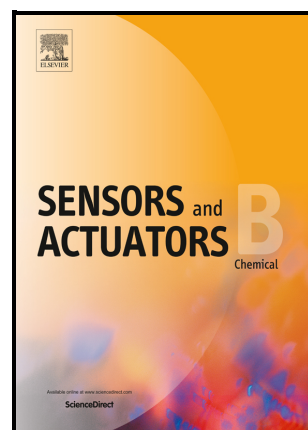


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PII: S0925-4005(21)01286-7

DOI: <https://doi.org/10.1016/j.snb.2021.130718>

Reference: SNB130718

To appear in: *Sensors and Actuators: B. Chemical*

Received date: 17 June 2021

Revised date: 27 August 2021

Accepted date: 5 September 2021

Please cite this article as: Chaolan Liang, Bochao Liu, Jinfeng Li, Jinhui Lu, Enhui Zhang, Qitao Deng, Ling Zhang, Ruiai Chen, Yongshui Fu, Chengyao Li and Tingting Li, A nanoenzyme linked immunochromatographic sensor for rapid and quantitative detection of SARS-CoV-2 nucleocapsid protein in human blood, *Sensors and Actuators: B. Chemical*, (2021) doi:<https://doi.org/10.1016/j.snb.2021.130718>

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# **A nanoenzyme linked immunochromatographic sensor for rapid and quantitative detection of SARS-CoV-2 nucleocapsid protein in human blood**

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## ABSTRACT

The establishment of a simple, low-cost, high-sensitive and rapid immunoassay for detecting SARS-CoV-2 antigen in human blood is an effective mean of discovering early SARS-CoV-2 infection and controlling the pandemic of COVID-19. Herein, a smartphone based nanozyme linked immunochromatographic sensor (NLICS) for the detection of SARS-CoV-2 nucleocapsid protein (NP) has been developed on demand. The system is integrated by disposable immunochromatography assay (ICA) and optical sensor devices. Immunoreaction and enzyme-catalyzed substrate color reaction were carried out on the chromatographic strip in a device, of which the light signal was read by a photometer through a biosensor channel, and the data was synchronously transmitted via the Bluetooth to the app in-stored smartphone for reporting the result. With a limit of detection (LOD) of 0.026 ng/mL NP, NLICS had the linear detection range (LDR) between 0.05 and 1.6 ng/mL NP, which was more sensitive than conventional ICA. NLICS took 25 min for reporting results. For detection of NP antigen in clinical serum samples from 21 COVID-19 patients and 80 healthy blood donor controls, NLICS and commercial enzyme linked immunosorbent assay (ELISA) had 76.2% or 47.6% positivity, and 100% specificity, respectively ( $P=0.057$ ), while a good correlation coefficient ( $r=0.99$ ) for quantification of NP between two assays was obtained. In conclusion, the NLICS was a rapid, simple, cheap, sensitive and specific immunochromatographic sensing assay for early diagnosis of SARS-CoV-2 infection.

**Keywords:** SARS-CoV-2; Rapid detection; Immunochromatography; Photometer; Smartphone.

## 1. Introduction

By the end of March 2021, the novel coronavirus pneumonia, named COVID-19, has been developed to a global pandemic, causing more than 3.7 million deaths.<sup>1</sup> The pathogen of COVID-19 is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),<sup>2</sup> which is highly contagious, causing symptoms such as fever, dry cough, fatigue or progressive dyspnea through upper respiratory tract infection, or

leading to death in severe cases.<sup>3-6</sup> Several COVID-19 vaccines have been approved for emerging use in human vaccination, the efficacy requires being monitored. Therefore, it is very important to develop rapid and reliable methods for early detection of SARS-CoV-2 infected patients and asymptomatic virus carriers.<sup>7-10</sup>

