Fabrication of SERS-Active Substrates using Silver Nanofilm-Coated Porous Anodic Aluminum Oxide for Detection of Antibiotics

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Abstract: We have developed a silver nanofilm-coated porous anodic aluminum oxide (AAO) as a surface-enhanced Raman scattering (SERS)-active substrate for the detection of trace level of chloramphenicol, a representative antibiotic in food systems. The ordered aluminum template generated during the synthesis of AAO serves as a patterned matrix on which a coated silver film replicates the patterned AAO matrix to form a 2-dimensional ordered nanostructure. We used atomic force microscopy and scanning electron microscopy images to determine the morphology of this nanosubstrate, and characterized its localized surface plasmon resonance by ultraviolet-visible reflection. We gauged the SERS effect of this nanosubstrate by confocal micro-Raman spectroscopy (782-nm laser), finding a satisfactory and consistent performance with enhancement factors of approximately $2 \times 10^4$ and a limit of detection for chloramphenicol of 7.5 ppb. We applied principal component analysis to determine the limit of quantification for chloramphenicol of 10 ppb. Using electromagnetic field theory, we developed a detailed mathematical model to explain the mechanism of Raman signal enhancement of this nanosubstrate. With simple sample pretreatment and separation steps, this silver nanofilm-coated AAO substrate could detect 50 ppb chloramphenicol in milk, indicating good potential as a reliable SERS-active substrate for rapid detection of chemical contaminants in agricultural and food products.

Keywords: food safety, contaminants, food analysis

Practical Application: This novel surface-enhanced Raman scattering substrate can be used for the detection of trace level of antibiotics in a fast and accurate manner.

Introduction
Raman scattering is often proposed as a spectroscopic means of measurement in many areas of chemistry, physics, material science, and biomedical engineering, owing to its unique label-free and nondestructive properties. However, conventional Raman spectroscopy suffers from a small cross section, yielding only one Raman-scattered photon for every $10^6$ photons delivered by the incident laser (Ellis and others 2012). This disadvantage has stimulated 40 y of research on the technique of surface-enhanced Raman scattering (SERS). SERS operates to varying degrees by quantum and classical mechanisms to amplify the signal of inelastically scattered light. In most cases, the major contribution comes from a classical electromagnetic enhancement of light scattering by a localized surface plasmon resonance (LSMR) that arises for molecules in close proximity to a roughened surface metal nanostructure. A smaller quantum contribution, termed chemical enhancement, results from charge transfer and/or electronic spectroscopic resonance formed by molecules absorbed on the metal surface (Kneipp and others 2008). To date, silver and/or gold colloids, easily generated by simple chemical reduction, have served as the most common and effective SERS substrates. However, poor spectral reproducibility made inevitable by variations in the properties of different batches and concentrations of silver and/or gold colloids have limited the quantitative effectiveness of colloid-based SERS techniques. Raman signal enhancement effects have been found to fluctuate even within the same batch of colloid owing to unpredictable aggregation properties of nanoparticles in colloidal suspension (Lu and others 2013).

By contrast, fabricated silver and gold nanostructures offer a unique set of advantages, including clean surfaces together with a well-defined, uniform structural morphology, critical for the production of highly reproducible SERS spectra (Haynes and others 2005; Lin and others 2009). Recent work has introduced porous anodic aluminum oxide (AAO) as a template for the fabrication of various nanostructured metal materials. Generally speaking, AAO features closely packed regular arrays of columnar cells, with well-controlled pore density and diameter. Electrochemical or metal-vapor deposition techniques fill pores in the ordered AAO matrix to yield size-selected silver and gold nanowires, nanorods, nanospheres, and nanofilms (Ko and Tsukruk 2008; Lu and others 2009; Liao and others 2009; Huang and others 2013). Material fabricated in this way forms an ideal matrix as SERS-active nanostructures.

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The AAO template has been applied for the development of ordered silver and gold nanostructure as SERS-active substrate. For examples, Wang and others (2006) fabricated a uniform and highly SERS-active substrate using silver nanoparticle arrays with small tunable gaps with the aid of porous AAO template. In another study, Lee and others (2006) developed a SERS substrate consisting of highly ordered and regular silver nanowires fabricated in porous aluminum oxide. Because the silver nanowires were sealed in the pores of aluminum oxide, this nanostructure was subsequently away from contamination until usage, at which point the alumina matrix could be etched. This could allow silver nanowires to collapse into bundles, forming “hot spots” and trapping interested molecules in these junctions. Ko and Tsukruk (2008) reported a 3-dimensional porous structure with nanocanal trapping interested molecules in these junctions. Ko and Tsukruk fabricated 3-dimensional nanostructure coated with silver nanoparticles for SERS using a “2-step anodization” approach coupled with alternating current electrochemical deposition technique. Recently, Terekhov and others (2011) prepared 3 types of silver-coated arrays from porous AAO as SERS active substrates, namely the porous side with open pores, the monolith side with open pores, and the monolith side with continuous barrier layer.

