Electronic Supplementary Information (ESI)

Extrinsic surface-enhanced Raman scattering detection of influenza A virus enhanced by two-dimensional gold@silver core-shell nanoparticle arrays

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1. Details of the SERS probe preparation

SERS probes were prepared in three steps: synthesis of 25 nm citrate-capped AuNPs, modification of AuNPs with TBBT (Raman reporter) and PEG molecules, and antibody immobilization. First, AuNPs were obtained by reducing $HAuCl_4$ with sodium citrate. In brief, 95 mL of an aqueous solution containing 5 mg of $HAuCl_4$ was refluxed. Then, 5 mL of 1 % (w/v) aqueous solution of sodium citrate was added to the boiling solution. The reflux was continued for 30 min.[1] An average diameter of obtained AuNPs was 25 ± 3 nm, which was determined from the SEM image (Fig. S1). Following the nanoparticle synthesis, AuNPs were simultaneously labeled with TBBT and thiolated PEG acid (COOH-PEG-SH) to avoid the particle aggregation and precipitation. A mixed solution of 5 μM ethanolic solution of TBBT (1.5 mL) and 1 μM aqueous solution of COOH-PEG-SH (1.5 mL) were freshly prepared and added to the colloidal solution of 25 nm citrate-capped AuNPs (9 mL) at room temperature. After 30 min incubation, 65 μL of a 1.5 mM aqueous solution of thiolated PEG (PEG-SH) was added to the colloidal solution, and then further incubation was performed for 2 h to acquire long-term stability of particles. To wash Au particles, centrifugation was conducted at 10,000 rpm for 10 min, and then the supernatant containing excess TBBT, COOH-PEG-SH and PEG-SH molecules was removed. Then, the remaining AuNP suspension was re-dispersed in water. This washing process was repeated twice for removal of unbound molecules. This colloidal solution can be stored at 4°C for more than 6 months. In the last step, influenza A antibodies were covalently immobilized on the TBBT-labeled AuNPs. Briefly, first we adjusted the

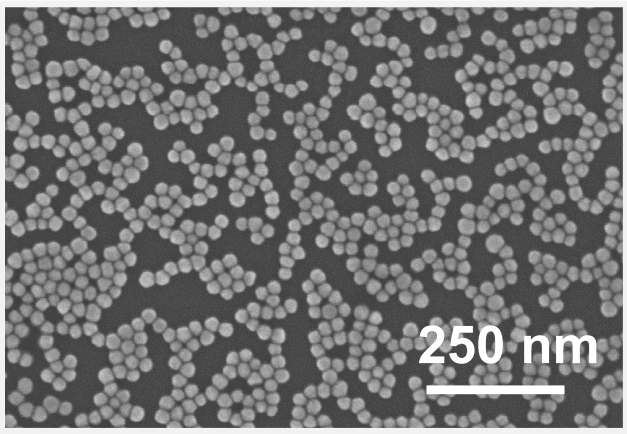

Fig. S1 SEM image of 25nm-citrated AuNPs drop-casted on a Si wafer substrate.

concentration of the aqueous solution of TBBT-labeled AuNPs, so that its optical density becomes unity at 523 nm (= absorption peak wavelength). Then, 0.5 mL of the TBBT-labeled AuNPs solution and 100 μL of an aqueous solution including 0.2 mg of EDC and 0.2 mg of NHS were mixed and kept at room temperature for 30 min to complete activation of carboxyl groups of COOH-PEG-SH molecules on AuNPs. Then, the excess amount of EDC/NHS was removed by three cycles of centrifugation (10,000 rpm, 10 min) and re-dispersion in water. In the last re-dispersion process, the particles were re-dispersed in 10 mM PBS solution (pH 7.4). Finally, 3 μL of a 5.3 mg/mL of Influenza A antibody was added to the colloidal solution and incubated for 3 h at room temperature. The particles were washed by two cycles of centrifugation at 10,000 rpm for 10 min and re-dispersion in PBS solution. These particles are called "SERS probes".