Currently, the diagnosis for SARS-CoV-2 infection is primarily dependent on reverse transcription polymerase chain reaction (RT-PCR) and serological methods.<sup>11-14</sup> RT-PCR is the most predominant method for detecting SARS-CoV-2.<sup>15</sup> Due to the limitations of sample preservation, testing reagents, requirement of the laboratory, professional operators and detection time (1-2 hours), RT-PCR is not suitable for rapid screening on-site. The high cost of RT-PCR also limits its popularization in many poor communities or health care systems. The serological testing of SARS-CoV-2 antibody is an auxiliary detection method for nucleic acid detection.<sup>15-17</sup> Some rapid methods such as lateral flow immunoassay for IgM/IgG antibody have been applied in clinic practice. However, the specific antibody against SARS-CoV-2 in blood is usually detected in 7-14 days of infection, which is not suitable for screening and diagnosis of COVID-19 in a window period of early infection.<sup>18</sup> Existing serological methods for antigen detection mainly include lateral flow immunoassay (LFIA) and double antibody sandwich ELISA.<sup>19-22</sup> Most LFIAs were to detect the NP antigen in nasopharyngeal swab samples, and provided only qualitative rather than quantitative results.<sup>23</sup> The double antibody sandwich ELISA assay was time-consuming with complicated washing and incubation steps.<sup>24</sup> Therefore, there is an urgent need for a simple, fast, reliable and cheap test for quantitative detection of SARS-CoV-2 antigen.

In previous studies, applications based on nanozyme catalyzed color development have been reported.<sup>25-26</sup> The nanozymes, such as Au@PtNPs, are nanomaterial with intrinsic enzyme mimicking activity, which have good catalytic activity and stability, presenting low cost and easy coupling, thus widely used in the various biosensors.<sup>27-29</sup> Compared with gold nanoparticles (AuNPs), the gold-platinum nanoparticles (Au@PtNPs) realized the signal amplification on color reaction, consequently the sensitivity was greatly improved. In comparison with

Au@PtNPs, natural protease, such as horseradish peroxidase (HRP), has low storage stability, complicated preparation process, and lower sensitivity.<sup>30-32</sup>

In the present study, a novel, rapid, sensitive, portable and low-cost nanoenzyme linked immunochromatographic sensor (NLICS) based on smartphone was developed for quantitative detection of the SARS-CoV-2 NP antigen in blood samples. Firstly, the NP antigen in blood sample was reacted with first specific monoclonal antibody (mAb1) coupled Au@PtNPs sprayed on conjugate pad of testing strip, and then migrated and captured by second mAb2 immobilized on the T-line (Test line) of NC membrane, forming a sandwich structure (Au@PtNPs-mAb1-NP-mAb2). Finally, the substrate solution was catalyzed by Au@PtNPs-mAb1-NP-mAb2 for color reaction. The laser permeated through the reacted substrate filtration to reach the photometer, and the light was measured by the photometer, which was inversely proportional to the NP concentration in the sample. The results measured by a photometer were synchronously transmitted to a smartphone through Bluetooth connection for data processing and analysis. As a novel point of care test (POCT), the NLICS would be of great significance for rapid screening of suspected SARS-CoV-2 infection, especially in the early stages of viral infection.

## 2. Materials and methods

### 2.1 Chemicals and materials

Phosphate buffer solution (PBS), chlorauric acid ( $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ ), chloroplatinic acid ( $\text{H}_2\text{PtCl}_6 \cdot 6\text{H}_2\text{O}$ ) were purchased from Guangzhou ZUOKE Biotechnology Development Co., Ltd., China. The polyvinyl alcohol (PVA) were purchased from Shanghai Yuanye Biological Technology Co., Ltd., China. Tween-20, bovine serum albumin (BSA), trisodium citrate, trehalose, sucrose, sodium casein, sodium azide and 3,3',5,5'- tetramethylbenzidine (TMB) were obtained from Sigma, USA. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 30 wt%) was purchased from Guangzhou Chemical Reagent Co., Ltd., China. Sample pad (Glass fiber G2), conjugate pad (Glass fiber SB06) and absorbent pad were obtained from Shanghai Jiening Biotechnology Co., Ltd., China. NC membrane (Sartorius CN95) and soft universal glue were purchased from

Shanghai Jinbiao Biotechnology Co., Ltd., China and the nucleocapsid protein (NP) of SARS-CoV-2 was purchased from Hangzhou Biotechnology Co., Ltd., China. SARS-CoV-2 (2019 nCoV) nucleocapsid protein detection ELISA kit was purchased from Beijing Biodragon immunotechnology Co., Ltd., China. The mAb1 (clone 5E4 as detection antibody) and the mAb2 (clone 4G11 as capture antibody) to NP were prepared in our laboratory.

