



## Silver nanotriangle array based LSPR sensor for rapid coronavirus detection

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## ABSTRACT

A rapid, portable, and cost-effective method to detect the infection of SARS-CoV-2 is fundamental toward mitigating the current COVID-19 pandemic. Herein, a human angiotensin-converting enzyme 2 protein (ACE2) functionalized silver nanotriangle (AgNT) array localized surface plasmon resonance (LSPR) sensor is developed for rapid coronavirus detection, which is validated by SARS-CoV-2 spike RBD protein and CoV NL63 virus with high sensitivity and specificity. A linear shift of the LSPR wavelength versus the logarithm of the concentration of the spike RBD protein and CoV NL63 is observed. The limits of detection for the spike RBD protein, CoV NL63 in buffer and untreated saliva are determined to be 0.83 pM, 391 PFU/mL, and 625 PFU/mL, respectively, while the detection time is found to be less than 20 min. Thus, the AgNT array optical sensor could serve as a potential rapid point-of-care COVID-19 diagnostic platform.

## 1. Introduction

The SARS-CoV-2 pandemic COVID-19 disease has led to unprecedented burden on national and international healthcare. This has motivated researchers to develop reliable tools to aid SARS-CoV-2 diagnostics. The current molecular diagnostic tests for SARS-CoV-2 can be classified into two categories, i.e., nucleic acid tests and serological/immunological tests. The identification of SARS-CoV-2 typically involves viral RNA based reverse transcriptase real-time polymerase chain reaction (RT-PCR) and nucleic acid hybridization strategies [1,2]. RT-PCR is the 'gold standard' and has excellent selectivity and sensitivity and is laboratory-based [3], but this detection requires viral RNA extraction and expertise in PCR which is time-consuming and requires qualified personnel. Similarly, immunological tests may take days-to-weeks after the onset of symptoms for a patient to develop a detectable antibody level [4]. Although the IgM/IgG rapid test kits are available, false-positive results may occur [5]. Recently, direct detection of SARS-CoV-2 using reverse transcription loop-mediated isothermal amplification (RT-LAMP) on heat-inactivated samples has become available [6]. However, the development of rapid and highly accurate biosensors for coronavirus (CoV) is still needed. Table S1 in the Supplementary Materials (SM) summarizes some of the recent development and the corresponding sensing performance. Among different proposed methods, localized surface plasmon resonance (LSPR)-based optical

sensors are one of the potential entrants as a rapid SARS-CoV-2 sensor, have attracted notable attention and been widely studied over the past decade [7–9]. The resonance absorbance wavelength of the LSPR sensor responds to changes in the local dielectric environment. These sensors are compact, durable, repeatable, and more reliable than traditional sensors, offering real-time and label-free chemical and biological detection. Label-free LSPR biosensors for SARS-CoV-2 screening have been reported for nucleic acids [10,11], surface protein subunits (spike, envelope, and membrane) [12], and SARS-CoV-2 virus [13,14], as well as antibodies (IgG, IgM) [15–17]. For example, Ventura et al. proposed a colorimetric sensor using gold nanoparticles for SARS-CoV-2 surface spike protein detection [12]. Huang et al. developed a double-antibody sandwich plasmonic resonance immunoassay for SARS-CoV-2 pseudovirus detection using Au nanocup array chip and gold nanoparticles, and reported a LOD of 370 virus particle/mL [14]. But this sandwich detection strategy and sensor preparations are complicated. Very recently, an aptamer-functionalized gold nanoparticle based sensor was reported and was able to detect 16 nM spike protein and  $3.54 \times 10^6$  genome copies/mL of inactivated SARS-CoV-2 [13]. However, silver should have a higher plasmonic effect compared to Au [18]. And the rapid and cost-effective LSPR sensor chips still need to be developed for direct detections of SARS-CoV-2 or coronavirus (CoV).

In this study, a human angiotensin-converting enzyme 2 protein (ACE2) functionalized silver nanotriangle (AgNT) array LSPR sensor is

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developed with high sensitivity and specificity of SARS-CoV-2 spike RBD proteins and CoV NL63. A linear shift of the LSPR wavelength versus the logarithm of spike RBD protein concentration is observed in the concentration region from 2.03 pM to 9420 pM. For NL63, the detection range extended from 625 to  $10^4$  PFU/mL in untreated saliva. The detection time is determined to be less than 20 min. This rapid optical sensor can be expanded as a potential point-of-care COVID-19 diagnostic platform for real sample analysis.

## 2. Materials and methods

### 2.1. Materials

Polystyrene nanospheres (PSNS) with 500 nm diameter (Polyscience, Lot# 679675) were used to form the colloid monolayer onto clean glass slides (Gold Seal, Part# 301). Sulfuric acid (Fisher Scientific, 98%), ammonium hydroxide (Fisher Scientific, 98%), and hydrogen peroxide (Fisher Scientific, 30%) were acquired to clean the glass slides. Silver (Kurt J. Lesker, 99.999%) and titanium pellets (Kurt J. Lesker, 99.995%) were purchased as the evaporation materials. Methanol, acetone, 1-hexanol (Tokyo Chemical Industry Co., >98%), chloroform (J.T. Baker, 99%), tetrachloromethane (Sigma-Aldrich, 99.9%), and toluene (Fisher Scientific, 99.8%) were used to characterize the refractive index (RI) sensing performance of the AgNT array. Human angiotensin-converting enzyme 2 protein (Sino Biological) and SARS-CoV-2 spike receptor binding domain protein (spike RBD; Sino Biological) were purchased for surface functionalization and sensing application. Bovine serum albumin (Sigma) was purchased for surface blocking. Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ ;  $\geq 99.0\%$ ), sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4$ ;  $\geq 99.0\%$ ), sodium chloride ( $\text{NaCl}$ ;  $\geq 99.0\%$ ), trehalose, mannitol, and Tween-80 were purchased from Sigma. PBS buffer (100 mM  $\text{Na}_2\text{HPO}_4$ ,