2. Synthesis of Au@AgNPs capped with a mixed alkanethiolate SAM

Here, we describe the synthesis of 40 nm Au core-5 nm thick Ag shell NPs and the surface modification with mixed alkanethiols. Firstly, 40 nm-AuNPs were synthesized by a seed-mediated growth method.[2,3] Briefly, 13 nm-Au seeds were synthesized by refluxing 20 mL of a 0.5 mM aqueous solution of $HAuCl_4 \cdot 3H_2O$ and then adding 1 mL of a 38.8 mM sodium citrate aqueous solution to the boiling solution. The reflux of the mixed solution was continued for 20 min to complete the reduction (solution turned wine-red). This solution was used as a seed solution in the next growth step. To grow the 13 nm-Au seeds up to 40 nm in size, 34 mL of water, and 1.37 mL of the seed solution, 0.8 mL of a 20 mM aqueous solution of $HAuCl_4 \cdot 3H_2O$, and 0.08 mL of a 10 mM aqueous solution of $AgNO_3$ were mixed and stirred. Then, 6 mL of a 5.3 mM aqueous solution of ascorbic acid was dropped into the stirred solution at a rate of 0.61ml/min.[4] The stirring was continued for 5 min after finishing the addition of ascorbic acid solution. To grow a 5 nm-thick Ag shell, 5 mM aqueous solution of $AgNO_3$ was added to a mixture of 13 mL of water and 15 mL of the 40 nm-AuNPs solution. Then, the reduction reaction was proceeded by dropping 1.2 mL of a 10 mM aqueous solution of ascorbic acid at a constant rate of 0.4 ml/min while stirring constantly. This solution was continuously stirred for 5 min to complete the reduction (solution turned orange). At this stage, we obtained a solution of citrated Au@Ag NPs. The size of Au@Ag NPs determined from SEM images were 51 ± 4 nm. The growth process of Au cores and Ag shells was performed at room temperature.

Next, the Au@Ag NPs were thiolated at room temperature as follows. A mixed solution of dodecanethiol and octadecanetiol in acetone was prepared at a molar ratio of dodecanethiol:octadecanetiol = 6:1 and at a total alkanethiol concentration of 11.3 mM. 10 mL of this mixed alkanethiol solution was gradually added to 10 mL of the citrated Au@AgNPs colloidal solution while stirring. Then, this stirring was continued overnight, until the solution became clear and Au@Ag NPs were floated. To collect mixed-alkanethiolate-capped Au@Ag NPs, a mixture of acetone and n-hexane was added to the colloidal solution. The thiolated Au@AgNPs concentrated at the interface between the hexane and acetone/water layers were collected with a pipette. This collection process was performed repeatedly until no Au@Ag NPs appeared at the interface. The collected particles were washed by three cycles of centrifugation and redispersion into a mixture of acetone and n-hexane; the acetone/hexane ratio was sequentially varied: 10:0, 1:9, and 0:10. After the supernatant was discarded in the final washing cycle, the Au@Ag NPs were dried under a nitrogen atmosphere. Before the arraying process, the powder of Au@Ag NPs was redispersed in 2 mL of a mixture of hexane-acetone (4:1 v/v). Using this solution, uniform and high coverage 2D arrays of the Au@Ag NPs were fabricated by the hybrid method.[5,6]

3. Functionalization of ITO substrates

A 10 nm-thick ITO film was formed on the quartz substrate (0.6 mm-thick) by radio frequency sputtering. The ITO film surface was functionalized at room temperature with 1% v/v 3-mercaptopropyl trimethoxysilane (MPTMS) in toluene for 42 h, rinsed with methanol three times, and dried under nitrogen blow. Then, the substrate was immersed in 1% v/v 1, 6-hexanedithiol in ethanol at room temperature for 12 h, rinsed with ethanol three times and dried under nitrogen blow. This substrate was used for arraying Au@Ag NPs by the hybrid method.[5,6]

4. Preparation of Au film substrates

Before Au film deposition, 0.6 mm-thick quartz substrates were immersed in piranha solution to remove organic matter and then rinsed with milli-Q water three times. A 10 nm-thick Cr adhesion layer and a 40 nm-thick Au film were sequentially deposited on the quartz substrate by thermal evaporation in vacuum ($<10^{-4}$ Pa). The deposition rate of both Cr and Au was 0.1 nm/s.

5. Functionalization of SERS substrates and immobilization of nucleoprotein and SERS probes on SERS substrates

The small-sized SERS substrates (5 × 5 mm^2) were used for immunoassay to reduce usage of recombinant nucleoprotein in the immunoassay, because its amount was limited. Therefore, a 1 μL of recombinant nucleoprotein solution was unavoidably used. Here, the detail of the surface treatment at each step of the immunoassay protocol shown in Scheme 1 was described.

In the EDC/NHS treatment, a 10 μL of EDC solution and a 10 μL of NHS solution were consecutively dropped on the small hydrophilic SERS substrate. The SERS substrate surface was fully covered with a 20 μL of EDC/NHS solution in total, and the solution was kept on the substrate due to the surface tension. See Figs. S2(a) and (b). The sample was kept in a dark moist chamber for 30 min to complete the reaction.

