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A nanoforest structure for practical surface-enhanced Raman scattering substrates

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Abstract
A nanoforest structure for surface-enhanced Raman scattering (SERS) active substrates is fabricated and analyzed. The detailed morphology of the resulting structure can be easily controlled by modifying the process parameters such as initial gold layer thickness and etching time. The applicability of the nanoforest substrate as a label-free SERS immunosensor is demonstrated using influenza A virus subtype H1N1. Selective binding of the H1N1 surface antigen and the anti-H1 antibody is directly detected by the SERS signal differences. Simple fabrication and high throughput with strong in-plane hot-spots imply that the nanoforest structure can be a practical sensing component of a chip-based SERS sensing system.

Online supplementary data available from stacks.iop.org/Nano/23/095301/mmedia
(Some figures may appear in colour only in the online journal)

1. Introduction
Surface-enhanced Raman scattering (SERS) is a powerful analytical technique that enhances the weak signal intensity of conventional Raman scattering [1–5]. The signal increase comes from the localized electric-field enhancement in the space (nanogap) between two metal surfaces. To achieve strong enhancement, gold or silver surfaces with sub-10 nm nanogaps are required [6, 18]. Numerous techniques for fabricating structures containing high-quality metal nanogaps have been investigated, such as electron-beam lithography [6, 7], nanoparticle aggregation [8, 9], nanowire bundles [10–12], nanoparticle-coated nanowires [13–15], on-wire lithography [16, 17], deposition and etching [18, 19], chemical or physical roughening [20, 21], and electrochemical synthesis [22, 23].

Three major factors determine the quality of SERS substrates: the enhancement factor, the reproducibility of the hot-spot, and the fabrication complexity. Early studies were primarily focused on enhancement factors [13, 24, 32]. Various SERS substrates with extremely high enhancement factors, up to ~10¹⁴, have been introduced; however, recent studies have revealed that an enhancement factor of the order of 10⁶ ~ 10⁸ is sufficient for single-molecule detection [24–27]. More recently, instead of attempting to achieve extremely high enhancement factors, researchers have focused on other factors such as structural reproducibility, fabrication complexity, on-chip integrability, throughput, and cost [7, 13, 14, 18–22, 24–27].

Among the methods for fabricating SERS substrates, top-down lithographic patterning techniques and bottom-up aggregation techniques have been most widely investigated. Top-down patterning techniques such as electron-beam lithography have shown the best reproducibility, but the throughput of the structure is low, and these methods are time- and labor-intensive. Bottom-up methods, such as nanoparticle aggregation, have shown high throughput with relatively simple fabrication; however, the reproducibility
of the hot-spot is problematic. In this respect, simply fabricated and highly reproducible SERS substrates with sufficiently high enhancement factors and high throughput are desirable. Here, we have analyzed nanoforest structures fabricated by a simple and reliable semiconductor process. The structures showed an enhancement factor greater than $10^7$ with wafer-scale throughput and high reproducibility. The properties of the nanoforest meet important standards for practical SERS substrates, which imply that the structure can act as a sensing component of a chip-based SERS analysis system.

A wide hot-spot area and a high surface area to volume ratio of the nanoforest are particularly advantageous for SERS immunosensor applications. A conventional SERS immunosensor requires gold nanoparticles to be labeled with both Raman reporter molecules and additional immobilized capture antibodies [28–31]. The overall process, which is referred to as the sandwich assembly method, utilizes hot-spots generated between the gold nanoparticles and the gold film below the molecules. However, SERS-based immunosensing has not yet been widely accepted as a common biosensing method compared to other methods, such as the enzyme-linked immunosorbent assay (ELISA), mainly because of its complicated sample preparation steps and unstable hot-spots associated with the mobile nanoparticles [32–34]. As an alternative solution to the complex sandwich assembly process characteristic of the conventional SERS biosensing method, the use of specially designed SERS-active substrates for the direct detection of pathogen molecules, which represents an intrinsic detection method, has been advantageous [32]. In particular, in 2011, Galarreta et al demonstrated the utility of this method for detecting biotin–streptavidin binding using a lithographically patterned nanotriangle array substrate [33].

Advancing these earlier studies, we here demonstrate that our nanoforest structure can be reliably used as a label-free SERS immunosensor by detecting the specific binding of the antigen of a real pathogen and its corresponding antibody.

