Electrochemical Synthesis of 3D Plasmonic-Molecule Nanocomposite Materials for In Situ Label-Free Molecular Detections

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For several decades, the precise positioning of chemicals and biomolecules at nanosized electromagnetic hotspots has posed a challenge to achieving ultrasensitive and reliable label-free molecular detection. Here, the authors report the rapid ultrasensitive direct detection of chemicals and biomacromolecules (i.e., enveloped virus) through the in situ electrochemical synthesis of a 3D plasmonic-probe molecule composite skin layer on a 3D Au nanopillar array. The bottom-up growth of plasmonic nanomaterials in the presence of target probe molecules provides intimate contact between the Au and the target molecules, thereby enhancing light–matter interactions in 3D spaces. These enhanced interactions result in highly sensitive and rapid direct detection of both small molecules and large influenza A virus (H1N1) with hierarchical complex structures. The virus serves as a structural motif for the formation of Au–virus nanocomposite structures through Au electrodeposition, which results in the in situ formation of hotspots for the surface-enhanced Raman spectroscopy (SERS) detection of spike proteins. The proposed SERS detection system with fast composite plasmon-molecule skin layer formation provides a general platform for highly sensitive and rapid label-free direct detection for chemical and biomedical applications.

1. Introduction

High-density plasmonic nanostructures with discrete nanoscale gaps enable ultrasensitive molecular detection only when the target molecules are located at the hotspots. The random positioning of target molecules results in poor reproducibility and reliability for surface-enhanced Raman scattering (SERS)-based molecular detection. Therefore, the precise placement of infinitesimal molecules in a 3D bulk solution at the hotspots remains a goal for attaining both ultrasensitive and reproducible label-free molecular detection. Some approaches for positioning target molecules have been proposed, including enhancing molecular interactions using bioreceptors and carrying out electrokinetic actuations. The receptor molecules provide specific binding sites for target molecules. However, because the binding events between receptor and target molecules are highly dependent on the molecular diffusion of target molecules in the bulk solution, real-time detection is difficult to achieve using this passive diffusion process. For solution-based detection systems, electrokinetic actuation is considered a promising method to concentrate charged small molecules at hotspot regions through electrophoresis. However, because of the size mismatch between nanoscale hotspots and large macrobiomolecules, these conventional SERS platforms are not well adapted to label-free and rapid detections of respiratory viruses. Although ≈100 nm virion particles can be attracted to a SERS-active surface via electrophoresis, they may not fit onto nanoscale hotspots because of their structural complexity and large size.

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To address these limitations and expand the applications of SERS-based detection for both small and large molecules, we developed an in situ ultrasensitive direct detection platform through the rapid formation of a 3D plasmonic-molecule nanocomposite skin layer on an Au nanopillar array. The in situ electrochemical deposition of Au in the presence of target molecules enhances light–matter interactions by fully utilizing intimate contact between the Au and the target molecules across the entire thickness of the 3D nanocomposite layer. This intimate contact results in highly sensitive and rapid direct detection of both small toxic molecules (e.g., thiabendazole, TBZ) and large influenza A (H1N1) virus. In the case of small uncharged TBZ molecules, in situ Au deposition led to the formation of an Au–TBZ nanocomposite skin layer on a 3D Au nanopillar array, which resulted in SERS signal amplification of TBZ molecules within 10 s after a potential was applied. To ascertain the applicability for macromolecular detection, influenza A virus subtype H1N1 was used as the probe target. Influenza viruses are common respiratory pathogens that pose a severe threat to human health worldwide, infecting more than 10% of the global population annually.[10] The rapid and accurate detection of viral infections in their early stages is critical to minimize seasonal outbreaks and control pandemics. The main conventional detection methods for viruses are the enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) methods; however, these techniques require long analysis times[11–13] and have limited applicability in point-of-care diagnostics. We found that applying an electric potential to an Au nanopillar electrode can attract viral particles to the Au nanopillar array via electrostatic interactions, with simultaneous electrodeposition of an Au–virus nanocomposite skin layer, providing ultrasensitive (i.e., limit of detection (LOD) of 0.05 ng mL⁻¹) and label-free direct detection of the virus within 1 min of electrodeposition. This bottom-up electrodeposition of Au–molecule composite nanomaterials leads to an enhanced SERS signal irrespective of the structural complexity and size of the target. The proposed method provides high versatility and expandability for rapid on-site analysis for chemical and biomedical detections.

