

# A Rapid and Sensitive Early Diagnosis of Influenza Virus Subtype via Surface Enhanced Raman Scattering

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## Abstract

We have developed a novel, rapid, highly sensitive biosensor protocol for diagnosis and characterization of influenza viruses in the early stage of the epidemic. Using Surface-Enhanced Raman Scattering (SERS) technique and nitrocellulose membrane, detection limit as low as 30 ng/mL was achieved with high selectivity to different subtypes of influenza virus tested. Moreover, the SERS measurement can be completed in less than 2 h, thus making the protocol useful in early diagnosis and impediment of propagation of potential influenza pandemic.

**Keywords:** Surface-enhanced raman scattering; Influenza virus; Membrane; Biosensors

**Abbreviations:** SERS: Surface-Enhanced Raman Scattering; HA: Hemagglutinin; NA: Neuraminidase; RT-PCR: Reverse-Transcriptase Polymerase Chain Reaction; NC: Nitrocellulose; VLP: Virus-like Particle; AuNP: gold nanoparticle; DNBA: 5,5'-Dithiobis(2-NitroBenzoic Acid); DSNB: 5,5'-Dithiobis(Succinimidyl-2-Nitrobenzoate); DCCD: 5,5'-Dithiobis(2-nitrobenzoic acid), NHS: N-hydroxysuccinimide; THF: TetraHydroFuran; DMSO: DiMethyl SulfOxide; PBS: Phosphate Buffer Saline; TBS: Tris Buffered Saline; TBST: TBS with 1% Tween-20; BSA: Bovine Serum Albumin; TMB: 3,3',5,5'-TetraMethylBenzidine; ELISA: Enzyme-Linked ImmunoSorbent Assay; TEM: Transmission Electron Microscopy.

## Introduction

In 1997, avian Influenza viruses have been shown to cross the species barrier and caused first human infection in Hong Kong. Influenza virus is one of *Orthomyxoviridae* family members and classified into three types: influenza A, B, and C. Among these three types, Influenza A virus (IAV) that can infect animals and humans continues to pose a major threat to public health and animal health worldwide. IAV is a negative single strand RNA virus and subdivided into different subtypes according to the antigenic property of their surface glycoproteins: hemagglutinin (HA), H1~H16 and neuraminidase (NA), N1~N9. All HA subtypes are identified in avians, but only H1, H2, H3, H5, H7 and H9 have been isolated in humans [1]. Currently, H1N1, H3N2 and influenza B virus are the dominant subtypes in seasonal outbreaks; the other subtypes H5N1, H7N7, H7N9 and H9N2 are transmitted from animals to human occasionally [2-4].

Influenza is an infectious disease with substantial morbidity and mortality in animal and human populations. As an RNA virus, the influenza virus is characterized by high mutational rate, necessitating frequent alterations in vaccine design for effective prevention of influenza. In addition, its segmented genome allows the re-assortment to occur in the host during co-infection, resulting in antigenic shift with a potential for flu pandemics. Therefore, a rapid and accurate identification of the virus subtype particularly in the early stage of the outbreak is epidemiologically crucial in fighting against the disease. Thus far there are a limited number of direct methods to accurately and quantitatively detect the existence of influenza virus at the early phase of infection, rendering difficult the search for the source and propagation pathway of an active and acute flu epidemic and aggravating public anxiety and/or panic, as exemplified by the recent H7N9 outbreak [3,4]. As a result, development of specific and sensitive methods for

the detection of influenza viruses has been the focus of many clinical investigations. According to Centers of Disease Control and Prevention [5], there are four common influenza virus testing methods to identify the infection in early stage: virus cell culture, immunofluorescence and antibody staining, Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR), and rapid influenza diagnostic tests.

