Near-Infrared Surface-Enhanced-Raman-Scattering-Mediated Detection of Single Optically Trapped Bacterial Spores

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A novel methodology has been developed for the investigation of bacterial spores. Specifically, this method has been used to probe the spore coat composition of two different Bacillus stearothermophilus variants. This technique may be useful in many applications; most notably, development of novel detection schemes toward potentially harmful bacteria. This method would also be useful as an ancillary environmental monitoring system where sterility is of importance (i.e., food preparation areas as well as invasive and minimally invasive medical applications). This unique detection scheme is based on the near-infrared (NIR) surface-enhanced Raman scattering (SERS) from single, optically trapped, bacterial spores. The SERS spectra of bacterial spores in aqueous media have been measured using SERS substrates based on 60-nm-diameter gold colloids bound to 3-aminopropyltriethoxysilane derivatized glass. The light from a 787-nm laser diode was used to trap and manipulate as well as simultaneously excite the SERS of an individual bacterial spore. The collected SERS spectra were examined for uniqueness and the applicability of this technique for the strain discrimination of Bacillus stearothermophilus spores. Comparison of normal Raman and SERS spectra reveals not only an enhancement of the normal Raman spectral features but also the appearance of spectral features absent in the normal Raman spectrum.

Index Headings: Optical tweezer; Surface-enhanced Raman scattering; Bacterial spore.

INTRODUCTION

Since the seminal work of Ashkin in 1970, based on a dual laser beam system,1 and later work in 1986 employing a single laser beam apparatus,2 the laser tweezer phenomenon has been broadly accepted as a powerful tool to study viruses,3 vegetative bacterial cells,4–8 mammalian cells,9 and colloidal crystallization in microgravity environments.10 More recently, this technique in combination with various Raman detection schemes has been applied to the investigation of inorganic gas bubbles,11 aerosols,12 emulsion particle polymerization,13 liquid–liquid extraction of toluene in water,14 organic nanoparticles,15 yeast cells,8 and solid-phase peptide synthesis.16 Although significant work has been devoted to using this unique tool to study vegetative bacterial cells, to our knowledge there have been no published reports that employ optical trapping to study bacterial spores. This is surprising in light of the fact that this technique is ideally suited for such an application and has been used extensively in the study of micrometer-scale dielectric particles.

Raman spectroscopy has been an invaluable technique in the study of various chemical systems and has become widely accepted as an analytical characterization methodology.17,18 The attractiveness of this technique stems from (1) its narrow spectral band structure, (2) its lack of interference from water, and (3) its relative insensitivity to the excitation wavelength employed. However, unenhanced Raman spectral features are considered to be relatively weak, thus requiring relatively lengthy collection times. In many applications, high quality spectra may be achieved with shortened acquisition times and improved spectral features by exploiting various amplification techniques, namely, resonance Raman scattering (RRS), surface-enhanced resonance Raman scattering (SERRS), or surface-enhanced Raman scattering (SERS).17–19 RRS and SERS are typically conducted using ultraviolet excitation to facilitate electronic excitation (i.e., strong absorption) in the analyte of interest. This precludes their implementation in a single-beam optical trapping configuration since the trapped particle experiences MW/cm² intensity levels. Further, UV intensities of this magnitude coupled with significant UV-light absorption would photo-decompose most analytes. In contrast, SERS is well suited for combination with the optical trapping phenomenon since this technique may be performed using near-infrared excitation. Moreover, this is attractive since most analytes do not absorb strongly in the near-infrared (NIR).

Currently, the mechanism of the SERS effect is not fully understood; however, a plausible explanation for at least a significant portion of the spectral amplification has been attributed to an increase in the electromagnetic field strength encountered by the analyte. Briefly, this intensified electromagnetic field is generated when a metal surface (typically, Au, Ag, Pt, Pd, or Cu) is irradiated with the requisite wavelength of light and metal conduction band electrons are excited to collective oscillation and produce a surface plasmon resonance. Additionally, with certain metal–analyte systems, enhancements from 4 to 14 orders magnitude are readily achieved.17 Since early reports on this technique in the late 1970s, its acceptance has not been as widespread as expected. This limited acceptance is in large part due to the lack of fabrication reproducibility in most SERS substrates. Relatively recent advances in SERS-active substrate fabrication have addressed this reproducibility issue. Specifically, these substrates are easily wavelength adjustable, durable, and biocompatible and possess a long shelf-life.19

