Optimized Silver Film over Nanosphere Surfaces for the Biowarfare Agent Detection Based on Surface-Enhanced Raman Spectroscopy

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ABSTRACT

This work presents the rapid detection of *Bacillus subtilis* spores, harmless simulants for *Bacillus anthracis*, using surface-enhanced Raman spectroscopy (SERS) on silver film over nanosphere (AgFON) substrates. Calcium dipicolinate (CaDPA), a biomarker for bacillus spores, can be extracted effectively from spores with nitric acid and successfully detected by SERS. The highly tunable nature of AgFON optical properties was exploited to establish general optimization conditions. AgFON surfaces optimized for 750-nm laser excitation have been characterized by UV-vis diffuse reflectance spectroscopy. The SERS signal from extracted CaDPA was evaluated over the spore concentration range 10^{-15}-10^{-12} M to determine the adsorption capacity of the AgFON surface and the limit of detection (LOD). These sensing capabilities have been successfully transitioned to an inexpensive, portable Raman spectrometer. Using the extraction method and this field-portable instrument, the anthrax infectious dose of 10^4 spores were detected with only a 5-second collection period on a one-month-old prefabricated AgFON substrate.

INTRODUCTION

Vibrational spectroscopic methods are valuable analytical tools because they yield not only quantitative information but also unique vibrational signatures for small molecule analytes. Raman spectroscopy, in all its forms, is a vibrational spectroscopic method that has the inherent ability to distinguish between molecules with great similarity. Unfortunately, high laser powers and long acquisition times are usually required to achieve high quality Raman spectra due to the

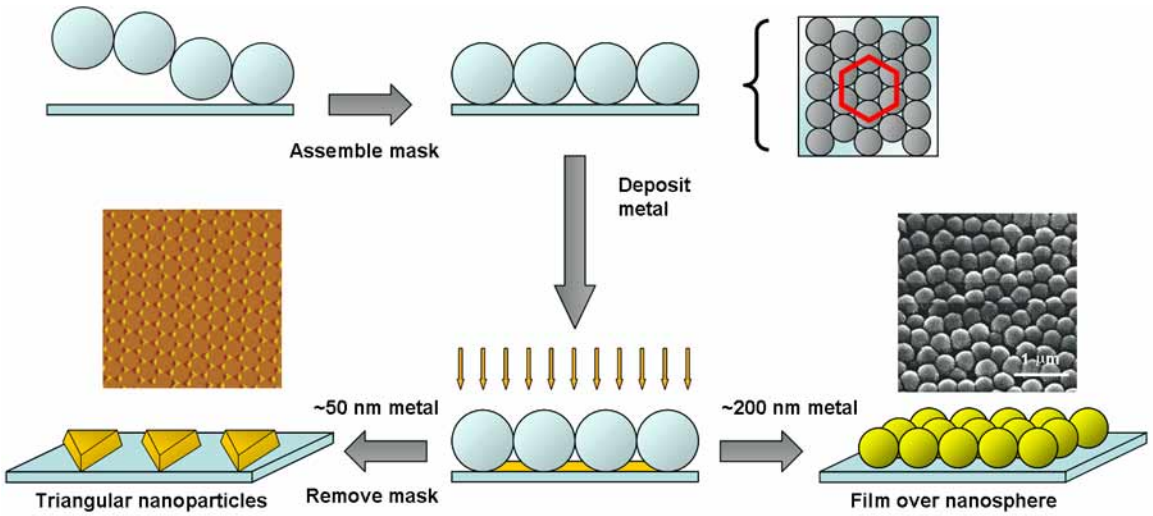

Figure 1. Nanosphere lithographic fabrication of nanoparticle arrays and film over nanosphere surfaces (FON).

inherently small normal Raman scattering (NRS) cross section of many molecules of interest.[1] Higher intensity Raman signals and lower detection limits can be achieved using SERS. SERS produces very large enhancements in the effective Raman cross section of species spatially confined within zone of the electromagnetic fields (*viz.* 0–4 nm)[2] generated upon excitation of the localized surface plasmon resonance (LSPR) of nanostructured noble metal surfaces. This large electromagnetic field induces a dipole in nearby molecules, thus enhancing Raman scattering from absorbed molecules. The Raman signals of ensemble-averaged molecules show enhancement of up to 8 orders of magnitude[3], while the signals from single molecules can show an increase by 14 to 15 orders of magnitude in special cases[4, 5]. In comparison with infrared and NRS spectroscopies, SERS enjoys the advantages of application in aqueous media and the sensitivity sufficient for trace level detection[6].

