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Surface-Enhanced Raman Detection on Metalized Nanostructured Poly(*p*-xylylene) Films**

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Surface-enhanced Raman spectroscopy (SERS) as the basis of rapid and reliable biosensing techniques has the significant advantages of minimal sample preparation and ease of operation, when compared with other methods. The SERS technique has received much attention over the years owing to its ability to reach single-molecule detection and for applications such as rapid DNA sequencing. Its use for biomedical analysis, however, has lagged behind because of the difficulties in finding effective substrates for accommodating biological entities that also provide high sensitivity and highly reproducible SERS responses from sample to sample. If these challenges are met, SERS could potentially be extremely useful in the field of medical diagnostics to detect infectious agents. Here, we report the development of SERS substrates that provide a highly uniform and reproducible bioanalysis surface and demonstrate examples of “fingerprint” signals from different types of bacteria, in which the signals differ based on variations in the cell membranes. Our method is unique in controlling surface morphology and topology prior to SERS-active metal deposition. As a result, we created a novel substrate that has a uniform and reproducible SERS signal over a large ($>1\text{ mm}^2$) surface. The advantage of using these new types of substrates is that no template or lithography is involved, thus providing a simple, inexpensive, and quick method to achieve highly sensitive and spatially uniform SERS signals for biomedical applications.

The surface-enhanced Raman phenomenon was first observed by Fleischmann et al. in 1974^[1] on an electrochemically roughened silver surface using pyridine as an analyte. Since then the SERS technique has received much attention^[2,3] because of its capability for single-molecule detection^[4,5] and a

variety of applications including rapid DNA sequencing,^[6] pathogen detection,^[7,8] nanostructure characterization,^[9] and food analysis.^[10] One of the most promising broad-based applications for advanced, high-quality SERS substrates would be in the field of diagnostic microbiology,^[11–13] with critical applications such as the detection of bacterial and viral pathogens where minimal sample preparation effort, low reagent costs, ease of operation, and rapid and reliable detection would offer a great advantage compared with traditional culture or amplification-based techniques.^[14] Some demonstrations of SERS detection have been reported for micro-organisms by using gold-nanoparticle-coated SiO_2 ,^[15] bacteria coated with deposited silver coatings,^[16] co-deposited bacteria and silver on inert substrates,^[17] and bacteria treated with a reducing agent for the formation of metal colloids.^[16] SERS has remarkable analytical sensitivity but practical diagnostic SERS probes have not been developed owing to the difficulty in preparing robust and uniform SERS substrates of correct surface morphology (i.e., nanoscale roughness) that provide maximum SERS enhancement.

Given our interest in both surface spectroscopy and biomedical diagnostics, we investigated methods for developing advanced SERS substrates that would lead to significant improvements in selectively detecting specific strains of bacteria. In this work, we report the development of a SERS substrate with high uniformity (ca. 5% signal variation across a 1 mm^2 area relative to the background) and sample-to-sample reproducibility while providing a surface capable of sustaining the integral structure of biospecies. Figure 1 summarizes our approach for SERS substrate preparation and infectious agent detection. First, nanostructured poly(*p*-xylylene) (PPX) films are prepared by using an oblique angle polymerization method.^[18–21] The film consists of a parallel assembly of nanowire arrays having a diameter of 150 nm and a column size of 10–50 μm . Second, SERS-active metals (e.g., Au and Ag) are deposited onto the nanostructured polymer by using thermal evaporation, which results in SERS-active films with high sensitivity and reproducibility that can serve as biosensing surfaces. Finally, we demonstrate the use of our substrate with a robust and reagentless detection of both Gram-positive (*Bacillus cereus*) and Gram-negative (*Escherichia coli*) bacteria.

Our nanostructured substrate provides a significant advantage over traditional SERS surfaces because the surface roughness of nanostructured PPX film is controllable over two length scales in the ca. 10 nm and 100 μm regimes. Figure 2

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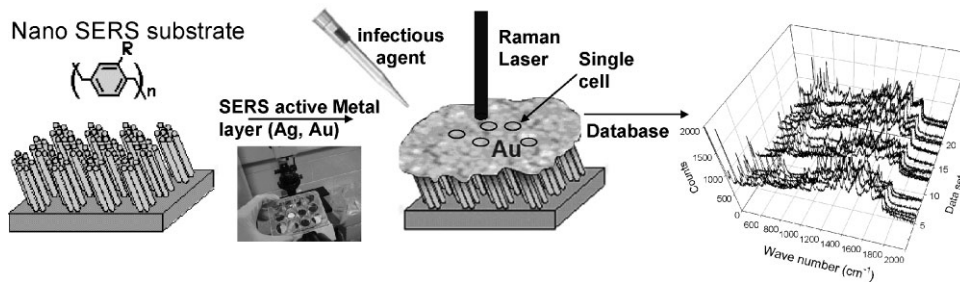


