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Surface-Enhanced Raman Spectroscopy for Bacterial Discrimination Utilizing a Scanning Electron Microscope with a Raman Spectroscopy Interface

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Surface-enhanced Raman scattering (SERS) utilizing colloidal silver has already been shown to provide a rapid means of generating "whole-organism fingerprints" for use in bacterial identification and discrimination. However, one of the main drawbacks of the technique for the analysis of microbiological samples with optical Raman microspectroscopy has been the inability to acquire preemptively a region of the sample matrix where both the SERS substrate and biomass are both present. In this study, we introduce a Raman interface for scanning electron microscopy (SEM) and demonstrate the application of this technology to the reproducible and targeted collection of bacterial SERS spectra. In secondary electron mode, the SEM images clearly reveal regions of the sample matrix where the sodium borohydride-reduced silver colloidal particles are present, Stokes spectra collected from these regions are rich in vibrational bands, whereas spectra taken from other areas of the sample elicit a strong fluorescence response. Replicate SERS spectra were collected from two bacterial strains and show excellent reproducibility both by visual inspection and as demonstrated by principal components analysis on the whole SERS spectra.

The development of physicochemical spectroscopic, or whole organism "fingerprinting"[1,2] methods within the field of bacterial systematics, has in part been driven by the need to find more cost-effective alternatives to conventional biochemical tests. Such methods can be exploited to generate large data sets rapidly, which are used in the formulation of mathematical (or chemometric) models for the discrimination and characterization of microorganisms. If they are to provide a viable alternative to traditional microbiological-based techniques, they need to obtain robust reproducible results with minimal sample preparation. Among the novel techniques that have proven popular for such studies are Fourier transform infrared spectroscopy,[3] and more recently mass spectrometry[4,5] and Raman spectroscopy.[6−8] In particular, Raman spectroscopy shows great promise since it is a reproducible and versatile technique that has already been used in many bacterial studies to obtain highly information-rich spectra. The unfortunate drawback of the method is the weakness of the inelastic scattering process with typically only ∼1 in 10^8 incident photons Raman scattered; thus the acquisition of spectra for biological samples can take many minutes.

Fortunately, several methods exist that provide a means of enhancing the magnitude of the Raman signal. Among those which have proved most popular for the study of biological systems are ultraviolet resonance Raman (UVRR) spectroscopy and surface-enhanced Raman scattering (SERS). In UVRR, the inelastic light scattering process can be enhanced when the laser is within the molecular absorption bands of the sample. Excitation of this type is in resonance with the electronic transition and yields Raman scattering that is resonance enhanced.[9] Deep UV resonance Raman (UVRR) has successfully been used to analyze bacteria[10,11] and more recently to identify and discriminate between bacterial isolates to the species level.[12] However, a drawback to this technique lies in the difficulties relating to sample presentation, since the high light energies involved can cause sample degradation. Thus, UVRR experiments have required the use of devices such as flow cells and spinning sample holders to avoid damage to samples. In addition, because only certain chromophoric segments of macromolecules absorb UV light, UVRR is a chemically selective technique.

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SERS[13] achieves enhancements of some 10^3-10^6-fold compared with the normal Raman scattering process and also exhibits a fluorescence quenching effect.[14] The latter is extremely advantageous when examining microorganisms that often exhibit a high fluorescence background under near-infrared to visible excitation. In contrast to UVRR, since excitation for SERS experiments is in this region of the electromagnetic spectrum there are no drawbacks associated with photochemical degradation of the sample.

The SERS process relies principally on either the adsorption or proximity of an analyte to a metal substrate.[15,16] Such substrates include roughened metal surfaces, colloidal solutions, or roughened electrodes. For the present study, a sodium borohydride-reduced silver colloid was used. The major SERS enhancement process is thought to be driven by an electromagnetic effect; this takes place on the nanometer scale and can be attributed to surface plasmon oscillations. These provide a higher "local" optical field, due to redistribution and concentration of electromagnetic energy. Coupled with the incoming electric field from the incident radiation, this generates a larger spectroscopic signal for an analyte "caught" in both fields. For colloidal SERS, the resonance frequency of the plasmon oscillations is dependent on the size and shape of the particles[17] as well as the wavelength of the incident radiation.