In the treatment with 1 μL of nucleoprotein solution, the SERS substrate surface was not

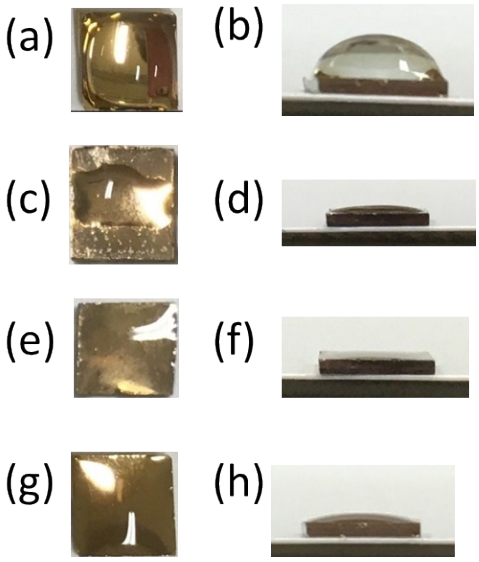

Fig. S2 Photographs of treament solutions on the substrate at each step of immunoassay protocol. The top (a) and side (b) view photographs of 20 μL of EDC/NHS solution on hydrophilic substrate. The top (c) and side (d) view photographs of 1 μL of nucleoprotein solution on EDC/NHS-treated substrate (before manually spreading). The top (e) and side (f) view photographs of 1 μL of nucleoprotein solution on EDC/NHS-treated substrate (after manually spreading). The top (g) and side (h) view photographs of 5 μL of SERS probe solution on nucleoprotein-immobilized substrate.

fully coverd with the 1 μL droplet as seen in Figs. S2(c) and (d). Thus we manually spread the droplet over the whole surface area, using micropipette. See Figs. S2(e) and (f). The thin solution layer spread over the whole substrate surface was stably kept during incubation for 30 min. To avoid the evaporation of water, the sample was kept in a moist chamber.

In the treatment with 5 μL of SERS probe solution, the SERS substrate surface was easily covered with the solution as seen in Figs. S2(g) and (h). The solution layer was stably kept during 1 h incubation process. To avoid the evaporation of water, the sample was kept in a moist chamber.

6. Extinction spectra of SERS probes

The solid, dotted, and dashed curves in Fig. S3 are the extinction spectra of the colloidal solutions of citrated AuNPs, PEGylated AuNPs labeled with TBBT, and SERS probes (after immobilization of Influenza A antibody), respectively. The narrow LSPR peak was observed at 523 nm for citrated AuNP solution. The peak position was red-shifted by a few nanometers by PEGylation, similar to the reports by X. Qian and M. Xiao groups.[7,8] This red-shift can be explained by change in the local dielectric constant surrounding AuNPs. No spectral change of the LSPR was observed by antibody decoration on PEGylated AuNPs. The single LSPR peak

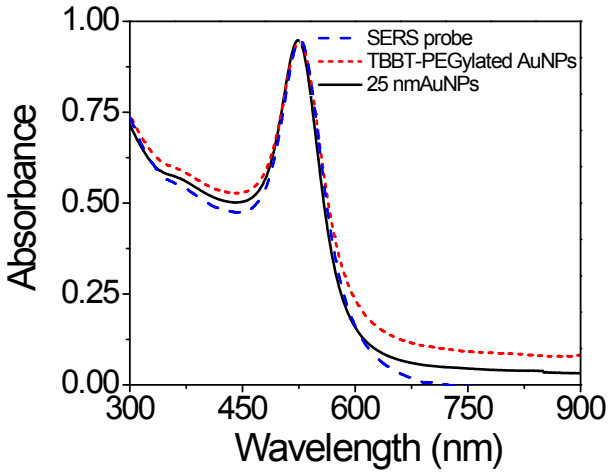

Fig. S3 Extinction spectra of colloidal solutions of citrated AuNPs, PEGylated AuNPs labeled with TBBT, and SERS probes.

with a narrow width was observed for the AuNP solutions in all three steps, indicating that the TBBT-PEGylated AuNPs and SERS probes are well dispersed and no aggregation occurs.