Viral cell culture is a conventional and sensitive technique, but it typically requires 3-10 days for testing [6,7]; for the rapid cell culture it takes 1-3 days. Therefore both techniques do not meet the requirement for the rapid test for early identification. RT-PCR detection for the viruses has progressed significantly in recent years and become more commonly available in clinical diagnostic laboratories [8-13]. RT-PCR has the following advantages: (1) amplification can be monitored in real time; (2) it has wider dynamic range (up to 10<sup>10</sup> fold); (3) distinction down to amino acid level can be achieved. However, it requires sophisticated laboratory facilities and expensive equipment. While the time to obtain preliminary results can be as little as 3h, more reliable results can only be obtained in 24 h by repeated measurements. More importantly, due to its extremely high sensitivity, it may exhibit high level variation with the same specimen leading to false negative or positive results and rendering sample handling a critical step in the analysis. The antigen capture immunoassays [14,15], such as the commercially available BinaxNOW, QuickVue, and Directigen tests, can provide rapid influenza test results in 10-15 min. However, the immunoassays suffer from poor detection limits afforded by lateral flow assay precluding diagnosis at the initial phase of infection during which only low antigen level is available for detection. For example, commercial lateral flow assays for influenza virus only provide 50-70% diagnostic sensitivity and 90-95% specificity with respect to culture-based diagnosis [16].

SERS probes have been widely used for detection and biosensors

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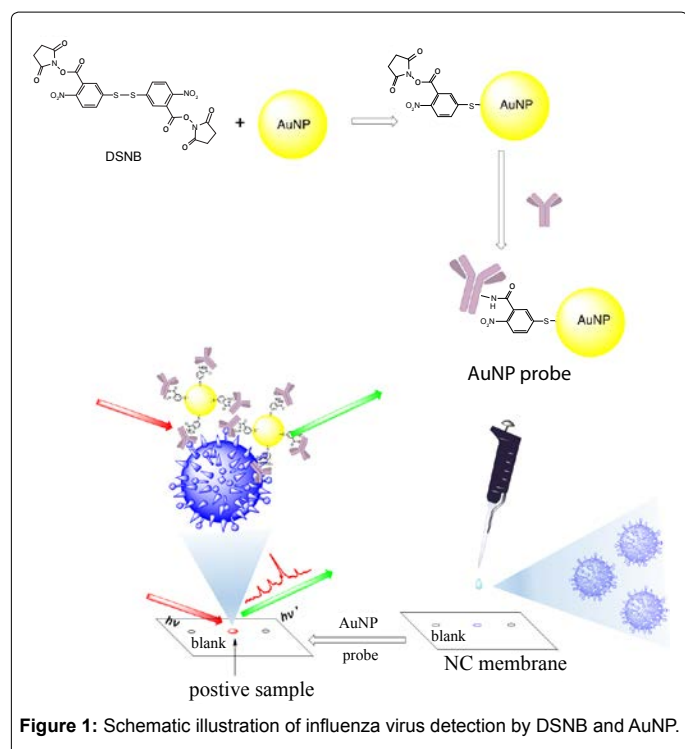
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in the past few years, due to their high sensitivity. Tracking the analyte with nanoparticles was often performed using sandwich bioassays [17,18]; however, the preparation of such samples is tedious and time-consuming. In the process of antibody-antigen conjugation in the sandwich bioassay, large volumes of analytes are needed to form the antibody-antigen complexes, as well as to obtain SERS signals.

In a previous study, Bishnoi et al. have developed a SERS-based immunoassay using a Nitrocellulose (NC) membrane for analyte capture by half-sandwich assay instead of the full version, which requires much less time for preparation and incubation [19]. The NC membrane has been considered as a good substrate with strong affinity to proteins, due to the hydrophobic interaction between NCs and proteins and has been extensively employed for protein isolation in Western blotting.

For biosafety concerns, the analytes we detected were influenza virus-like particles (VLP), which emulate the virus particles in structure, size, and surface composition, and even glycosylation pattern of viral antigens [20]. In the conventional sandwich bioassay the incubated substrate in analyte-containing solution flows through the immobilized antibody for a long period of time, thus demanding a large quantity of antigen. In contrast, a volume as small as 0.1  $\mu\text{L}$  of VLP solution dropped onto the NC is sufficient for antigen detection. Another component is the self-assembled monolayer gold nanoparticle (AuNP) as a probe for analyte recognition and a SERS reporter. Gold nanoparticles were conjugated with Raman tags and specific antibodies for SERS data reading and analyte binding, respectively. It has been shown that the probe coated with the bifunctional molecule, 5,5'-dithiobis(succinimidyl-2-nitrobenzoate) (DSNB), to covalently couple to antibody by forming a stable amide bond and to the AuNP probe by sulfide bond, can be used as a strong Raman reporter [14] as shown in Figure 1.