2. Experimental details

2.1. Fabrication of the nanoforest structure

Nanoforest fabrication was based on a metal-induced chemical etching process [35]. First, a 70 Å gold layer was deposited on a 4 in (100) silicon wafer by thermal evaporation. At this thickness, the gold layer does not exhibit continuous film morphology, but rather forms an island structure (figures 1(A) and (D)). The thickness of the gold layer determines the size of the island segments [36]. After the initial gold deposition, the substrates were etched using a solution containing HF and H$_2$O$_2$ (HF:H$_2$O$_2$:DI water \((v/v/v) = 2:1:7\) [36, 37]. The etching was performed at room temperature in the absence of light. During the etching, the silicon under the gold island was removed and a nanogap was formed. As a result, a porous silicon surface with vertically standing silicon bundles, in the form of forest-like pillars, was made (figures 1(B) and (E)). To make the nanogap, a SERS-active hot-spot, an additional gold layer was deposited. A 50 Å layer of Cr was first deposited to serve as an adhesion layer, and 500 Å of gold was subsequently deposited on top of this layer. After the secondary gold deposition, silicon bundles formed dense hot-spots (figures 1(C) and (F)). A cross-sectional transmission electron microscopy (TEM) image of the final nanoforest is shown in supporting information (figure S3 available at stacks.iop.org/Nano/23/095301/mmedia). Although silver generates higher signal
enhancement than gold, gold was used because of its higher bio-compatibility and chemical stability [38].

2.2. SERS spectra measurement

SERS spectra measurements were performed using a dispersive Raman spectrometer based on the LabRAM HR system (HORIBA Jobin Yvon, France). Brilliant cresyl blue (BCB, Sigma-Aldrich) was used as the SERS test molecule. Twenty microliters of $10^{-4}$ M BCB solution was dropped and dried on the experimental substrate. A He–Ne laser with a wavelength of 633 nm was then focused on the sample, and Raman signals were collected for 10 s. The magnification of the optical microscope was $\times 100$, so the beam spot size of the laser was approximately 500 nm in diameter. When Raman spectroscopy was initially analyzed on glass, an unfiltered laser was used. Its output power was 17 mW. In contrast, a neutral density filter was applied for the SERS measurement of the nanoforest substrate. The neutral density filter reduced the output power from 17 mW to 17 $\mu$W, i.e. 1000-fold. Therefore the resulting output power of 17 $\mu$W was used for the subsequent SERS measurements.

2.3. Simulation of the electric field

Simulations of electric-field profiles and enhancement factors in figures 3(C) and (D) were obtained using commercial software (COMSOL Multiphysics). The purpose of the simulation is to find the relationship between an enhancement factor and a nanogap depth, not to extract exact values of the enhancement factor. Among the modules of the software, the RF module was used; specifically, a scattered harmonic propagation of the TM wave mode was employed. The nanoforest structure was modeled as a periodic array of nano-pillars with an inter-pillar distance of 20 nm. Transverse magnetic waves with a wavelength of 633 nm were propagated vertically with respect to the nanoforest, and the scattered electric field was presented as a color map (figure 3(C)). The electric field of the incident wave was ignored in this map. With the values obtained through the simulation, the enhancement factors for different nanogap depths were calculated using the simple equation given in section 3. The calculated curves were linearly scaled and plotted (figure 3(D)).

2.4. Immunosensing experiment

The immunosensing experiment using influenza A virus subtype H1N1 was performed according to the conventional procedure used in the study of antigen–antibody interactions (figure 4(A)). To attach the antigen to the gold surface, we fused gold-binding polypeptide (GBP) with the surface hemagglutinin H1 antigen of the H1N1 influenza virus (see supporting information available at stacks.iop.org/Nano/23/095301/mmedia) [39–41]. The nanoforest samples were immersed in the GBP–H1 antigen solution for 50 min at room temperature and were then rinsed twice with deionized (DI) water for 10 min (each treatment). After antigen immobilization, the nanoforest substrate was immersed in a solution containing the antibody against the H1N1 virus for 50 min. The anti-H1 antibody molecules were anticipated to selectively bind to GBP–H1 because of their specificity for the antigen molecule. As a control, anti-rabbit immunoglobulin G (IgG) molecules from whole sera, which cannot specifically bind antigen H1, were used. Finally, the substrate with the antigen–antibody complex was rinsed with DI water for 10 min and subsequently dried by blowing with N$_2$.