2. Results and Discussion

2.1. Electrochemical Synthesis of Au–Molecule Nanocomposite Materials

Figure 1a shows a schematic of the experimental setup for the simultaneous electrochemical synthesis of an Au–molecule nanocomposite layer and in situ acquisition of the Raman spectra. The fabrication process of the Au nanopillar array (NPA) using maskless plasma etching of a polyethylene terephthalate (PET) substrate and Au thermal evaporation has been reported elsewhere.[14,15] An Au NPA electrode was inserted at the bottom of an electrochemical cell containing an Au precursor (i.e., tetraethylchloroauric acid, HAuCl₄) and probe molecules. The incident laser was precisely focused onto the Au NPA surface during the entire electrochemical synthesis, and the Raman spectrum was recorded every 2 s to monitor the SERS signal on the Au NPA substrate. The application of an electric potential induced the deposition of a 3D Au–molecule nanocomposite skin layer onto the Au NPA electrode by reducing the Au precursor to clustered Au atoms (i.e., Au nanoparticles [NPs]) while trapping probe molecules on the growing Au nanostructures. Cathodic currents from the solution containing probe molecules and HAuCl₄ were measured during application of external electric potential and confirmed the Au electrodeposition process (see details in Figure S1, Supporting Information).

As proof-of-concept, TBZ, a small fungicide molecule, was used as a probe molecule for analysis. Figure 1b shows the temporal evolution of the SERS spectra during the entire Au electrodeposition process onto the 3D Au NPA electrode. Initially, the Au NPA electrode in the 0.1 m NaCl aqueous solution containing 10 ppb TBZ and the Au precursor exhibited a weak SERS signal; however, the spectrum showed several distinct characteristic TBZ SERS peaks at 1142, 1270, 1392, and 1542 cm⁻¹.[16–19] The electrochemical-SERS response of TBZ was further analyzed as an external electric potential was applied to the electrode. Upon application of an electric potential (+0.3 V versus Ag/AgCl) to the 3D Au NPA electrode, the SERS
signal of TBZ was amplified and new strong peaks appeared in the Raman spectrum. New characteristic SERS peaks at 863, 1010, 1270, and 1602 cm$^{-1}$ were amplified within 15 s and remained stable and strong as the potential was applied for an additional 15 s (peak assignments for TBZ is summarized in Table S1, Supporting Information). The SERS signal obtained by application of +0.3 V in the presence of HAuCl$_4$ was enhanced ≈24 times compared with the initial signal intensities of TBZ measured for the pristine 3D Au NPA substrate. The strong SERS signal remained stable even after the potential was terminated, as shown in Figure 1b. We speculated that, because TBZ contains a S atom in its molecular structure, strong Au–S bonds formed, enabling the stable SERS signal to be detected even after completion of the Au electrochemical deposition. We investigated the SERS response of other molecules containing S atoms (e.g., methylene blue and tricyclazole) and compared the observation with a molecule without S (e.g., bisphenol A) in order to support the hypothesis. It is observed in Figure S2, Supporting Information, that methylene blue and tricyclazole exhibit similar response as TBZ on potential termination, as their respective SERS signal intensities were strongly maintained. In the case of bisphenol A, signal intensities rapidly decreased on potential termination, which is attributed to the molecular diffusion of molecules from the electrode surface. It confirms that the Au–S bonds contribute to SERS signal stabilization after the potential termination.