The linker DSNB has several distinct advantages: (1) DSNB has succinimidyl groups which can covalently bind to the antibodies or other amine-containing groups; (2) DSNB can tightly bind to the gold surface to form the thiolate layer by breakage of the disulfide bond; (3) the symmetric stretching of the nitro groups in DSNB gives rise to an intense Raman signal due to the large Raman cross section of the nitro group. These “sandwich” probes have successfully detected a prostate-specific antigen in human serum on the femtomolar scale [12].

In the following, we demonstrate a half-sandwich bioassay based on a modification of AuNP surface to achieve a highly sensitive response to the presence of virus through the use of SERS and NC membrane as schematically shown in Figure 1. Such a bioassay is characterized by rapid and easy sample preparation requiring only small amount of analytes [19].

## Materials and Methods

### Materials and chemicals

5,5'-dithiobis(2-nitrobenzoic acid) (DCCD), N-hydroxysuccinimide (NHS), tetrahydrofuran (THF), acetonitrile, dimethyl sulfoxide (DMSO), 5,5'-dithiobis(2-nitrobenzoic acid) (DNBA), phosphate buffer packs, and bovine serum albumin were purchased from Sigma and used without further purification. NC membranes (BA85 Protran, 0.45  $\mu\text{m}$ , blotting membrane) were obtained from GE Healthcare. 40 nm gold nanoparticles solution was obtained from Ted Pella Inc. (Redding, CA) and Tween-20 was acquired from Bio-Rad (Hercules, CA). Mouse anti influenza A H1N1 Hemagglutinin antibody (5315-2907) was purchased from AbD Serotec (Kidlington, UK). Anti-Influenza A Virus Hemagglutinin 3 antibody (ab62485) and (H5N1/HA1) antibody (ab135382) were acquired from Abcam Inc. (Cambridge, UK). Tris buffered saline (TBS) was obtained from Amresco (Solon, OH).

### Synthesis of gold nanoparticles

In the SERS bioassays, the homemade 40 nm AuNPs were prepared with Turkevich's citrate reduction method [21]. Briefly, 1% HAuCl<sub>4</sub> was added to 100 mL of Milli-Q water, and the solution was brought to boil. 1.5 mL of 1% sodium citrate was then added. The surface plasmonic peak absorbance of the synthesized AuNP is at 524 nm [22]. The commercial 40 nm AuNP was also used in the SERS bioassay for comparison with the homemade one. As similar results were obtained, the homemade AuNPs were used for the work presented herein (see the Supplementary Information for details; Figure S1).

### DSNB synthesis

The DSNB linker was synthesized according to the previously published method using a one-pot carbodiimide coupling of 1,3-dicyclohexylcarbodiimide (DCCD) [18]. Briefly, 5,5'-dithiobis(2-nitrobenzoic acid) (DNBA, 0.5 g, 1.3 mmol), 1,3-dicyclohexylcarbodiimide (DCCD, 0.52 g, 2.5 mmol), and N-tetrahydrofuran (THF) were mixed in a 100 mL round-bottom flask equipped with a drying tube. The mixture was magnetically stirred at room temperature overnight, filtered, and rotoevaporated to remove the solvent. Crude product was recrystallized from acetone/hexane to yield DSNB. Then, the DSNB solution was added to the AuNP suspension and antibody to form the bioconjugates as the probe solution.

### Immunogold transmission electron microscopy / Raman spectroscopy / ELISA

1 mL of DSNB-labeled AuNP solution was conjugated with the anti-influenza virus H5N1/HA1 antibody on the ice overnight. 1  $\mu\text{L}$  of this

