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Facile and sensitive detection of influenza viruses using SERS antibody probes†

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It is highly important to identify influenza viruses rapidly and accurately for the prevention of future pandemic outbreaks. We developed an easy and sensitive influenza virus detection method using surface-enhanced Raman scattering (SERS) antibody probes. The SERS antibody probes can be prepared easily by mixing Au nanoparticles, gold binding peptide-protein G, and antibodies without complicated chemical or biological reactions. They also retain the optimal conformation for the antibody's interaction with Influenza A/CA/07/2009 (pH1N1), allowing us to detect pH1N1 sensitively and selectively. This method provides a detection limit of 4.1×10^3 TCID per mL and is highly selective for pH1N1. We anticipate that this method can be useful in a wide range of immunoassays.

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Introduction

The influenza virus is a member of the orthomyxoviridae family, which is divided into three types: influenza A, B, and C.^{1,2} Of these, influenza A virus is a major threat to human infection and has caused pandemics such as swine flu (H1N1) in 2009 and bird flu (H5N1) in 2004.^{3,4} Influenza A virus contains eight segments of single-stranded RNA and can mutate at a high rate,⁵ thus inducing antigenic shifts with the potential to cause pandemics.^{6,7} Therefore, it is critically important to rapidly and accurately identify influenza viruses for the prevention of future pandemic outbreaks. Many researchers have focused on the development of influenza virus-sensing methods, and four typical methods are currently in use for clinical diagnostics: cultivation of viruses in cell culture, immunofluorescence, reverse transcriptase-polymerase chain reaction (RT-PCR), and rapid influenza diagnostic testing.^{8,9} Although each of these methods has advantages and all are widely used depending on the application, an easy and sensitive detection method for influenza virus is still needed.

Recently, metallic colloid nanoparticles (NPs) have been widely used for the development of ultrasensitive detection probes because of their long-term stability, easily controlled size, unique optical properties, and biocompatibility.^{10–14} Particularly in surface enhanced Raman scattering (SERS),

a fascinating phenomenon in which Raman signals of molecules are increased by a factor of up to 10^8 or more, metal NPs have played important roles as detection probes.^{15–17} SERS-based assays have the advantages of single-molecule level sensitivity, molecular specificity, and reduced photobleaching.^{18–22} Numerous NP-based SERS probes have been developed to detect DNA,²³ RNA,²⁴ proteins,^{25–28} pathogens,^{29,30} cancer cells,³¹ and chemicals,³² among other molecules. However, few reports have described the direct detection of viruses using SERS probes.^{30,33,34}

Here, we report the easy and sensitive detection of influenza viruses through SERS antibody probes. Influenza A/CA/07/2009 (pH1N1) was captured on a substrate by immunoreaction, and SERS antibody probes were applied. After Ag enhancement of the probes, pH1N1 was detected at a low concentration of 10^3 TCID per mL by the measurement of SERS. Furthermore, pH1N1 was clearly distinguished from other types of influenza viruses. The present method has several distinct advantages. First, SERS antibody probes can be prepared easily by mixing Au NPs, gold binding peptide (GBP)-protein G, and antibody without a need for complicated chemical or biological reactions, because GBP can bind to Au NPs and protein G can bind to the Fc region of an antibody.^{35–37} Second, the protein G-antibody retains the optimal conformation for its interaction with pH1N1, thus enabling efficient detection compared with that by a randomly labelled antibody. Third, the sensitive and quantitative detection of pH1N1 is achieved by Ag enhancement and SERS measurement. Compared with previous immunoassays,^{5,38,39} this method offers a wider dynamic range. We expect that the present method can be applied to a wide range of immunoassays by changing the probe antibody.