Over the past three decades Raman spectroscopy has been employed to study various Bacillus spore species. Due to the relatively meager Raman scattering cross-sec-

Received 3 April 2003; accepted 17 June 2003.
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tions exhibited by many biological materials, a majority of this reported work has been conducted using various Raman amplification techniques. Specifically, early work in this area was based on the resonance Raman scattering (RRS) technique utilizing ultraviolet (i.e., 222 nm) to green (i.e., 514.5 nm) laser excitation sources. More recently, reports have been published describing the implementation of femtosecond adaptive spectroscopic techniques applied to coherent anti-Stokes Raman spectroscopy (FAST-CARS), as well as SERS microscopy. To date, these various Raman spectroscopies have been applied to the study of bulk bacterial spore samples. In contrast, the methodology described in this report can be used for the spectral investigation of single bacterial spores. Further, this method facilitates an estimation of biodiversity in a given spore population.

We report here, to our knowledge, the first simultaneous exploitation of the optical trapping and surface-enhanced Raman scattering phenomena to detect a single bacterial spore. Further, a method based on their tandem application has been used to detect and discriminate between two different *Bacillus stearothermophilus* variants, namely, *Bacillus stearothermophilus* (ATCC 10149) and *Bacillus stearothermophilus* (ATCC 7953). In addition, we describe a near-infrared Raman tweezer detection system (NIR-RTDS) that may be used to detect single optically trapped biologically important particles (i.e., bacterial spores), as well as solutions occupying the trapping volume. Preliminary Raman and SERS spectra measured using this method have been examined for uniqueness and the applicability of these spectra for the discrimination of *Bacillus stearothermophilus* spores at the strain level.

**EXPERIMENTAL**

**Instrumentation.** As shown in Fig. 1, the near-infrared Raman tweezer detection system (NIR-RTDS) used in this study is similar in concept to those described previously. As illustrated, 787-nm light from a commercially available temperature-controlled laser diode (LD, Thor Labs) capable of 50-mW output power was collimated through a Faraday optical isolator (OI, Electro-Optics Technology, Inc., Model # LD381780) and a circularizing anamorphic prism pair (PP, Thor Labs) before passing through an interference filter (IF, Omega Optical, p/n 785DF10) with better than 70% transmission at 785 nm. The circular beam was then reflected by a holographic SuperNotch-Plus® filter (SNF, Kaiser Optical Systems, Inc., p/n HSPF-785.0AR–1.0) centered at 785.0 nm into a Nikon TMS inverted microscope. A 50-mm focal-length lens (FL, Thor Labs, p/n LA1131-B) was placed before the microscope to expand the laser beam efficiently fill the back aperture of the focusing objective. This expanded beam was then focused by a 100× oil immersion microscope objective (100× OBJ, Nikon TE 300, N.A. 1.25) into the sample with a spot size of 1–2 μm at the focus (with an intensity of ~0.5 MW/cm² at the beam focus). A dichroic mirror (DM) was placed inside of the microscope housing before the sample to deflect the laser light into the microscope objective. The position of the sample at the microscope stage was con-

![Fig. 1. Schematic diagram of the near-infrared Raman tweezer detection system (NIR-RTDS). See text for description.](image-url)
trolled in three dimensions using piezoelectric-driven actuators (Model 8302, New Focus, Inc.) having a minimum linear motion of less than 30 nm.

Both Rayleigh and inelastic scattered light from a single trapped particle was collected by the same 100× oil immersion objective and collimated onto and directed by the dichroic mirror through two SNFs with optical densities better than 6.0 (to remove most of the Rayleigh scattered light) onto the entrance slit of a 0.275-m polychromator (SpectraPro®-275, Acton Research Corporation) and liquid-nitrogen-cooled, back-illuminated charge-coupled device (CCD) (Spec-10:100BR, Roper Scientific) for spectral analysis. Light coupled into the polychromator was laterally dispersed across the 1340×100 pixel CCD chip by a 1200 grooves/mm grating blazed at 750 nm. The polychromator was controlled through a digital-to-analog converter (D/A) and data was collected from the charge-coupled device (CCD) by a personal computer though a 16-bit interface card using the WinSpec/32® software package (Version 2.5.8.1, Roper Scientific). To increase the signal-to-noise (S/N) ratio, the output of the CCD was vertically binned prior to readout. The area immediately surrounding the optical trap in the sample was imaged through the microscope objective and imaging optics (Edmund Industrial Optics) by an NIR-sensitive video camera (NEC TI-24A). A reflective neutral density filter (ND) was placed before the camera to further reduce the amount of laser light reaching the camera. Images from this video camera were displayed on a video monitor for observation and digitized for later inspection using an 8-bit analog-to-digital (A/D) framegrabber (PixelSmart, Lewiston, NY).