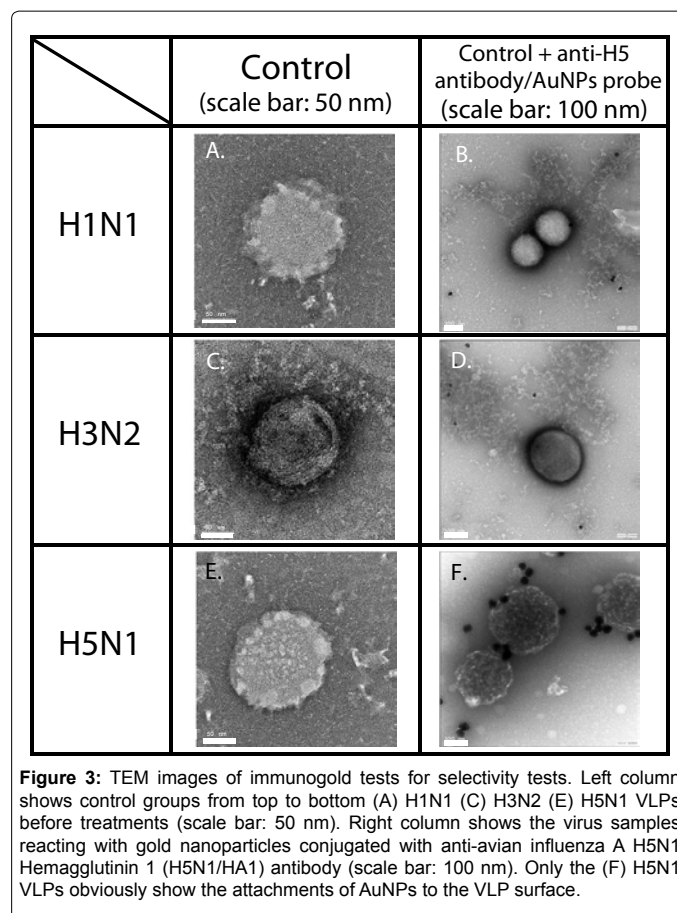
solution was adsorbed onto formvar/carbon-coated 200 mesh copper grids (Agar Scientific Ltd, Essex UK). The grids were then washed twice with TBS, fixed with 1% glutaraldehyde, water, and negatively stained with 2% uranyl acetate for 30s. The images of stained mammalian VLPs were acquired using a JEOL transmission electron microscope.

A portable Raman instrument (Portable TE-cooled Raman spectrometer system QE65Pro from Ocean Optics Inc., Dunedin, FL; schematic illustration and photographs of Raman spectroscopy are shown in Figure 2) was set up with a 20 mW/785 nm diode laser for 30 s of exposure time. Ten spectra were collected for averaging and statistical analysis.

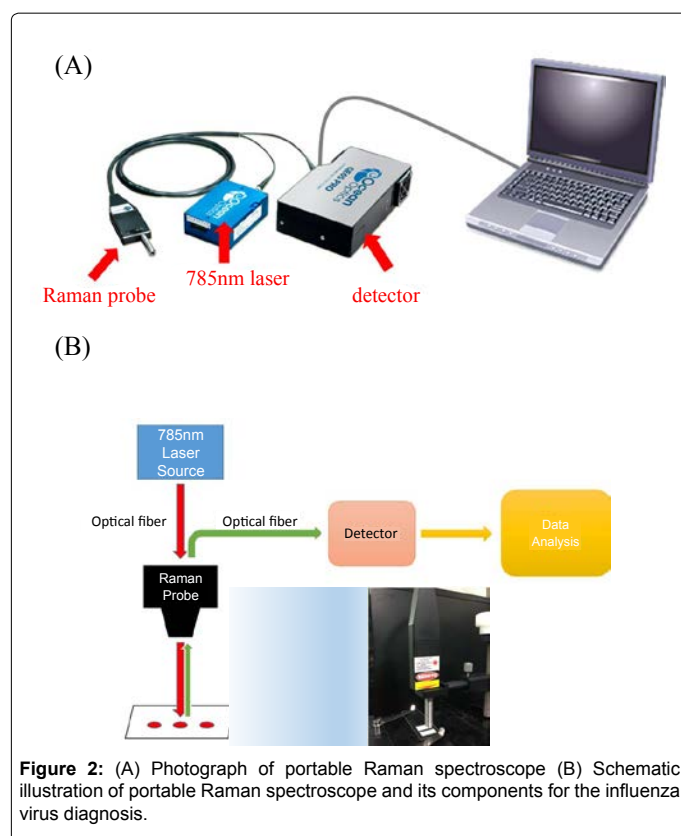
To compare with the conventional method, the Enzyme-Linked Immunosorbent Assays (ELISA) for the H5N1 virus were performed in which viral samples were diluted in PBS and coated in a 96-well plate at 4°C for 16 h with shaking. Wells were washed three times with PBS and blocked with 3% BSA in PBS at 37°C for 1 h. Anti- HA antibody was added and incubated at 37°C for 2 h and then rinsed six times with PBS containing 0.1% Tween-20. HRP-conjugated antibody that targets the primary antibody was diluted in blocking buffer and added to the wells following incubation at 37°C for 1 h. The color was developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate for 30min at room temperature with shaking and the reaction was stopped by adding 2 M H<sub>2</sub>SO<sub>4</sub>. The absorbance of the conjugate was recorded at 450 nm.