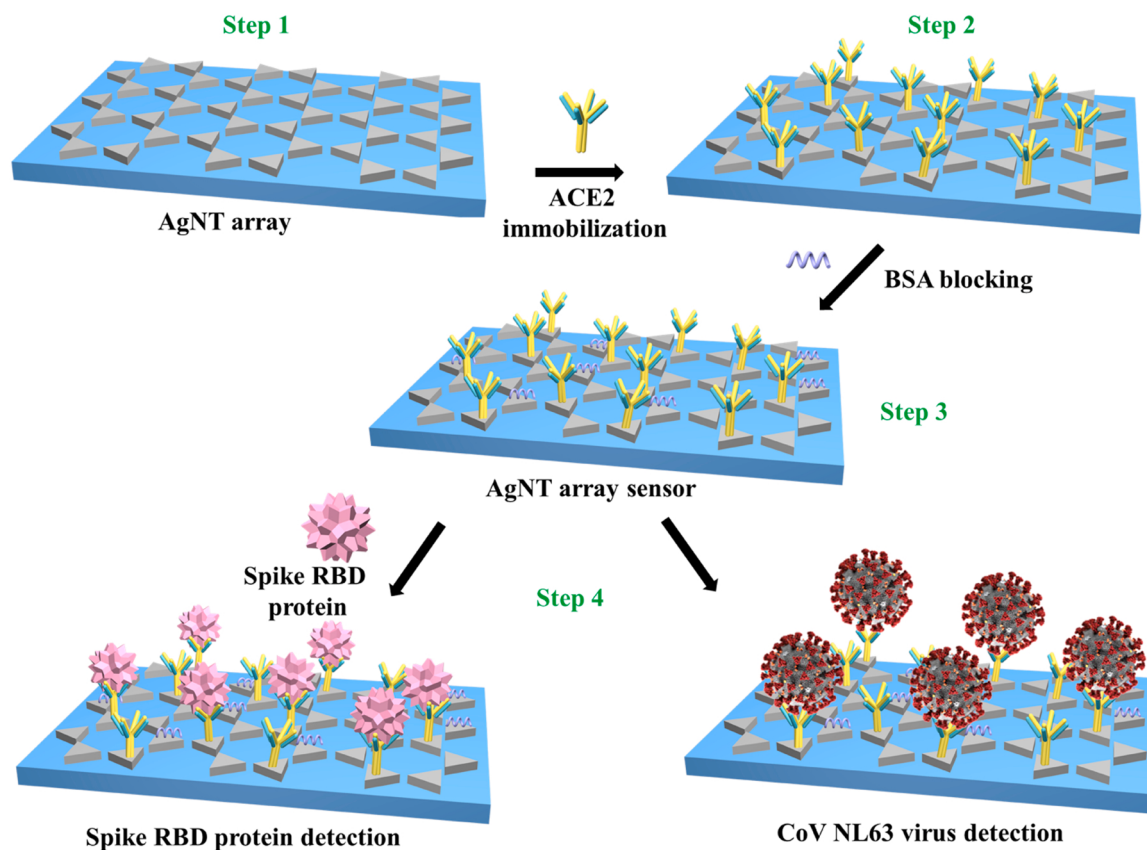
100 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM  $\text{NaCl}$ , pH = 7.4), phosphate buffer (100 mM  $\text{Na}_2\text{HPO}_4$  and 100 mM  $\text{NaH}_2\text{PO}_4$ , pH = 7.4) and dilution buffer (1 mL PBS containing 0.05 g trehalose, 0.05 g mannitol, 0.1  $\mu\text{L}$  Tween-80) were prepared and used for the treatment of proteins. Dulbecco's Modified Eagles Medium (DMEM; GIBCO BRL laboratories, Grand Island, NY) supplemented with 1% fetal bovine serum (FBS; Hyclone Laboratories, Salt Lake City, UT) was used as cell culture media. Deionized (DI) water (18 M $\Omega$  cm) was used throughout all the experiments. All chemicals and materials were used without further purification.

### 2.2. Sensing strategy

The general sensing strategy is shown in Fig. 1. It consists of four steps: Step 1: AgNT array fabrication. Step 2: ACE2 immobilization. Step 3: BSA blocking. Step 4: spike RBD protein or virus detection.

### 2.3. Fabrication of AgNT array

The general fabrication procedure is outlined in Fig. S1A. Prior to PSNS monolayer formation, the glass substrates (2.54 cm  $\times$  0.9 cm) were cleaned with sulfuric acid, ammonium hydroxide, and hydrogen peroxide. All substrates were then rinsed in deionized water. The uniform PSNS (diameter 500 nm) monolayers with large monocrystalline domains were prepared on glass substrates via an air-water interface method [19]. The PSNS monolayer-coated substrates were loaded into a custom-designed electron beam deposition system with the substrate normal antiparallel to the incident vapor direction. The vacuum chamber was pumped down under a base pressure of  $< 10^{-6}$  Torr. A 3 nm layer of Ti was deposited at a rate of 0.2 nm/s followed by a 60 nm Ag film deposited at 0.3 nm/s under a high vacuum condition. The



**Fig. 1.** Proposed strategy for spike RBD protein or virus detection. Step 1: AgNT array fabrication. Step 2: ACE2 immobilization. Step 3: BSA blocking. Step 4: spike RBD protein or CoV detection.

deposition rate and vapor thickness on the substrate were monitored by a quartz crystal microbalance. After the Ag deposition, the monolayer template was removed using Scotch tape, and the substrates were rinsed in toluene, acetone, and 2-propanol to remove PSNS residue. Subsequently, a PDMS layer with arrayed small wells (3 wells, with the well diameter of 4 mm, well depth of 1 mm) was molded on the AgNT array to restrict the effective sensing areas (Fig. S1B), and we refer them as AgNT wells.

#### 2.4. Immobilization of ACE2 on AgNT array

The ACE2 protein was immobilized on the AgNT array via electrostatic and hydrophobic interactions. First, 200  $\mu\text{L}$  ACE2 protein at 0.62 mg/mL was dialyzed in phosphate buffer (100 mM  $\text{Na}_2\text{HPO}_4$  and 100 mM  $\text{NaH}_2\text{PO}_4$ , pH = 7.4). The dialyzed ACE2 solution was diluted to 66  $\mu\text{g}/\text{mL}$  with phosphate buffer. 20  $\mu\text{L}$  ACE2 solution was transferred into each AgNT well and incubated for 2 h at room temperature. Then, the wells were washed with DI water 3 times. Subsequently, 20  $\mu\text{L}$  of 1 mg/mL BSA solution was transferred to a AgNT well and incubated for 2 h in order to block the ACE2 uncovered area of AgNT and avoid nonspecific binding of spike RBD protein or virus particles. Subsequently, the wells were rinsed with DI water and air-dried. The ACE2 protein modified AgNT array substrates (AgNT sensors) were now ready for the detection. The corresponding optical transmission spectra of the same AgNT well was measured after each step of surface modification.

