Black silicon as a platform for bacterial detection

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Surface-enhanced Raman scattering (SERS) shows promise for identifying single bacteria, but the short range nature of the effect makes it most sensitive to the cell membrane, which provides limited information for species-level identification. Here, we show that a substrate based on black silicon can be used to impale bacteria on nanoscale SERS-active spikes, thereby producing spectra that convey information about the internal composition of the bacterial capsule. This approach holds great potential for the development of microfluidic devices for the removal and identification of single bacteria in important clinical diagnostics and environmental monitoring applications. © 2015 AIP Publishing LLC.

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Plasma etching of silicon can be used to produce inexpensive, large surface area, nano-textured surfaces known as black silicon. Recently, it has been shown that black silicon nano-needles can impale bacteria¹ and that it can be used as a sensor in microfluidic devices.² When coated by a plasmonic metal, such as gold, the nano-textured surface of black silicon is ideal for use as a surface-enhanced Raman scattering (SERS) sensing platform.³ This work aims to investigate whether gold-coated black silicon nano-needles can be used to both impale bacteria and identify them by SERS. This combination of properties would promote the development of microfluidic devices for the removal and monitoring of bacteria in a wide range of medical, environmental, and industrial applications.⁴

Black silicon was fabricated by a reactive ion etching technique,⁵ resulting in pyramidal-shaped spikes of height 185 ± 30 nm, full width at half height of 54 ± 10 nm, and 10 ± 2.4 nm radius of curvature at the tip. Samples were then magnetron sputter coated with 200 nm of gold, as this coating thickness was found to provide a suitable compromise between SERS enhancement and impalement efficiency. *E. coli* (ATCC 25922) from -80°C stock was isolated on a nutrient agar plate (Difco nutrient broth, Becton Dickinson) for approximately 12 h. A single *E. coli* colony was then inoculated from the plate into 20 ml of nutrient broth media and incubated overnight at 37°C with orbital shaking at 200 rpm. The total biomass of overnight culture was adjusted to an optical density of 0.3 at $\lambda = 600$ nm by adding fresh sterile nutrient broth (Cary 50 spectrophotometer, Agilent). The *E. coli* planktonic cells were washed three times by centrifugation at 12 000 rpm (Centrifuge 5804 R, Eppendorf) for 2 min. The washed cells were then re-suspended in a low strength minimum medium (Dulbecco A, phosphate buffered saline). A volume of 100 μl of solution was pipetted onto substrates and left to incubate for 1 h on the bench. Separate sets of samples were created for scanning electron microscope (SEM) imaging, live/dead staining, and SERS. Three sets were needed as each of these measurements altered the samples and left them unsuitable for further analysis.

The first set of samples was washed three times with milliQ water after incubation, allowed to dry and then immediately sputter coated with gold using the Emitech K975x (operating

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current 35 mA, sputter time 32 s, stage rotation 138 rpm, and vacuum of 1×10^{-2} mbar). SEM imaging was performed with a Zeiss Supra 40VP in high vacuum mode with a working distance of 5 mm and an accelerating voltage of 3 kV. Figure 1 shows an example of the different levels of impalement that occurred on the black silicon surface. All cells showed signs of damage, but in some cases, the damage was limited to the perimeter of the cell and the main body appeared whole. In other cases, the entire cell had collapsed onto the spikes.

The second set of samples was used for live/dead staining (Invitrogen BacLight Bacterial Viability Kit L7012) with 3.34 mM SYTO 9 (green fluorescence) and 20 mM propidium iodide (red fluorescence). Equal volumes of both dyes were mixed thoroughly in a tube and added to the sample in a ratio of 3 μ l of mixed dye to 1 ml of bacterial suspension. After mixing, a volume of 100 μ l of the solution was pipetted onto the substrates, which were then incubated at room temperature in the dark for 15 min, before the staining solution was removed by pipetting. The substrates were then washed three times with milliQ water and mounted on a microscope slide for fluorescence imaging. The substrates were not allowed to dry and were stored in phosphate buffered saline at 4 °C when not in use. An epifluorescence microscope (Olympus IX71) with a mercury lamp source and a 60 $\times$ water immersion objective was used to collect live/dead images from the substrates. Two filter blocks were used to collect the images: U-MNIBA2 blue excitation narrow band delivered green emission (live) and U-MWIG2 green excitation wide band provided red emission (dead).

