and further with a 1:3 mixture of 30% H_2O_2/H_2SO_4 (piranha solution) for 30 min under continuous sonication. *Caution!* Piranha solution is a very strong oxidizing agent and reacts violently with organic compounds. It should be handled with extreme care. The slides were modified in a 1% solution of poly(vinylpyridine) for several hours and following thorough rinsing with reagent alcohol were placed into an oven at 100 °C for 20 min. The polymer-modified slides were then placed in the vacuum deposition apparatus (Edwards, model E306A) for the

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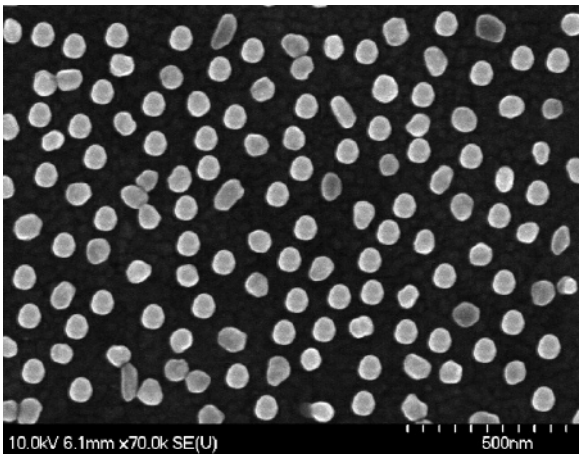

Figure 1. Scanning electron microscopy image of M3S fabricated using 100-nm Ag nanoparticles.

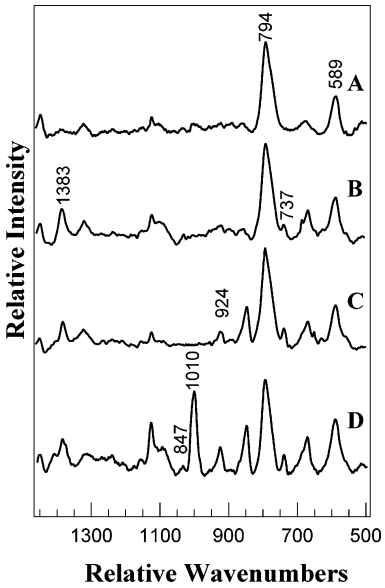

Figure 2. SERS spectra of (A) M3S in water; (B) *B. subtilis* spores adsorbed onto M3S; (C) M3S with spores in 100 mM L-alanine (t = 0 min); and (D) M3S with spores after germinating in 100 mM L-alanine at 55 °C (t = 35 min).

evaporation of Ag mirror films. The silver mirror films were further modified in a 1% aqueous PDDA solution overnight. After thorough rinsing with water, the films were dried at 100 °C for ~2 h and placed into an aqueous suspension of Ag nanoparticles with diameters of 100 nm. The films were exposed to the Ag nanoparticle solution for one week to allow saturation of the surface with the nanoparticles. The films were then examined with a field emission scanning electron microscope (SEM) S-4700 (Hitachi) to determine the size distribution, shape, and aggregation state of the nanoparticles. The continuous Ag films provide the electrical conductivity required for SEM measurements. A typical SEM image of the substrates is shown in Figure 1.

SERS Spectroscopy of *B. subtilis* Spores. Spores were sandwiched between M3S and a blank microscope slide in a solution with the germinant, L-alanine and heated to the required temperatures. The sandwich slides were immersed into a small preheated vial containing ~1 mL of water that was fitted into a large aluminum block with a heating element. The temperature was monitored using a thermometer with 0.5 °C precision, and the aluminum block provided the stabilization so that no temperature fluctuation during the measurements was observed.

SERS spectra were directly measured from the sandwich using a spectrograph (Spex, Triplemate 1377) interfaced to a liquid nitrogen-cooled CCD detector (Princeton Instruments model LN1152). The spectra were excited with 568.2-nm radiation from an Innova 100 (Coherent) krypton ion laser. The laser power was ~20 mW at the sample, and the total acquisition time was 100 s for each measurement. The scattered light was collected in a backscattering geometry, and the instrument was calibrated using indene. All spectra were processed and figures prepared using Spectra-Solve for Windows (LasTek Pty. Ltd).