#### 2.5. Spike RBD protein detection

20  $\mu\text{L}$  spike RBD protein ranging from 2.03 pM to 9420 pM in dilution buffer were transferred into different ACE2 functionalized AgNT wells and incubated for 2 h at room temperature. Then, the AgNT array sensors were washed with DI water and air-dried for optical transmission measurements.

#### 2.6. Virus incubation

All the virus experiments, including virus preparation, characterization, and LSPR measurements, were conducted in a biosafety level 2 (BSL-2) environment. CoV NL63, CoV 229E, and CoV OC43 were propagated in Vero E6 cells which were maintained in DMEM supplemented with 1% heat-inactivated (56 °C) FBS. Briefly, cells were infected using a multiplicity of infection (MOI) = 0.1. After 48 h, the viruses were harvested in serum-free DMEM followed by two freeze-thaws (−70 °C/4 °C), after which the contents were collected and centrifuged at 4000 g for 15 min at 4 °C. The virus titers were similar, i.e.,  $10^5$  PFU/mL, determined by immunostaining plaque assay as previously described [20]. Table S2 lists the different kinds of coronavirus as well as corresponding receptors. There are three kinds of coronavirus, CoV NL63, SARS-CoV, SARS-CoV-2, which are specific to ACE2 protein. The experiments on SARS-CoV and SARS-CoV-2 must be conducted in BSL-3 lab, while CoV OC43, CoV 229E, and CoV NL63 can be handled in BSL-2 environment.

#### 2.7. Coronavirus (CoV) detection

CoV NL63, CoV 229E or CoV OC43 cell-free supernatant suspensions ranging from 391 to  $10^5$  PFU/mL in PBS buffer were transferred into different ACE2 functionalized AgNT wells and incubated for 2 h at room temperature. Subsequently the AgNT wells were washed 3x with DI water and air-dried for optical transmission measurements. Virus spiked saliva samples were prepared by adding different concentrations of CoV NL63 to saliva to achieve final concentrations ranging from 625 to  $10^4$  PFU/mL for detection. To mimic the non-pretreated saliva sample, saliva samples for each AgNT well were prepared by adding 2  $\mu\text{L}$  of different concentrations of coronavirus solutions to 18  $\mu\text{L}$  of saliva, so the original coronavirus solutions were diluted 10 folds, i.e., the

maximum viral concentration in the saliva sample for detection was only  $1 \times 10^4$  PFU/mL.

#### 2.8. Instruments

The morphology of the AgNT array was characterized by atomic force microscopy (AFM, Park Systems NX-10 AFM). The optical transmission spectra of the as deposited AgNT arrays were measured by an ultraviolet-visible spectrophotometer (UV-Vis, Jasco-750). For virus detection in a BSL-2 environment, a portable UV-Vis spectrophotometer was used for transmission measurement of CoV detection in a biosafety level-2 hood and a pair of pinholes were added between the incident light and sample surface to restrict the incident light to a smaller area. The setup also consisted of a halogen source (Tungsten Halogen HL-2000), a spectrophotometer (Ocean Optics USB2000) and two optical fibers. The transmission spectra  $T_s(\lambda)$  and  $T_r(\lambda)$  of AgNT wells and reference sample (bare glass slide) were measured separately, and the final transmission spectra  $T(\lambda)$  was calculated as  $T(\lambda) = T_s(\lambda)/T_r(\lambda)$ .

### 3. Results and discussion

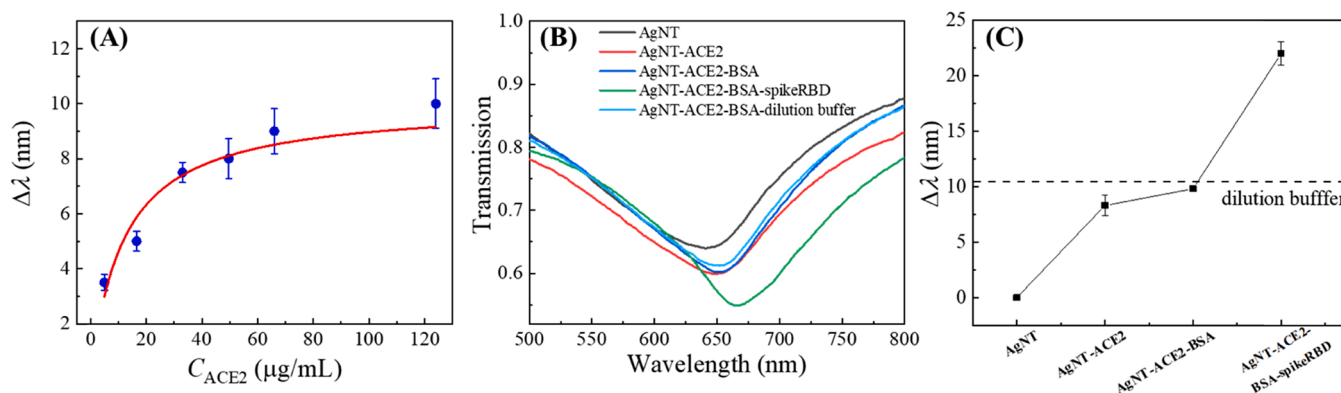
#### 3.1. Characterization of AgNT array

Fig. S2A shows a representative AFM image of AgNT array fabricated via the 500 nm PSNS monolayer. As expected, equilateral nanotriangle arrays with the side length of  $152 \pm 3$  nm are formed, and the height of each triangle is  $60 \pm 1$  nm. A distinct dip  $\lambda_0$  in UV-Vis transmission spectrum (Fig. S2B) is found at  $\lambda_0 = 641 \pm 2$  nm, taking from 8 different AgNT samples (Fig. S3). The refractive index (RI) sensitivity of the AgNT array is evaluated by measuring the  $\lambda_0$  shift when the AgNT array is immersed in solutions with different refractive indices, and the corresponding transmission spectra are shown in Fig. S2C. The change of  $\lambda_0$  versus refractive index (RI) is plotted in Fig. S2D, and the slope gives the sensitivity of the LSPR sensor,  $S = 210 \pm 10$  nm/RIU, where RIU refers to refractive index unit. A similar sensitivity of 191 nm/RIU has been reported for a similar structure[21]. Even though this AgNT array does not have a high RI sensitivity, the AgNT array is straightforward to fabricate and has large uniform area. The nanofabrication procedure for AgNT array is relatively inexpensive compared to gold nanostructures or other nanostructures fabricated either by focus ion beam method or by electron beam lithography method.