The live/dead image in Figure 2 shows a mix of both live and dead cells on the black silicon sample. The prevalence of live cells could be due to the incomplete impalement seen under SEM for some cells. It can also be explained by the sample still being wet during live/dead staining. The cells are dried prior to imaging in the SEM and this could weaken the cell wall and allow capillary forces to draw the cells onto the spikes for impalement. This hypothesis is supported by the large number of cells on the stained sample and the presence of cell groupings and cells imaged during mid-division. If the cells were immediately impaled, then such activity would not have been visible and a greater proportion of red cells would be expected.

The third set of samples was washed three times with milliQ water after incubation and allowed to dry prior to spectral analysis. SERS spectra were collected with a Renishaw inVia Raman spectrometer operating at 785 nm with a 1200 l/mm grating. Power at the sample was 150 mW focused with a 100 $\times$ /0.85 NA objective to obtain a diffraction limited laser spot. The resulting spot size ($\leq 2 \mu$ m in diameter) is well matched to the size of the bacterial cells. Spectra were collected with three accumulations of 10 s. Data were background subtracted⁶ and normalised to unity for ease of plotting. A great deal of variability was observed in the resulting spectra, as shown in Figure 3.

FIG. 1. A typical SEM image showing *E. coli* cells with different levels of impalement on gold-coated black silicon.

FIG. 2. Epifluorescence image showing live (green) and dead (red) *E. coli* cells after incubation on gold-coated black silicon.

It should be noted that *E. coli* SERS is known to produce a high level of variability,^{7–12} depending on the experimental setup.¹³ However, the variability seen in the SERS spectra of Fig. 3 is unusual for measurements performed under consistent experimental conditions. This increased level of variability may be related to the different levels of impalement seen in Fig. 1, which results in the probing of different internal components. SERS is a surface sensitive technique, with the signal primarily arising within 2 nm of the metal surface.¹⁴ Note that unlike apertureless nanoprobes¹⁵ or conical plasmonic nanotips,¹⁶ the SERS signal in black silicon arises primarily from “hot spots” between the spikes, where the plasmon resonance field is

FIG. 3. SERS spectra of *E. coli* after incubation on a gold-coated black silicon substrate. The spectrum numbers represent single cells at different locations and different levels of impalement.

particularly strong.¹⁷ Therefore, depending on the depth and location of impalement, different biomolecules are expected to be excited by this novel substrate.

Some peaks occur in the same position for multiple spectra (e.g., peak positions 420, 893, 1001, 1285, and 1307 cm⁻¹), but there are also a lot of unique peaks. The vertical lines in Fig. 3 indicate peaks which have appeared in the literature for SERS of *E. coli*.^{7–12} Spectrum 3 has a high proportion of peaks matching published values. This is also the case for spectrum 5, which shares a few peak positions with spectrum 3. Preliminary peak allocations have identified carbohydrates¹¹ (420 cm⁻¹), tyrosine¹¹ (650 cm⁻¹), adenine^{10,11} (706 and 735 cm⁻¹), hypoxanthine⁷ (722 and 1373 cm⁻¹), phenylalanine⁹ (1001 cm⁻¹), amide III (Ref. 10) (1285 cm⁻¹), CH₂ deformation¹² (1556 cm⁻¹), and C=C¹⁰ (1587 cm⁻¹).

Given the varying levels of impalement observed in the SEM, it appears that the spike shape and Au coating should be further optimized to ensure that the entire cell is consistently pierced and the internal biomolecules are more comprehensively probed. In this way, it may be possible to obtain a more reproducible SERS spectrum of the internal biomolecular constituents of single bacterial cells, thereby providing rapid identification for medical and environmental diagnostic applications. Given that SERS is insensitive to water,⁴ future work should aim to achieve impalement in an aqueous environment, so that the full capability of microfluidics can be used to separate and concentrate suspended bacteria before presenting them to the substrate for rapid analysis.⁴ This suggests a broad range of potential applications in the detection, monitoring, and control of bacterial contamination.

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