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Diagnosis of Tamiflu-Resistant Influenza Virus in Human Nasal Fluid and Saliva Using Surface-Enhanced Raman Scattering

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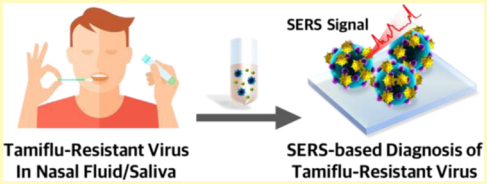
Supporting Information

ABSTRACT: Influenza viruses cause respiratory infection, spread through respiratory secretions, and are shed into the nasal secretion and saliva specimens. Therefore, nasal fluid and saliva are effective clinical samples for the diagnosis of influenza virus-infected patients. Although several methods have been developed to detect various types of influenza viruses, approaches for detecting mutant influenza viruses in clinical samples are rarely reported. Herein, we report for the first time a surface-enhanced Raman scattering (SERS)-based sensing platform for oseltamivir-resistant pandemic H1N1 (pH1N1) virus detection in human nasal fluid and saliva. By combining SERS-active urchin Au nanoparticles and oseltamivir hexylthiol, an excellent receptor for the pH1N1/H275Y mutant virus, we detected the pH1N1/H275Y virus specifically and sensitively in human saliva and nasal fluid samples. Considering that the current influenza virus infection testing methods do not provide information on the antiviral drug resistance of the virus, the proposed SERS-based diagnostic test for the oseltamivir-resistant virus will inform clinical decisions about the treatment of influenza virus infections, avoiding the unnecessary prescription of ineffective drugs and greatly improving therapy.



KEYWORDS: *Tamiflu, influenza, nasal fluid, saliva, surface-enhanced Raman scattering*

Since the first emergence of pandemic swine flu (H1N1) in 2009, influenza A viruses have become a major threat to human health.[1] To relieve the symptoms of influenza virus infection and treat influenza virus-infected patients, oseltamivir phosphate (Tamiflu) has been widely used since receiving Food and Drug Administration approval in 1999.[2,3] Unfortunately, the abuse of oseltamivir phosphate triggered the emergence of oseltamivir-resistant viruses, and the occurrence of these mutant viruses has sharply increased, with such viruses quickly spreading worldwide and threatening global public health.[4,5] The World Health Organization even warned that oseltamivir phosphate should not be used for flu prevention because the administration of oseltamivir phosphate induces resistance to influenza viruses.

Influenza viruses have typically been detected by using immunoassays and rapid molecular assays after nasal and throat swab sampling.[6] Since influenza viruses are mainly transmitted to other people through nasal secretions, analysis of nasal fluid samples has been considered as the most accurate way to diagnose influenza viruses, although nasal swab sampling is uncomfortable and painful.[6] Saliva has often been employed as an alternative[7,8] because saliva collection is noninvasive, minimally disturbs patients, and can be performed by an untrained person.[9,10] However, saliva contains a lower concentration of virus-infected cells than the nasal fluid. Although the current influenza virus diagnostic tests can identify subtypes of influenza viruses, they cannot provide information about antiviral drug resistance. Although polymerase chain reaction (PCR) techniques and enzyme assays have been employed to detect mutant viruses, these techniques are time-consuming and complicated, and they occasionally provide false-positive errors.[11−13] In this regard, we think that there is room for the development of a sensitive and specific detection method for oseltamivir-resistant influenza viruses in nasal fluid and saliva samples.

Surface-enhanced Raman scattering (SERS) is a powerful technique for the sensitive and selective detection of biomolecules because of its single-molecule-level sensitivity and molecular specificity.[14] During the past two decades, several kinds of SERS-based sensors have been developed to detect various types of influenza viruses, and these sensors exhibit high sensitivity and selectivity.[15−17] However, there are few reports of the identification of mutant viruses due to the lack of a receptor for mutant viruses. Recently, it was reported that oseltamivir hexylthiol (OHT) has a 250-fold higher binding affinity for the oseltamivir-resistant virus than the wild-