For sensors, it is important that the optical properties of the substrate be designed to fully maximize SERS intensities, which accordingly lowers analytical limit of detection (LOD). Early SERS substrates contained a random distribution of roughness feature sizes produced by oxidation-reduction cycling on a metal electrode[7] or evaporation of a thin metal film onto a flat substrate[8]. In recent years, researchers have explored the optimal size, shape, spacing, and pattern of noble metal nanoparticles on surfaces to optimize SERS enhancements. One of the most robust SERS substrates in use today are the metal film over nanospheres substrates prepared by nanosphere lithography (NSL) (Figure 1)[9-11]. The diameter of the colloidal nanosphere cores and the thickness of the metal film shell determine the size distribution of the roughness features and, hence, the optical response. Even though the nanoscale roughness features are not homogeneous in size; but, are instead driven by the larger scale templating, they are homogeneous enough to generate a relatively narrow LSPR (FWHM ~200 nm). Recent experiments have conclusively demonstrated that metal film over nanospheres (MFON) substrates are stable for months[12] (unlike many other nanostructured surfaces) and remain SERS-active even when exposed to large temperature[13] and potential excursions[10]. The utility of the MFON substrate is demonstrated herein as a robust SERS substrate used inthe rapid detection of *Bacillus subtilis* spores, harmless simulants for *Bacillus anthracis*. A bacillus spore structurally consists of several protective layers and a core cell. CaDPA exists in these protective layers and can be used as the spore biomarker because other potentially interfering species lack this particular molecule in such high proportions [14, 15].

EXPERIMENTAL SECTION

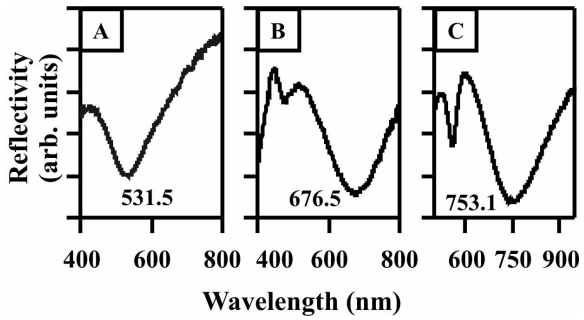

Figure 2. UV-vis diffuse reflectance spectra of different AgFON substrates in air. (A) D = 390 nm, dm = 200 nm, (B) D = 510 nm, d_m = 200 nm, and (C) D = 600 nm, d_m = 200 nm.

Materials. All the chemicals used were of reagent grade or better. Ag (99.99%) was purchased from D. F. Goldsmith (Evanston, IL). Glass substrates were 18 mm diameter, No. 2 cover slips from Fisher Scientific (Pittsburgh, PA). Pretreatment of substrates required H_2SO_4, H_2O_2, and NH_4OH, all of which were purchased from Fisher Scientific (Fairlawn, NJ). Surfactant-free white carboxyl-functionalized polystyrene latex nanospheres with diameters of 390, 510, 600, and 720 nm were obtained from Duke

Scientific Corporation (Palo Alto, CA) and Interfacial Dynamics Corporation (Portland, OR.). Tungsten vapor deposition boats were purchased from R. D. Mathis (Long Beach, CA). Nitric acid 70% (Fisher Scientific), dipicolinic acid (2,6-pyridinedicarboxlic acid, DPA), and calcium hydroxide (Aldrich Chemical Co., Milwaukee, WI) were used as purchased. Water (18.2 MΩ/cm)was obtained from an ultrafilter system (Milli-Q, Millipore, Marlborough, MA). Calcium dipicolinate (CaDPA) was prepared from DPA and calcium hydroxide according to the method of Beiley and co-workers.[16]

Spore Samples. *B. subtilis* was purchased from the American Type Culture Collection (Manassas, VA). Spore cultures were cultivated by spreading the vegetative cells on sterile nutrient agar plates (Fisher Scientific), followed by incubating at 30 °C for 6 days. The cultures were washed from the plates using sterile water and centrifuged at 12000 g for ten minutes. The centrifuging procedure was repeated five times. The lyophilized spores were kept at 2-4 °C prior to use. Approximately 1 gram of sample was determined to contain 5.6×10^{10} spores by optical microscopic measurements (data not shown). The spore suspension was made by dissolving spores in 0.02 M HNO_3 solution and by sonicating for 10 minutes.