#### 3.2. Optimize ACE2 protein immobilization on the AgNT array

ACE2 is the cellular receptor for NL63, SARS-CoV, and SARS-CoV-2 [22]. Previous studies have demonstrated that the S1 domains of coronaviruses contain the receptor-binding domains (RBDs) that directly bind to the cellular receptors [23]. To achieve specific detection of spike RBD protein and NL63, ACE2 protein is used to functionalize the AgNT array. However, Ag is not chemically stable in NaCl solution [24], rather is found to be stable in phosphate buffer (see Section S1 in SM). Thus, phosphate buffer was used to dialyze the ACE2 solution to remove the chloride ions and for further ACE2 immobilization. To optimize the ACE2 protein immobilization on the AgNT array, 20  $\mu\text{L}$  ACE2 solutions of a series of concentrations ranging from 4.96 to 124  $\mu\text{g}/\text{mL}$  were transferred into different AgNT wells. Transmission spectra of AgNT wells were measured before and after the ACE2 immobilization. The redshift of  $\lambda_0$ , i.e.,  $\Delta\lambda = \lambda_{ACE2} - \lambda_0$ , versus different concentration  $C_{ACE2}$  of ACE2 protein is plotted in Fig. 2A. The  $\Delta\lambda$  initially increases rapidly with  $C_{ACE2}$  when  $C_{ACE2}$  is small; then approaches to a constant when  $C_{ACE2} > 40$   $\mu\text{g}/\text{mL}$ . This relationship follows the Langmuir isotherm adsorption model for proteins [25],

$$\Delta\lambda = \Delta\lambda_{\max} \left( \frac{kC_{ACE2}}{1 + kC_{ACE2}} \right), \quad (1)$$



**Fig. 2.** (A) The plot of the shift of LSPR wavelength  $\Delta\lambda$  of AgNT array versus the different concentration  $C_{ACE2}$  of ACE2 protein. (B) The transmission spectra of AgNT array at different stages of surface functionalization and detection: immobilized with ACE2 protein, BSA blocking, spike RBD protein detection at  $C_{spikeRBD} = 675$  pM as well as AgNT array sensor incubating with dilution buffer. (C) Corresponding LSPR wavelength change  $\Delta\lambda$ .

where  $\Delta\lambda_{max}$  is the maximum LSPR shift measured when the ACE2 protein is fully covering the AgNT and  $k$  is the surface binding constant of the ACE2 to Ag surface. The solid curve in Fig. 2A shows the best fitting with  $k = 0.08 \pm 0.03 \text{ mL}/\mu\text{g}$  and  $\Delta\lambda_{max} = 10 \pm 1 \text{ nm}$ . The adsorption of ACE2 protein on the silver surface is mainly dependent on electrostatic interaction and the Ag-S covalent bond [26]. ACE2 proteins can bind to silver surfaces through either free amine groups or cysteine residues in the proteins and via the electrostatic attraction of negatively charged carboxylate groups. Those amino acids with residues containing sulfur atoms can form covalent bonds with silver. The binding constant is influenced by these factors as well as the surface coverage of the Ag film. According to the fitting, for  $C_{ACE2} > 66 \mu\text{g/mL}$ , the  $\Delta\lambda$  ( $= 8.3 \text{ nm} \times C_{ACE2} = 66 \mu\text{g/mL}$ ) does not change too much. Therefore,  $C_{ACE2} = 66 \mu\text{g/mL}$  of ACE2 has been selected as the optimized immobilization concentration. The success of the ACE2 immobilization on Ag surface was further confirmed by the surface enhance Raman scattering (SERS) spectroscopy as shown in Section S2 of SM.

The spike RBD detection is performed following the four steps shown in Fig. 1, and the corresponding transmission spectra and  $\Delta\lambda$  change for each step are shown in Fig. 2B. When AgNT array is incubated with  $66 \mu\text{g/mL}$  ACE2 protein, the  $\Delta\lambda$  red shifts  $8.3 \pm 0.9 \text{ nm}$  (measured from 20 AgNT wells, see Fig. S5), indicating that ACE2 proteins have been immobilized on the AgNT array. The assembly of ACE2 protein results in a change of the dielectric constant of microenvironment. After BSA blocking,  $\lambda_0$  further red shifts  $1.5 \text{ nm}$ . Such a small change in  $\Delta\lambda$  indicates that the BSA only blocks a small fraction of the AgNT surface area. When adding spike RBD protein of a concentration  $C_{spikeRBD} = 675$  pM,  $\lambda_0$  further increases  $12.0 \pm 0.4 \text{ nm}$ , indicating that the spike RBD proteins bind to ACE2 proteins. Compared  $\Delta\lambda$  between adding spike RBD protein and BSA blocking, this AgNT array sensor shows good specificity for spike RBD protein detection. Here, prior to the spike RBD detection, the buffer for spike RBD protein incubation has been optimized as shown in Fig. S6. The dilution buffer (1 mL PBS contains 0.05 g trehalose, 0.05 g mannitol, 0.1  $\mu\text{L}$  tween-80) gave the maximum LSPR shift and was used in all reported results for spike RBD protein incubation. When only adding dilution buffer without spike RBD protein,  $\lambda_0$  only red shifts around  $1 \text{ nm}$  (the dashed line in Fig. 2C), which is within the accuracy of the detection system. Also, with only the PBS buffer, the LSPR wavelength  $\Delta\lambda$  does not blue shift, which demonstrates that ACE2 and BSA protein can protect the silver surface from degradation in the biological environment. So, the functionalized AgNT array sensor is reliable for biosensing applications.