SERS has received much attention recently within biomedicine[14] and genomics[18] and is emerging as a very powerful (bio)-chemical detection method.[19,20] The technique has also been exploited for the purposes of discrimination and identification of urinary tract infection bacteria using a silver colloid and 785-nm excitation.[21] In this latter study, reproducibility of spectra and consequently the overall spectral collection times would have been improved considerably had it been possible to locate regions of the sample where colloidal silver and bacterial biomass were both present.

However, it is now possible to address this problem by using a scanning electron microscope (SEM) in conjunction with suitably interfaced Raman optics. With the powerful magnification afforded by SEM, it is possible to target "SERS-active" regions of the sample matrix (areas where the biomass and SERS substrate are both present) in order to ensure collection of SERS spectra. In this study, we demonstrate for the first time the reproducible collection of information-rich bacterial SERS spectra using a Renishaw structural and chemical analyzer (SCA), which is a SEM interface for Raman spectroscopy.

EXPERIMENTAL SECTION

Bacterial Strains and Growth Conditions. Two bacterial strains were selected for the study, *Bacillus subtilis* (B0014^T) and *Escherichia coli* (UB5201). Both were cultivated axenically and aerobically for 18 h at 37 °C on LabM blood agar base (IDG Plc, Lancashire, U.K.). After subculturing three times, the biomass was carefully collected from single colonies using sterile plastic inoculating loops. These samples were then prepared for SERS as described below, before being spotted on to aluminum SEM specimen stubs and dried at 50 °C.

Silver Colloid Preparation. The sodium borohydride-reduced silver colloid utilized in this study was prepared as described in Zeiri et al.,[22] an adaptation of the Lee and Meisel[23] method. Briefly, the bacterial biomass was centrifuged and washed twice in distilled water before being resuspended in 0.05 M sodium borohydride (Sigma, Dorset, U.K.) solution and allowed to soak. This mixture was then spun and washed again in distilled water, after which 5×10^{-4} M silver nitrate (Sigma) solution was introduced. At this stage, a reduction of the silver nitrate takes place to form small colloidal silver particles of ~10 nm in diameter, which in some cases become aggregated to form larger colloidal clusters or otherwise adsorb on to the bacterial cell surface.

Scanning Electron Microscopy. A JEOL JSM-5610LV SEM was used to observe the samples. SEMs focus an energetic (1−25 keV) electron beam onto the sample, this focused spot is then scanned, and typically, secondary electrons are detected for each point scanned on the sample from which a display is scanned in synchrony with the beam to build up an image. Changing the scanned area on the sample causes a change in the magnification.

SEM has a number of advantages over optical microscopy, principally greater spatial resolution and depth of field, and normally better discrimination due to a range of contrast mechanisms. Renishaw's SCA (Figure 1) enables laser light to be focused onto a micrometer order sized spot on a sample in the SEM, which is governed by the diameter of the laser beam. Raman spectra can then be collected from this spot, while maintaining the ability to view the secondary electron image (SEI). The imaging power of the SEM can be used to identify regions of interest on samples; these can then be analyzed using Raman spectroscopy. This approach therefore has benefits when applied to colloidal SERS of bacteria, whereby the small analyte and substrate structures can be clearly observed and targeted prior to capture of spectra via the Raman interface.

The SCA briefly comprises elements 3−5 shown in Figure 1. This allows for the incident laser beam to be directed into the SEM chamber at an angle orthogonal to that of the electron beam; the path is redirected off a right-angled mirror to interact with the sample (element 5). Raman scattered photons are then collected via the same beam path geometry.

For these studies, an accelerating voltage of 15 keV was used so that the contrast between the colloidal silver-labeled regions of the sample matrix and the unlabeled regions was clearly obvious. It was recognized that these conditions are not optimal for observation of individual bacteria but were biased toward identifying areas that were likely to exhibit the SERS effect.