Figure 1. Description of SERS substrate preparation and pathogen detection: i) nanostructured poly(*p*-xylylene) (PPX) films are prepared by using an oblique-angle polymerization method, ii) SERS-active metals (e.g., Au and Ag) are deposited onto the nanostructured PPX by using thermal evaporation, and iii) a robust and reagentless detection of bacteria is demonstrated.

shows the root mean square (rms) surface roughness of the PPX film as a function of polymer film thickness. The increase in surface roughness as the film becomes thicker is inherent to oblique-angle polymerization. Additionally, this technique does not require any template or lithography for its preparation, thus making it simple and quick method to prepare uniform nanostructured SERS-active films. Proper selection of the deposition geometry and conditions and the PPX derivative permits simultaneous control of film morphology, topology, and surface chemistry, yielding nanostructured PPXs having well-organized porous structures. The details of the procedure for preparing the nanostructured PPX film have been explained previously.^[18–21]

To test the uniformity, enhancement factors (EFs), and reproducibility of the SERS signals, 4-fluorobenzenethiol (FBT) molecules are adsorbed onto the gold and silver substrates. First, the FBT C–F stretching mode signals are used for evaluating the effects of the metal layer thickness. Figure 3 shows the data for 60 and 90 nm silver films. Gold films show similar effects but with the expected ca. 100 times lower intensities (see Fig. S1 of Supporting Information). Second, the

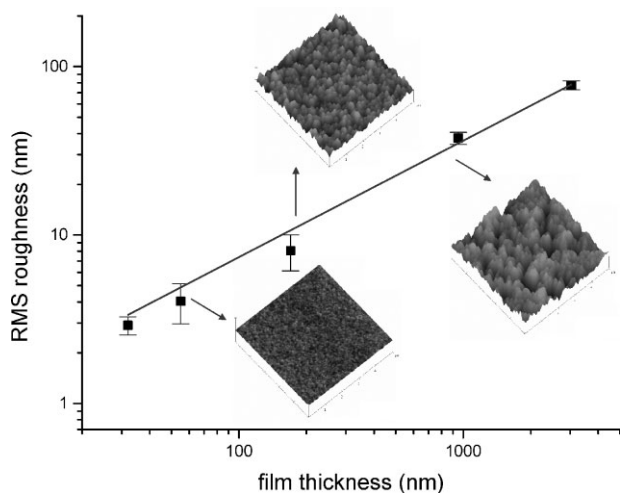


Figure 2. The rms surface roughness of the PPX film as a function of polymer film thickness. The scale bars for the atomic force microscopy scans are X: 1 $\mu\text{m div}^{-1}$, Y: 1 $\mu\text{m div}^{-1}$, and Z: 300 nm div^{-1} .

C–F stretch mode intensity is used for quantitative estimates of the EFs using the following equation:

$$EF(1077 \text{ cm}^{-1}) = \frac{N_{\text{bulk}} I_{\text{poly}}}{N_{\text{poly}} I_{\text{bulk}}} \quad (1)$$

where I_{bulk} and I_{poly} are the measured intensities of pure bulk FBT and the adsorbed molecules on the metal-coated polymer substrates, and N_{bulk} and N_{poly} are the volume number density of bulk and adsorbed molecules, respectively. The resultant EFs for gold and silver are 10^4 and 10^6 , respectively (see Supporting Information data). The uniformity of FBT SERS data on Ag and Au substrates as a function of substrate area show consistent uniformity over a large area: ca. 5% signal variation across a 1 mm^2 area relative to the background. We also note that both types of metalized SERS substrates remain stable after two months storage in ambient conditions without light. The enhancing effect of the Raman spectra are generally attributed to two mechanisms: electromagnetic and chemical.^[22] The former arises from the nanoscale roughness of the underlying PPX film, which promotes excitations of localized surface plasmon resonances with amplified local electromagnetic fields. Chemical enhancement arises from coupling of the

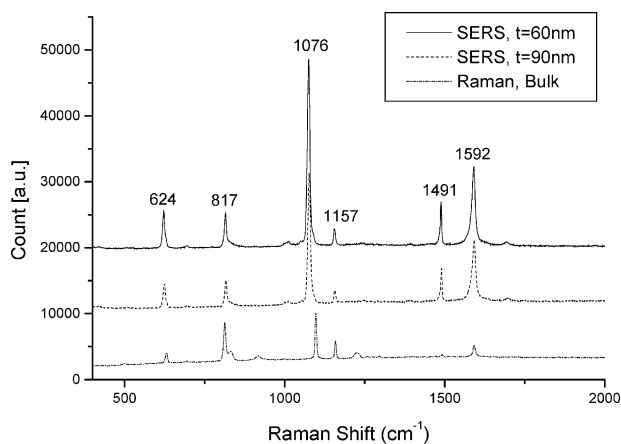


Figure 3. Raman spectra of FBT and SERS spectra of FBT for 60 and 90 nm Ag films.