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Experimental

Materials

pH1N1 and influenza B/Yamagata/Florida/07/06 1/50 (IBV) suspended in cell culture media (MEM medium, MDCK cells) were provided by the Korea Centers for Disease Control and Prevention, Korea. Influenza A/canine/Korea/MV01/2012 (H3N2) and influenza A/aquatic bird/Korea/w351/2008 (H5N2) suspended in cell culture medium (MEM, MDCK cells) were obtained from KRIBB. Each virus titer of the stock solutions was similar and ranged between 10^7 and 5×10^8 TCID₅₀ per mL, as determined by real-time reverse transcription polymerase chain reaction according to a previous report.⁴⁰ Monoclonal anti-influenza A H1N1 antibody (ab128412) was purchased from Abcam. Polyclonal anti-influenza A H1N1 (Swine Flu 2009) antibody (11055-RP02) was purchased from Sinobiological. Gold binding peptide (GBP)-protein G was purchased from Bioprogen (Daejeon, Korea), and Cys3-protein G was purchased from Micobiomed (Daejeon, Korea). Carboxylated glass substrate (SMA2) was purchased from Arrayit. *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (NHS), rhodamine B isothiocyanate (RBITC), Tween 20, Au NPs in phosphate-buffered saline (PBS), bovine serum albumin (BSA), and Ag enhancer solution A, B (S5020, S5145) were purchased from Sigma-Aldrich.

Preparation of SERS antibody probes

GBP-protein G (0.1 mg mL^{-1}) was applied to 20 nm Au NPs in PBS (0.1 mM , pH 7.0) for 16 h at 4°C . Unbound GBP-protein G was separated by centrifugation at 12 000 rpm for 10 min. Au NP-GBP-protein G probes were resuspended in 10 mM PBS containing 0.05% Tween 20. Monoclonal antibody ($10 \mu\text{g mL}^{-1}$) against pH1N1 was added to Au NP-GBP-protein G probes in 10 mM PBS containing 0.05% Tween 20 for 2 h at room temperature. After separation of unbound antibody by centrifugation at 12 000 rpm for 10 min, Au NP-GBP-protein G-antibody probes were obtained.

Preparation of capture substrate

A carboxylated glass substrate was immersed in the mixture of EDC and NHS solution to activate the carboxyl group of a glass substrate. Cys3-protein G (0.1 mg mL^{-1}) was added to the activated glass substrate to couple Cys3-protein G and the substrate *via* amide linkage. Polyclonal antibody (0.1 mg mL^{-1}) against pH1N1 was added to the Cys3-protein G-attached glass substrate for 2 h at room temperature. To prevent non-specific binding, BSA (0.1 mg mL^{-1}) was applied to the antibody-attached glass substrate.

Detection of pH1N1

pH1N1 solution ($3.5 \mu\text{L}$) was dropped into the capture substrate for 2 h at room temperature, and the substrate was washed with 100 mM PBS containing 0.1% Tween 20. The pH1N1-captured substrate was exposed to SERS antibody probes for 1 h at room temperature and washed with ultrapure water. Ag

enhancer solution A (Ag salt) and B (Initiator) were then mixed 1 : 1 and applied to the substrate for 20 min. The resultant Ag-enhanced substrate was washed with ultrapure water and dried under nitrogen gas. After the addition of RBITC ($80 \mu\text{M}$) to the Ag-enhanced substrate, SERS spectra were measured.

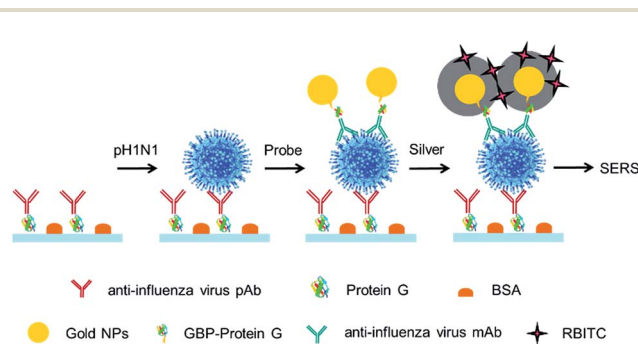
Instrumentation

SERS spectra were measured with a Raman spectrometer (XperRam 200, Nanobase Inc., Korea). A 632 nm visible diode-pumped solid-state laser was used as an excitation source. The exposure time was 1 s, and ten SERS spectra were collected for averaging and statistical analysis. The scanning electron microscopy (SEM) images were obtained with a Quanta 250 FEG (FEI Co., USA) operated at 20 kV. Grayscale images were obtained by using an optical flatbed scanner (SCX-4210) with a resolution of 600 dpi, and the data were analyzed with an 8 bit grayscale histogram using ImageJ software (NIH; Bethesda, Maryland, USA).