2.2. SERS Enhancement by 3D Au–Probe Molecule Composite Formation

To confirm the formation of a 3D Au–molecule nanocomposite layer on the Au NPA, we analyzed the surface morphologies resulting from different fabrication process and the chemical compositions of the electrode after the electrodeposition of Au in the presence of 1 ppm TBZ. Scanning electron microscopy (SEM) images of the pristine Au NPA electrode (Figure 2a) and the Au NPA electrode (Figure 2b,c) after electrodeposition in the presence of TBZ and HAuCl$_4$ were compared. The pristine Au nanopillars exhibit a high aspect ratio of 3.7 and a diameter of ≈120 nm at an areal density of 23 ± 3 μm$^{-2}$ (Figure 2a). The Au nanopillars electrodeposited under an applied potential of +0.3 V for 30 s in the presence of 1 ppm TBZ and 3 mM HAuCl$_4$ exhibit a lower areal density of 14 ± 2 μm$^{-2}$ because of merging and clustering among adjacent Au nanopillars as a result of the electrodeposition of additional Au (Figure 2b,c).

The formation of a highly porous Au electrodeposited layer on the Au nanopillars was confirmed by high-resolution SEM images showing a) a tilted view of the as-prepared Au NPA electrode and b) top and c) tilted views after a +0.3 V potential was applied for 30 s in the presence of TBZ (1 ppm) and HAuCl$_4$ (3 mM). d) Transmission electron microscopy (TEM) images of 3D nano-composite nanostructures after Au electrodeposition in the presence of TBZ and HAuCl$_4$. e) A zoomed-in TEM image of the yellow dashed box in (d). f) High-resolution TEM image of a 3D Au–TBZ nanocomposite shell layer formed on the Au NPA. An interconnected 30 nm-thick Au layer was formed on the 3D Au nanopillar. g) Depth profiling of chemical compositions for 3D Au–TBZ composite nanostructures using time-of-flight secondary-ion mass spectrometry. h) Electric-field distribution of 3D Au–TBZ composite nanostructures for the wavelength of 785 nm, as calculated by FDTD simulation.
transmission electron microscopy (TEM) images (Figure 2d–f). The electrodeposited Au layer is ≈30 nm thick and densely interconnected Au NPs with a diameter of ≈3 nm are distributed throughout the thickness (Figure 2e). The 30 nm-thick Au electrodeposited shell contains six or seven stacked layers of interconnected and densely packed Au NPs with nanogaps (Figure 2f). The interparticle and interlayer spaces in the shell measure ≈1 nm, which implies that the shell is occupied by TBZ molecules. To verify the incorporation of TBZ molecules into the electrodeposited shell layer on the Au nanopillars, samples were analyzed by time-of-flight secondary-ion mass spectrometry (ToF-SIMS) after being subjected to sputtering for various times (Figure 2g). Because a pulsed Bi⁺-ion beam removed molecules from the outermost surface of the 3D electrodeposited nanostructures at the initial sputtering times, we concluded that highly concentrated S⁻ ions were main components of the outer composite shell layer (red dotted square region in Figure 2g). High concentrations of S⁻ ions were detected on the top layer of the 3D composite nanostructures because TBZ molecules were intercalated and trapped during the Au electrodeposition process. The Cl⁻ ions originate from the NaCl electrolyte and the S⁻ ions are detected from the 1 ppm TBZ probe molecules, whose S atoms form Au–S bonds in the composite shell layer. The fact that Au⁺ ions are present at low areal density (compared with Au⁻ ions in the Au nanopillar region) is further evidence that the outermost shell region contains composite materials that include Au- and S/C-containing molecules (i.e., TBZ molecules).

The dense nanogaps among the multistacked Au NPs in the Au–TBZ nanocomposites on the surface of nanopillars function as strong hotspots for amplification of the Raman signal mainly due to localized surface-plasmon resonance (LSPR) coupling effects.⁴,¹⁴,¹⁵ We used the finite-difference time-domain (FDTD) method to calculate the electric-field distribution in the nanostructure modeled from the TEM observations (Figure 2f), as shown in Figures 2h. For plane-wave radiation with a wavelength of 785 nm, the electric field is intensified at the nanogaps between randomly distributed 3 nm Au NPs by LSPR plasmonic coupling effects. In particular, the intensification is stronger at the side surface of the nanopillar than at the top surface. We attribute this greater intensification to efficient LSPR coupling as a result of the parallel alignment of nanogaps along the polarization direction of the incident light.²⁰ Notably, the dense hotspots in the entire cross-section of the 30 nm-thick nanocomposite layer are fully utilized to amplify the Raman signal.