In this study, we have fabricated a SERS-active substrate by the deposition of silver onto an AAO template to form a nanoporous structure, and applied this substrate to the detection of chloramphenicol. Chloramphenicol is a broad-spectrum antibiotic used to treat bacterial infections in humans and its abuse is a major factor that results in its residues present in foods. Numerous countries have banned the use of chloramphenicol in food-producing animals (Cerkvenik 2002). Current detection methods include chromatographic separation coupled with mass spectrometry (Posnyiak and others 2003) and enzyme-linked immunosorbant assay (Impens and others 2003). Both methods require time- and labor-intensive effort by highly trained personnel. Recently, a silver nanorod array fabricated by oblique angle deposition has served successfully as a SERS substrate for the detection of aflatoxins (Wu and others 2012). Here we demonstrate that a nano-patterned AAO template, coated with silver serves effectively as a SERS-active substrate for the rapid detection of chloramphenicol with reliability and good limit of detection.

Materials and Methods

Chemicals and materials
Chloramphenicol (HPLC grade) was purchased from Sigma-Aldrich. Aluminum foil (99.9999% purity) was purchased from Beijing General Research Inst. for Nonferrous Metals (Beijing, China). Silver sputtering target (99.99% purity) was obtained from Kurt J. Lesker Inc. (Jefferson Hills, Pa., U.S.A.). Other chemicals, including 85% phosphoric acid, 70% perchloric acid, and 99.99% chromium trioxide, were purchased from the Tianjin Chemical Plant (Tianjin, China). All the reagents except aluminum were used as received without further purification. All aqueous solutions were prepared using water processed by a Milli-Q system to have resistivity greater than 18 MΩ/cm.

Preparation of patterned aluminum templates and SERS-active substrates
Figure 1 illustrates the overall preparation and fabrication procedure. We first prepare a patterned aluminum template using the first step of a “2-step anodization” method. Briefly, a piece of pure aluminum foil is electro-polished in a mixture of perchloric acid and ethanol (1:4, v/v) at 5 °C for 3 min to remove surface irregularity and potential contamination. Anodization is then carried out using 0.1 M phosphoric acid at 140 V and 2 °C for 2 h. A long anodization is necessary to increase the surface regularity (Lee and others 2005). The first anodization forms an oxide layer on the surface and begins the development of random pores. Continued anodization greatly improves the regularity in pore arrangement, yielding a straight pore array and ordered cell configuration. After the first anodization, we etch away the porous alumina layer by an alumina etchant (1.8 wt.% chromic acid and 6 wt.% phosphoric acid) at 65 °C for 4 h, excavating an ordered concave pattern on the surface of the aluminum specimen. A second anodization conducted for 30 min using conditions identical to the first anodization completes the synthesis of the AAO template.

We next deposit a thin silver film on this template using a magnetron sputtering system (Lab 18, Kurt J. Lesker Inc., Pa., U.S.A.) operated in pulsed DC mode with a frequency of 30 kHz. The chamber is evacuated to 8 × 10⁻⁶ Torr before deposition. The process pressure, substrate temperature, and power density are kept at 2 mTorr, 23 °C and 0.04 W/cm², respectively. One minute of deposition produces a 25-nm silver film coating the patterned AAO template.

Characterization of SERS-active nanosubstrates
The morphology and surface characteristics of our AAO templates and silver nanofilm–coated AAO substrates have been determined using atomic force microscopy (AFM) and scanning electron microscopy (SEM). AFM images were obtained in tapping mode by using SPA 400 AFM (SII Nanotechnology, Inc., Tokyo, Japan). For SEM, the AAO template was mounted onto SEM stubs and sputter coated with a thin layer of gold. The substrate was observed under a SUPRA 55VP scanning electron microscope (Carl ZEISS AG, Jena, Germany) using an accelerating voltage of 15 kV.

We have taken ultraviolet-visible (UV-vis) spectra in the range of 300 to 1100 nm to observe the LSPR of nanosubstrates using a Carry 5000 spectrophotometer (Varian Co., Palo Alto, Calif., U.S.A.) equipped with an integrating sphere.