Results and discussion

The influenza virus detection method using SERS antibody probes is illustrated in Scheme 1. The capture substrate was prepared by the coupling of protein G and a carboxylated glass substrate *via* amide linkage, and subsequent treatment with a polyclonal antibody against pH1N1. BSA was used to prevent non-specific binding. To detect pH1N1, the solutions containing pH1N1 were dropped onto the capture substrate, and SERS antibody probes were applied. The probes were then enhanced by using an Ag enhancer solution, and SERS signals were measured exclusively from RBITC immobilized on the probes. Only in the presence of pH1N1, SERS antibody probes could be pulled down onto the substrate, and strong SERS signals were obtained after Ag enhancement.

The SERS antibody probes were prepared by mixing Au NPs, GBP-protein G, and an anti-pH1N1 monoclonal antibody. The GBP-protein G was synthesized, cloned, expressed, and purified as described previously.³⁸ The probes were heated at 95°C for 7 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the formation. We prepared four types of probes as shown in Fig. 1. First, pure Au NPs showed no band by SDS-PAGE (*lane 2* of Fig. 1). Second, we



Scheme 1 Schematic illustration of influenza virus detection using SERS antibody probes.

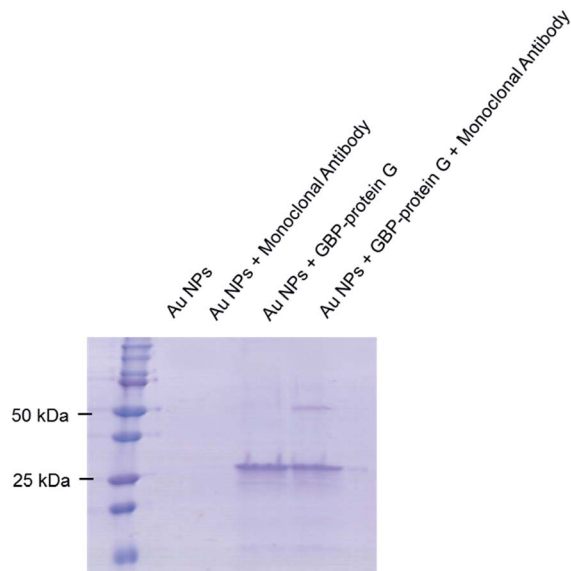


Fig. 1 SDS-PAGE analysis of SERS antibody probes [lane 1 size marker, lane 2 Au NPs, lane 3 Au NPs + monoclonal antibody, lane 4 Au NPs + GBP-protein G, lane 5 Au NPs + GBP-protein G + monoclonal antibody].

prepared the probes by mixing Au NPs and a monoclonal antibody against pH1N1, centrifugation, and resuspension. These probes were not observed by SDS-PAGE (lane 3 of Fig. 1) because Au NPs and the antibody cannot bind to each other by simple mixing. Third, Au NP-GBP-protein G probes were analyzed. A clear band of ~ 25 kDa was observed by SDS-PAGE (lane 4 of Fig. 1), corresponding to the size of GBP-protein G.³⁸ This result indicates that GBP-protein G successfully bound to Au NPs. Finally, SERS antibody probes were analyzed. Two bands at ~ 25 kDa and ~ 50 kDa were observed (lane 5 of Fig. 1). The band at ~ 25 kDa corresponds to both GBP-protein G and the antibody light chain. The band at ~ 50 kDa corresponds to the antibody heavy chain. From this result, we confirmed the successful binding of the antibody to Au NPs.

We investigated the pH1N1 concentration-dependent SERS signals from the probes on capture substrates. The pH1N1 solutions were prepared by 1/10 serial dilution, and a blank

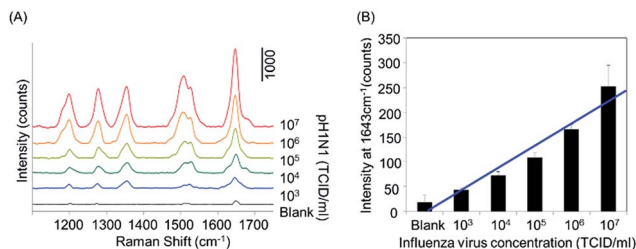


Fig. 2 (A) Influenza virus detection results using SERS antibody probes at various concentrations (blank, 10^3 , 10^4 , 10^5 , 10^6 , and 10^7 TCID per mL). (B) Plot of SERS intensity at the 1643 cm^{-1} band versus the influenza virus concentration. The blue line shows the linear fit line. Data represent the mean plus standard deviation of ten measurements.

