Ultra-highly sensitive detection of influenza virus by Localized surface-plasmon resonance sensor

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Abstract

H5N1 is a highly pathogenic influenza virus that causes severe respiratory disease in birds and humans. The development of a highly sensitive detection method for H5N1 is vital to control spread and even terminate the outbreak of pandemic H5N1. Herein, we develop a highly sensitive local surface plasmon resonance (LSPR) sensor based on a gold-dot pattern chip for H5N1 detection. The LSPR sensor chip is fabricated by nano-imprint lithography, and it can detect and discriminate hemagglutinin H5 protein at femtogram-level sensitivity. Based on the results of the finite-difference time-domain simulation, the LSPR spectrum in the near-infrared region is suitable and appropriate to obtain the desired sensor sensitivity. The detection sensitivity of the sensor is 10^9 times higher than the immunochromatography method and is comparable to RT-PCR. Therefore, our sensor chip is very useful to provide valuable information for protection from pandemics.

Introduction

Over the past decades, globalisation has driven social and economic changes that deepen the threat of disease emergence and promote the spread of new viruses or diseases. For example, the world has experienced the new virus outbreak, normally referred to as 'coronavirus' (COVID-19) since the beginning of 2020. Global cooperation and support are normally needed to respond to such a serious epidemic or pandemic. The containment of new viruses requires early diagnosis using detection methods and rapid implementation of subsequent control measures. The highly pathogenic avian influenza subtype—H5N1—is a zoonosis with a mortality rate of 60% and has caused numerous hospitalisations and deaths¹. Specifically, H5N1 virus mutation may result in the ability to infect people easily and spread among humans, causing a pandemic^{2,3}. Such a virus not only poses a serious threat to the global public health systems but also has significant impacts on economic and social functions. In this regard, an early diagnosis that leads to appropriate medical treatment, such as antiviral drug administration, can boost the chances of survival for patients. For instance, the active metabolite of oseltamivir phosphate selectively inhibits neuraminidase (NA) of human influenza viruses and proliferation of the viruses by preventing the release of newly formed viruses from infected cells. The antiviral drug should be administered within 48 h of infection to achieve its optimal effectiveness⁴. Therefore, accurate early diagnosis of influenza virus in point of care testing is important to ensure the immediate administration of the antiviral agent if necessary.

Commercial rapid influenza diagnostic tests (RIDTs) are widely used in medical practice due to their high specificity and positive predictive values⁵. Such a rapid test can significantly ease the appropriate allocation of limited antivirals' supplies early in a pandemic outbreak. However, RIDTs were reported to have a high false-negative rate, especially among patients with a small amount of viral load at the early stages⁶. The resulting failure to initiate infection control precautions for patients hospitalised with influenza can result in the spread of the pandemic and increase the disease severity in the patients. Although RT-PCR is currently the most sensitive detection method of influenza virus, the high device cost

and long detection time of RT-PCR have limited its use for rapid virus confinement. Improvement of detection sensitivity for RIDTs is thus strongly required in the medical field.

Metal nano-structures, such as gold and silver, are known to exhibit local surface plasmon resonance (LSPR) in which the electromagnetic field of the light creates a collective coherent oscillation on the surface conduction electrons of the nano-structure in resonance with the light's specific frequency. The wavelength of the LSPR spectrum relies upon metal species, nano-structure and refractive index near the metal nano-pattern^{7–12}. The binding of biomolecules on the nano-pattern surface typically stimulates a change in the local refractive index, which results in a wavelength shift of the LSPR spectra^{13–15}. The LSPR-based biosensors have been considered as promising tools for simple test chips for detecting biological interaction due to their rapid, easy operation and relatively low sensitivity to temperature^{16–22}.