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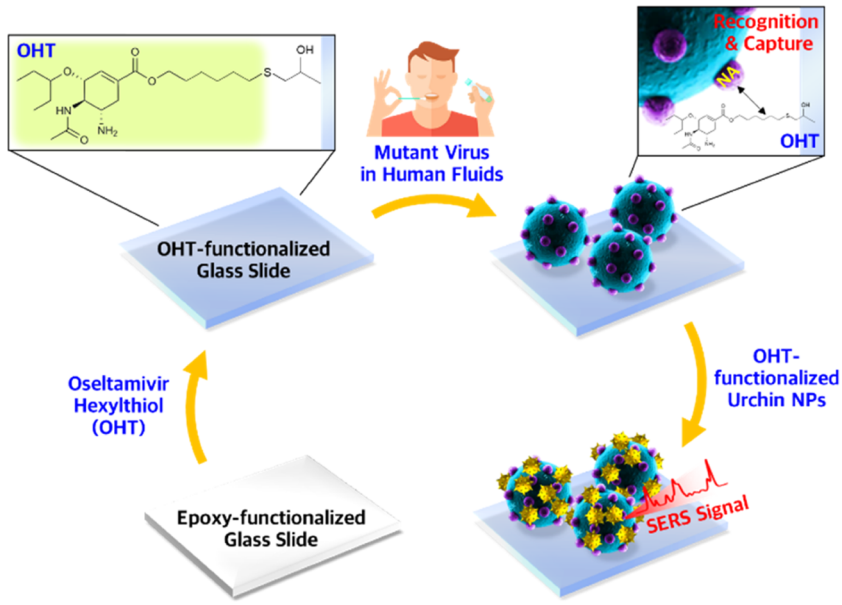

Figure 1. Schematic illustration of the pH1N1/H275Y mutant virus detection procedure using OHT-functionalized glass slides and urchin Au NPs. OHT-functionalized glass slides were incubated with pH1N1/H275Y mutant virus samples. Next, OHT-functionalized urchin Au NPs were reacted with the mutant virus captured on the glass slides, and SERS signals were measured.

type (WT) virus.[18,19] Herein, by combining this oseltamivir-resistant virus-specific receptor with urchin Au nanoparticles (NPs), we developed a novel SERS-based sensing method that can identify drug-resistant viruses with high sensitivity and accuracy in human saliva and nasal fluid samples. Only in the presence of oseltamivir-resistant virus, OHT-functionalized Au NPs bound to the mutant virus and strong SERS signals were obtained. In the presence of the wild-type influenza virus, weak SERS spectra were observed. We could detect the mutant virus at a low concentration of 1 PFU by using the method. Furthermore, the present SERS-based assay can detect the mutant virus specifically in the mixture of mutant and wild-type viruses. Most importantly, the oseltamivir-resistant viruses were detectable in the saliva and nasal fluid samples. To the best of our knowledge, this is the first report of the SERS-based diagnosis of drug-resistant influenza virus infection in clinical samples.

EXPERIMENTAL SECTION

Materials and Reagents. Epoxy-functionalized glass slides were purchased from Nanocs, Inc. Malachite green isothiocyanate (MGITC) was purchased from Setareh Biotech. NaOH was purchased from Sigma-Aldrich. OHT was synthesized by 4Chem Laboratory (Suwon, South Korea).

Preparation of OHT-Functionalized Glass Slides. To prepare OHT-modified glass slides, epoxy-functionalized glass slides (1″ × 3″) were immersed in a mixture of 135 μM NaOH and 135 μM OHT in deionized (DI) water at room temperature. After 12 h, the glass slides were washed with DI water.

Preparation of OHT-Functionalized Urchin Au NPs. To synthesize urchin Au NPs, 0.25 mL of 20 mM $HAuCl_4$ solution was added to 10 mL of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). Without shaking, the color of the solution changed from light yellow to dark burgundy within 30 min.[22,23] To prepare OHT-functionalized urchin Au NPs, the OHT solution (135 nM, 100 μL) was added to the urchin Au NP solution (1 mL) and reacted for 1 h at room temperature. Then, the NP solution was centrifuged at 15 000 rpm for 20 min at 4 °C and resuspended in DI water. All chemical solutions were prepared in Ultrapure Millipore water (18.2 M).