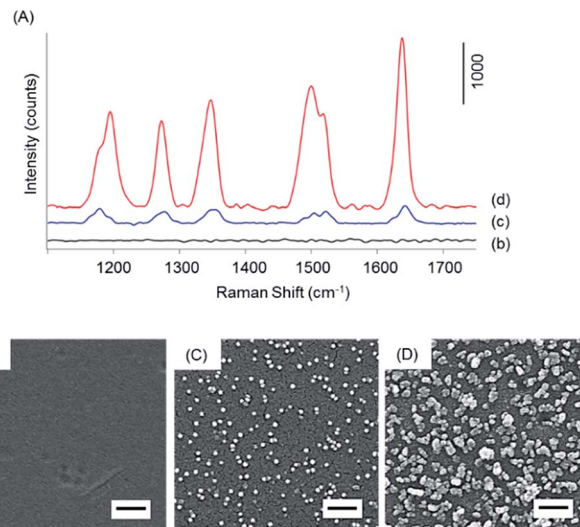


Fig. 3 (A) SERS spectra of RBITC without SERS antibody probes (spectrum b), and before (spectrum c) and after Ag enhancement (spectrum d). (B–D) SEM images of the substrates which we obtained the SERS spectra b–d in (A). Scale bars denote 200 nm.

solution was prepared as a control. As shown in Fig. 2A, the SERS signals intensified as the pH1N1 concentration increased. This result was further analyzed by plotting the intensity of a 1643 cm^{-1} band as a function of the pH1N1 concentration (Fig. 2B). The SERS signal intensity increased linearly within a concentration range of 10^3 to 10^7 TCID per mL, enabling the quantitative detection of pH1N1. We estimated the detection limit of this method to be 4.1×10^3 TCID per mL (65 ng mL^{-1}). PCR-based assays shows the detection limit of 10^2 TCID per mL level^{41–43} and immunoassay kits provide the detection limit of 10^3 or 10^4 TCID per mL level (Table S1†).^{44–46} Compared to the previously reported immunoassays, the present method has comparable detection limit and wider dynamic range.^{5,38,39}

The low detection limit of this method may be attributed to the optimal conformation of the antibody and Ag enhancement. Protein G can bind specifically to the Fc region of an antibody, and thus properly orient the antibody for optimal pH1N1 binding. Moreover, protein G can capture an antibody without

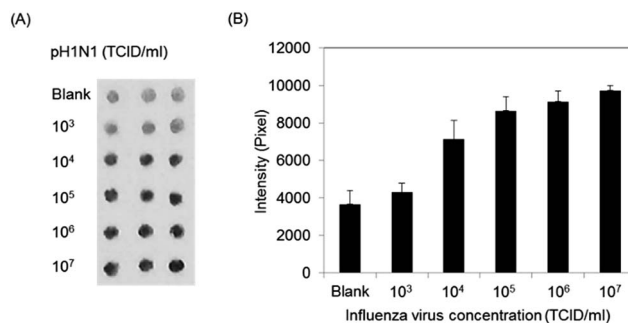


Fig. 4 (A) Grayscale image of the Ag-enhanced substrate after the detection of pH1N1 (0 to 10^7 TCID per mL). (B) Plot of 8 bit grayscale values depending on the pH1N1 concentration. Data represent the mean plus standard deviation from three measurements.