In general, the localised surface plasmon (LSP) field of isolated metal nano-structures has the short characteristic electromagnetic field decay length of around 5–15 nm^{23–27}. Consequently, LSPR sensors are very sensitive to refractive index changes occurring within 5–10 nm of the metal nano-structure surface^{24,25}. The LSPR biosensors with the antigen–antibody reaction are not suitable for influenza virus detection since the short LSP field is occupied by relatively large (4–14 nm) sized antibodies. When antibodies are immobilised on the metal nano-structure for antigen recognition, a large shift in the LSPR spectrum occurs by filling the LSP field with the antibody. Even if the antigen reacts with the antibody later, the LSPR spectrum shifts marginally due to a little difference between the permittivity of the antibody and the antigen. Such behaviour makes it difficult to detect the target biomolecules, especially at low concentrations. Several reports indicated that the detection limit of label-free protein by LSPR improves to around 100 pg mL⁻¹ by reducing the size of the receptor molecule^{28–30}.

In this study, we chose a relatively simple sugar acid (~ 1 nm) as receptor molecules due to the small sensitivity area (5–10 nm) of LSPR sensors. The capture of the influenza virus with small sugar could impose a huge impact on the LSPR spectrum shift, considering the increase of analyte mass to be close to the nano-pattern surface. There are two spike glycoproteins, which are hemagglutinin (HA) and NA, in influenza virus membranes, and they play important roles in the formation of virus infections, proliferation and budding in host cells^{31,32}. Influenza infection is initiated by the viral HA binding to sialic acid receptors on the surface of the host cell. Human influenza virus HA and avian influenza virus HA bind to a sugar chain having Neu5Acα2-6Gal (human receptor) and Neu5Acα2-3Gal (avian receptor), respectively^{33–35}. Therefore, the identification of the human-avian influenza virus using selective receptors on the sensor surface is possible.

The gold (Au)-dot pattern chip was manufactured using the nano-imprint method. The nano-imprinting method can form all nano-scale structures at once and exhibits high reproducibility. Owing to the simple and inexpensive apparatus configuration in the nano-imprint technology, components with various features can be manufactured at a lower cost when compared to conventional technologies such as electron beam lithography³⁶. Herein, we attempted high sensitivity detection of human/avian influenza

virus by immobilising Neu5Ac α 2-6Gal and Neu5Ac α 2-3Gal chains on the surface of Au-dot pattern chip produced by the nano-imprint method.

Results And Discussion

Design of gold-dot pattern with high sensitivity

Figure 1 displays the schematic illustration of the finite-difference time-domain (FDTD) simulation model for the Au-dot pattern chip. To improve the sensitivity of the LSPR sensor, it is important to understand, for each Au-dot pattern, how the LSPR peak shifts under the presence of the influenza virus (model protein). The FDTD simulation was therefore used to determine the diameter (D) and pitch (P) of Au-dot pattern structure suitable for the influenza virus detection from the peak shift of the LSPR spectra when a single model protein was placed on the Au-dot surface. The result of the FDTD simulation in **Supplementary Fig. S1** suggests that the Au-dot pattern structure with the peak position in the near-infrared region (780–2500 nm) is useful for detecting protein capture. The LSPR spectrum is heavily affected by water vapour in the atmosphere because water absorption bands occur at 1400 and 1800 nm. In this study, the pattern of D400P800 (**Supplementary Fig. S1**) with a peak wavelength of about 1200 nm was used to prevent deformation of the LSPR peak profile caused by moisture adsorbed on the sensor surface.

Fabrication of gold-dot pattern chip by the nano-imprint method

Several techniques have been developed for fabricating nano-scale structures, including the Au-dot pattern. Self-organisation techniques, such as colloidal lithography³⁷ and nano-sphere lithography^{38,39}, are well known for their low cost; however, these methods are unable to provide high precision nano-structures over large areas. On the other hand, the direct writing techniques such as electron beam lithography⁴⁰ requires high manufacturing cost due to the long processing time. To put it simply, these methods are not suitable for the mass production of nano-scale structures. Nano-imprint lithography^{41,42} has thus been a promising approach for fabricating uniform nano-structures over a large area. Au-dot pattern chips in this study were thus prepared by the thermal nano-imprinting method (**Fig. 2**).