### 3.3. Spike RBD protein detection

The above result shows that the AgNT LSPR sensor can specifically detect spike RBD protein. Further  $C_{spikeRBD}$  dependence experiments

have been conducted and the  $\Delta\lambda$  versus  $C_{spikeRBD}$  plot is shown in Fig. 3A along with the original UV-Vis spectra. In a semi-log plot, the  $\Delta\lambda$  seems to increase linearly with  $\log_{10} C_{spikeRBD}$  (Fig. 3B). A good linear relationship is found in the  $C_{spikeRBD}$  region from  $2.03 \text{ pM}$  to  $9420 \text{ pM}$ , and the best fitting gives  $\Delta\lambda_{spikeRBD} = 3.7 \times \log[C_{spikeRBD}] + 1.8 \text{ (nm)}$ , where the goodness of fitting  $R^2$  is  $0.978$ . Defining the limit of detection (LOD) as the lowest detected concentration whose signal is higher than the blank control signal plus three standard deviations [27,28], the LOD of spike RBD protein is estimated to be  $0.83 \text{ pM}$ . This is comparable with those obtained by other methods, including commercial ELISA kits, that usually fall in the pM concentration range [29]. In addition, the total detectable range spans in 4 orders of magnitude, from  $\sim 1 \text{ pM}$  to  $10^4 \text{ pM}$ , and the actual amount of spike RBD molecules in the detection light beam area is estimated to be from  $3 \times 10^4$  to  $2 \times 10^9$  molecules shown in Table S4.

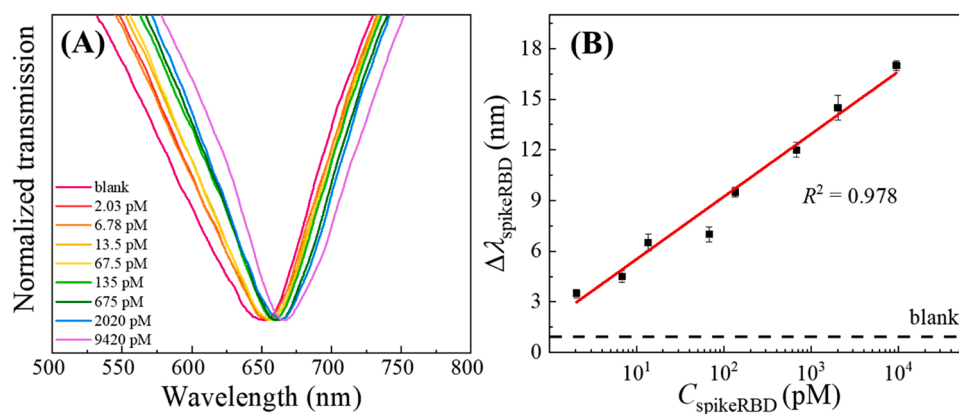
The stability of the AgNT array sensor has also been examined. ACE2 immobilized AgNT array sensors were stored in sealed opaque pouches filled with argon gas and kept at  $20^\circ\text{C}$  for three weeks, then the optical transmission spectra of the ACE2 modified AgNT samples (Fig. S8A) as well as treated by spike RBD protein ( $C_{spikeRBD} = 2020 \text{ pM}$ , Fig. S8B) were measured. As shown in Fig. S8A, there is almost no change in  $\lambda_0$  in AgNT samples after stored for one and three weeks, compared to that of the freshly prepared AgNT samples. Similar result is found for the samples incubating with spike RBD protein solutions (Fig. S8B), and the corresponding redshifts  $\Delta\lambda$  were found to be  $14.0 \pm 0.5 \text{ nm}$ ,  $12.5 \pm 0.6 \text{ nm}$ , and  $14.5 \pm 0.3 \text{ nm}$ , respectively (Fig. S8C), which are comparable to the result reported in Fig. 3B.

### 3.4. Specificity of AgNT array sensor for CoVs

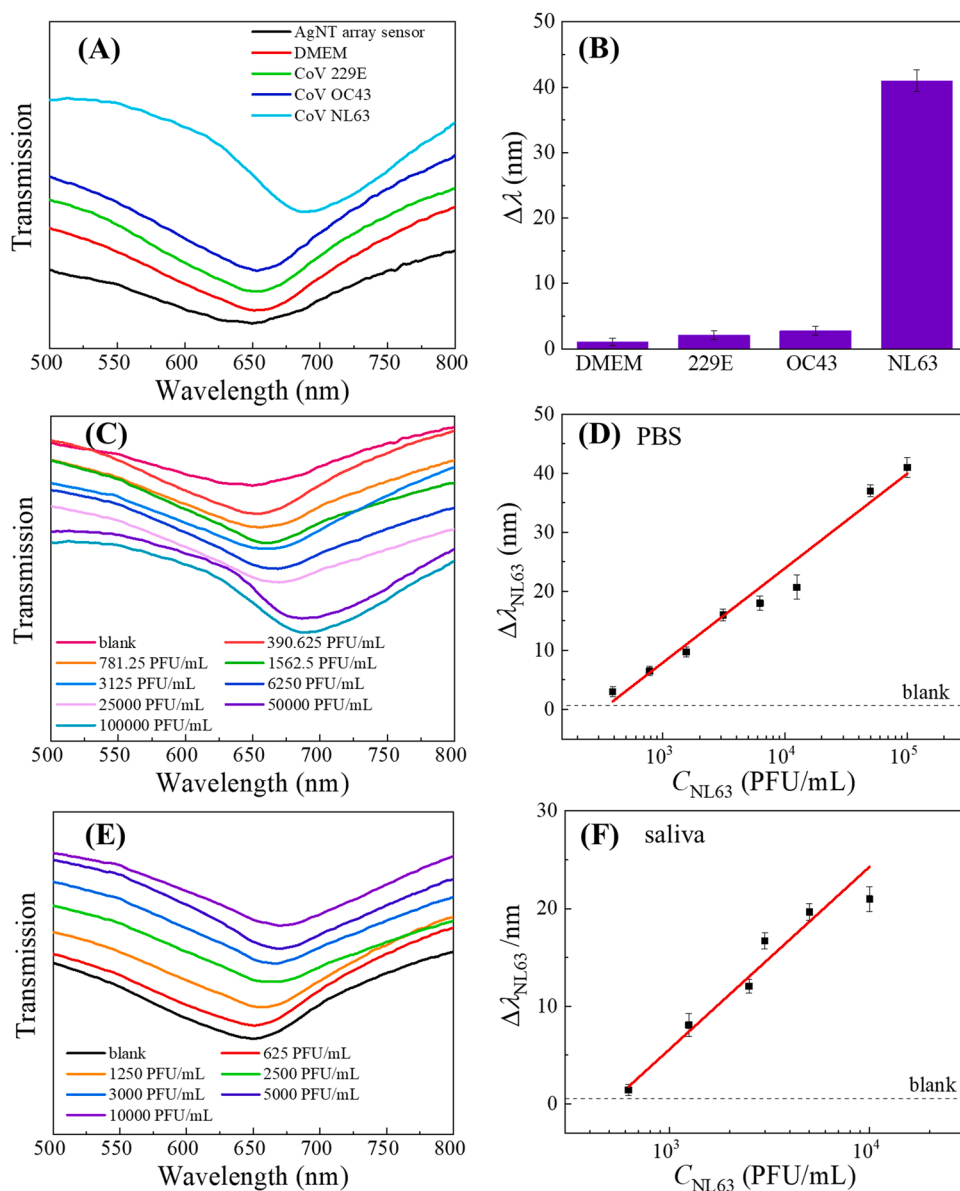
To test the specificity of AgNT array sensor, similar detection experiments have been performed on the three strains of CoV, i.e., 229E, OC43, and NL63 at the same concentration of  $10^5 \text{ PFU/mL}$  in DMEM with 1% FBS. Fig. 4A shows the corresponding transmission spectra and the  $\Delta\lambda$  is plotted in Fig. 4B. The  $\Delta\lambda$  caused by DMEM buffer (with 1% FBS) is around  $1 \text{ nm}$ , and the average  $\Delta\lambda$  for 229E and OC43 is around  $2 \text{ nm}$ , which is significantly lower compared to  $\Delta\lambda = 40 \text{ nm}$  for NL63. Such a difference demonstrates that the ACE2 is highly specified to CoV NL63.