SERS spectral collection
To obtain SERS spectral features of chloramphenicol, we deposited 2 μL chloramphenicol solution samples with a series of concentrations (10 to 100 ppm) onto the surface of nanosubstrates. We illuminated samples and collected backscattered light using a confocal micro-Raman spectroscopic system, which includes a Raman spectrometer (Renishaw, Gloucestershire, U.K.), a near-infrared (λ = 782 nm) laser (Coherent, Santa Clara, Calif., U.S.A.), and a Leica microscope (Leica Biosystems, Wetzlar, Germany) equipped with a 50× Nikon objective. The spectrometer has an entrance aperture of 50 μm and a focal length of 300 mm and is equipped with a 1200-line/mm grating. The SERS signal was dispersed by a diffraction grating, and recorded as a spectrum spanning a Raman shift interval from 1800 to 400 cm⁻¹ by a 576- by 384-pixel charge-coupled device (CCD) array detector, with a pixel size of 16 by 16 μm. The measurement was performed with a 10-s exposure time at from 8 to 10 random locations on each sample with approximately 0.3 mW of incident laser power. WIRE 3.4 software (Renishaw) was used for spectral collection. Raman wavenumbers were calibrated using the Raman peaks of pyridine. Each measurement was conducted at least in triplicate.
Enhancement factor

We compute SERS enhancement factors according to the following equation:

\[
\text{Enhancement factor} = \frac{I_{\text{SERS}} \times C_{\text{RS}}}{I_{\text{RS}} \times C_{\text{SERS}}}
\]

where \(I_{\text{SERS}}\) and \(I_{\text{RS}}\) denote the peak intensities of SERS and normal Raman spectrum, respectively. The magnitude of \(I_{\text{SERS}}\) arises for the most part from a single layer of molecules covering the nanosubstrate. Additional layers of molecules contribute little to the intensity (Haynes and others 2005). \(C_{\text{RS}}\) and \(C_{\text{SERS}}\) represent the concentrations of SERS molecules and normal Raman concentrations, respectively.

SERS spectral sensitivity, reproducibility, and consistency

We determined the sensitivity of SERS for this analysis by depositing different concentrations of chloramphenicol solution onto nanosubstrate and collecting spectra. The limit of detection (LOD) was determined using a partial least squares regression (PLSR) model described in a previous paper (Strickland and Batt 2009). The SERS signal intensity at 1345 cm\(^{-1}\) has correlation to the concentration of chloramphenicol. LOD is equal to \(3\sigma/m\), where \(\sigma\) is the standard error in the \(y\)-intercept and \(m\) is the slope of the PLSR cross-validation curve.

A 3-dimensional principal component analysis (PCA) model was constructed to distinguish chloramphenicol samples on the basis of analyte concentration, resulting in determination of limit of quantification (LOQ). The Mahalanobis distance determines the separation between PCA groups in units of within-group standard deviation, reflecting the spectral reproducibility of each group in relation to the precision of measurement of chloramphenicol concentration (Lu and others 2012). The consistency of SERS spectra was determined by calculating the relative standard deviation of the intensity of the highest band in the SERS spectrum as observed in 3 individual experiments.

Results and Discussion

Preparation and characterization of SERS-active nanosubstrate

The morphology of AAO templates and AAO templates coated by silver nanofilms were characterized by SEM. Figure 2A illustrates a typical top view of AAO template micrograph. The AAO substrate forms a hexagonal honeycomb structure with the average pore diameter and interpore distance of 200 and 500 nm, respectively. A scraggly surface with nano-protuberances can be observed clearly at the oblique view of AAO template as shown in Figure 2B. Silver films deposited onto the AAO template create no obvious difference in the head face morphology, as shown in Figure 2C and D.

We also used AFM to characterize the microstructure on the surface of AAO template with and without coating by silver nanofilms. Figure 3A and C show 3-dimensional morphological features. Consistent with the SEM data, a quasi-periodic structure with each pore surrounded by 6 protuberances at each hexagon corner was observed. The vertexes at hexagon corners sit at the center of 3 adjacent nano-holes. The AFM data show that silver layer coating does not alter surface roughness as characterized by the root mean square (RMS) variation (64 nm). Further longitudinal structural information could be extracted from AFM height profile, as shown in Figure 3B and D. Several typical sites are marked. In Figure 3B, labels 2 and 4 stand for 2 adjacent protuberances. Label 1 signifies the position between 2 protuberances and label 4 points to the peak of the pit. As extracted from AFM data, the horizontal distance between 2 adjacent protuberances is 578 nm, and the altitude difference between adjacent protuberance and pit is 298 nm. Similarly, Figure 3D represents the height profile of the AAO template coated by silver nanofilms. The horizontal distance and altitude difference are 565 and 301 nm, respectively. These structural parameters indicate that the silver deposition produces little if any change in the AAO surface structure.