RESULTS AND DISCUSSION

Immobilization of Ag nanoparticles on continuous Ag films provides substrates that are capable of enhancing Raman scattering of adsorbed molecules. SERS comes from the enhanced local electromagnetic field originated from coupling between the Ag nanoparticles and the underlying continuous Ag film as well as coupling between the Ag nanoparticles themselves.[20] The role of PDDA was to attach the Ag nanoparticle to the mirror film as well as to attract DPA molecules that were released from the *B. subtilis* spores. Once the acid is released from the spores, the positively charged polymer captures the negatively charged DPA molecules between the Ag nanoparticles and the mirror film yielding enhanced Raman spectra. The M3S substrates provide additional advantages such as stronger SERS of DPA as compared to electrochemically roughened Ag surfaces and the possibility for internal calibration using PDDA bands.

SERS spectra of M3S and the *B. subtilis* spores before and after germination are shown in Figure 2. The spectrum of the substrate alone is relatively featureless with the exception of two broad peaks at 589 and 794 cm^{-1} from PDDA (Figure 2A). These peaks remain constant throughout the experiments and were selected as an internal standard to which all other SERS spectra were normalized. The presence of *B. subtilis* spores on the surface of the M3S resulted in additional weak spectroscopic features, the most prominent of which are at 737 and 1383 cm^{-1} (Figure 2B). No attempt to assign these features was made as they are not significant for monitoring the germination. After the addition of L-alanine (the germinant), new bands appeared in SERS spectra at 847 and 924 cm^{-1} that were attributed to this amino acid (Figure 2C), and this assignment was further confirmed by additional experiments.

This spectrum was taken immediately after the addition of the germinant before the process of germination of the spores begins.

Heating in the presence of L-alanine initiates the germination, and the SERS spectrum acquired new features with the most important band at 1010 cm^{-1} that progressively increased as the germination proceeded (Figure 2D). The growth of this band is attributed to the release of DPA from the core of the spores onto the SERS surface thereby characterizing the germination kinetics. To illustrate the emergence and growth of DPA peaks characteristic of the germination of *B. subtilis* spores, a three-dimensional SERS plot is shown in Figure 3. Under experimental conditions chosen to construct the plot, the onset of the germination began after several minutes, after which the prominent peak at 1010 cm^{-1} and several other peaks started to grow.

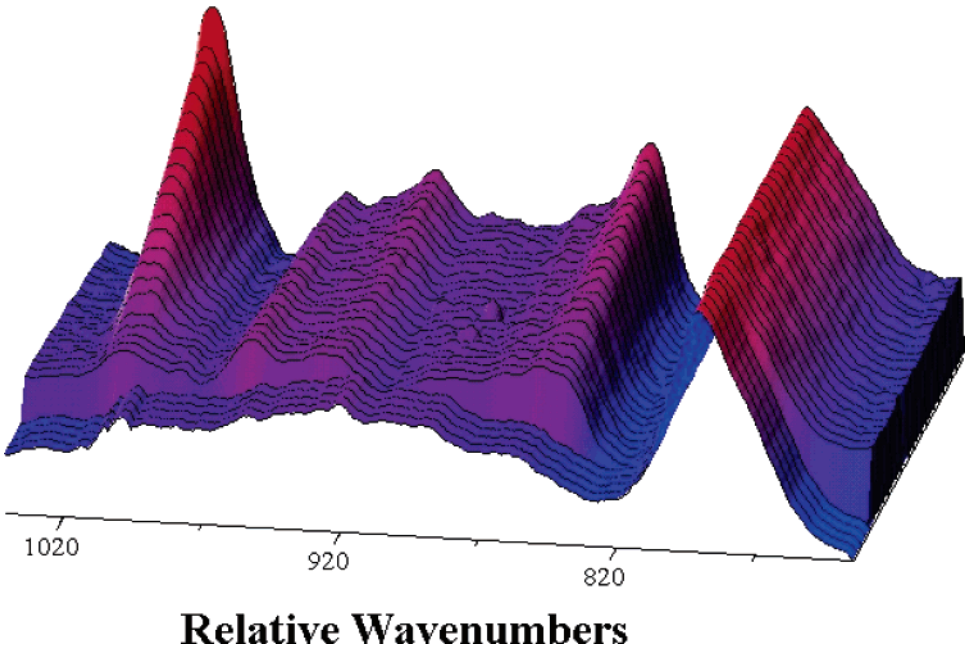

Figure 3. Three-dimensional representation of germination as a function of time. The evolution and growth of most prominent peak at 1010 cm^{-1} is illustrated.