The main issue in manufacturing the Au-dot pattern is weak adhesion between Au and quartz substrate. Generally, a metal adhesion underlayer such as silicon (Si), titanium and chromium is interposed between Au and quartz substrate to enhance their adhesion. However, the metal underlayer considerably reduces the plasmon dephasing time, causing degradation of LSPR sensitivity⁴³. Jo et. al.²⁷ reported that LSPR sensitivity could be improved by partially removing the metal underlayer through piranha treatment despite the decrease of the mechanical and chemical stabilities of the Au-dot pattern chip. In this study, we simultaneously improved LSPR sensitivity and mechanical strength by oxidising the Si underlayer via heating at 450 °C; Si forms a eutectic with Au at more than 370 °C⁴⁴. The improvement of the mechanical strength is likely attributed to the occurrence of Au–Si eutectic reaction.

Detection experiments of influenza virus using the gold-dot pattern chip

A sialic sugar chain with a small molecular weight was used as a recognition molecule for detecting HA with high sensitivity herein. Polysaccharides play an important role as ligands specifically recognisable by various HA variants in influenza virus infections into host cells. The receptor specificity of influenza viruses has been primarily stratified by recognition of sialic acid species (N-acetyl- or N-glycolylneuraminic acid) and the type of glycosidic linkage between sialic acid and penultimate galactose ($\alpha 2,3$ or $\alpha 2,6$)^{45, 46}; $\alpha 2,3$ sialic acid is known to strongly bind H5 HA and $\alpha 2,6$ sialic acid to H1 HA. We observed that H5 and H1 HAs can be distinguished with high sensitivity by immobilising $\alpha 2,3$ and $\alpha 2,6$ sialic acids on the Au-dot pattern chip, respectively.

Figure 3a represents the LSPR spectral change of the $\alpha 2,3$ sialic acid-coated chip that was employed to detect 1 fg mL^{-1} of avian influenza virus (H5N1). To investigate the ability to distinguish virus type and the possibility of false-positive occurrence, 1 pg mL^{-1} of human influenza virus (H1N1) with a 1000-fold higher concentration than H5N1 virus was added dropwise on the chip. The peak shift of the LSPR spectrum barely occurred when 1 pg mL^{-1} of H1N1 was dripped on the chip. Subsequently, 1 fg mL^{-1} of H5N1 was dripped on the chip, resulting in the red-shift of the peak by about 10 nm.

Figure 3b represents the LSPR spectral change of the $\alpha 2,6$ sialic acid-coated chip that was employed to detect 1 fg mL^{-1} of H1N1. H5N1 (1 pg mL^{-1}) was dripped on the chip, followed by 1 fg mL^{-1} of H1N1. Although the peak shift of the LSPR spectrum barely occurred after the dripping of 1 pg mL^{-1} H5N1, the dripping of 1 fg mL^{-1} H1N1 generated the red-shift of the peak by about 10 nm. As the evaluation of other protein contaminants, the change in the LSPR spectrum was observed when $1 \text{ }\mu\text{g mL}^{-1}$ bovine serum albumin was dripped on the chip. The LSPR spectrum was barely shifted. It is evident that the influence of contaminants was rather low. These results show that the Au-dot pattern chip can discriminate between H1 and H5 HAs with high sensitivity.

Figure 3c shows the shift amount of the LSPR spectrum at H5N1 concentrations ranging from 1 ag mL^{-1} to 1 ng mL^{-1} . The shift amount of the LSPR spectrum was about 8 nm at the concentration above 1 fg mL^{-1} , and about 0.96 nm at the concentration of 100 ag mL^{-1} . The shift of the LSPR spectrum could not be observed at the concentration of 1 and 10 ag mL^{-1} . These results indicate that the detection limit of the Au-dot pattern chip is 1 fg mL^{-1} of HA. The HA included in the dropped solution ($10 \text{ }\mu\text{L}$ of 1 fg mL^{-1} HA) was estimated to have 100 HA molecules. Such HA amount is smaller than that in a single influenza virus containing approximately 500 HA. This means that the Au-dot pattern chip can detect a single influenza virus in about 10 min. Since the detection sensitivity of our chip is 10^9 times higher than currently used immunochromatography (detection sensitivity: viral protein concentration $10 \text{ }\mu\text{g mL}^{-1}$), our Au-dot pattern chip is extremely useful as an inspection chip.