Preparation of the pH1N1 and pH1N1/H275Y Mutant Viruses. The pandemic H1N1 (pH1N1) virus (A/California/07/2009) (pH1N1) was obtained from the BioNano Health Guard Research Center (H-GUARD) of Korea. The pH1N1/H275Y mutant virus (H275Y mutation; A/Korea2785/2009 pdm: NCCP 42017) was provided by the National Culture Collection for Pathogens (NCCP), which is operated by the Korea National Institute of Health. All virus titers were determined using a one-step real-time PCR kit (Promega) in accordance with the manufacturer's instructions.

Diagnosis of Mutant Virus Infection Using SERS. WT virus and pH1N1/H275Y mutant virus samples were prepared after dilution to the desired virus concentration in DI water. The OHT-functionalized glass slides were immersed in the virus sample solution for 4 h at room temperature and washed with DI water three times. Then, the mutant virus captured on the slide was immersed in the OHT-functionalized urchin Au NP solution for 1 h at room temperature and washed with DI water. Finally, SERS signals were measured from the urchin Au NPs on the glass slides. Nasal fluid and saliva samples collected from healthy persons were purchased from Lee BioSolutions, Inc. The human fluid specimens were unprocessed and stored at −20 °C immediately after collection. To spike influenza viruses into the human fluid samples, nasal fluid and saliva were thawed at room temperature, and 10^4 PFU of influenza virus (10 μL) was spiked into each human fluid specimen (90 μL). Next, the virus-spiked human fluid specimens were filtered with a syringe filter (0.2 μm pore size, DISMIC-13cp, ADVANTEC, Japan). The filtered samples were diluted 10-fold with DI water (1000 μL total volume) and diagnosed through the same procedure as above.

Instrumentation. SERS measurements were carried out using a micro-Raman system based on an Olympus BX41 microscope. The excitation source was a He−Ne laser operating at λ = 633 nm, and the laser power was 20 μW. The laser spot was focused on a glass slide through a 50× objective lens. The laser spot diameter was about 1 μm. The SERS signals were recorded with a thermodynamically cooled electron-multiplying charge-coupled device (Andor) mounted on a spectrometer with a 1200-groove/mm grating. Scanning electron microscope (SEM) images were obtained on a Nova 230 system at an accelerating voltage of 15 keV. Transmission electron microscope (TEM) images were obtained on a Tecnai G2 F30 S-Twin microscope operated at 300 kV. Absorbance spectra were obtained from a UV/vis spectrometer (Beckman Coulter). The extinction spectrum was obtained from a UV/vis spectrometer (PerkinElmer).

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RESULTS AND DISCUSSION

Influenza viruses infect cells through the steps of adsorption, endocytosis, replication, assembly, and release. At the release step, neuraminidase (NA) on the viral surface promotes the release of progeny viruses from infected cells and facilitates viral movement within the respiratory tract.[6] When influenza virus-infected patients take oseltamivir phosphate, the drug strongly binds to the NA proteins of viruses, blocking the enzyme active sites and thus inhibiting the release of progeny viruses from infected cells.[20] However, the histidine-to-tyrosine mutation at the 275 position (H275Y mutation) of the influenza virus interrupts the binding of oseltamivir phosphate to the NA protein of the pH1N1/H275Y mutant virus.[21] Therefore, the pH1N1/H275Y mutant virus becomes 1500-fold less sensitive to oseltamivir phosphate than the WT pH1N1 virus.[22] To prevent the widespread transmission of the pH1N1/H275Y mutant virus and to treat the mutant virus, we previously developed OHT as a novel NA inhibitor for the pH1N1/H275Y mutant virus. OHT was synthesized by substituting the ethyl ester group of oseltamivir phosphate with a hexylthiol group. The synthesized OHT showed a 3.3 kcal/mol lower binding free energy with the pH1N1/H275Y mutant virus than with WT pH1N1, enabling us to identify the mutant virus specifically (Figure S1).[19] This unique property of OHT prompted us to develop a method for detecting the pH1N1/H275Y mutant virus by combining this tool with SERS.