All spectra were collected with a laser excitation power of 20 mW (before the microscope), 500-ms integration, and 100 coadditions at a spectral resolution of 7.5 cm⁻¹ unless otherwise stated.

**Bacterial Spores.** *Bacillus stearothermophilus* (ATCC 10149 and ATCC 7953) were purchased from Raven Biological Laboratories, Inc. (Omaha, NE) as 40% ethanol-deionized water suspensions. According to the manufacturer, these spores were prepared by incubation in soybean-casein digest broth at 55–60 °C for 7 days. Samples for measurement were prepared by diluting 100 μL of the spore suspension in 40 mL of 18 MΩ deionized water to give a final concentration of 7.5×10⁴ CFU/mL (CFU = colony forming units).

**Surface-Enhanced-Raman-Scattering-Substrate Fabrication.** The SERS-active substrates were prepared by a method similar to that suggested by Grabar, Freeman, Hommer, and Natan using 60-nm gold colloids synthesized in-house. Briefly, standard glass microscope slides were cleaned for 1 h in a bath consisting of 1:4 (v/v) 30% H₂O₂ to sulfuric acid at 60 °C. Subsequent to cleaning, the microscope slides were profusely washed in methanol and stored in fresh CH₃OH until needed. A self-assembled-monolayer (SAM) of 3-aminopropyltriethoxysilane (APTES, Aldrich Chemical Company) was formed on the cleaned glass substrates by submerging the glass slides in a solution of the silane diluted 1:4 (v/v) in methanol. After 24 h, the APTES derivatized glass was removed from the silane solution and washed repeatedly (~15×) with methanol to remove any unbound silane from the surface. The APTES derivatized glass was stored in fresh methanol until needed.

Gold colloids of 60-nm size, freshly prepared by a previously published method, were coupled to the silanized substrate by immersing the washed glass in the aqueous colloidal suspension for 24 h at room temperature. Subsequent to the colloidal coupling, the SERS substrates were washed with and stored in deionized water until needed for analysis. UV-visible absorbance measurements (not shown) of these substrates reveal a surface plasmon resonance (SPR) optimally excited with 715- to 775-nm light. SERS substrates fabricated through this method may be near-optimally excited using the NIR-RTDS.

**RESULTS AND DISCUSSION**

*Bacillus stearothermophilus* (ATCC 10149 and ATCC 7953), the two *Bacillus* spore strains selected for this study, were chosen for their taxonomic closeness to each other. In addition, the ubiquitous occurrence of bacterial spores makes their rapid determination of interest in various applications (namely, food preparation, medicine, and agriculture). Further, the genetic similarity between these two variants poses a challenge in their determination since they are expected to be similar in shape, size, and immunoassay reactivity. Additionally, a spectroscopic investigation of single spores is attractive since it: (1) is noninvasive; (2) is nondestructive; (3) potentially, facilitates the development of novel detection schemes toward potentially pathogenic biological agents; and (4) allows an estimation of the biodiversity among individual spores in a given population.

The performance of the near-infrared Raman tweezer detection system (NIR-RTDS) was estimated by collecting the Raman spectrum (not shown) of a single optically trapped 2.0-μm-diameter polystyrene microsphere suspended in deionized water. The background-corrected spectra were in good agreement with published data. Further, the SERS activity of a representative immobilized Au colloid substrate was ascertained by measuring the spectrum of neat pyridine (relative to deionized water) at an immobilized colloidal mass (~50 μm × 50 μm) and subsequently measuring the same neat pyridine solution in a vacant (optically transparent) region of the substrate (a few micrometers away from the colloidal mass). As illustrated in Fig. 2, the SERS spectrum shows the appearance of new bands at 626, 989, 1568, and 2082 cm⁻¹, which are not present in the un-enhanced pyridine Raman spectrum. The central band in the ring breathing region at 989 cm⁻¹ is especially interesting since it has been reported to be indicative of surface enhancement of pyridine. That is, the central band in this triplet is present in the pyridine SERS spectrum but not in the pyridine normal Raman spectrum. Therefore, the appearance of the 989 cm⁻¹ band is a good indicator of a substrate's SERS activity. Moreover, the appearance of the band in the pyridine SERS spectrum verifies that the fabricated substrates are in fact SERS active. Further, these data illustrate a 50-fold signal enhancement in the pyridine SERS spectrum over the pyridine normal Raman spectrum using the prepared substrates. Depicted in the inset of this figure is a scanning electron microscope (SEM)
image of a Au colloid mass immobilized on glass. The individual gold colloid particles composing the mass are apparent in the image.