electronic polarizability of the molecule and underlying metal conduction electrons.^[22]

The high spatial uniformity and reproducibility of the SERS signal, at millimeter length scales, as shown above, is ideal for bacterial detection. Thus, the SERS technique potentially provides a noninvasive and nondestructive method for bacterial analysis without amplification of cultures and with the capability of a reusable, inexpensive, and easily fabricated substrate. As a demonstration of this capability, we have selected Gram-positive and Gram-negative bacteria as test organisms for pathogen detection. Diluted bacterial samples are analyzed on the SERS substrate (ca. 10^4 cells in 1 mm^2 area). The results definitively show detection with clear differentiation between the two species. The differentiation is related to the different cell wall structures; both organisms possess a peptidoglycan layer but Gram-negative organisms have an additional structure of lipopolysaccharides (LPS).

Table 1. SERS peak positions of *E. coli* and *B. cereus* on the nanostructured silver substrate.

Assignment	<i>E. coli</i>	<i>B. cereus</i>
	SERS shift [cm^{-1}]	SERS shift [cm^{-1}]
Guanine, tyrosine (nucleic acid)	653	656
Adenine	724	720
C=C deformation	958	937
C–C stretching (phospholipids or carbohydrates)	–	1022
O–P–O– symmetric stretching (DNA)	1091	1096
Amide III (random)	1242	1236
Adenine, guanine (protein), CH deformation	1330	1332
COO–stretching	1372	–
CH ₂ deformation	1456	1477
Amide II	–	1559
Adenine, guanine (ring stretching)	1593	1594
Amide I	1710	1650

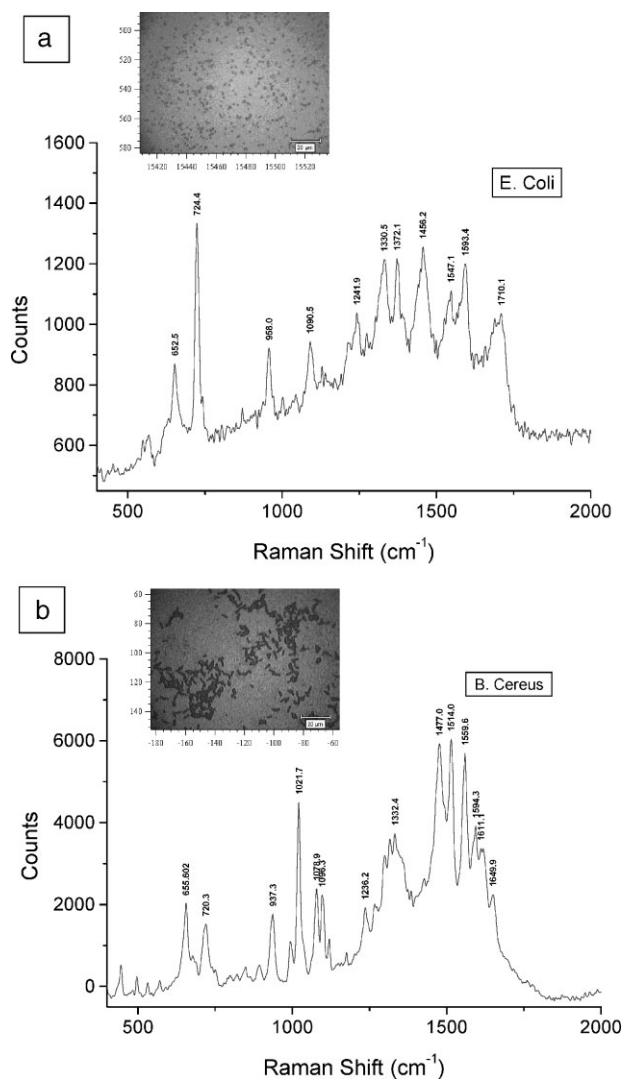


Figure 4. SERS spectra of a) *E. coli* and b) *B. cereus*. The insets show optical images of bacteria on nanostructured PPX film coated with a thin Ag layer.

Figure 4 shows the SERS signal of *E. coli* and *B. cereus* on the nanostructured silver substrate. Typical Raman bands of membrane proteins, phospholipids (C–C stretching^[23]), and polysaccharides^[17] can be observed on the spectra. The SERS spectra match well with the literature,^[15] and the peak positions are listed in Table 1. The peak assignments for *E. coli* and *B. cereus* are very similar, however, the intensities of the spectra vary drastically, which is the key for differentiating these two test organisms. SERS spectra on silver substrates are highly reproducible for the two bacteria. From spot to spot (i.e., individual cells), the intensity variation of the signal at 1372 cm^{-1} is calculated as 15% for the *E. coli* data (see Fig. S3 of the Supporting Information).