### SERS immunoassay

The VLP suspension was dropped into the circle drawn on the NC membrane. The solution was dried for 15 min and the membrane was subsequently immersed in blocking buffer (5% BSA in TBS with 1% Tween-20, TBST) and agitated for 45 min to avoid non-specific binding to the regions outside of the circle. After blocking, the membrane was



**Figure 3:** TEM images of immunogold tests for selectivity tests. Left column shows control groups from top to bottom (A) H1N1 (C) H3N2 (E) H5N1 VLPs before treatments (scale bar: 50 nm). Right column shows the virus samples reacting with gold nanoparticles conjugated with anti-avian influenza A H5N1 Hemagglutinin 1 (H5N1/HA1) antibody (scale bar: 100 nm). Only the (F) H5N1 VLPs obviously show the attachments of AuNPs to the VLP surface.



**Figure 2:** (A) Photograph of portable Raman spectroscopy (B) Schematic illustration of portable Raman spectroscopy and its components for the influenza virus diagnosis.

immediately placed in a solution containing the AuNP probe solution (gold nanoparticles conjugated with Raman-active dye molecules DSNB and antibody). The membrane in the probe solution was shaken for 35 min to prevent probe sedimentation and then immersed in cold TBST and agitated for 15 min two to three times to remove nonspecifically bound gold probe particles. The photograph of final samples is shown in Figure S2 with different total amounts of VLP on the NC membrane surface. The membrane with the stained samples was directly used in the subsequent SERS measurements, rendering our method amenable to scale-up and automation. The -NO<sub>2</sub> symmetric stretching vibration of DSNB has a signature peak at ~1340 cm<sup>-1</sup> that allows for distinct and quantitative determination of the binding specificity of influenza virus.

## Results and Discussion

### H5-HA1 immunogold assay and immunoblotting

To determine whether anti-HA1 antibodies can recognize the virus, we performed a western blot analysis for various influenza viruses (including H1N1, H3N2, and H5N1) in supplementary information. Figure S3 showed that the anti-H5N1/HA1 antibody could only specifically recognize the H5N1 virus. Moreover, immunogold tests were carried out by adding different kinds of influenza virus into AuNP probe solution for Transmission Electron Microscopy (TEM) images. To detect the H5N1 influenza virus, the AuNPs were conjugated with the anti-H5N1 hemagglutinin antibody. All samples were incubated overnight on the ice and followed by centrifuge and filtration to remove the unbound gold nanoparticles in solution. Figure 3A,3C and

3E display the TEM images of H1N1, H3N2, and H5N1 virus samples without adding gold nanoparticles for the control, while Figures 3B,3D and 3F show the virus images after reacting with AuNPs probe. Only the H5N1 viral sample in Figure 3F clearly exhibited distinct conjugation with the probe particles. The result justified our strategy to fabricate the SERS biosensor that could identify different subtypes of influenza virus.