Figure 1a shows a schematic illustration of the pH1N1/H275Y mutant virus detection procedure using OHT and SERS-active urchin Au NPs. First, an epoxy-functionalized glass slide was immersed in a mixture of NaOH and OHT to produce an OHT-functionalized glass slide. Thiolate anions on OHT can react with the epoxy groups of the glass slide.[23] Next, the OHT-functionalized glass slide was incubated with the pH1N1/H275Y mutant virus samples and washed. The virus samples were prepared by spiking the mutant viruses into nasal fluid and saliva samples collected from healthy individuals. Last, the urchin Au NPs were reacted with the mutant virus captured on the glass slide, and SERS measurements were performed. The surface of urchin Au NPs was modified by OHT and MGITC simultaneously. The number densities of particles are calculated to be 3.26×10^9 particles/mL. MGITC is a widely used Raman reporter that has a strong absorbance peak near 630 nm and provides a distinctive SERS signal. Because the OHT-functionalized glass slide can provide a well-exposed and densely fixed oseltamivir moiety (indicated by a green shadow), the pH1N1/H275Y mutant viruses can be captured on the glass slide efficiently. Moreover, the OHT-functionalized urchin Au NPs can recognize the mutant viruses on the glass slide effectively because a single mutant virus particle contains over 200 NA proteins, which is sufficient to bind to several OHT molecules.[24] Consequently, we could detect the pH1N1/H275Y mutant virus using this procedure.

Figure 2a is a TEM image of the synthesized urchin Au NPs. The urchin-like shapes are clearly observed, and the diameter of the NPs is ~60 nm. The extinction spectrum of urchin Au NPs shows that the NPs are well-dispersed without aggregation (Figure S2). Urchin Au NPs can exhibit strong SERS enhancement because highly enhanced local electromagnetic fields are generated in the urchin-like nanostructures.[25] Thus, the use of urchin Au NPs can be advantageous for the sensitive detection of mutant viruses. After the attachment of OHT to the urchin Au NPs through Au−S bonds, we obtained the absorbance spectra of the Au NPs before and after mixing with influenza viruses (10^5 PFU). As shown in Figure 2b, the bare OHT-functionalized urchin Au NPs showed an absorbance peak at 545 nm (black curve), and the peak shifted to 554 nm after mixing with the pH1N1/H275Y mutant virus (purple curve). This red shift is because the urchin Au NPs come very close to each other after the interaction between OHT and NA of the pH1N1/H275U mutant viruses. After mixing the urchin Au NPs with the WT influenza virus, the peak of the NPs remained at 545 nm (orange curve). This result clearly indicates that the OHT-functionalized Au NPs could selectively recognize the oseltamivir-resistant virus.

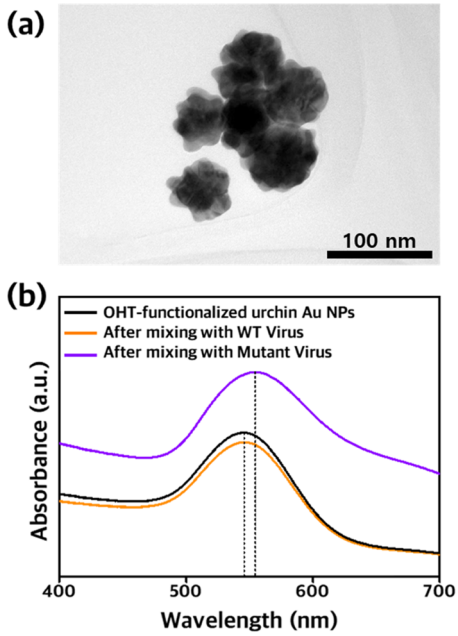

Figure 2. (a) TEM image of urchin Au NPs. The diameter of NPs is ~60 nm. Scale bar denotes 100 nm. (b) Absorbance spectra of OHT-functionalized urchin Au NPs before (black curve) and after mixing with the WT virus (orange curve) and the pH1N1/H275Y mutant virus (purple curve).