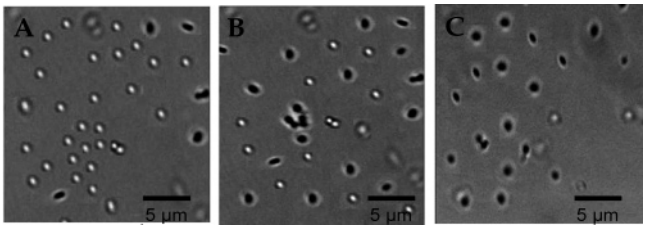

Figure 4. Optical microscopy images of *B. subtilis* endospores (A) before, (B) after 10 min of germinating, and (C) after 35 min of germinating.

The spores go through a series of changes during the germination process that can be monitored using light microscopy. *B. subtilis* endospores are highly refractile in their nongerminated form and appear as bright, ~1-μm-diameter spots with differential interference contrast microscopy (Figure 4A). After spore germination, they are no longer refractile and appear as dark, slightly enlarged bodies (Figure 4C). There were a small number of spores that appear to have lost their refractivity before the beginning of the germination experiment most likely representing residual heat-killed cells that had not sporulated during spore preparation.

To further illustrate the ability of SERS to measure the germination kinetics, experiments were performed to determine the effect of L-alanine concentration and temperature on the germination of *B. subtilis* spores. The results are shown in Figure 5 and Figure 6. Four different concentrations, 50, 75, 100, and 150 mM, of L-alanine were selected and each experiment was repeated at least three times with different SERS substrates to determine the reproducibility of the technique. Spores treated with 50 mM L-alanine did not induce noticeable germination, as determined from the absence of the 1010-cm^{-1} DPA peak after heating for 30 min. This experiment serves as the negative control. Germination was observed with all other concentrations as determined by the appearance of the DPA peak (1010 cm^{-1}) over time. The time course of the DPA peak intensity resembled a characteristic sigmoidal shape that is indicative of the extent of

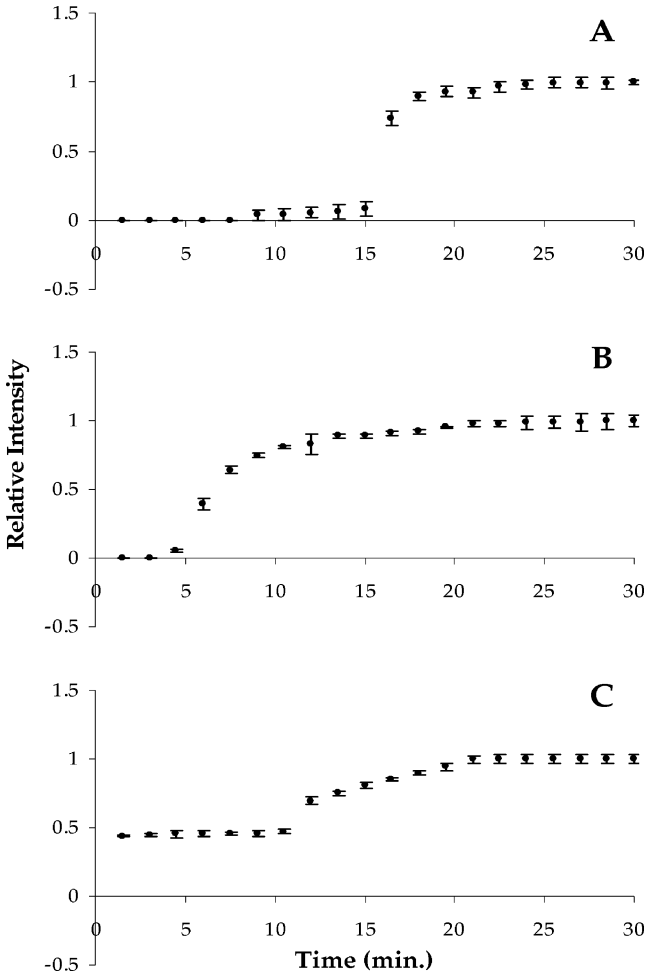

Figure 5. Relative peak intensity as a function of germination time: (A) 75, (B) 100, and (C) 150 mM L-alanine.