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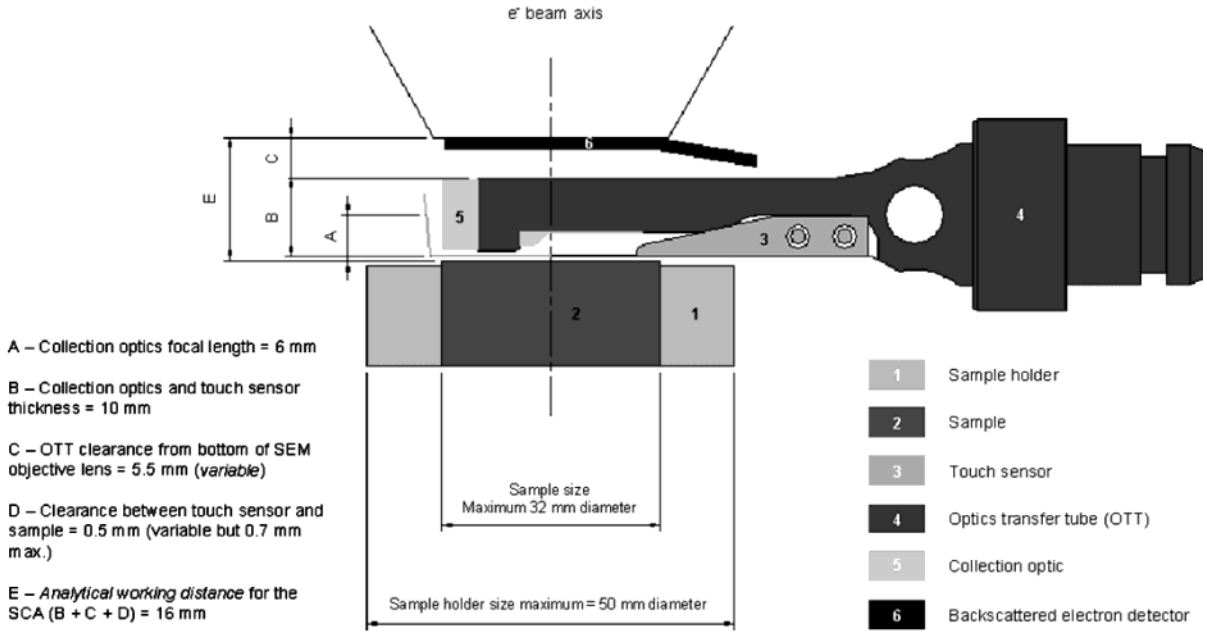

Figure 1. Diagram showing the design of the SCA interface for SEM-Raman spectroscopy.

Raman Microscopy. SERS spectra were collected using a Renishaw RA-200 compact Raman spectrometer (Renishaw plc., Gloucestershire, U.K.) with a 60-mW, 532-nm laser resulting in power at the sampling point of ~8 mW. The instrument was wavelength calibrated with a silicon wafer focused under the SCA interface and collected as a static spectrum centered at 521 cm^{-1} for 1 s.

Each sample was dried on to a SEM specimen stub, focused under the collection assembly, and a spectrum collected for three accumulations of 10 s. For the purpose of assessing the reproducibility of this approach, nine replicate spectra were obtained from each of the bacterial samples from different points on the sample matrix.

The GRAMS WiRE software package (Galactic Industries Corp., Salem, NH) running under Windows 95 was used for instrument control and data capture. Stokes Raman spectra were collected over the wavenumber range of 26−3884 cm^{-1}. The resolution was ~10 cm^{-1}, and the bin size used was ~3.6 cm^{-1}. Each sample was represented by a spectrum containing 1066 points, and spectra were displayed in terms of the Raman scattered photon count. A subset of the wavenumber range from 220 to 1770 cm^{-1} (421 data points) was then extracted for further analysis since this represents the portion of the bacterial spectra expressing the SERS signal.