The presence of HA on the chip surface was validated by using a nano-search microscope. **Figure 4a** shows the atomic force microscopy (AFM) image of the surface after dropping 1 fg mL^{-1} of H5. The AFM image demonstrates that a sphere of about 50 nm was present on the surface. The oxygen plasma treatment was implemented to prove that this sphere was HA. The plasma treatment got rid of all traces of organic matter and left no residues unless excessive inorganic contaminants were present on the sample. If this sphere disappeared after the plasma treatment, the sphere was likely an organic matter. **Figure 4b** shows the LSPR spectrum before and after the oxygen plasma treatment. The LSPR spectrum shifted by about 10 nm after dropping 1 fg mL^{-1} of H5, but it returned to its original state upon the oxygen plasma treatment. In addition, comparing AFM images, spherical bodies disappeared due to oxygen plasma treatment. These results suggest that a wavelength shift in the LSPR spectrum occurred due to the capture of HA on the surface of the Au dot.

What led to the detection of the influenza virus with ultra-high sensitivity?

This study suggests that the Au-dot pattern chip can detect influenza viruses with ultra-high sensitivity. The FDTD simulations reveal the reasons for the high sensitivity. **Figure 5b** shows the FDTD simulations of the electromagnetic field intensity distribution in the simulation area presented in **Fig. 5a**. The electric field was remarkably enhanced in the rim of the Au dot along the electric field polarisation direction. The electric field intensity enhancement ($|E|^2$) can achieve a maximum of 24.8, 27.49, 29.78 and 26.97 at the wavelengths of 1204.05, 1329.85, 1442.91 and 1576.99 nm, respectively. These simulations suggest that electric field intensity enhancement depends on the wavelength. **Figure 5c** shows the spectrum of the normalised electric field intensity in the near-infrared wavelength range from 0.4 to 1.8 μm . Sharp peaks were found at the wavelength between 0.5 and 0.65 μm , which are attributed to the size of the single Au dot. A broad peak was observed at the wavelength between 0.65 and 1.8 μm , which can reach up to 34.4 times compared with the incident electric field intensity.

The FDTD simulation results also indicate that the Au-dot pattern structure clearly affected the electric field intensity around the Au dot (**Fig. S6**). The D and P/D are important for controlling the peak position of the electric field intensity at the top edge of the Au dot. As the D of the Au dot increased, the peak position shifted to the higher wavelength, and the peak area increased. For the same D of the Au dot, the Au-dot P was affected by the peak area. To understand the correlation between the electric field intensity and the detection sensitivity of model proteins, we investigated the peak area change of the electric field intensity relative to the peak shift in the LSPR spectrum when a single model protein was placed on the Au-dot pattern surface (**Fig. 6**). The results show that the peak shift in the LSPR spectrum is negligible ($\sim 2 \text{ nm}$) when the peak area was below 15 ($D \leq 300$). When the peak area is more than 15 ($D \geq 350$), a positive correlation between the peak area and the amount of shift was displayed. At the peak shift of about 10 nm, which was comparable to that observed in the detection experiments of the influenza virus, the peak area was about 19. These results imply that the enhancement and localisation of the electric

field intensity in the Au-dot pattern are significant for the sensitive detection of the dielectric constant change associated with model protein adsorption.

Conclusion

Our work successfully demonstrates that the LSPR sensor chip can detect and discriminate H1 and H5 hemagglutinins at the femtogram level, which shows the ultra-high sensitivity of the sensor. Moreover, the sensor is capable of distinguishing the human influenza virus and highly pathogenic avian influenza virus. The detection sensitivity of the sensor is 10^9 times higher than the immunochromatography method. The detection ability is almost the same as the RT-PCR. Therefore, the sensor chip is very useful in providing valuable information to the medical diagnostic field in preventing serious pandemics such as the highly pathogenic avian influenza virus and COVID-19.