the homogeneity of the spore samples in terms of their ability to germinate. Deviations from the sigmoidal behavior imply the

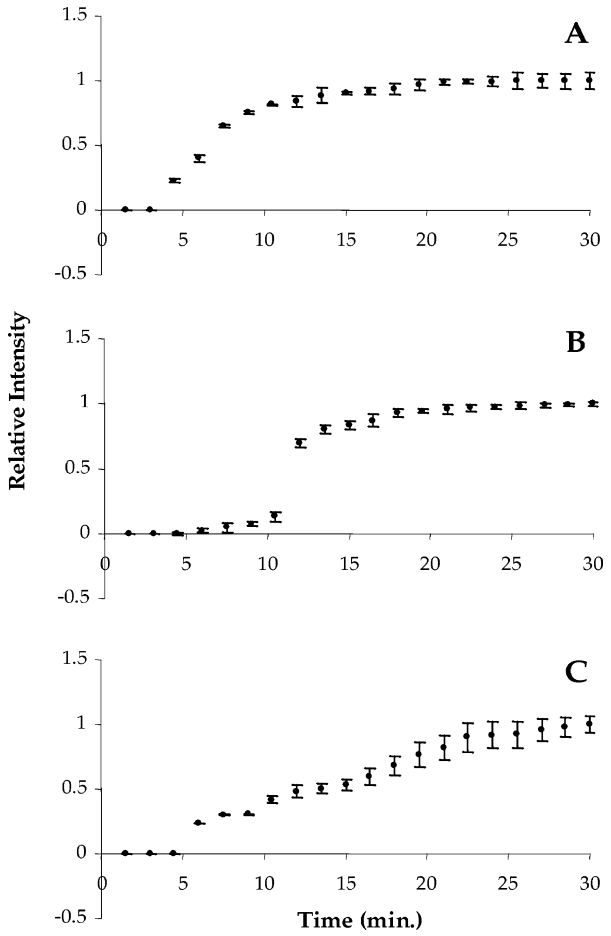

Figure 6. Relative peak intensity as a function of germination time: (A) 55, (B) 37, and (C) 30 °C.

presence of different spore populations with separate characteristic germination kinetics. For perfectly sigmoidal behavior, the germination time can be defined as the time at which the onset of germination occurs and the kinetics curve starts to rise or as a point at which half of the spores are already germinated. Earlier germination is expected at higher concentration of the germinant. Indeed, at 75 and 100 mM L-alanine concentrations, the onset of germination appears at 15 and 4 min, respectively, after increasing the temperature to 55 °C; however, evidence of the sample heterogeneity can be noticed as a small fraction of the spores started to germinate after ~8 min at 75 mM (Figure 5A). This heterogeneity was even more pronounced at 150 mM concentration of L-alanine, at which half of the spores were already germinated at zero time and the rest of the population started to germinate at 10 min. These data suggest that the spores vary not only in their ability to germinate but also in their response to different concentrations of the germinant. Notably, all kinetics curves exhibit low standard deviation between different M3S substrates, and this fact is attributed to the normalization of the DPA peak intensity to the intensity of the 794-cm^{-1} peak, which was selected as an internal standard. Heterogeneity of germination time was previously reported for *Clostridium* species.[23]