ASCII data were exported from the GRAMS WiRE software into Matlab version 6.5 (The Math Works, Inc., Natick, MA). Although the spectral reproducibility was extremely good, to correct for any quantitative error due sample size differences, the following procedure was performed: (i) a linear mean filter was applied column wise, across a sliding bin (data point) width of 15; (ii) the spectra were then normalized so that the highest photon count was set to +1 for each spectrum.

In order to assess the reproducibility of the analysis, principal components analysis (PCA) was performed on the processed spectra and an ordination plot of the first two principal component scores was produced.[24]

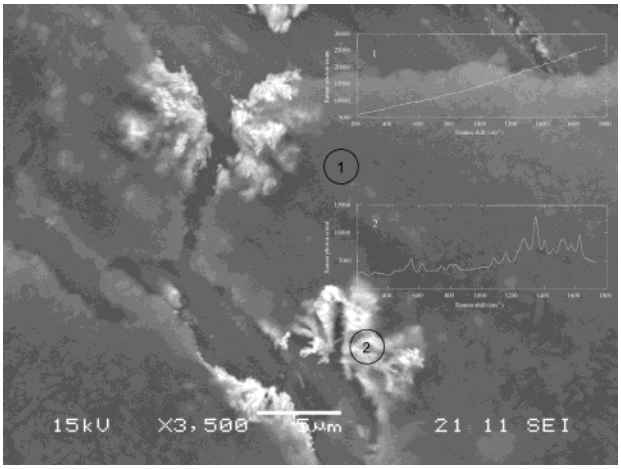

Figure 2. SEM image in SEI mode of a thin film of *E. coli* (typically one cell thick); prepared for SERS using a $NaBH_4$-reduced silver colloid and spotted onto an aluminum sample stub. In SEI mode, any accumulations of colloidal silver are clearly visible as light areas on the image. By focusing the laser at point 1 where there is a clear absence of silver, a strong fluorescence spectrum is observed. However, when focusing on a lighter area (i.e., point 2), strong SERS spectra are obtained.

RESULTS AND DISCUSSION

Figure 2 shows a secondary electron image of a thin film of *E. coli*, typically of a single layer of cells. The bacterial sample has been prepared for colloidal SERS as detailed above, with the presence of aggregated silver particles easily identified as the lighter areas on the image—the silver particles have higher secondary electron and backscattered electron yields than the surrounding organic matter, making them appear brighter in the SEM images. When a spectrum is collected from a region of the sample where colloidal silver is absent, such as at point 1, a strong

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Table 1. Tentative Wavenumber Assignments for the Bacterial SERS Spectra

wavenumber (cm^{-1})	strength[a]	vibration[b]	compound[b]
1627	m	[1,3]ν(CC)	olefinic
1572	m	[1,3]δ(NH) & ν(CN)	amide II
1521	m	[3]ν(CC) ?	pigment
1459	m	[3]$\delta(CH_2)$ scissoring	
1401	m	[2]$\nu(CO_2^-)$ stretch	α-amino acids
1344	s	$\nu(NH_2)$ stretch ?	[4]adenine and guanine
1268	m	[1,3]$\delta(CH_2)$?	amide III
1150	m	[1,2]ν(CC) stretch	*n*-alkanes
1078	m	[3]ν(CC)	skeletal ?
828	w	[1]δ(CCH)	aliphatic
798	mw	[1,2]ν(CC)	ring breathing
739	mw	[1]$\rho(CH_2)$	
615	m	[2]δ(CCC) ring deformation ?	monosubstituted benzenes
543	m	[1]δ(COC)	glycosidic ring
318	w	[1]δ(CCC) ring deformation	

[a] s = strong; m = medium; w = weak; δ = deformation; ρ = rocking; ν = stretching. [b] Assignments are taken from [1]Williams and Edwards,[27] [2]Grasselli et al.,[30] [3]Edwards et al.,[28] or [4]Britton et al.[29]