7. Confirmation of hydrophilization of hydrophobic 2D arrays of Au@Ag NPs

The hydrophobic 2D arrays of Au@Ag NPs prepared by the hybrid method were hydrophilized by annealing at 90°C for 1 h and then immersing in an ethanolic solution of MHDA at 50°C for 12 h. These reactions were confirmed by infrared (IR) absorption spectra taken at each step, which were measured with a FTIR spectrometer (Nicolet 4700) in an attenuated total reflection (ATR) geometry using a diamond prism. Fig. S4(a) is the IR spectrum of the hydrophobic 2D array. Four absorption bands were clearly obserbed in υ(C-H) region. The absorption bands at 2847 and 2913 cm^{-1} are assigned to the methylene symmetric and asymtetric C-H stretching vibrations, respectively. The 2883 and 2960 cm^{-1} bands are assigned to the methyl symmetric and asymtetric C-H stretching vibrations, respectively[9]. Since the alkanethiols (docecanethiol and octadecanethiol) have both a metyl end group and a metylene chain, the appearance of the four bands shows that the Au@Ag NPs are capped with alkanethiols. The FTIR spectrum of the annealed hydrophobic 2D array is shown in Fig. S4(b). The absorption intensity of the four bands was decreased by annealing, indicating that the alkanethiol capping molecules were partly desorbed from the Au@Ag NPs. After the MHDA treatment, an

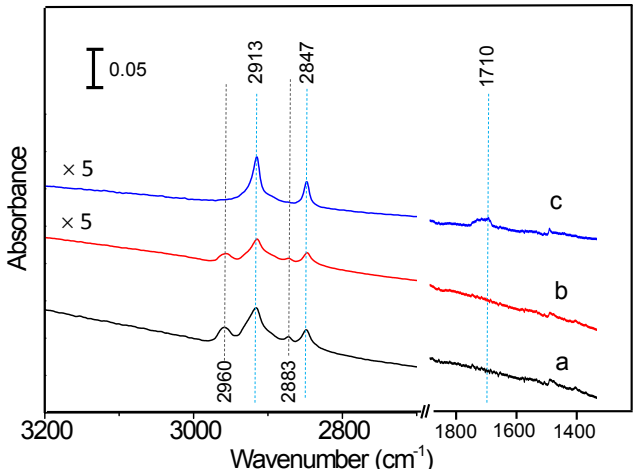

Fig. S4 ATR-FTIR spectra of the 2D array of Au@Ag NPs: (a) as-prepared hydrophobic 2D array, (b) after annealing at 90°C for 1 h, and (c) after MHDA treatment.

absorption band assigned to the carboxyl C=O stretching vibration was observed around 1710 cm^{-1}, and the 2883 and 2960 cm^{-1} bands assigned to the methyl C-H stretching vibrations disappeared as shown in Fig. S4(c). Since MHDA has a carboxyl end group but has no methyl end group, this spectral change shows that the alkanethiol capping molecules were replaced with MHDA.

8. Raman peak assignment of the control sample using a SERS substrate

Fig. S5 shows the Raman spectrum of the control sample using a SERS substrate, which is the same as Fig. 5, together with that of the EDC/NHS-activated 2D array of Au@Ag NPs. A lot of peaks were observed for the control sample, and the peak positions were coincident with those of the Raman spectrum of the EDC/NHS-activated 2D array. Thus the Raman peaks of the control sample can be assigned to the vibrational modes of MPTMS and EDC/NSH-activated