The UV-vis absorption spectrum of the nanosubstrate shown in Figure 4 is converted from the total reflection spectrum (collected with integrated sphere at an incident angle of 5°). The absorption cross section of AAO template is relatively high (that is, approximately 0.6) at wavelength below 800 nm and then decreases tremendously. This absorption property indicates that photons with short wavelength can travel along the AAO hollow hexagonal prism at an incident angle of 5°. Therefore, the AAO hollow hexagonal prism serves as a nano/microcavity that traps a substantial amount of light.

With the deposition of a silver nanofilm onto the AAO template, the absorption spectrum of the nanosubstrate shows prominent surface plasmon properties. The wide range absorption with the maximum at 494 nm relates directly to the LSPR. (Huang and others 1999). We can conclude from the intensity of the absorption spectrum that the silver nanostructure exhibits a strong LSPR. The relatively strong interaction between plasmons produces a broad
absorption spectrum. For this reason, the intensity at 782 nm is still strongly affected by the LSPR, reflecting a substantial interaction between photons from incident light and surface electrons.

The spectral breadth of the observed resonance originates from the various geometries present on the sample surface. The hexagonal honeycomb structure has the capability to simultaneously support both propagating and localized surface plasmon resonances. A shallow grating of grooves or holes in silver nanofilms (shown in AFM and SEM images) is well-known to facilitate excitation of propagating surface plasmon polarization (known as grating coupling; Nielsen and others 2011), and the grating of interconnected hollow hexagonal prisms might well support such plasmon modes (Yu and others 2006).

To verify the electromagnetic (EM) field distribution in this patterned silver nanostructure, we simulated the steady-state EM distribution by using a 2-dimensional finite-difference time-domain method. Figure S1 shows a 3-dimensional sketch of the AAO template. We model the AAO structure by a hexagon with circular corners and a cylindrical hole to simplify the simulation. Our simulated structure has a height of 2000 nm, radius of 285 nm, and inner, cylindrical hole radius of 120 nm, all of which are based upon the SEM profiles as shown in Figure 2. A uniform 25-nm silver film coating on the patterned AAO template, preserves the surface profile. We calculate the frequency-dependent complex relative permittivity of this silver coating by the Drude model:

\[
\varepsilon_\omega(\omega) = \varepsilon_\infty - \omega_p^2/\omega(\omega + i\gamma)
\]

where \(\omega_p = 9.1\) eV is the bulk plasma frequency, \(\gamma = 0.018\) eV is the damping frequency of the oscillations, \(\omega\) is the angular frequency of the incident electromagnetic radiation, and \(\varepsilon_\infty = 3.7\) is the dielectric constant at infinite angular frequency (Han and others 2007). We take the refractive index of AAO to be \(n_{\text{AAO}} = 1.76\) and the refractive index of air to be \(n_{\text{air}} = 1\). To avoid reflection from the boundary of the simulation area, we assume a perfectly matched layer that serves effectively to reduce the numerical reflection. A plan wave emitted from the top of the template simulates the surface plasmon polarization. A monitor installed on
the top surface of the patterned template detects the EM field distribution. At the LSPR wavelength (494 nm), the EM field of the silver coated AAO template is substantially enhanced compared to that of the AAO template, as shown in Figure 5A and B. The increasing field intensity arises from the excitation of the plasmon polarization on the surface of silver structure. Surface plasmon resonances persist at the infrared wavelength (782 nm), as shown in Figure 5D. The total EM field intensity of the silver-coated AAO at 782 nm was less than the EM field intensity of the same substrate at a 494 nm incident wavelength. However, even at 782 nm, the focusing and localizing of surface plasmons close to sharp holes can lead to extreme light concentration in nanometer-sized regions creating electromagnetic “hot-spots,” contributing greatly to SERS signal enhancement (Bozhevolnyi and others 2006).

Raman scattering arises because nuclear motion modulates the polarization of the electronic charge distribution. Local EM field enhancement enlarges this polarization, particularly in “hot-spots” caused by surface plasmon polarization and near-field coupling. The significant enhancement of the calculated EM field (Figure 5D) demonstrates that even incident light at 782 nm produces a significant localized surface plasmon polarization. Model calculations further predict that a pore diameter and interpore distance altered to tune the $\lambda_{\text{max}}$ of LSPR spectra to match the excitation wavelength could result in a substantial further increase of enhancement by the nanosubstrate.