2.3. Optimization of In Situ Au Electrochemical Deposition

The operation parameters (i.e., the applied potential and precursor concentration) of the Au–TBZ nanocomposite shell layer on the Au NPA electrode were investigated and optimized (Figure S3, Supporting Information). Because the redox potential of AuCl₄⁻ is approximately +0.79 V, AuCl₄⁻ can accept electrons from the Au electrode at a potential lower than its redox potential (i.e., potentials more negative than +0.79 V) and grow into Au NPs as a flux of AuCl₄⁻ near the electrode is reduced to Au atoms. Therefore, we varied the applied potential from +0.5 to −0.3 V in the presence of 3 mM HAuCl₄ and 1 ppm TBZ for the Au reduction. The application of a potential of +0.3 V in the presence of 3 mM HAuCl₄ resulted in the strongest TBZ SERS signal (Figure S3, Supporting Information). Under these operating conditions, we performed sensitivity tests for different TBZ concentrations. The characteristic SERS peaks of TBZ at 1185 (ring stretch), 1295 (ring stretch), and 1644 cm⁻¹ (C=N stretch) were clearly observed even at a very low TBZ concentration of 0.05 ppb (Figure 3a) and exhibited good linear correlation at TBZ concentrations between 10 and 0.05 ppb, with R² values of 0.995, 0.995, and 0.991, respectively (Figure 3b). The LOD of 0.05 ppb shows superiority to the LOD of other reported approaches for TBZ detection as summarized in Table S2, Supporting Information. These results demonstrate that the SERS monitoring technique combined with in situ 3D Au–TBZ nanocomposite shell formation can reliably improve the sensitivity for rapid and label-free direct molecular detection.

Figure 3. SERS performance of the 3D Au–TBZ composite shell layer on the Au NPA. a) SERS spectra of TBZ recorded at various concentrations at a +0.3 V electric potential and b) the linear correlation between the SERS signal intensities at 1185, 1295, and 1644 cm⁻¹ as a function of the TBZ concentration. The error bars indicate the standard deviations for five replicate measurements.
2.4. In Situ SERS Detection of Influenza A (H1N1) Virus

We further investigated the direct molecular detection technique using in situ electrochemical synthesis of an Au–probe molecule nanocomposite shell for respiratory viral pathogens. The typical size of influenza A virus is ≈100 nm, and it is enveloped in glycoproteins on its outer surface membrane. Thus, conventional SERS substrates with dense nanogaps cannot generate a strong Raman signal from these large macromolecules. To address this problem, we directly grew Au nanostructures in the presence of 100 nm-sized whole virus particles, which is unprecedented. Because the hierarchical virions provide a structural motif or template for the Au reduction and growth, the bottom-up Au deposition intrinsically forms 10 nm-scale Au–envelope protein nanocomposites on the Au nanopillars during the Au electrodeposition process.

Influenza virus detection was carried out in the presence of 3 mM HAuCl₄ under an applied potential of +0.3 V. During the electrodeposition process, Raman measurements were performed every 30 s using a 785 nm laser. First, SERS spectra were recorded for the virus solution on a pristine Au NPA electrode; the resultant spectra showed no SERS signal. Upon addition of HAuCl₄, no change in the SERS signal was observed (Figure 4a-i). A potential of +0.3 V was then applied to the electrode, and the SERS spectra were collected. The SERS signals emerged and were clearly discernable after the potential had been applied for 60 s. The potential was applied for an additional 10 min, and the peak intensities gradually increased and saturated at 3 min (Figure 4a-ii). To accurately assign Raman signals characteristic to the influenza virus and eliminate interferences, the Raman spectra were compared with the background signals of the buffer analyte (i.e., IX PBS). In Figure S4, Supporting Information, the influenza A (H1N1) virus signal was differentiated and peaks that overlapped with PBS were not considered for qualitative and quantitative analyses. SERS peak assignment of the target virus particles⁹,⁰,¹¹,²¹–²³ is summarized in Table S3, Supporting Information. When the potential was terminated, the SERS response was observed to be inconsistent with that of TBZ and the SERS intensities of the enhanced signals decayed with time (Figure 4a-iii). From this temporal evolution for an entire Au electrochemical deposition process in the presence of influenza virus, the mechanism of Raman signal enhancement can be elucidated.