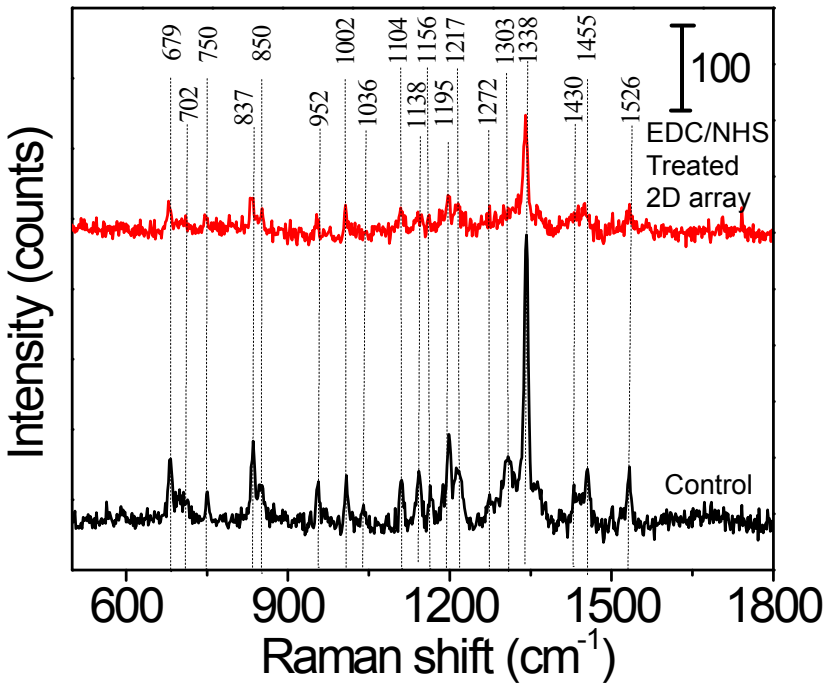

Fig. S5 Raman spectra of the control sample using a SERS substrate (bottom) and the EDC/NHS-activated 2D array of Au@Ag NPs formed on ITO substrate (top).

MHDA. MPTMS was used for the surface modification of ITO substrates, and MHDA was used for hydrophilization of 2D-array of Au@Ag NPs.

Table S1 Possible peak assignment of the Raman spectrum of the EDC/NHS-activation 2D array.

Observed peak (cm^{-1})	**Possible assignment**
679	$\nu(Si\text{-}C)_T$[10]
702 sh	$\nu(S\text{-}C)_T$[10,13]
750	CH_2 rock[13, 14]
837	CH wagging[12]
850	CH_2 rock [14]
952	$\nu(Si\text{-}OH)$ [10]
1002	$\nu(C\text{-}C)_T$[10,14]
1036	CH_2 rock [10,14]
1104	$\nu(Si\text{-}O\text{-}Si)$[10] or $\nu(C\text{-}C)_T$[13]
1138	CH_2 twist[10] or $\nu(C\text{-}C)_T$[13]
1156	C-O strech[12]
1195	CH_2 twist[10,14]
1217	CH_2 twist[10] or wagging[14]
1272	CH_2 twist[10] or wagging[14]
1303	CH_2 twist[10,14]
1338	CH_2 twist[10] or wagging[14]
1455	CH_2 bending[11,12,14]

sh: shoulder, ν: streatching, T: trans.

9. Definition of the limit of detection used in this study

We define the limit of detection (LOD) used in this study. The linear sensitivity curve can be expressed by:

$$I^{ob} = ax + b, \qquad \text{(S-1)}$$

where I^{ob} is the observed Raman intensity, and x is the concentration of target bio-analytes. When the mean value and the standard deviation of the Raman intensity for the control sample are denoted by $I^{ave}_{control}$ and $\sigma_{control}$, respectively and the standard deviation of the Raman intensity for the immunoassay at a target concentration near and higher than LOD is denoted by σ_{LOD}, we defined the LOD by the following equation:

$$LOD = x_{LOD} = \frac{\left\{I^{ave}_{control} + 1.645\left(\sigma_{control} + \sigma_{LOD}\right) - b\right\}}{a}. \qquad \text{(S-2)}$$

Table S2 Calculation of LOD of recombinant nucleoprotein.

Peak (cm^{-1})	$I^{ave}_{control}$ (counts)	$\sigma_{control}$ (counts)	σ_{LOD} (counts)	LOD (ng/mL)
SERS substratre				
1565	16	4	6 at 13.4 ng/mL	8
Au film				
1565	22	5	7 at 67 ng/mL	59

Table S3 Calculation of LOD of infected allantoic fluid.

Peak (cm^{-1})	$I^{ave}_{control}$ (counts)	$\sigma_{control}$ (counts)	σ_{LOD} (counts)	LOD ($TCID_{50}$/mL)
1565	74	11	10 at 5.6 $TCID_{50}$/mL	6

10. Limit of detection of representative sensors for detecting infected allantoic

Table S4 Summary of LOD of representative sensors for detecting infected allantoic fluid.

Probe	Biomarker	Type of assay	Detection method	Linear range	LOD	ref
Cy5- doped SiO_2NPs	infected allantoic fluid	sandwich	Fluorescence based-ELISA	50-1000 $TCID_{50}$/mL	11 $TCID_{50}$/mL	[15]
Dual mAb-immunogold	infected allantoic fluid	sandwich	Color based Lateral flow immunoassay	NA	47 $TCID_{50}$/mL	[16]
mAb-immunogold	infected allantoic fluid	sandwich	Absorbance based LFIA biosensor	NA	5×10^2 $TCID_{50}$/mL	[17]
PEGylated, TBBT-labeled immunogold	infected allantoic fluid	direct	SERS-based biosensor	5-56 $TCID_{50}$/mL	6 $TCID_{50}$/mL	This work

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