SERS performance of nanosubstrate

Figure 6 shows SERS spectral features owing to background and scattering by chloramphenicol. For solutions in a series of chloramphenicol concentrations, we observe Raman features distinctive of analyte still discernible at levels as low as 10 ppb. As the method described earlier, the LOD was calculated to be 7.5 ppb. Furthermore, the background adds no obvious structure, underlining the feasibility of this nanosubstrate for SERS. Three major bands at 1598 cm$^{-1}$ (ring stretching), 1345 cm$^{-1}$ ($\text{NO}_2$ symmetric stretching; Lai and others 2011), and 1107 cm$^{-1}$ (N-H in-plane bending; He and others 2010) conform to the molecular structure of chloramphenicol shown in Figure S2. The relatively strong intensity and sharp features of the SERS bands indicate that chloramphenicol molecules are tightly adsorbed onto the SERS substrate.

A 3-dimensional unsupervised PCA model clearly differentiates chloramphenicol samples by concentration as shown in Figure S3. Principal component coordinates form tight clusters with interclass distances ranging from 4.82 to 15.31 based upon
Mahalanobis distance measurements computed between the centroids of different classes. Clusters with interclass distance values higher than 3 are considered to be significantly different from each other. Therefore, these results demonstrate very good spectral reproducibility within each group (Lu and others 2012). On the basis of this, we estimate the LOQ to be about 10 ppb.

Triplicate experiments with spectral collection from different locations on the nanosubstrates test the spectral consistency of this measurement. The residual standard deviation was calculated to be 8.3%. This spectral reproducibility and consistency speak to the homogeneity of the SERS-active substrates. The signal enhancement effect is subject to significant change owing to minor variations in the surface morphology and shape. Taken together, the nanosubstrates fabricated in this study show good reproducibility.

SERS enhancement factor was calculated using the SERS spectra of 1 ppm chloramphenicol on nanosubstrate and Raman spectra of 1000 ppm chloramphenicol on a normal substrate. Comparing the intensity of the nanosubstrate chloramphenicol SERS band at 1345 cm\(^{-1}\) with the same feature observed using a normal gold-coated microarray glass slide (validated to show no SERS effect; Lu and others 2012), the enhancement factor was calculated to be about \(2 \times 10^4\) using Equation (1) (Figure S4). The concentration of the molecules (that is, chloramphenicol) in this SERS measurement may not fully reflect the total number of the adsorbed molecules onto the nanosubstrate due to the “first layer” effect.\(^1\) Therefore, the real signal enhancement factor could be larger than the calculated one.

![Figure 6–SERS spectral features of a series of concentrations of chloramphenicol (10 to 100 ppm).](image)

For testing of chloramphenicol in real food samples, whole milk (2 mL) was spiked with chloramphenicol for a series concentration of 10 to 10 ppm. Ethyl acetate (5 mL) was added, followed by 30 s vortex and centrifugation at 10000 \(\times \) g for 2 min. Ethyl acetate layer was run through an anhydrous sodium sulfate column and dried down by rota-vap and then dissolved in 10% methanol (Gao and others 2014). The resultant solution was loaded onto solid-phase extraction filled by synthesized molecularly imprinted polymers, as described in our previous publication (Gao and others 2014), before SERS detection. We confirmed that this SERS substrate could detect 50 ppb chloramphenicol in whole milk after simple sample pretreatment (Figure S5). Recovery of chloramphenicol from milk and remaining milk residues after solid phase extraction are 2 major factors (Gao and others 2014) that can affect the sensitivity of using this SERS substrate to detect chloramphenicol in real food samples. Taken together, this developed SERS substrate can achieve a fast and high-throughput screening of chloramphenicol contamination in milk after aforementioned sample pretreatment.

Conclusions

In conclusion, the current results indicate that the nanostructure we have developed could be applied as an active and reliable SERS substrate for the detection and quantification of chloramphenicol at ppb levels. Future study will focus on the development of 3-dimensional SERS-active substrate, considering that it may contain more “hot spots” than the currently developed 2-dimensional substrate. In addition, we shall explore other preseparation and enrichment techniques, such as aptamer and microfluidics before SERS spectral collection, for the detection of antibiotic residues in real food samples. This SERS-active nanosubstrate shows satisfactory spectral stability and reproducibility and has the potential to be applied for rapid detection of trace level of chemical hazards in agricultural and food products.

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References


Fabrication of SERS-active substrates


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Figure S1–The sketch of the 3-D structure.
Figure S2–Molecular structure of chloramphenicol.
Figure S3–Principal component analysis plot of different concentrations of chloramphenicol.
Figure S4–SERS enhancement factor.
Figure S5–Representative SERS spectrum of extract of chloramphenicol-spiked (50 ppb) whole milk (n = 3).