Method

Chemicals. The influenza hemagglutinin (HA) (A/H1N1/New Caledonia/20/1999, A/H5N1/Vietnam/1203/2004, and A/H5N1/Indonesia/05/2005; recombinant, full-length, and maintaining the oligomeric structure of the protein) was purchased from Protein Sciences Co. (Meriden, CT, USA). The glycans, α 2,3 sialic acid (3'-sialyllactose, Neu5Ac α 2,3GalGlc), and α 2,6 sialic acid (6'-sialyllactose, Neu5Ac α 2,6GalGlc) were purchased from Carbosynth Limited (Berkshire, UK). 3-Aminopropyltrimethoxysilane (APTMS), 3-aminopropanethiol and [(tert-Butoxycarbonyl)aminooxy]acetic acid were purchased from Tokyo Chemical Industry Co., Ltd. Bovine serum albumin was purchased from Sigma-Aldrich Corporation. All other chemicals were purchased from Tokyo Chemical Industry Co., Ltd.

Fabrication of Au-dot pattern chip by nanoimprint method. The Au-dot pattern was fabricated on a quartz substrate by thermal nanoimprint lithography. The substrate was first immersed in piranha solution (70% H₂O₄ and 30% H₂O₂), sonicated for 30 min, and then rinsed with Milli-Q water. A thermoplastics resin (mr-I 7030R) was spin coated onto the substrate, and then the substrate was baked for 2 min at 140°C. A mold with nanopattern was pressed into the resin coated onto the substrate surface at 150 °C using nanoimprint equipment (X-300, SCIVAX Co., Ltd.), followed by removal of the mold. The residue resin in pattern holes was removed by Ar milling treatment (EIS-220E, ELIONIX Co., Ltd.). We used the ECR sputtering equipment (EIS-230P, ELIONIX Co., Ltd.) to deposit a 2nm-thick Si layers, subsequently a 50-nm-thick Au layer on the patterned substrate. Finally, the residual resin pattern was removed by Ar milling treatment and sonicating the substrate in acetone for 3 hours and 2-propanol for 1 min. and then rinsed with Milli-Q water. Surface topographic images of the Au-dot pattern chip were acquired with an atomic force microscope (AFM, SFT-3500; Shimadzu Corp., Japan). A cross section and plane view of the chip was observed using a field-emission scanning electron microscope (FE-SEM, S-4800; Hitachi High-Technologies Corp., Japan).

Fabrication of recognition domain of influenza virus. The Au-dot pattern chip was heated for 1 hour in a furnace at 450°C and cleaned by 10 min UV/ozone irradiation. The SiO₂ surface of the chip was

immersed in 5:1:1 mixture of Milli-Q, H₂O₂ (30%) and NH₃ (25%) for 10 min at 70 °C to introduce hydroxyl groups on the surface, followed by coating with SAM of 3-aminooxypropyltriethoxysilane (APTES). The SAM was formed on the SiO₂ surface by immersing in 0.1% (v/v) APTES in Milli-Q water at room temperature for 1 hour, rinsed with Milli-Q water, and then heat treated in an oven for 1 hour at 100°C. On the other hand, the SAM was formed on the Au-dot surface by immersing in 1mM of 2-aminoethanethiol in methanol for 12 hours. Next, the amino group coated chip was immersed in 0.1% (v/v) [(*tert*-butoxycarbonyl) aminooxy] acetic acid solution including 0.1% (v/v) WSC at room temperature for 17 hours, rinsed with Milli-Q water. Removal of the *tert*-butoxycarbonyl protecting group was accomplished with 25% trifluoroacetic acid/methanol at 40°C. Subsequently, trisaccharides terminating in α2,3 sialic acid sialic and in α2,6 sialic acid (100 μM in acetic acid, pH 5.3) were allowed to react with the aminooxy group of the SAM on the chip surface at 60°C for 90 min.

Detection experiments of influenza hemagglutinin. 10 μL of H1 or H5 hemagglutinin solution ranging from 1 ag/ml to 1 μg/ml was dropped onto the surface of Au-dot patterned chip immobilized with α2,3 or α2,6 sialic acid, respectively, for 10 minutes, and then rinsed with Milli-Q water and naturally dried. The peak shift of LSPR spectra was observed by sensing system with the transmittance mode. The light source was a tungsten halogen lamp (LS-1, Ocean Optics) optimized for the visible-NIR (VIS–NIR) range (360–2500 nm). LSPR spectra were measured using a real-time CCD-array NIR spectrometer (NIRQuest, Ocean Optics).