2.5. SERS Signal Enhancement Mechanism of Au–Influenza A Virus Composites Layer

The initial absence of SERS signals from the viral particles is presumed to be due to the weak light–matter interactions between virions in the bulk solution and Au nanopillars. At +0.3 V in the presence of HAuCl₄, A/H1N1 virions in the solution were drawn closer to the 3D Au NPA electrode via electrophoretic attraction. The electrophoretic movement is explained by the isoelectric point (IEP) of the virus, which establishes its net surface charge.²⁴,²⁵ In solution, viruses maintain a

Figure 4. Label-free influenza virus A (H1N1) detection through close interaction of electrodeposited Au and envelope proteins of influenza virus. a) Temporal evolution of the influenza virus Raman spectra. The influenza virus solution and HAuCl₄ were added consecutively to a 0.1 M NaCl electrolyte solution at 2 min interval and electric potential was applied for 10 min. The potential was then terminated to complete the Au electrodeposition process. b) Cross-sectional view of the electric-field distribution of 3D Au–influenza virus composite nanostructures for the wavelength of 785 nm. Inset shows a modeling of 100 nm virion-forming envelope proteins with a cylindrical shape bridged the two Au nanopillars. A 60 nm-thick electrodeposited Au layer was modeled as the undercoating of the virion. c) Top-view of the electric-field distribution of the nanocomposite structures sectioned along the white solid line in (b). d) SERS signal comparison between influenza virus and its envelope proteins; HA and NA. e) SERS spectra of influenza virus dissolved in PBS buffer solution at various concentrations, as denoted. f) SERS intensity at 1583 and 1639 cm⁻¹ as a function of the concentration of influenza virus solution. The error bars indicate the standard deviations for five replicate measurements.
pH-dependent charge that influences their adsorption and movement under electric fields. A distinct factor affecting the equilibrium of a virus particle in solution is the ability to switch its surface charge in correlation to the pH of the specified solution; the pH at which this switch occurs is defined by its IEP.\(^{[26]}\)

For the influenza A (H1N1) virus, the reported IEP values range between 4 and 4.5.\(^{[26]}\) The virus is therefore assumed to possess a net negative charge at pH values greater than the IEP and a positive charge at lower pH values. In the Au electrodeposition system, the pH of the analyte was recorded as 3.1, indicating that the net surface charge of the virus particles is positive. Consequently, for the virus to be attracted to the electrode, a negatively charged surface is required. As shown in Figure S1, Supporting Information, the open-circuit potential (OCP) of the electrode in the presence of HAuCl\(_4\) was +0.8 V (versus Ag/AgCl); with reference to the OCP, the electrode surface charge is made negative\(^{[27, 28]}\) at +0.3 V, leading to preconcentration of the virus particles at the Au electrode surface as a result of electrophoretic attraction. The virions with hierarchical structures subsequently adsorb onto the Au NPA electrode surface and are confined, forming an Au–virus nanocomposite layer during the electrodeposition process. We calculated the electric-field distribution in the Au–virus nanocomposite structure bridging two Au nanopillars using the finite-difference time-domain (FDTD) method (Figure 4b,c). While the virus particles were structurally enveloped with ∼10 nm-sized surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), the 10 nm gaps between electrodeposited Au nanostructures exhibited a strong electric-field intensity due to LSPR effects, which resulted in sensitive SERS detection of the surface spike proteins of the virus. To confirm this effect, the SERS spectra of purified envelope proteins of A/H1N1 virus, that is, HA and NA, were individually recorded. In Figure 4d, the virus signal (background subtracted) is traced to both viral proteins, with HA showing a more dominant factor for influenza A virus SERS detection. This observation is consistent with our expectations because HA is the most abundant glycoprotein on the surface of influenza virus.\(^{[29]}\) In addition, HA is a cylindrical-shaped integral membrane protein (type I transmembrane glycoprotein) with an approximate length of 13.5 nm and varying between 3.5 and 70 nm along its radial direction, resulting in spikes that project externally.\(^{[30, 33]}\)