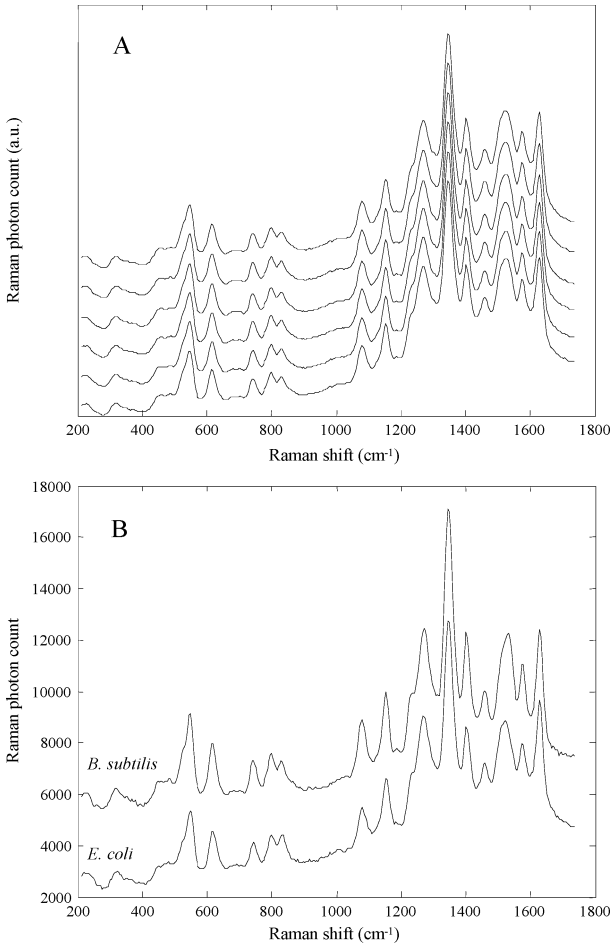

Figure 3. (A) Processed replicate spectra of five *E. coli* samples, demonstrating the high degree of spectral reproducibility that can be obtained by SEM-SERS. (B) Typical SERS spectra of *B. subtilis* and *E. coli* at 532-nm excitation.

fluorescence spectrum is observed. This is typical of our attempts to obtain normal Raman spectra of biological samples using Raman microspectroscopy with 532-nm excitation. By contrast, acquiring spectra from regions of the sample where silver is clearly present (i.e., point 2) results in a SERS response that produces information-rich spectra representative of the bacterial sample. The benefit of using the SEM interface to capture SERS spectra is that it is immediately obvious where to aim the laser in order to be guaranteed a SERS response. Considering that a single layer of bacteria were analyzed and that the diameter of the laser beam spot is $\sim$1 μm, it is likely that we are typically collecting data from four bacterial cells.

Given the high magnification factor afforded by SEM, it would be preferable to acquire SERS spectra from individual bacteria. Unfortunately, two challenges exist that make this problematic; first there is a significant charging effect when analyzing bacterial samples. This can be minimized or even eliminated by sputter coating the sample, but there is a possibility of SERS enhancement from these nanoparticles that would introduce an additional element of error in to the experiment and thus why we used SEI. Second, low concentrations of biomass would need to be used to ensure that isolated organisms were present. Since the colloid preparation used for this study did not result in a distribution of colloid over all of the bacterial biomass, it would be time-consuming to locate individuals on the sample matrix that are also in the presence of the colloidal substrate. Given that a limited amount of time on the instrument was available for this study, we chose to use greater concentrations of biomass that resulted in the formation on the SEM stub of bacterial films of a single cell layer rather than of isolated cells. This offered far greater certainty that SERS could be observed from the samples; however, future studies will certainly focus on the attainment of SERS from single bacterial cells.

The problem of spectral reproducibility is widely acknowledged within the SERS community.[25] For colloidal SERS, the variables that can impact upon this include the size and shape of colloidal particles and the geometry of the SERS substrate in relation to the analyte. To demonstrate the high degree of reproducibility obtainable by the technique, five replicate spectra of *E. coli* were collected from five different areas of the sample identified as likely to be SERS active. Typical SERS spectra are shown in Figure 3A, where it is clear that there are great similarities between replicates.