### 3.5. Specific detection of NL63 in PBS and in untreated saliva

Fig. 4C shows that the LSPR wavelength  $\lambda_0$  redshifts continuously after being incubated with NL63 of different concentrations, ranging from  $391$  to  $10^5 \text{ PFU/mL}$  in PBS buffer. PBS solution with no NL63 was used as the blank. The semi-log concentration dependent  $\Delta\lambda_{NL63}$  plot in



**Fig. 3.** (A) The normalized concentration-dependent transmission spectra for  $C_{\text{spikeRBD}} = 2.03 \text{ pM}$  to  $9420 \text{ pM}$ . (B) A semi-log plot of the LSPR wavelength shift  $\Delta\lambda_{\text{spikeRBD}}$  versus  $C_{\text{spikeRBD}}$ . The solid line is the fitting result.



**Fig. 4.** (A) The transmission spectra of the AgNT wells treated with DMEM buffer (with 1% FBS), 229E, OC43, and NL63 at the concentration of  $10^5 \text{ PFU/mL}$ , and (B) the corresponding  $\Delta\lambda$  plot. (C) The concentration-dependent transmission spectra of NL63 detection ranging from  $391$  to  $10^5 \text{ PFU/mL}$  in PBS buffer, and (D) the semi-log plot of  $\Delta\lambda_{\text{NL63}}$  versus  $C_{\text{NL63}}$ . (E) The concentration-dependent transmission spectra of NL63 detection ranging from  $625$  to  $10^4 \text{ PFU/mL}$  in untreated saliva, and (F) the corresponding semi-log plot of  $\Delta\lambda_{\text{NL63}}$  versus  $C_{\text{NL63}}$ .

Fig. 4D also demonstrates a linear relationship, which can be written as  $\Delta\lambda_{NL63}(\text{nm}) = m\log[C_{NL63}] + \Delta\lambda_0$ , where  $m$  characterizes the apparent sensitivity of the AgNT sensor and  $\Delta\lambda_0$  is the initial LSPR wavelength shift. For NL63 detection in PBS buffer,  $m^{PBS} = 16 \pm 1 \text{ nm}/\log(\frac{\text{PFU}}{\text{mL}})$ ,  $\Delta\lambda_0 = -40 \pm 4 \text{ nm}$  with  $R^2 = 0.981$ , and the LOD is 391 PFU/mL. The good reproducibility of AgNT array sensor has been demonstrated as shown in Section S3 of SM. Considering the serious matrix effects of high concentration saliva, NL63 was added in 90% saliva to prepare NL63 spiked saliva samples with different concentrations. Saliva without adding NL63 was used as the blank. The good detection ability of the AgNT array sensor is again demonstrated in untreated saliva. As shown in Fig. 4F, a linear relationship in the semi-log plot is found ranging from 625 to  $10^4$  PFU/mL with  $m^{saliva} = 19 \pm 2 \text{ nm}/\log(\frac{\text{PFU}}{\text{mL}})$ ,  $\Delta\lambda_0 = -51 \pm 6 \text{ nm}$  with  $R^2 = 0.965$ . The LOD is 625 PFU/mL. These results show that the apparent sensitivities of the AgNT sensor,  $m^{PBS}$  and  $m^{saliva}$ , for NL63 detection in different environments, are very similar, which indicates that the AgNT array sensor has good performance in complex biological environments. For the LOD, as shown in Table S1 for different sensors, the LODs are given in different units and there is no definite relationship between different units. For example, PFU/mL, copies of RNA, or number of viral particles/mL. In general, one tends to assume that one PFU corresponds to one viral particle and one viral particle only contains one RNA [30,31]. However, there are exceptions, for example, the PFU/viral particle ratio for varicella-zoster virus is 40,000:1 [32] and the RNA copies to viral particle varies for different variants of CoV-SARS-2 viruses [33]. In order to have a systematic comparison, a side-by-side PCR measurements and LSPR measurements should be conducted in order to obtain a more qualitative relationship.