We varied the influenza virus concentration to investigate the detection sensitivity of the technique. When a potential of +0.3 V was applied in the presence of 3 nM HAuCl\(_4\), a LOD of 0.05 ng mL\(^{-1}\) was obtained (Figure 4e). The concentration dependence of the SERS signal intensities shows a non-linear Hill isotherm at 1583 and 1639 cm\(^{-1}\) (Figure 4f) by the equation:\(^{[32]}\)

\[
Q = \left( \frac{Q_s \cdot C^n}{K + C^n} \right)
\]

where \(Q\) represents amount of adsorbate on the substrate surface at equilibrium, \(Q_s\) is the surface saturation, \(C\) is the adsorbate concentration, and \(K\) and \(n\) are the Hill's constant and the adsorption coefficient, respectively. At 1639 cm\(^{-1}\), a linear correlation was obtained for concentrations between 100 and 0.05 ng mL\(^{-1}\) with an \(R^2\) value of 0.970. With respect to the bulk solution volume, the detection limit of H1N1 virus is considered ≈10\(^4\) PFU/mL. A comparison of the detection limit with other reported works for influenza virus detection is summarized in Table S4, Supporting Information. These results demonstrate the sensitivity of the developed technique and its reliability in detecting large biomolecules in various solution-based SERS systems.

2.6. Confirmation of SERS Temporal Response for Au–Influenza Virus Nanocomposite Formation

The signal decay after the applied potential is terminated is attributed to the desorption of trapped virus particles from the 3D Au NPA electrode surface. To support this hypothesis, we analyzed the 3D Au electrode surface by SEM after terminating the applied potential. Figure 5a shows the morphology of the 3D NPA electrode after the potential was applied for 10 min (+0.3 V) in HAuCl\(_4\) without virus particles. The Au nanopillars were highly connected and merged after the 10 min of additional Au electrodeposition. In comparison, the surface morphology after the potential was applied when virus particles (1 μg mL\(^{-1}\)) were present exhibited numerous cavities or empty pits (white circles representing single viral particles and black circles representing clustered viral particles) in the thickened Au layer, as depicted in Figure 5b. The empty pits measured ∼100 nm in diameter and were considered the adsorption sites of the virus particles under an applied potential (Figure 5c).

We justified our assumption by measuring a lower virus concentration (1 ng mL\(^{-1}\)). As shown in Figure 5d, the proportion of empty pits corresponding to a 1 ng mL\(^{-1}\) virus concentration was unequivocally smaller than that corresponding to a 1 μg mL\(^{-1}\) virus concentration, confirming that the empty pits were indeed sites occupied by the H1N1 viruses. Thus, the virions adsorbed onto the 3D Au nanopillar surface when a potential was applied but desorbed after the potential was terminated. Our hypothesis was further confirmed in experiments using 10 nm NA proteins (Figure 5e) and 50 nm SiO\(_2\) NPs (Figure 5f). The surface morphology after potential termination for the 3D NPA electrodes with NA proteins present showed ∼10 nm empty pits (white circles) and several tens of nanometers of spherical-shaped NPs budding on the 3D Au NPA, which indicates that some proteins can be completely covered by the electrodeposited Au layer and cannot escape from the electrode surface even after the potential is terminated. This situation also applies to inorganic SiO\(_2\) colloidal particles. The 50 nm SiO\(_2\) NPs function as a structural motif for the Au–SiO\(_2\) nanocomposite shell layer on the 3D Au electrode during the electrodeposition process, and most of the SiO\(_2\) NPs leave the nanocomposite shell after the potential is terminated, which results in half-sphere empty pits on the electrodeposited Au layer. Notably, a small portion of the SiO\(_2\) NPs is captured on the thickened Au layer (Figure 5f). These Au–SiO\(_2\) composite nanomaterials can also be used for the plasmon-enhanced spectroscopy.\(^{[33]}\)
3. Conclusions