AgFON Substrate Fabrication. Glass substrates were pretreated in two steps: (1) piranha etch (CAUTION: piranha solution should be handled with great care), 3:1 H_2SO_4: 30% H_2O_2 at 80 °C for one hour, was used to clean the substrate, and (2) base treatment, 5:1:1 H_2O: NH_4OH: 30% H_2O_2 with sonication for one hour, was used to render the surface hydrophilic. Approximately 2 µL of the nanosphere suspension (4% solids) was drop coated onto each substrate and allowed to dry in ambient conditions. The metal films were deposited in a modified Consolidated Vacuum Corporation vapor deposition system.

UV-vis Diffuse Reflectance Spectroscopy. Measurements were carried out using an Ocean Optics (Dunedin, FL) SD2000 spectrometer coupled to a reflection probe (Ocean Optics) and a halogen lamp (Model F-O-Lite H, World Precision Instruments, Sarasota, FL). All reflectance spectra were collected against a mirror-like Ag film over glass substrate as a reference.

SERS Apparatus. A battery-powered Raman spectrometer (model Inspector Raman, diode laser excitation wavelength λ_{ex} = 785 nm) was purchased from DeltaNu (Laramie, WY), which was used to demonstrate the feasibility of a field-portable device for spore detection. The remaining data were acquired using a macro-Raman system. This system consists of an interference filter, a 1”

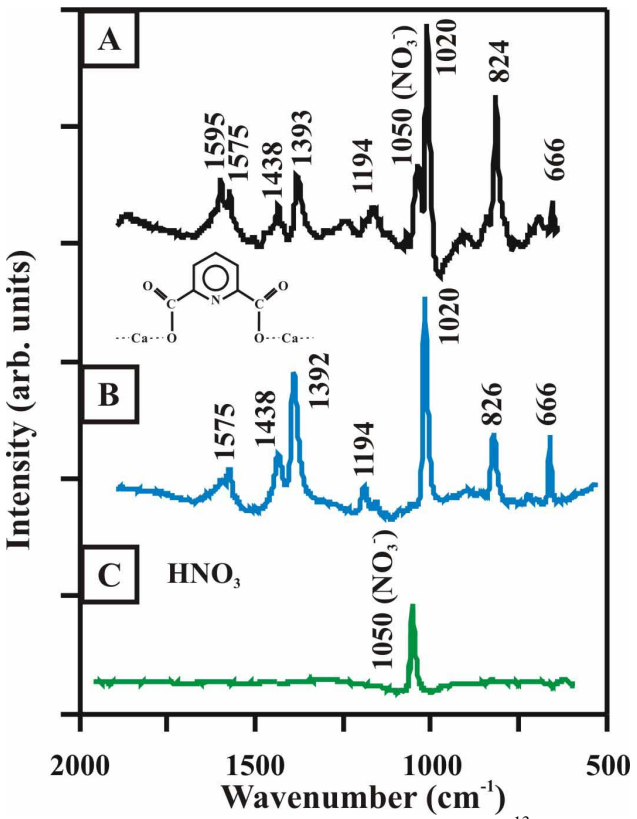

Figure 3. (A) SERS spectrum of 3.1×10^{-13} M spore suspension on a AgFON substrate. (B) SERS spectrum of 5.0×10^{-4} M CaDPA. (C) SERS spectrum of 0.2 µL 0.02 M HNO_3. λ_{ex} = 750 nm, P_{ex} = 50 mW, acquisition time = 1 min.