### 3.6. Estimation of the number of detected viruses on AgNT

Based on the detection strategy shown in Fig. 1, the whole detection process can be considered as coating the AgNT with two dielectric layers as shown in Fig. 5A. The first coated layer is a uniform layer of ACE2 protein with an RI  $n_p = 1.45$  [34,35]. BSA blocking can be ignored or included in the first layer, because the  $\Delta\lambda$  of BSA blocking is only 1.5 nm. The second layer is NL63 with refractive index of each particle  $n_v = 1.8$  [36]. According to Li et al., the electric field in the surrounding layers decays exponentially from the surface of AgNT with a characteristic decay length  $\delta$  [37]. Therefore, the effective refractive index of each coated layer is integrated by the local refractive index from zero to infinity [21,38],

$$n_{eff} = \frac{2}{\delta} \int_0^\infty n(x) e^{-\frac{2x}{\delta}} dx, \quad \text{with } n(x) = \begin{cases} n_p, & 0 < x \leq d_p \\ n_t, & d_p < x \leq d_p + d_t \\ n_a, & x > d_p + d_t \end{cases}, \quad (2).$$

where  $n_p$  is RI of ACE2 layer,  $n_t$  is RI of NL63 viral particle layer,  $n_a$  is RI of air,  $d_p$  is the thickness of ACE2 layer, and  $d_t$  is the thickness of NL63 layer. Based on the ACE2 immobilization,

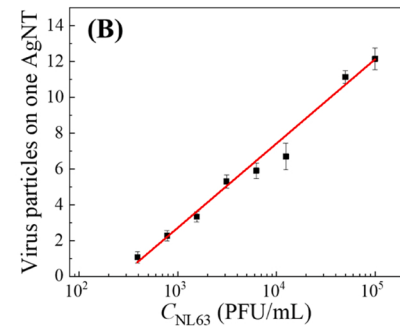
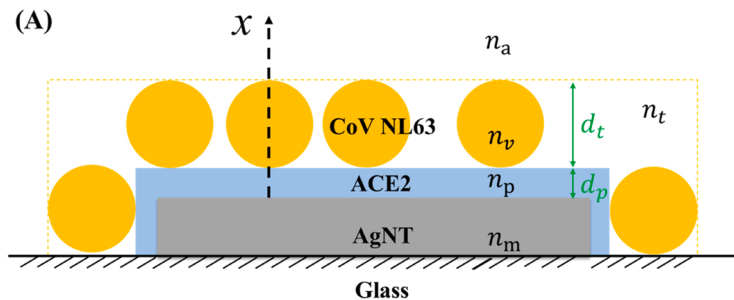


Fig. 5. (A) The scheme of coronavirus detection model. (B) The semi-log plot and linear fitting of the virus particles on one AgNT versus  $C_{NL63}$ .

$$\Delta\lambda_{ACE2} = S(n_p - n_a)(1 - e^{-\frac{2d_p}{\delta}}), \quad (3)$$

where  $\Delta\lambda_{ACE2} = 10 \pm 1 \text{ nm}$ ,  $S = 210 \pm 10 \text{ nm}/\text{RIU}$ ,  $n_p = 1.45$ ,  $n_a = 1$ ,  $d_p = 5 \text{ nm}$  [22], then  $\delta$  can be estimated to be 89.4 nm, which is similar to the value reported in Ag nanohole array [39]. When the sensor captures the virus,  $\Delta\lambda$  can be written as,

$$\Delta\lambda = S(n_t - n_a)e^{-\frac{2d_p}{\delta}}(1 - e^{-\frac{2d_t}{\delta}}) \quad (4)$$

Therefore, the effective RI of the virus layer can be estimated as,

$$n_t = \frac{\Delta\lambda_{LSPR}}{S e^{-\frac{2d_p}{\delta}}(1 - e^{-\frac{2d_t}{\delta}})} + n_a. \quad (5)$$

Here we take  $d_t = 100 \text{ nm}$ , since the transmission electron microscopic (TEM) studies of NL63 infected LLCMK2 cells revealed that virions were spherical, spiked, and range from 75 to 115 nm in diameter [40]. Based on Fig. 4D, the effective  $n_t$  versus  $C_{NL63}$  is estimated, as plotted in Fig. S12A. The viral layer is a porous layer, not totally covered by the virus particles. Its RI can be estimated by the effective medium theory, the Bruggeman's equation [41],

$$\delta_v \left( \frac{\epsilon_v - \epsilon_t}{\epsilon_v + \epsilon_t} \right) + (1 - \delta_v) \left( \frac{\epsilon_a - \epsilon_t}{\epsilon_a + \epsilon_t} \right) = 0, \quad (6)$$

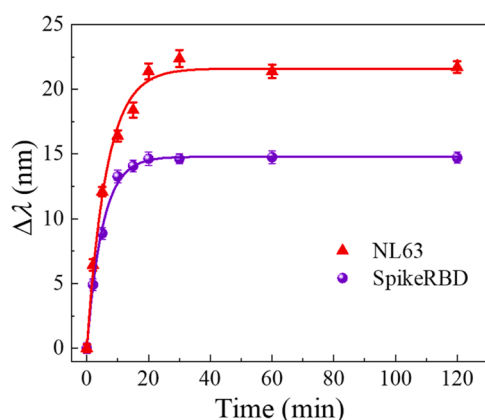
where  $\epsilon_t$ ,  $\epsilon_v$  and  $\epsilon_a$  are the effective dielectric constants of the medium, the dielectric constant of the NL63 virus, and the dielectric constant of air, respectively, and  $\delta_v$  is the volume fraction of the NL63. Also,  $\epsilon_t = n_t^2$ ,  $\delta_v$  can be written as

$$\delta_v = \frac{\left( \frac{n_a^2 - n_t^2}{n_a^2 + n_t^2} \right)}{\left( \frac{n_a^2 - n_t^2}{n_a^2 + n_t^2} \right) - \left( \frac{n_v^2 - n_t^2}{n_v^2 + n_t^2} \right)}. \quad (7)$$

The experimentally obtained  $\delta_v$  versus  $C_{NL63}$  is plotted in Fig. S12B. If the NL63 viral particle is assumed to be a sphere, then the number of viral particles that are detectable on each individual AgNT can be estimated based on Fig. 4D. As shown in Fig. 5B, at the LOD, every AgNT has an average of one viral particle bonded. At the highest detectable concentration ( $10^5$  PFU/mL), each AgNT has an average 12 virus particles bonded. According to the area of an AgNT, the total number of virus particles available on an AgNT is estimated to be around 32. So, there is still room for the sensor to detect higher virus concentrations.