We developed a new technique of using in situ electrochemical synthesis of nanocomposite shells to improve light–matter interactions for sensitive direct molecular detection. We concluded that our electrochemical synthesis of 3D Au–probe molecule composite nanomaterials is highly relevant for use in in situ label-free molecular detection with high sensitivity for both small uncharged molecules and large complex biomolecules such as whole viral particles. We further provided direct evidence of the formation of a plasmonic-molecule nanocomposite shell layer during the electrochemical synthesis. The results presented here shine light on the practical and diverse application of electrochemistry in SERS platforms, and we believe that our proposed approach represents a novel path for further exploring the electrochemical-SERS technique for improved molecular sensing applications.

4. Experimental Section

Materials: Thiabendazole (TBZ), gold chloride trihydrate (HAuCl₃·3H₂O), potassium phosphate monobasic (KH₂PO₄), potassium phosphate dibasic trihydrate (K₂HPO₄·3H₂O) and silica nanospheres (SiO₂, 50 nm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl) and deionized water were purchased from Samchun (Pyeongtaek, South Korea). Phosphate-buffered saline (PBS) was purchased from Tech and Innovation (Gangwon, South Korea). Hemagglutinin (ab217652) and neuraminidase (ab219893) were purchased from Abcam (Cambridge, UK). Phosphate buffer solution (0.1 m, pH 7.18) was prepared using KH₂PO₄ and K₂HPO₄·3H₂O.

Fabrication and Characterization of Substrates: Au SERS substrates were fabricated using 250 µm-thick PET substrates. Nanopillar structures were first generated on the PET substrates by treating the substrates with CF₄ plasma (plasma power: 100 W) flowing at 3 standard cubic centimeters per minute (sccm) under a pressure of 56 mTorr for 2 min.

The substrates were further treated with Ar plasma for 1 min (plasma power: 100) using a 13.56 MHz radio-frequency ion etching instrument (SNTEK). Au was deposited onto the generated nanopillars to a thickness of 200 nm at a deposition rate of 2.0 Å s⁻¹ under a pressure of 7 mTorr using a sputtering system (SNTEK). The morphologies of the Au SERS substrates were characterized by field-emission scanning electron microscopy (FE-SEM; Joel JSM-6700F).

Virus Preparation: Influenza A virus strain A/Puerto Rico/8/34 (H1N1; PR8) was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and propagated in 10-day-old embryonated chicken eggs at 37 °C for 3 days. Virus was purified using ultracentrifugation according to the authors’ previously described method. Viral titer was determined by plaque assay in Madin–Darby canine kidney (MDCK; ATCC) cells. For biosafety, the virus was inactivated using 0.02% formalin at 4 °C overnight, followed by ultracentrifugation to remove the formalin. The concentration of virus dissolved in PBS was determined to be 0.73 mg ml⁻¹, corresponding to 2.86 × 10⁸ plaque forming units (PFU)/mL. Inactivated viral stocks were stored at −80 °C before use.

Instrumentation: A ZIVE SP2 Wonatech potentiostat was synchronized with an Ocean Optics portable probe spectrometer system (UQEPRO-Raman) to conduct simultaneous electrodeposition and SERS measurements. The wavelength of the spectrometer laser source was 785 nm (theoretical spot size: 158 µm). A custom-built electrochemical cell that held the PET/Au nanopillar working electrode, Ag/AgCl (in 3 M NaCl) reference electrode, Pt counter electrode, and an analyte was employed. The cell had a perforation on its upper side that exposed the PET/Au electrode to the analyte solution. The total analyte volume in the cell was 400 µL.