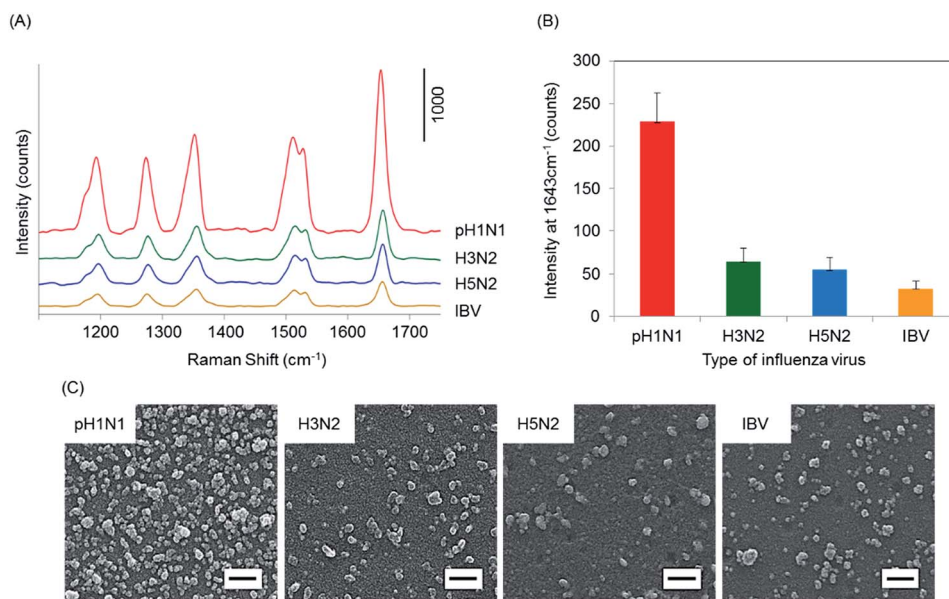


Fig. 5 (A) SERS spectra of RBITC in the presence of pH1N1, H3N2, H5N2, and IBV, respectively. (B) Plot of the SERS intensity at the 1643 cm^{-1} band versus the type of influenza virus. (C) SEM images of the substrates which we obtained the SERS spectra in (A). Scale bars denote 200 nm.

chemical modification, allowing the antibody to completely retain its function. The Ag enhancement technique markedly increased SERS signals and eliminated the flaws of Au NPs in SERS.¹¹ By combining these advantages, sensitive detection of pH1N1 was possible. Fig. 3A shows the SERS spectra of RBITC without the probes (spectrum b) and before (spectrum c) and after Ag enhancement (spectrum d). Before Ag enhancement, SERS signals were weak and fluctuated (Fig. S1A†). After Ag enhancement, the signals intensified significantly and stabilized (Fig. S1B†), allowing us to sensitively and quantitatively detect pH1N1. Fig. 3B–D show SEM images of the substrates, from which we obtained the SERS spectra b–d shown in Fig. 3A. SERS antibody probes were captured on the substrate in the presence of pH1N1 (Fig. 3C) and enlarged to ~ 100 nm by Ag enhancement (Fig. 3D). Since the enlarged NPs were visible, pH1N1 was detectable with naked eye as reported previously.³⁸ Fig. 4 shows the naked eye detection results of pH1N1. At the low concentration of 10^4 TCID per mL, dark spots were clearly observed. The plot of grayscale values versus pH1N1 concentration shows that the value increases from 10^3 to 10^5 TCID per mL and saturates. Although naked eye detection is simple, more sensitive and quantitative measurements of pH1N1 were feasible by SERS measurement.

To verify the selectivity of this approach, four different types of influenza viruses (pH1N1, H3N2, H5N2, and IBV) were tested. The concentration of each virus was 10^7 TCID per mL. Fig. 5A shows the SERS spectra of probes in the presence of four types of influenza viruses. Strong signals were observed only in the presence of pH1N1, whereas weak signals were observed in the presence of H3N2, H5N2, and IBV. The plot of the 1643 cm^{-1} band intensity provided a clearer confirmation of the specificity of this method for pH1N1 (Fig. 5B). The SEM images in Fig. 5C also showed that many enlarged probes were present only in the

presence of pH1N1. Because the SERS antibody probes can be simply modified with various influenza virus-specific antibodies, we expect that the present method will be useful for the identification of influenza virus subtypes.

Conclusions

In summary, we developed an easy and sensitive influenza virus detection method using Au NP-GBP-protein G-antibody probes. The probes were successfully conjugated to pH1N1 with high efficiency and selectivity. After simple Ag enhancement, pH1N1 was sensitively detected by the measurement of SERS signals from the enlarged probes. The detection limit was 4.1×10^3 TCID per mL, and the high selectivity of this method was clearly verified. The optimal conformation of antibody and highly enhanced SERS signals enabled the accurate and sensitive detection of pH1N1. The SERS antibody probes can be prepared easily by direct labelling of antibody and Au NPs to GBP-protein G without further chemical or biological steps, suggesting their wide applicability to various immunoassays. We expect that this method may be used for the detection of many biohazardous substances and for disease diagnostics.

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