### 3.7. The time-dependent detection of spike RBD and NL63

The detection time of AgNT array sensor is mainly determined by the ACE2-SpikeRBD or ACE2-virus binding time. To assess the real detection time for the AgNT LSPR sensor, systematic investigations have been performed. First, the AgNT array sensor was incubated with 20  $\mu\text{L}$  spikeRBD at  $C_{\text{spikeRBD}} = 2020 \text{ pM}$  for varied time duration  $t$ . The purple



**Fig. 6.** The time-dependent detection of spike RBD (circles) at  $C_{\text{spikeRBD}} = 2020$  pM and NL63 (triangles) at  $C_{\text{NL63}} = 12,500$  PFU/mL. The solid curves are fitting results based on Eq. (8).

circles in the Fig. 6 plot  $\Delta\lambda_{\text{spikeRBD}}$  as a function of  $t$ . When  $t < 20$  min,  $\Delta\lambda_{\text{spikeRBD}}$  red shifts almost monotonically with  $t$ , while when  $t \geq 20$  min, the  $\Delta\lambda_{\text{spikeRBD}}$  reaches a saturation value,  $\Delta\lambda_{\text{spikeRBD}}^S = 14.7 \pm 0.5$  nm. At  $t = 5$  min, a distinct  $\Delta\lambda_{\text{spikeRBD}}$  ( $= 8.9 \pm 0.5$  nm) can be observed, while at  $t = 10$  min,  $\Delta\lambda_{\text{spikeRBD}}$  ( $= 13.3 \pm 0.5$  nm) is near 90% of the saturation  $\Delta\lambda_{\text{spikeRBD}}^S$ . Such a trend is consistent with molecular binding kinetics on a surface, which can be written as

$$\Delta\lambda = \Delta\lambda^S (1 - e^{-t/t^0}), \quad (8)$$

where  $\Delta\lambda^S$  is the saturation wavelength shift and  $t^0$  is the binding time constant. For spike RBD protein detection at  $C_{\text{spikeRBD}} = 2020$  pM,  $\Delta\lambda_{\text{spikeRBD}}^S$  can be obtained as  $14.8 \pm 0.2$  nm, and  $t_{\text{spikeRBD}}^0$  is  $5.1 \pm 0.2$  min. Similarly, the AgNT array sensor was incubated with 20  $\mu\text{L}$  NL63 at  $C_{\text{NL63}} = 12,500$  PFU/mL for various  $t$ . The red triangles in the Fig. 6 plot  $\Delta\lambda_{\text{NL63}}$  as a function of  $t$ , and a trend similar to that of  $\Delta\lambda_{\text{spikeRBD}}-t$  is observed. Based on Eq. (8),  $\Delta\lambda_{\text{NL63}}^S = 21.6 \pm 0.4$  nm and  $t_{\text{NL63}}^0 = 6.4 \pm 0.4$  min are obtained. The  $t_{\text{spikeRBD}}^0$  is slightly smaller than  $t_{\text{NL63}}^0$ , indicating that spike RBD protein of SARS-CoV-2 binds faster to ACE2. Both results indicate that the AgNT based sensor has a detection time of less than 20 min

#### 4. Conclusions

In summary, an ACE2 functionalized AgNT array LSPR sensor has been developed and shown to have a high specificity to SARS-CoV-2 and NL63. The LSPR sensor can detect the SARS-CoV-2 spike RBD protein and NL63 virus with high sensitivity and selectivity. For all these detections, the shift of the LSPR wavelength follows a linear relationship with the logarithm of the concentration of spike RBD proteins and NL63. For the spike RBD protein, the detection is observed in the concentration region from 2.03–9420 pM. For the NL63, lowest detection concentration (LOD) is found to be 391 PFU/mL in PBS buffer. This sensor can also detect NL63 in untreated saliva with a slightly higher LOD of 625 PFU/mL. The detection time is governed by the ACE2-virion particle binding time, and systematical experiments have shown it to be  $< 20$  min for both spike RBD protein and NL63 detections. This LSPR sensor configuration is very simple and many of the measurements can be performed using a handheld UV-Vis spectrometer (Ocean Optics USB2000). In principle, any LSPR sensor can adopt the proposed strategy in Fig. 1 for spike RBD protein or coronavirus detection.

In fact, most works reported in the literature (see Table S1) are concentrated on spike protein detection, and only few really report the results on real virus detection [42–44]. Among them, only four works

focused on plasmonic based sensors [12–14,45]. Though the LOD of our sensor on spike RBD detection is significantly higher than the graphene-based field-effect transistor sensor [42] and cell-based sensor [46] (but lower than that of the electrochemical immunoassay [47]), the fabrication and detection instrument for our sensors are much more simplified and cost effective. For the virus sensing, the LOD of our sensor is comparable to or better than that of the plasmonic sensor [14] and the electrochemical immunoassay [48]. In addition, the viral particles used in Refs. [14,49] are SARS-CoV-2 pseudotyped viral particles or inactivated virus, not the real virus. Our estimation on the number of viral particle binding shows that at the LOD, every AgNT has an average of one viral particle bonded, which further confirms the reliability of our detection.

Clearly this AgNT sensor has the following advantages: first, the fabrication procedure for AgNT array is straightforward and inexpensive; second, the AgNT array can be fabricated into a large and uniform area; and finally, the sensor measurement can be fulfilled by a handheld UV-Vis spectrometer. In addition, AgNT should have a higher plasmonic effect compared to Au. However, the structure of the AgNT is not optimized for sensitivity. By varying the composition and size of the AgNT, one could significantly improve the sensitivity of the LSPR sensor from  $\sim 210$  nm/RIU to  $\sim 700$  nm/RIU or possibly better [50,51], thus lowering the LOD. With improvements, a fast and cost-effective optical sensor can be expected as a potential point-of-care SARS-CoV-2 diagnostic platform for sample analysis.

#### CRedit authorship contribution statement

**YanJun Yang:** Conceptualization, Methodology, Investigation, Writing. **Jackelyn Murray:** Methodology, Investigation. **James Haverstick:** Methodology, Investigation. **Ralph A. Tripp:** Conceptualization, Methodology, Writing, Funding acquisition, Supervision. **Yiping Zhao:** Conceptualization, Methodology, Writing, Funding acquisition, Supervision.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Supplementary materials

Supplementary data to this article can be found online at: A diagram of the AgNT array fabrication procedure; the basic characterization of the LSPR AgNT sensor; the transmission spectra of multiple AgNT wells; the exploration of the optimized buffer for spike RBD protein detection; the stability test of the AgNT array sensor; the calculation of the number of spike RBD molecules in the light beam during the detection via AgNT well; modeling for virus detection; estimation of the full coverage of virus on a single AgNT.

#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.131604.

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