**Surface-Enhanced Raman Scattering Spectra of Single Optically Trapped Bacterial Spores.** Figure 3A illustrates a schematic diagram of the method used to measure the SERS spectrum of a single bacterial spore. As shown, a three-dimensionally trapped spore is captured and vertically translated towards a gold colloid coated microscope slide. NIR light transmitted through and around the spore subsequently excites the downward-directed surface plasmon resonance (SPR) generated electric field of an adjacent immobilized colloidal mass. The SPR-generated electric field extends only a short distance (hundreds of nanometers) away from the substrate surface and only partially overlaps the trapped spore. In fact, the amplified electric field is expected to only influence constituents of the spore coat and not the entire spore. That is, this methodology can be used to selectively probe the spore coat. This is attractive since it facilitates the isolation and study of bacterial spore coat constituents through nonphysical means. In addition, the unique spore isolation capability inherent in optical trapping facilitates studying the same bacterial spore in different environments (i.e., normal Raman versus SERS conditions). This allows the investigation of Bacillus spores while eliminating spore-to-spore composition variability.

Depicted in Fig. 3B are the normal Raman and SERS spectra for the same optically trapped *Bacillus stearothermophilus* (ATCC 10149) spore. These spectra are dominated by bands at 1005, 1345, 1434, and 1553 cm$^{-1}$ and 1005, 1135, 1246, 1360, 1675, and 1860 cm$^{-1}$ in the normal Raman and SERS spectra, respectively. Further, the SERS spectrum is enhanced by approximately two orders of magnitude over the normal spectrum. This enhancement is admittedly smaller than those previously reported for various analyte–metal systems (typically, 4 to 14 orders). However, it should be kept in mind that these enhancements were achieved in analyte–substrate systems where the enhancement is due to a combination of (1) a substantially intensified electric field and (2) a charge transfer (i.e., chemical) mechanism. Further, in the current scheme (Fig. 3A), the latter enhancement mechanism is most probably inactive since the optically trapped spore is well separated (tens of nanometers) from the enhancing feature and does not facilitate charge transfer between the trapped particle and the metal surface. The complexity of these samples (i.e., bacterial spores) makes it difficult if not impossible to make band assignments. The only exception is the dipicolinic acid (DPA) band in the “ring-breathing” region. Specifically, the common band in these spectra at 1005 cm$^{-1}$ is most likely due to dipicolinic acid (DPA), a major constituent of bacterial spores. A comparison of the SERS and normal spectra illustrates that the far-Stokes-shifted bands in the SERS spectrum are enhanced more than the DPA ring-breathing band at 1005 cm$^{-1}$. This can be understood in light of the fact that the moieties responsible for these spectral features most probably experience a stronger electric field than DPA. Since the DPA band and the far-Stokes-shifted bands in the normal (i.e., whole spore) spectrum are comparable in intensity (i.e., Raman cross-section), this implies that the far-Stokes-shifted constituents are held closer to the spore surface than DPA.

Plotted in Fig. 4A are representative SERS spectra for two different optically trapped *Bacillus stearothermophilus* (ATCC 10149) spores. Only two spectra are shown for clarity. Both spectra are dominated by bands at 1005, 1360, 1675, and 2092 cm$^{-1}$, as well as a pronounced shoulder at 1860 cm$^{-1}$. In general, the shape of these spectra are similar; however, distinct bands at 1140, 1195, 1245, and 1537 cm$^{-1}$ are present in the spectrum labeled B but absent in spectrum A. Additionally, the band intensity in spectrum B at 1360 cm$^{-1}$ is much smaller than this band’s intensity in spectrum A. These intensity differences, as well as the appearance of pronounced bands in spectrum B in the spectral region from 1060 to 1250...
Fig. 3. (A) Schematic diagram of the method used to measure SERS spectrum of a single optically trapped bacterial spore. (B) Normal Raman and surface-enhanced Raman scattering (SERS) spectra of a single optically trapped Bacillus stearothermophilus (ATCC 10149) spore. Only two spectra are shown for clarity.