TBZ SERS Measurements: The PET/Au electrode surface was first electrochemically cleaned using cyclic voltammetry (CV). The potential was cycled between 0 and 1.1 V at a scan rate of 0.05 V s⁻¹ in 400 µL phosphate buffer solution (0.1 m). The buffer solution was changed after the potential was cycled and the measurement was repeated until a reproducible cyclic voltammogram was obtained. The cell was then rinsed several times with deionized water and filled with 0.1 M NaCl aqueous solution (384 µL) as the supporting electrolyte. Four microliters of 1 ppm TBZ solution (dissolved in ethanol) was added to the electrolyte and SERS spectra were recorded for 10 s. Afterwards, 12 µL
of 100 mM HAuCl₄ (dissolved in deionized water) was added to the TBZ analyte. After careful mixing, the solution was exposed to the laser. The final concentrations of TBZ and HAuCl₄ in the solution were 10 ppb and 3 mM, respectively. SERS spectra were collected for 10 s, after which a constant potential (+0.3 V) was applied to the electrode for 30 s. SERS data were acquired in real time during entire potential application period and continuously for 30 s after the potential was terminated. The laser power used for measurements was 40 mW. All electrode potentials were reported versus Ag/AgCl reference electrode.

Protein/Virus SERS Detection: The PET/Au electrode surface was electrochemically cleaned using the CV method previously described. After the cleaning procedure, 5 µL of 0.1 µg mL⁻¹ protein or virus (dissolved in 1× PBS) solution was dropped onto the electrode and 380 µL of 0.1 M NaCl solution was added. 12 µL of 100 mM HAuCl₄ was then added to the analyte and carefully mixed. The solution was exposed to the laser and SERS spectra were acquired for 90 s. Afterward, +0.3 V was applied to the electrode for 10 min and SERS spectra were recorded at 30 s integration time and 40 mW laser power.

ToF-SIMS Depth Profiling: ToF-SIMS experiments were performed with a ToF-SIMS V (ION-TOF, Münster, Germany) instrument in Korea Basic Science Institute (KBSI), Busan center using a pulsed 30 keV Bi⁺ primary beam with a current 0.58 nA. For depth profiling, a Cs⁺ ion sputter beam (2 keV, 104 nA) and Ar gas cluster source were operated in dual-beam mode (polarity: negative mode). The analysis area and sputter area were 200 µm × 200 µm and 500 µm × 500 µm, respectively. Negative-ion spectra were internally calibrated using H⁺, C⁺, C₂⁺, C₃⁺, and C₄⁺ peaks normalized to the respective secondary total ion yield. The chemical images of the analyzed area were recorded with 128 × 128 pixel resolution during the data acquisition.

Numerical Simulations: The numerical simulations were carried out using the FDTD Solution software (Ansys, Inc. 2021 R1.2). The nanostructure was sketched using Autodesk Fusion 360 on the basis of TEM observations and the sketch was exported to the FDTD software. The 30 nm-thick Au–TBZ nanocomposite layer contained randomly distributed 3 nm Au NPs. The linearly polarized 785 nm plane-wave illumination beam was directly incident onto the 3D Au–TBZ-encapsulated Au nanopillar. In the case of the Au–virus nanocomposite layer, two vertically elongated oval-shaped Au nanopillars with a long axis length of 180 nm and a short axis length of 120 nm were separated a distance of 140 nm. A 100 nm virus particle-forming spike with a cylindrical shape and approximate dimensions of 14 nm (length) × 8 nm (diameter) bridged the two Au nanopillars. A 60 nm-thick electrodeposited Au layer was modeled as the undercoating of the viral particle. The linearly polarized 785 nm plane-wave illumination beam was directly incident onto the 3D Au–molecule nanocomposite layer. The mesh override region was fixed at 0.5 nm. In the calculation, the dielectric constant of Au was taken as εAu = 8.962364 + 1.164i at 785 nm, and the refractive index of A/N1N1 and H₂O was set to 1.60 and 1.33, respectively.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords
composite nanomaterials, electrochemical synthesis, respiratory viral pathogens, surface-enhanced Raman scattering (SERS)
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