Numerical simulation. The simulations were performed with FDTD Solutions (Lumerical Inc.). To calculate LSPR spectra and electric field distribution of Au dot pattern, a normally incident light source polarized along the x-axis covering a wavelength range from 400 to 1800 nm was used. The perfectly matched layer (PML) boundary condition was used along the x, y, and z axes. The grid sizes in the x, y and z directions (Dx, Dy and Dz) of the simulation domain were 1 nm respectively. The dielectric function of Au was selected from the materials database of the Lumerical FDTD solutions and the background relative dielectric constant was assumed to be 1.

Declarations

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Author contributions

A.M. wrote the manuscript, developed the concept, fabricated and characterized LSPR sensor and calculated FDTD simulation. Y.T. and K.N. provided Au-dot pattern using nano-imprint lithography. T.N.

and T.G. performed detection experiments of influenza hemagglutinin. All authors contributed to fruitful discussions and corrected the manuscript. A.M. supervised the research.

Competing interests

The authors declare no competing interests.

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Figures

Fig.1

Figure 1

Schematic illustration of the finite-difference time-domain simulation model for the gold (Au)-dot pattern chip. The diameter of the Au dot and the distance between the centres of two Au dots are represented by diameter (D) and pitch (P), respectively. The incident light was perpendicular to the chip. The adsorption of model protein led to a red-shift of the LSPR peak as a result of a refractive index change occurring at the Au surface.

Fig.2

Figure 2

Fabrication method of gold (Au)-dot pattern chip by nano-imprint lithography method. A thermoplastics resin was spin coated onto the quartz glass (1). A nano-pattern mould was pressed into the resin coated onto the thermoplastics resin (substrate) surface at 150 °C (2), followed by removal of the mould (3). The residue resin inside pattern holes was removed via reactive ion etching (4). The silicon (Si) film of 1 nm (first layer) and Au film of 50 nm (second layer) were then deposited on the patterned substrate by ion beam sputter deposition (5). Finally, the residual photoresist pattern was removed via sonication of the substrate in acetone for 15 min (6). SEM, scanning electron microscopy

Fig.3a

Fig.3b

Fig.3c

Figure 3

(a) Local surface plasmon resonance (LSPR) spectral change of the α 2,3 sialic acid-coated chip that was employed to detect 1 fg mL⁻¹ of avian influenza virus (H5N1). (b) LSPR spectral change of the α 2,6 sialic acid-coated chip that was employed to detect 1 fg mL⁻¹ of H1N1. (c) LSPR spectrum at H5N1 concentrations ranging from 1 ag mL⁻¹ to 1 ng mL⁻¹. The shift of the LSPR spectrum was 8.11 ± 1.52 nm at the concentration of 1 fg mL⁻¹, 8.46 ± 0.87 nm at the concentration of 1 pg mL⁻¹ and 8.24 ± 1.73 nm at the concentration of 1 ng mL⁻¹.

Fig.4a

Fig.4b

Figure 4

(a) Atomic force microscopy images of the gold (Au)-dot surface (dot A and dot B) with the dripping of 1 fg mL⁻¹ of H5, and those that experienced oxygen plasma treatment after the dripping. (b) Local surface plasmon resonance spectrum before and after the oxygen plasma treatment.

Figure 5

(a) Schematic illustration of finite-difference time-domain (FDTD) simulation area (X–Y plane and cross-section) on the gold (Au)-dot pattern surface. (b) FDTD simulations of the electromagnetic field intensity distribution in the X–Y planes (D400P800) on the top of the Au dot ($Z = 0.05\mu\text{m}$) and the Z section (A–B) at the wavelength of 1204.05, 1329.85, 1442.91 and 1576.99 nm. (c) The change in the normalised electric field intensity ($|E|^2$) at the top edge of the Au dot (D400P800) in the near-infrared wavelength ranging from 0.4 to 1.8 μm .

Fig.6

Figure 6

Relationship between the peak area of the electric field intensity and the peak shift amount of the local surface plasmon resonance spectra with adsorption of a single model protein. D is the Au-dot diameter.

Supplementary Files

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