Fig. 4. (A) Surface-enhanced Raman scattering (SERS) spectra of two different single optically trapped Bacillus stearothermophilus (ATCC 10149) spores. Only two spectra are shown for clarity. (B) Surface-enhanced Raman scattering (SERS) spectra of two different single optically trapped Bacillus stearothermophilus (ATCC 7953) spores.

1344 cm\(^{-1}\), infer that the distribution and/or relative concentration of Raman-active analytes are different in spore A and spore B. That is, the analytes responsible for the bands between 1060 and 1250 cm\(^{-1}\) in spectrum B are held closer to the spore surface and penetrate deeper within the SPR-generated electric field in spore B than in spore A. Further, the pronounced appearance of these bands in spectrum B illustrates the biodiversity present in this spore population.

Shown in Fig. 4B are SERS spectra representing two different Bacillus stearothermophilus (ATCC 7953) spores. Only two spectra are shown for clarity. These spectra are made up of pronounced bands at 585, 995, 1348, and 1668 cm\(^{-1}\). Although the plotted spectra are similar in shape over the measured spectral range (300 to 2200 cm\(^{-1}\)), large intensity variations are obvious between spectra A and B, especially in the short-Stokes-shifted (from 300 to about 1300 cm\(^{-1}\)) spectral features. Additionally, a strong band at 1575 cm\(^{-1}\) is present in spectrum A but absent in spectrum B. Further, a band in the “ring-breathing” region at 995 cm\(^{-1}\), which is attributed to dipicolinic acid, is blue shifted 10 cm\(^{-1}\) from the spectra in Fig. 4A. This shift infers that dipicolinic acid occurs in a slightly different chemical environment in the two different Bacillus stearothermophilus strains (namely, ATCC 10149 and ATCC 7953) studied. Moreover, the pronounced band at 585 cm\(^{-1}\) as well as the DPA band shift around 1000 cm\(^{-1}\) implies that the composition of Bacillus stearothermophilus (ATCC 7953) is significantly different than that of Bacillus stearothermophilus (ATCC 10149).

The SERS spectral differences between the Bacillus stearothermophilus spores studied are more apparent in Fig. 5. Shown in this figure are averaged SERS spectra for Bacillus stearothermophilus (ATCC 7953) and Bacillus stearothermophilus (ATCC 10149), intensity normal-
Fig. 5. Intensity normalized surface-enhanced Raman scattering (SERS) spectra of single optically trapped *Bacillus stearothermophilus* (ATCC 10149) and *Bacillus stearothermophilus* (ATCC 7953) spores. The spectra are displayed in an arbitrary unit vs. Raman Shift (cm⁻¹). The bands at 7953, 565, 1348, and 1670 cm⁻¹ are prominent features in both spectra. However, the ATCC 7953 spectrum shows additional bands at 10149, 1000, and 2100 cm⁻¹ that are not present in the ATCC 10149 spectrum. These bands can be used to distinguish between the two bacterial strains.

**CONCLUSION**

In summary, a near-infrared Raman tweezer detection system (NIR RTDS) has been constructed and applied to the development of a novel methodology for the detection of single optically trapped *Bacillus* spores. This method is based on the simultaneous exploitation of the optical trapping and surface-enhanced Raman scattering (SERS) phenomena. Two different *Bacillus stearothermophilus* spore strains have been studied using this methodology, namely, *Bacillus stearothermophilus* (ATCC 7953) and *Bacillus stearothermophilus* (ATCC 10149). Results show that this method facilitates detection of the SERS spectrum from a single optically trapped bacterial spore. Further, spectral features measured using this method demonstrate that it is possible to discriminate between the two *Bacillus stearothermophilus* strains studied.

Over the past thirty years, various Raman spectroscopic techniques have been employed to study bulk bacterial spore samples. To our knowledge, this is the first demonstration of the optical trapping and SERS phenomena used in tandem to study single bacterial spores. The results reported here show that Raman spectroscopy, under the proper conditions, can be made highly sensitive for the investigation of individual bacterial spores through nonphysical means.