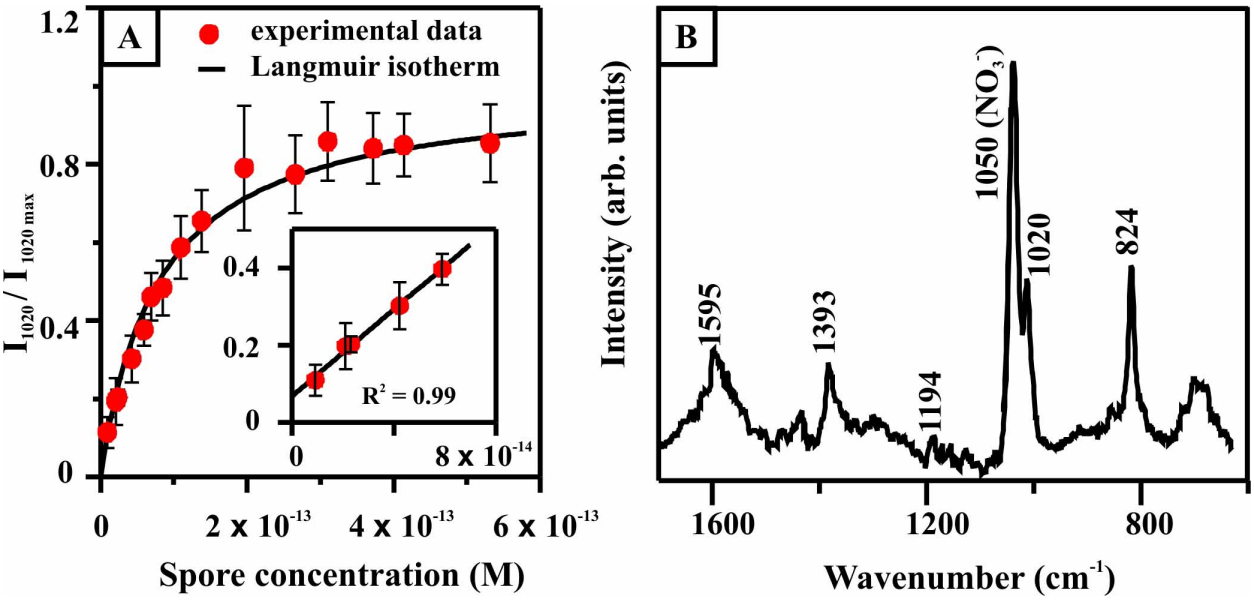

Figure 4. (A) Adsorption isotherm for *B. subtilis* spore suspension onto a AgFON substrate. I_{1020} was taken from SERS spectra that correspond to varying spore concentrations in 0.2 µL, 0.02 M HNO_3 on AgFON substrates. λ_{ex} = 750 nm, P_{ex} = 50 mW, acquisition time = 1 min, D = 600 nm, and d_{Ag} = 200 nm. A Langmuir curve was generated with the adsorption constant for CaDPA from spores, $K_{spore} = 1.3\times 10^{13}\ M^{-1}$. The inset shows the linear range that is used to determine the LOD. Each data point represents the average value from three SERS spectra. Error bars show the standard deviations. (B) SERS spectrum of 2.1×10^{-14} M spore suspension (2.6×10^3 spores in 0.2 µL, 0.02 M HNO_3) on AgFON. λ_{ex} = 750 nm, P_{ex} = 50 mW, acquisition time = 1 min.

holographic edge filter, a single-grating monochromator with the entrance slit set at 100 µm, a liquid-N_2-cooled CCD detector, and a data acquisition system. A titanium-sapphire laser (CW Ti: Sa, model 3900, Spectra Physics, Mountain View, CA) pumped by a solid-state diode laser (model Millenia Vs, Spectra Physics) was used to generate λ_{ex} of 750 nm.

RESULT AND DISCUSSIONS

AgFON substrates for SERS measurements using 750 nm laser excitation were optimized by first measuring the dependence of the LSPR spectral position on nanosphere diameter. Figure 2 shows the UV-vis diffuse reflectance spectra of AgFON substrates with nanospheres having diameters of 390, 510, and 600 nm. A AgFON sample was also fabricated using 720 nm-diameter spheres, however, the spectrum is not shown because the reflectance minimum is shifted beyond the red limit (~ 900 nm) of the CCD detector. In Figure 2, the reflectance spectrum of AgFON substrate C (nanosphere diameter, D = 600 nm, and mass thickness of Ag film, d_m = 200 nm) shows a reflectivity minimum near 753 nm, attributable to the excitation of the LSPR of the silver film. This substrate is expected to show the largest intensity for 750 nm laser excitation. Further confirmation has been shown in our previous work.[12]