### Selectivity of half sandwich assay for different subtypes of influenza viruses

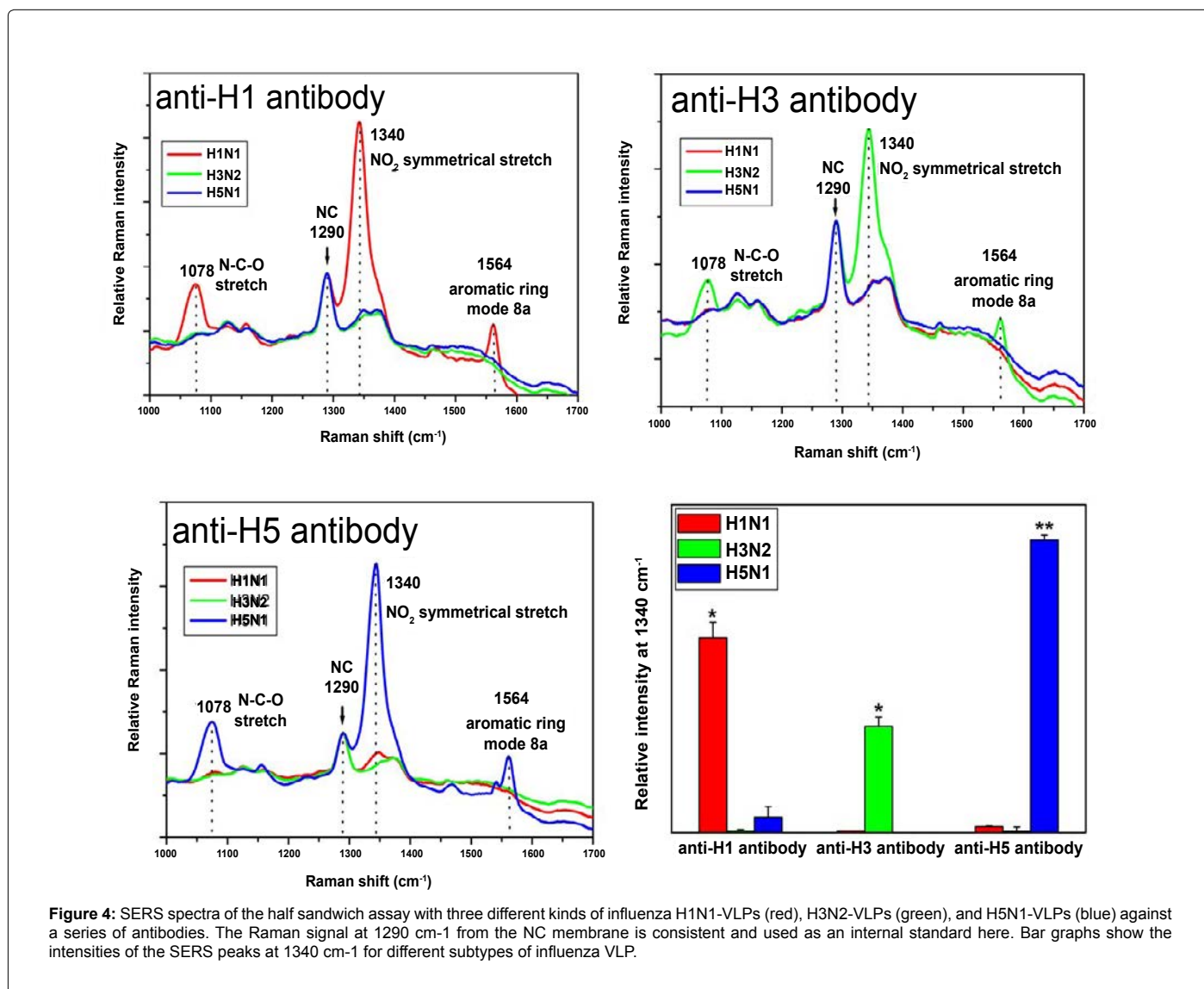
To examine the selectivity of the assay, 10ng of each of the three different types of VLPs (H1N1, H3N2, and H5N1) or PBS as blank was used. The Raman signal from the NC membrane is consistently found at 1290  $\text{cm}^{-1}$ , which was used as the internal standard for normalization of all the spectra. From the measurements (Figure 4), it is clear that we can identify the different subtypes of VLP by the assay while the other two negative VLP controls do not exhibit a prominent SERS peak at 1340  $\text{cm}^{-1}$ . The bar graphs for each antibody are significantly different from negative VLP controls at  $p < 0.05$  (\*) for H1N1 and H3N2 and  $p < 0.01$  (\*\*) for H5N1.