Temperature studies at 30, 37, and 55 °C and at the same 100 mM concentration of the L-alanine germinant were performed in order to determine the role of temperature on the germination kinetics (Figure 6). The kinetic data in Figure 6A correlate well with the data from Figure 5B as both measurements were performed under the same experimental conditions but at different times on different substrates, further emphasizing the reproducibility of the SERS approach for studying the germination process. As expected, the onset of germination occurred at longer times as the temperature was decreased, specifically at 10 min when the temperature was set to physiological 37 °C as compared to ~3 min at 55 °C. The kinetics curve at this temperature also revealed the heterogeneous composition of the samples as a small fraction of the spores started to germinate at 6 min (Figure 6B) after the application of heat, similar to that in Figure 5A. The heterogeneous composition of the spore samples can be clearly noted in the kinetics data obtained at 30 °C, at which the kinetic curve deviates significantly from the sigmoidal shape (Figure 6C). In this plot, the maximum intensity measured at 30 min was normalized to unity in order to emphasize the early kinetics; however, the actual DPA signal was not at the saturation level at this time and continued to increase. In comparison, all other kinetic transients shown here were normalized to unity at 30 min. In these cases, the DPA signal ($t = 30$ min) was already saturated. The absolute intensity of the DPA signal at 30 min in Figure 6C was at least four times weaker than that in all other measurements. The time course of germination at 30 °C appears to proceed in a staircase manner although the noise level is significantly higher as compared to other temperatures. As noted for the previous measurements, a small fraction of spores that germinate early in time, at ~5 min, is evident at this temperature. Germination studies were also performed at higher temperatures and concentrations of the germinant, and they revealed fast kinetics that could not be resolved using our current experimental setup. The method or substrate does not exclude the possibility of increasing temporal response and sensitivity to monitor fast kinetics.

The timing for onset of germination and the observed kinetic rates depend on the bacterial strain, germinant composition, heat activation, and temperature.[24−29] Therefore, the exact comparison of previously reported kinetics is difficult due to variations of these parameters. However, the overall shape and kinetic trends we observed with SERS during *B. subtilis* germination correlate with previous reports using other methods.[25,27−29] At higher temperatures and concentrations of the same germinant, earlier onset times were observed as expected. These onset times typically varied between minutes and tens of minutes. Observed kinetic transients representing fast and efficient germination follow a characteristic sigmoidal shape from which the rate constant for germination can be recovered using nonlinear least squares. By changing conditions to promote slower germination, we could distinguish small differences in the kinetics attributable to variability of spores. As compared to other methods for monitoring germination kinetics, SERS provides several other advantages. Previously used methods include light microscopy,[24] UV−visible[25] and IR spectroscopy,[26] fluorescence spectroscopy,[27] and radioactive

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labeling with ^{45}Ca.[28] Light microscopy is based on observing changes in refractility of the spores or changes in the spore shape, and it is not a quantitative method. UV−visible spectroscopy measures decline of the absorbance in the visible spectral range due to change of refractility or increase of the absorbance at 270 nm due to free dipicolinic acid. The method is insensitive and inconsistent and requires high concentrations of spores. IR spectroscopy that was performed in the ATR configuration also required a large number ($\sim 10^8$) of spores. The IR spectra reflected DPA and the degradation of spore proteins that takes place during the germination; however, no kinetics data were reported. Fluorescence monitoring is based on the increase of fluorescence of specific dyes when they bind with nucleic acids that become accessible as the endospore coat degrades during germination. This method allows germination monitoring in real time; however, the free dye in the solution produces the background that reduces the overall sensitivity. In addition, the presence of other species capable of binding these dyes, specifically cells, leads to increase of the fluorescence background, making this method less specific to germination. In another approach, the enhancement of Tb^{3+} fluorescence[29] was monitored as it was complexed with DPA released during the germination. This method may be prone to interferences from other transition metal ions that compete for the complex formation with DPA.[30] Germination kinetics was monitored using radioactive labeling with ^{45}Ca that was introduced during sporulation. Even though this method is sensitive, it cannot offer fast real-time monitoring because the released ^{45}Ca should be separated from that in the dormant spores. Our SERS method does not require labeling and can potentially provide time resolution on the order of several seconds, allowing small differences in the effects on germination of different conditions to be readily distinguished. It produces quantitative information with high precision due to the internal standard, high sensitivity, and low detection limits. In fact, the low detection limit makes it well suited for monitoring germination kinetics of individual spores using micro-Raman imaging instrumentation.

In conclusion, SERS from the optimized M3S substrates provides a convenient and powerful method for measuring the kinetics of germination of *B. subtilis* spores by following the Raman peak at 1010 cm^{-1} characteristic of DPA that is released during germination. Several hundred spores appeared to be a sufficient amount to generate kinetics curves with low noise at different temperatures and concentrations of germinant. It is expected that this method has general utility for studying the kinetics of other *Bacillus* and *Clostridium* species, including *B. anthracis* that contain dipicolinic acid as a characteristic marker.

Received for review November 11, 2005. Accepted December 19, 2005.

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