Next, we attempted to detect the mutant virus as depicted in Figure 1 by using the OHT-functionalized substrates and urchin Au NPs. Figure 3a shows SEM images of the OHT-functionalized glass slides after the detection of pH1N1/H275Y mutant virus (10^2 PFU), WT virus (10^2 PFU), and control samples. The SEM image of the control sample shows the nonspecific bindings of urchin Au NPs. In the WT virus sample, the number of NPs slightly increased compared to that in the control sample, but the urchin Au NPs were still sparsely distributed. Note that the number of NPs in the pH1N1/H275Y mutant virus sample was significantly larger than that in the WT virus and control samples. This finding suggests that the current method enabled the accurate identification of the pH1N1/H275Y mutant virus.

As shown in Figure 3a, the urchin Au NPs were densely assembled on the glass slide in the presence of the pH1N1/H275Y mutant virus. Since the assembled urchin Au NPs can provide strong SERS signals, we could detect the pH1N1/H275Y mutant virus sensitively and quantitatively through the measurement of SERS signals. Figure 3b shows a plot of the SERS intensity for the 1616 cm^{-1} band versus the concentration of influenza virus particles (0, 1, 10, 10^2, and

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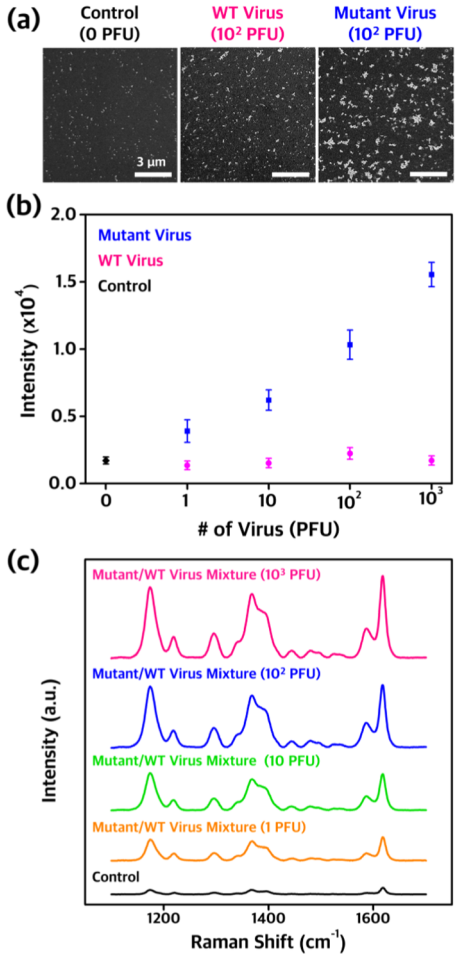

Figure 3. (a) SEM images of OHT-functionalized glass slides after the detection of control, WT virus (10^2 PFU), and pH1N1/H275Y mutant virus (10^2 PFU) samples. The scale bars denote 3 μm. (b) Plot of SERS intensity for the 1616 cm^{-1} band versus the concentration of influenza viruses (0, 1, 10, 10^2, and 10^3 PFU). The data represent the average plus standard deviation from five measurements. (c) SERS spectra of MGITC obtained from glass slides after the detection of mutant and WT virus mixture samples.

10^3 PFU). The control sample showed no distinct signals (black spot). When the sample contained the WT virus, weak SERS signals were obtained at all concentrations (blue spots). When the sample included the pH1N1/H275Y mutant virus, the SERS signals of MGITC increased as the concentration of the mutant virus increased (magenta spots). The full SERS spectra corresponding to the experiment presented in Figure 3b also show the selective detection of mutant viruses (Figure S3). This result agrees well with the SEM images shown in Figure 3a. Surprisingly, we could distinguish the pH1N1/H275Y mutant virus even at the low number of 1 PFU. This sensitivity may indicate that several OHT-functionalized urchin Au NPs were attached to a single pH1N1/H275Y mutant virus.