In conclusion, a noninvasive and nondestructive method for bacterial analysis without amplification of cultures is provided. The SERS substrate is reusable and inexpensive as well as easy to fabricate. In the case of gold, the substrate is highly cleanable for continued use in operator-independent situations. The results show clear differentiation between Gram-positive and Gram-negative species. The potential for SERS to produce spectra that can be used for rapid whole-organism fingerprinting is extremely important for outbreak prediction, reducing the mortality rate from misdiagnosis of infectious diseases in hospitals, and understanding the fundamental strain epistemology in infectious diseases.

Experimental

Materials: All chemicals were of ACS reagent grade and were used as-received. Deionized water of $18.1\text{ M}\Omega\text{ cm}$ was used for all experiments using Barnstead Nanopure Diamond dispenser. Poly (chloro-*p*-xylylene) (PPX-Cl) films were prepared from dichloro-[2,2] paracyclophane (DCPC), purchased from Parylene Distribution Services and deposited on p-type Si (100) wafers (Wafernet, Inc., San Jose, CA, USA).

Structured PPX-Cl Film Preparation: Silicon wafers were first sonicated in acetone. Afterwards, the wafers were washed in water and dried using nitrogen gas. The wafers were then transferred to a 1:1 v/v solution of HCl and methanol. After 30 min, they were removed and

washed with copious amounts of water and dried under nitrogen gas. The wafers were then kept in concentrated sulfuric acid for another 30 min, after which they were washed and sonicated in water for 10 min. The wafers were thoroughly dried under nitrogen gas. The self-assembled monolayer (SAM) solution was prepared by adding 1% allyltrimethoxysilane (Gelest, PA, USA) in toluene containing 0.1% acetic acid. The cleaned wafers were transferred to this solution and left for SAM formation for 60 min at 25 °C. The wafers were removed after 60 min and sonicated in anhydrous toluene for 10 min. They were then dried on a hot plate at 140 °C for 5 min for solvent removal. Nanostructured PPX-Cl films were deposited onto the wafers using 0.3 g of DCPC. The vaporizer and the pyrolysis chamber temperatures were maintained at 175 and 690 °C, respectively. The angle between the substrate and the flux was held constant at 10° during the deposition for all the samples.

Metal Deposition: The preparation of substrate for SERS experiments was started from the structured PPX-Cl film templates. The gold and silver were thermally deposited from resistively heated tungsten and tantalum boats onto the surface at about 1×10^{-8} Torr (1 Torr = 1.333×10^2 Pa) base pressure in a cryogenically pumped deposition chamber. The thickness of gold deposition was determined by a parallel QCM. Nitrogen adsorption isotherm measurements were performed at 77 K and a base pressure of ca. 1×10^{-5} Torr using a locked loop employing an HP 8656B frequency generator and an EG&G 5202 lock-in amplifier. One side of a 4 MHz AT-cut quartz crystal (Lap-Tech, Inc., Bowmanville, ON, Canada) was coated with nanostructured PPX-Cl film, over which a 600 Å layer of Ag was deposited. Nitrogen adsorption isotherms were used to calculate the monolayer adsorption surface area of the Ag-coated PPX-Cl film. The deposited metal films (100, 300, and 600 Å) were immersed into 1 mM FBT solution for 24 h. After that, the sample was rinsed with pure ethanol for 1 min to remove additional physical adsorbed FBT, and then dried under nitrogen gas.

SERS Measurements: A Renishaw inVia microRaman instrument was used for studying the SERS substrate. The instrument consisted of a 35 mW HeNe laser (632.8 nm) as the source, a motorized microscope stage sample holder, and a CCD detector. The motorized microscope stage allowed SERS maps of the surface to be formed. The instrument parameters were 50× objective and 10 s acquisition time. In addition, a fixed silicon wafer was used as a reference for normalizing the variation of power in different scans. To test the stability and photometric reproducibility of the instrument setup, we measured the uniformity of the silicon phonon peak across a 40 μm^2 area of a highly uniform single crystal silicon substrate. The measurements show a ca. 4–5% spot-to-spot reproducibility owing to fluctuations of the instrument optics, electronics, and laser power. An area map was selected for uniformity calculations with 10% laser power (see Fig. S2 of Supporting Information).

Bacterial Strains and Growth Conditions: *E. coli* was purchased from Lucigen (BL21-DE3) and *B. cereus* (ATCC #9818) was kindly provided by Dr. Stephanie Doores. Both bacteria were cultivated for 16 h at 37 °C on an LB agar base. After subculturing, single colonies were collected using sterile plastic inoculating loops. The cells were

suspended in L. B., grown for an additional 4h, and 10 μL aliquots were placed on SERS substrate for immediate characterization.

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