3. Results and discussion

To confirm the signal-enhancing behavior of the nanoforest structures, we prepared two reference samples: pristine glass and bare gold film. On the pristine glass, $10^{-2}$ M BCB solution was used to obtain a detectable Raman signal. The BCB solution was drop-evaporated on the nanoforest structures and sampled using a Raman spectrometer with 633 nm laser excitation. A detailed description of the measurement is given in section 2. Compared to the signals generated by the pristine glass, those from the gold film were one order of magnitude stronger even at a 100-fold lower concentration of analyte. Moreover, the nanoforest generated more than $10^4$ times the signal intensity of the gold film (figure 2(A)). We quantified the enhancement factor using the following equation [42]:

$$EF = \frac{C_{ref} I_{SERS}}{C_{SERS} I_{ref}},$$

where $C_{ref}$ and $C_{SERS}$ indicate the concentration of the molecules, and $I_{ref}$ and $I_{SERS}$ indicate the signal intensity at a Raman shift of 581 cm$^{-1}$, which is the position of the main peak of BCB. The calculated enhancement factor of the nanoforest was of the order of $10^7$ relative to pristine glass, and $500$ relative to bare gold film.

Because the porous silicon surface after etching originates from the voids in the initial thin gold layer, we can control the density of the nanogaps by controlling the initial thickness of the gold layer. A thicker initial gold layer produces a smaller void density, which results in lower gold nanogap density. A primary purpose of this experiment is to find out whether the nanogap is acting as a hot-spot and to provide a guideline for the optimization of the structure. We prepared three samples with different initial gold layer thicknesses, namely 70, 100, and 150 Å. Scanning electron microscopy (SEM) images of the samples before etching (i.e. after initial gold deposition) are shown in supporting information available at stacks.iop.org/Nano/23/095301/mmedia, and the resulting nanoforest samples are shown in the insets of figure 2(B). We compared the enhancement factor calculated at a Raman shift of 581 cm$^{-1}$. The etching time was fixed to 30 s for all the samples, so the density of the nanogap was only different among samples. The results showed that a larger density of the nanoforest generates a higher enhancement factor. This trend strongly suggests that the gold nanogaps act as the main hot-spots in the substrates as expected. Fewer gold nanogaps clearly imply a lower hot-spot density, which results in weaker enhancement.
Samples with different nanogap depths were also fabricated to determine the optimum nanoforest height (figure 3(A) and figure S2 available at stacks.iop.org/Nano/23/095301/mmedia). The depth of the nanogaps can be controlled by etching time in the HF/H$_2$O$_2$ solution. The etch rate was about 3.0 nm s$^{-1}$ for the first 30 s and increased to about 13.8 nm s$^{-1}$ at longer etching times (figure 3(B)). Prior to the SERS measurement, the sample electric field was simulated using a commercial package (COMSOL) [43]. The cross-sectional geometry of the nanoforest structure was modeled as an array of periodic nano-pillars. The nanogap widths and inter-nanogap distances were assumed to be 20 nm. The coverage of evaporated gold at the nano-pillar steps was considered zero, so the gold at the side walls was ignored [20]. The wavelength of the light was 633 nm and the light was projected vertically to the surface, which is the same as the condition in real experiments. A transverse magnetic (TM) wave was used for the simulation. It should be noted that in an actual device there are other uncontrollable structural effects such as random sized nano-crevices and nano-size fractal shapes. Even though the simplified model did not reflect the random effects on the simulations property, the overall enhancement trend can be extracted with high accuracy due to the averaging effect. Since the diameter of the laser spot is approximately 500 nm, more than a few hundred of the hot-spots contribute to determining the measured enhancement factor. The simulation snapshots for the modeled nanoforest are shown in figure 3(C). The color map represents the ‘scattered electric field’, which is a crucial factor in determining the signal enhancement. The enhancement factor (EF) was extracted using the following well-known equation [17]:

$$EF \sim \frac{\int_C |E_{\text{scat}}|E_{\text{inc}}|^4 \, ds}{\int_C |E_{\text{inc}}|^4 \, ds}.$$  

The integration path ‘C’ was defined as a square-shaped closed line along the side wall and bottom floor inside the nanogap. $E_{\text{inc}}$ represents the electric field of the incident light and $E_{\text{scat}}$ represents the scattered electric field obtained from the simulation. The results illustrate that two types of hot-spots exist: inter-pillar hot-spots along a horizontal plane and inter-layer hot-spots along a vertical plane. The strength of the inter-pillar hot-spot was determined by the width of the nanogap and did not change with the depth of the nanogap. However, the strength of the inter-layer hot-spot varied with depth of the nanogap. When the depth was shallow, the top and bottom layers were not separated, and hence the inter-layer hot-spot was absent (figure 3(C), left). After the two layers were separated, the inter-layer interaction was triggered, which produced a strong hot-spot (figure 3(C), center). As the distance between the two layers increased, the strength of the inter-layer hot-spot decreased and eventually disappeared (figure 3(C), right). As a result, the total enhancement factor curve showed a maximum with respect to etched depth. The experimental results matched well with the relationship obtained from the simulation (figure 3(D)), in which one data group contains seven measurements at random points. The maximum enhancement factor was approximately 3 x 10$^7$ for an etching time of 30 s and was reduced to factors of 10$^6$ with further etching. This value of enhancement factor implies that it is possible to detect a single resonant molecule using this optimized nanoforest substrate.