For the practical application of oseltamivir-resistant virus diagnostic methods, such methods should be able to identify mutant viruses in an environment in which mutant and WT viruses coexist. We investigated the selective detection of the pH1N1/H275Y mutant virus in a mixture of WT and mutant viruses by using the SERS-based method. Figure 3c shows the SERS spectra of MGITC obtained from the glass slides after the detection of mutant and WT virus mixture samples. The SERS signals of MGITC gradually increased as the concentration of the pH1N1/H275Y mutant virus increased, and 1 PFU of the mutant virus could be detected even in the presence of the WT virus. This result clearly confirms that the present SERS-based method can specifically detect the pH1N1/H275Y mutant virus.

Finally, we examined the detection of the pH1N1/H275Y mutant virus in human nasal fluid and saliva samples. Current influenza diagnostic tests employ the clinical specimens collected from the nasal and throat swab sampling; therefore, the clinical applicability of the proposed SERS-based method can be validated through the diagnostic test of the pH1N1/H275Y mutant virus in human nasal fluid and saliva samples. As shown in Figure 4a, nasal fluid and saliva were collected from healthy individuals, and the pH1N1/H275Y mutant viruses were spiked into the collected fluid samples. The prepared virus-spiked nasal fluid and saliva samples were filtered and diagnosed by the SERS-based method. To mimic the real clinical environments of influenza virus-infected patients, the concentrations of the influenza viruses in the nasal fluid and saliva samples were set at 10^4 PFU/mL. The mean viral load was ~3 × 10^4 PFU/mL in respiratory specimens obtained from 2009 pandemic H1N1 virus-infected patients immediately after symptom onset.[26] Since the prepared concentration of virus is approximately 3-fold lower than that in patients with symptoms of infection, we estimated that the samples were similar to real clinical samples before symptom onset.

Figure 4b,c show the SERS spectra of MGITC obtained from the glass slides after treatment with the pH1N1/H275Y mutant virus (blue spectrum), the WT virus (magenta spectrum), and no virus (black spectrum) in nasal fluid and saliva samples. Strong SERS signals clearly appeared in the mutant virus-spiked nasal fluid and saliva samples, whereas weak SERS signals were obtained in the WT virus-spiked and bare nasal fluid and saliva samples. Furthermore, we directly observed the mutant virus particles captured on the OHT-functionalized glass slides through SEM measurement (Figure 4d). In both nasal fluid and saliva samples, the pH1N1/H275Y mutant viruses were clearly visible on the glass slides. In contrast, the WT viruses were rarely observed on the OHT-functionalized glass slides (Figure S4). The size of the viruses is ~95 nm, which is similar to the previously reported size of influenza virus A. From the SEM results, the total number of captured viruses on the whole surface was estimated to be 2.0 × 10^6 in nasal fluid and 2.5 × 10^6 in saliva. These numbers correspond to a virus particle-to-PFU ratio of 200:1. Considering that the virus particle (counted by electron microscopy)-to-PFU (counted by plaque-forming assay) ratio is typically 100:1, the ratio in this work is acceptable.[27] The SERS and SEM results demonstrate the excellent diagnostic ability of the developed method for the pH1N1/H275Y mutant virus, even in human fluids. As a specific receptor for the pH1N1/H275Y mutant virus and as SERS-active nanoprobes, OHT and urchin Au NPs, respectively, contribute synergistically to the sensitive and selective identification of the oseltamivir-resistant virus. Meanwhile, for the practical use of the influenza virus-sensing methods, the price and time should be reasonable. We estimated the cost of this technique to be $50 according to the prices of materials. Although the present mutant virus identification method is more expensive than the commercial influenza virus KIT (~$10), the price may become