CaDPA was extracted from spores by sonicating a spore suspension in 0.02 M HNO_3 solution for 10 min. A 3.1×10^{-13} M spore suspension (3.7×10^4 spores in 0.2 µL, 0.02 M HNO_3) was deposited onto a AgFON substrate for the SERS measurement. A high signal-to-noise ratio (S/N) SERS spectrum was obtained in a 1-minute data acquisition period (Figure 3); this spectrum is dominated by bands associated with CaDPA (Figure 3B). The bands due to HNO_3 in the suspension were identified as well (Figure 3C). The peak at 1050 cm^{-1} in Figure 3C

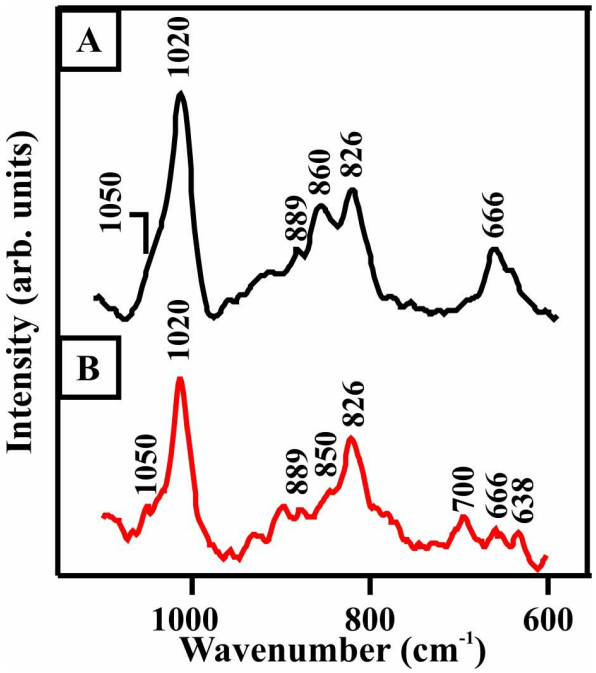

Figure 5. SERS spectra obtained by portable Raman spectrometer. (A) SERS spectrum of 8.3×10^{-14} M spore suspension (1.0×10^4 spores in 0.2 µL, 0.02 M HNO_3) on 30-day old AgFON. (B) SERS spectrum of 10^{-4} M CaDPA in 0.2 µL 0.02 M HNO_3 on 30-day old AgFON substrate. λ_{ex} = 785 nm, P_{ex} = 35 mW, acquisition time = 5 sec, resolution = 15 cm^{-1}, D = 600 nm, and d_m = 200 nm.

is from the symmetrical stretching vibration of NO_3^-, which was selected as the internal standard to reduce the sample to sample deviations.

The SERS signal from extracted CaDPA was measured over the spore concentration range 10^{-14} – 10^{-12} M to determine the saturation binding capacity of the AgFON surface and to calculate the adsorption constant ($K_{spore} = 1.7 \times 10^{13}$ M^{-1}). In Figure 4A, each data point represents the average intensity at 1020 cm^{-1} (a ring breathing mode) from three samples with the standard deviation shown by the error bars. At low spore concentrations, the peak intensity increases linearly with concentration (Figure 4A inset).

Herein, the LOD is defined as the concentration of spores for which the strongest SERS signal of CaDPA at 1020 cm^{-1} is equal to three times the background SERS signal within a one-minute acquisition period. The LOD for *B. subtilis* spores was found to be 2.1×10^{-14} M (2.6×10^3 spores in 0.2 µL, 0.02 M HNO_3), as calculated by extrapolation of the linear concentration range of the adsorption isotherms. Furthermore, a similar spore concentration 2.1×10^{-14} M was used to test the LOD prediction. A one-minute acquisition yields a SERS spectrum that clearly demonstrates the spore Raman features (Figure 4B) in comparison with Figure 5A. These data demonstrate that the SERS LOD is well below the anthrax infectious dose of 10^4 spores[17].

As a first step in this direction, the Raman spectrum from 10^4 *B. subtilis* spores dosed onto a one-month-old AgFON substrate was readily acquired using a commercially available portable Raman instrument. A high S/N spectrum was achieved within five seconds (Figure 5A). The SERS peak positions and intensity pattern for the spore sample was similar to those of CaDPA recorded utilizing the same device (Figure 5B). This is the first example of using a compact, portable Raman spectrometer for the detection of bacillus spores.

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