### Sensitivity of the SERS-based bioassay

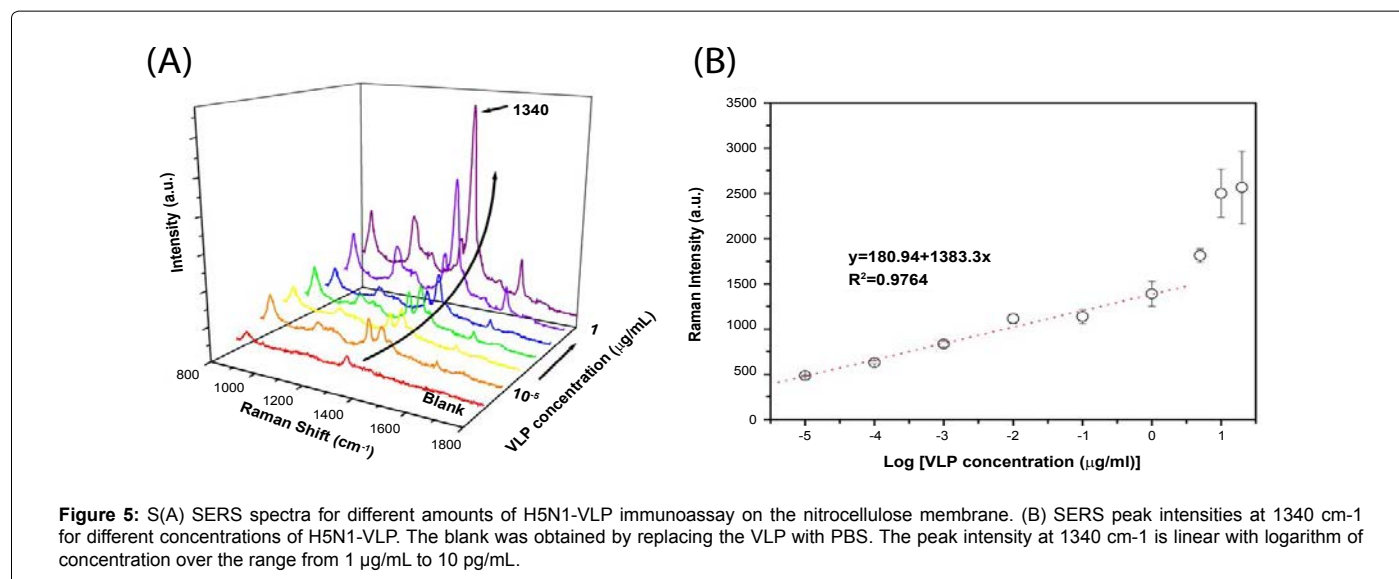
The sensitivity of the proposed strategy to viral detection was investigated by adding a series of different concentrations of VLP on the NC membrane. Figure 5A demonstrates that the intensity of the signature peak at 1340  $\text{cm}^{-1}$  in SERS spectra displays a high correlation with the concentration of VLP bound on the NC membrane. From Figure 5B, the correlated peak intensity at 1340  $\text{cm}^{-1}$  is linear with the logarithm of concentration from 1  $\mu\text{g/mL}$  to 10  $\text{pg/mL}$  ( $y = 180.94 + 1383.3x$ ,  $R^2 = 97.64\%$ ). The calculated detection limit (LOD) for the H5N1 VLP was 30  $\text{ng/mL}$ , which is obviously much more sensitive than that obtained with the current, rapid tests for influenza virus (data acquired by ELISA are shown in Figure S4 for comparison; the LOD from the ELISA method is 1  $\mu\text{g/mL}$ ).

### Conclusions

In conclusion, we have demonstrated a rapid, membrane-based ultra high sensitivity and selectivity, and highly portable biosensor to detect influenza virus through the use of SERS on the NC membrane. It was found that the detection limit was achieved as low as 30  $\text{ng/mL}$  with



**Figure 4:** SERS spectra of the half sandwich assay with three different kinds of influenza H1N1-VLPs (red), H3N2-VLPs (green), and H5N1-VLPs (blue) against a series of antibodies. The Raman signal at 1290  $\text{cm}^{-1}$  from the NC membrane is consistent and used as an internal standard here. Bar graphs show the intensities of the SERS peaks at 1340  $\text{cm}^{-1}$  for different subtypes of influenza VLP.



high selectivity to different subtypes of influenza virus tested herein. This resulted in better early detection of influenza virus than the ELISA method. The SERS characterization can be completed in less than 2h after sample collection without any further preparation, making our method easier and less-time consuming than the RT-PCR method. Furthermore, the highly portable equipment in our assay affords a practical means for on-site viral detection. In addition, the bioassay can be scaled up to multi-sample bioarray system to save more time and automatically identify the influenza virus subtypes in the future. This will provide an alternative for early identification of influenza virus leading to the timely treatment of infection and more effective flu outbreak management.

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