The nanoforest structure with optimized morphology was then used to demonstrate its practical application as a SERS immunosensor. The resonant molecules such as BCB or Rhodamine 6G generate a sufficiently high signal that even normal Raman spectroscopy can easily detect; however, direct detection of real pathogen molecules is more important in practical applications such as proteomics and medical diagnostics and is considered to be a more difficult task. Since the nanoforest has a very large surface to volume ratio and strong enhancement factor, we can directly capture the signal difference from antigen–antibody specific binding. Figure 4(A) describes the overall biosensing procedure, which was explained in section 2. An initial gold layer thickness of 70 Å and an etching time of 40 s were used for the experiment. The SERS spectrum of GBP–H1 ([39–41]; see supporting information available at stacks.iop.org/Nano/23/095301/mmedia) showed a main band at a Raman shift...
Figure 3. (A) Cross-sectional SEM images for the nanoforest before secondary gold deposition with various etching times, namely 10, 30, 50, and 60 s. (B) Relationship between the etching time and the etched depths of the nanogaps. The etchant solution was composed of HF/H_{2}O_{2}/DI water (2:1:7, v/v/v). (C) Schematic depiction of the simulation results. Silicon and gold sections of the substrates are illustrated differently for clarity. The color map represents the scattered electric field. (D) Experimental and simulated results from samples with different nanogap depths. The magnitude of the simulation result was linearly scaled using a fixed constant.

of 1578 cm\(^{-1}\), which corresponds to the \(\nu_{as}(\text{COO}^-)\) peak, and several minor peaks in the range of 900–1600 cm\(^{-1}\) (figure 4(B)). The spectrum is similar to previous spectra from other viruses, particularly rhinovirus [34]. After the GBP–H1 was immobilized, anti-rabbit IgG purified using a protein A column from the whole sera (negative control) and anti-H1 antibody molecules (experimental group) were added to two different nanoforest samples that contained the GBP–H1 antigens. The control sample exhibited almost the same spectrum as that with only GBP–H1 antigens. Because the anti-rabbit IgG molecules cannot specifically bind to the H1 antigens, the molecules were washed out during the rinsing step, and the SERS spectrum therefore did not change. However, when the anti-H1 antibodies were combined with the GBP–H1 antigens, the spectrum underwent a significant change. The largest change occurred at Raman shifts of 993 cm\(^{-1}\) and 1525 cm\(^{-1}\), which correspond to the phenylalanine (Phe) and tryptophan (Trp) peaks, respectively. Quantitative analysis of the normalized Raman intensity was performed on these peaks. Seven spectra for each sample were measured and compared. The spectra were normalized to the intensity of the main peak (1578 cm\(^{-1}\)). The average and standard deviations for the seven sample spectra are presented as bar graphs with error bars (figure 4(C)). At a Raman shift of 993 cm\(^{-1}\), the peak intensity decreased after anti-H1 immobilization, whereas the anti-rabbit IgG molecules caused no change in peak intensity. On the other hand, at a Raman shift of 1525 cm\(^{-1}\), a new minor peak appeared after anti-H1 immobilization. As a result, we were able to ascertain the presence of anti-H1 antibodies by comparing the differences in the SERS peak intensities at this spectral position.

4. Conclusions

In summary, we have proposed and characterized a nanoforest structure as a practical SERS-active substrate. The fabrication process was based on metal-induced chemical etching. The density of the nanogaps in the resulting porous surface was
Figure 4. (A) Schematic of the immunosensing procedure. First, GBP–H1 viral antigens were attached to the gold walls of the nanoforest structure. Then, anti-H1 antibodies or anti-rabbit IgG antibodies were added to the GBP–H1 molecules. Anti-rabbit IgG molecules cannot bind to the GBP–H1 antigens and were washed out during the subsequent rinsing step, whereas the anti-H1 antibodies remained specifically bound to the H1 antigen. (B) SERS spectra of the three preparations. The black spectrum (bottom) was observed with only GBP–H1 molecules bound to the substrate. The subsequent addition of anti-rabbit IgG molecules and anti-H1 antibodies resulted in the SERS spectra shown in blue and red, respectively. (C) Relative intensities of the SERS bands at 993 cm$^{-1}$ and 1525 cm$^{-1}$, respectively. The intensity at 1578 cm$^{-1}$ was set to 1.0 and all spectra were normalized relative to this value. Seven samples were used for each condition, and standard deviations of the sample sets are presented as error bars.

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