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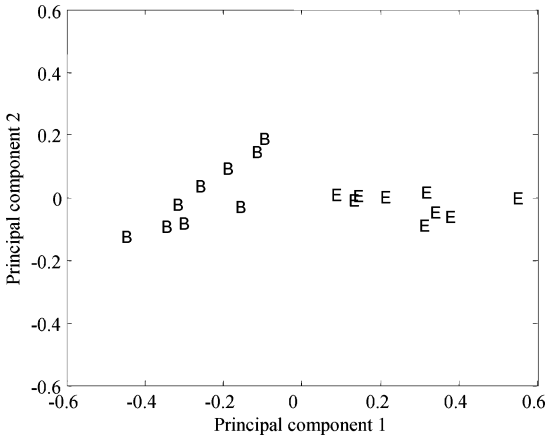

Figure 4. A principal component analysis ordination plot (PC 1 vs PC 2) showing two clearly resolved clusters for *E. coli* (E) and *B. subtilis* (B). This further demonstrates the reproducibility of the SERS technique and shows that there is some potential for bacterial discrimination.

Figure 3B compares raw SERS spectra of *B. subtilis* and *E. coli*. In a previous SERS study of bacteria at 514.5-nm excitation, Zeiri et al.[22] attributed the spectral characteristics to the cell wall component flavin adenine dinucleotide. The SERS spectra presented in this study were acquired at 532-nm excitation, although very similar in profile to those reported by Zeiri and colleagues,[22] and those collected in an earlier study at 514.5 nm by Efrima and Bronk,[26−30] the Raman shifted wavenumber assignments are quite different. Wavenumber allocations for the major Raman bands exhibited in the SERS spectra are listed in Table 1, where vibrations can be assigned to amino acids, nucleic acids, and sugars as well as lipids. Regardless of the provenance of the observed peaks, it is immediately obvious that there is little to distinguish between the *B. subtilis* and *E. coli* SERS spectra; although on closer inspection there are very small peaks present in *B. subtilis* at ∼700 and 1050 cm^{-1} (both unassigned) that are absent in *E. coli*, and the peak shapes of the amide III band at 1344 cm^{-1} and pigmentation band at 1521 cm^{-1} are clearly quantitatively different.

To investigate the reproducibility of this approach further at the full spectrum level, nine SERS spectra from *E. coli* and nine from *B. subtilis* were collected. This number of replicates was chosen arbitrarily and simply reflects the total number of spectra that were obtained from each preparation. PCA was used directly on the subset of 421 data points from all 18 bacterial samples, and the resultant ordination plot is shown in Figure 4. It is obvious from this figure that two separate clusters for *B. subtilis* (coded B) and *E. coli* (coded E) are clearly resolved, which demonstrates that the reproducibility of the spectral collection was good and that there is potential for the application of this technique to classification problems. An inspection of the PC 1 loadings plots (data not shown) indicated quantitative differences in the 1400−1600-cm^{-1} region of the spectra, which is also clear from the raw data. Since only two organisms were analyzed, it will be necessary to conduct a more comprehensive discrimination exercise in order to determine the full extent of the ability of the SCA to provide a basis for bacterial discrimination and identification.

CONCLUSIONS

Analysis of bacteria by colloidal SERS using a SEM interface has clearly been demonstrated and allows for SERS-active regions of the sample matrix to be easily identified from which reproducible spectra are obtained. Utilizing this approach with 532-nm excitation and $NaBH_4$-reduced Ag colloid substrates resulted in information-rich spectra that show great potential for use in bacterial discrimination and characterization studies. Future work will focus on the discriminatory modeling of larger bacterial culture collections to prove the effectiveness of the technique for the rapid characterization of microorganisms. Finally, the technology and experimental design will be developed further for the analysis of bacteria at the single-cell level.

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