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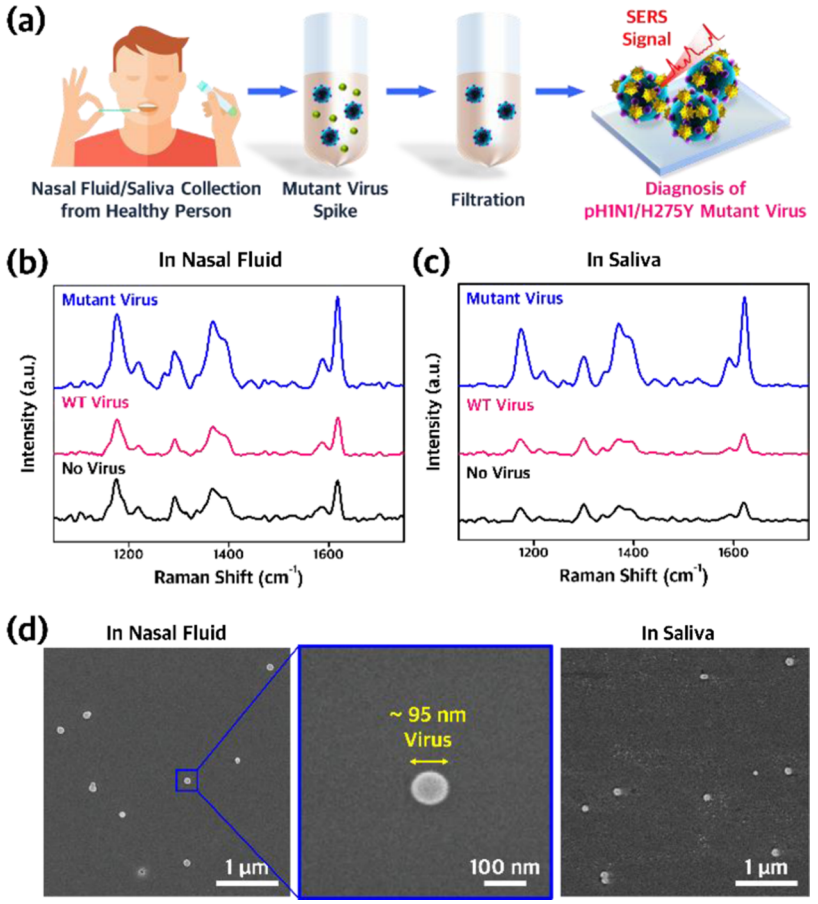

Figure 4. (a) Schematic illustration of pH1N1/H275Y mutant virus detection in human nasal fluid and saliva collected from healthy persons. Samples were prepared by spiking the pH1N1/H275Y mutant virus into the collected human fluids, followed by filtration and diagnosis by the SERS-based method. (b, c) SERS spectra of MGITC obtained from glass slides after the detection of the pH1N1/H275Y mutant virus (blue spectra), WT virus (magenta spectra), and no virus (black spectra) in (b) nasal fluid and (c) saliva. The concentrations of the viruses were 10^4 PFU/mL. (d) SEM images of mutant virus particles captured on OHT-functionalized glass slides. A magnified SEM image shows the captured pH1N1/H275Y mutant virus with a diameter of ~95 nm (blue box).

cheaper when it commercializes. In addition, the identification of the mutant virus takes ~5 h including virus incubation, NP reaction, washing, and measurements. The commercial influenza virus KIT requires 30 min for sensing. However, it should be noted that the commercial influenza virus KIT dose does not identify the mutant virus. Compared with the PCR-based methods, the price and detection time of this method could be advantageous.

CONCLUSIONS

In conclusion, we developed a SERS-based diagnostic method for the oseltamivir-resistant pH1N1/H275Y mutant virus in human nasal and saliva specimens. OHT-functionalized Au NPs bound to the mutant virus, and strong SERS signals were obtained only in the presence of the pH1N1/H275Y mutant virus. In the presence of the WT influenza virus, weak SERS spectra were observed. We detected the pH1N1/H275Y mutant virus at a low concentration of 1 PFU by using this method. Furthermore, the present SERS-based assay can detect the mutant virus specifically in a mixture of mutant and WT viruses. Most importantly, the oseltamivir-resistant viruses were detectable in saliva and nasal fluid samples. We anticipate that the proposed SERS-based approach could be used for the practical diagnosis of pH1N1/H275Y mutant virus infection and that it could further contribute to preventing the global transmission of drug-resistant viruses and effectively treating influenza virus-infected patients.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.9b00697.

Plot of absorbance values depending on the type of influenza virus (WT virus, mutant virus, and control) (Figure S1); extinction spectrum of urchin Au NPs (Figure S2); full SERS spectra corresponding to Figure 3b (Figure S3); SEM images of OHT-functionalized glass slides after treatment of nasal fluid and saliva samples with WT virus or no virus (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest.

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