Microplate reader (Bio-Tek Instruments, Inc.), transmission electron microscope (TEM, Philips, Netherlands), UV curable 3D printers and consumables (Novartis Intelligent Technology Co., Ltd., China), fused deposition molding (FDM) 3D printer and consumables (Heli Technology Co., Ltd., China) were also used during the experiment. Laser ( $\lambda=450$  nm) was purchased from Shenzhen Bluestar Microelectronics Co., Ltd. The hand-held photometer was obtained from Sanfu Intelligent Technology Co., Ltd. (Shenzhen, China).

## **2.2 Design and assembly of U-shaped immunochromatography assay device and optical reading device**

The smartphone based NLICS included an immunochromatographic assay (ICA) device and a result reading device. The ICA device was a coin sized U-shape strip to generate immunoreactive and colorimetric signal. The U-shaped ICA had a total size of 22 mm\*11 mm\*17 mm and was composed of eight components: shell, U-groove, silica gel stopple, optical fiber, sample pad, conjugate pad, NC membrane, and absorbent pad (Fig. S1A). The U-groove and shell based on wedge-shaped structures were 3D-printed with UV light cured opaque photosensitive resin materials and could slide up and down after assembly. To prevent leakage, a silica gel stopple was pasted on the bottom of the U-groove, and an optical fiber with a diameter of 1.5 mm was inserted at the center of the silica gel stopple. The sample pad was 6 mm\*15 mm glass fiber G-2 (pre-treated with 10 mmol/L Tris-HCl solution containing 0.1% Tween-20, pH 8.0). The conjugate pad was 6 mm\*10 mm glass fiber SB06 (pre-treated with 10 mmol/L Tris-HCl solution containing 0.1% PVA, 2.5% trehalose, 2.5% sucrose, 0.5% sodium casein, 0.1% Tween-20, 0.03% sodium azide, pH8.0), sprayed with

Au@PtNPs-mAb1. The 6 mm\*25 mm NC membrane was placed in the middle of the U-groove, on which the T-line was coated with mAb2 (1 mg/mL). The sample pad, conjugate pad, NC membrane, and absorbent pad (6 mm\*17 mm) were assembled sequentially with glue. In addition, the insertion plate and supporting plate specially designed for U-shaped ICA equipment were also fabricated by 3D-printing.

Result-reading device included an optical reading device and a handheld photometer for reading and transmitting results. The shell of the optical reading device was made of 3D printing with the total size of 46 mm\*46 mm\*73 mm, and the reading device consisted of switch, USB port, adjustable resistor and 450 nm laser (Fig. S1B). The USB port was used to connect the power supply. The laser was a low power laser transmitter with an excitation wavelength of 450 nm and a power of 10 mW. The hand-held photometer received the light signal and converted it into a digital signal (Fig. S1C).

### 2.3 Preparation of NP paired antibody

According to the previously reported method,<sup>33</sup> the monoclonal antibodies to SARS-CoV-2 NP were produced and the paired antibodies were selected. Briefly, splenocytes of NP-immunized BALB/c mice and mouse myeloma cells SP2/0 were fused, and positive hybridoma cells were identified by indirect ELISA. Hybridoma cells were intraperitoneally injected into BALB/c mice to prepare ascites. The purified monoclonal antibodies were obtained through the purification of ascites with protein G agarose gel preloaded column. Then double antibody sandwich ELISA was used to select the paired antibodies and to determine the titer of antibodies.

### 2.4 Preparation of Au@PtNPs-mAb1

Au@PtNPs were synthesized according to the method reported in the previous study.<sup>34</sup> Firstly, 13 nm AuNPs were synthesized. 100 mL of ultrapure water were added to a clean erlenmeyer flask, and heated to boiling with stirring, then 1 mL of 1% HAuCl<sub>4</sub> solution was added. And after boiling again, 4 mL of 1% sodium citrate were

added quickly and continuously stirred for 30 min. When heating stopped, the stirring was continued to cool the AuNPs to room temperature and stored at 4°C. 597  $\mu\text{L}$  of  $\text{H}_2\text{PtCl}_6$  (20 mmol/L) were added to 8.603 mL of AuNPs solution and heated to 90°C by stirring. Then 800  $\mu\text{L}$  of ascorbic acid (20 mmol/L) were slowly dropped into the mixture and heated for 30 min by stirring continuously. Stopped heating after 30 min, the mixture was cooled naturally to room temperature and stored at 4°C.

The antibody labeling was carried out in the laboratory according to the relevant literature with modification<sup>35</sup>. Briefly, 20  $\mu\text{L}$  of 0.2 mol/L  $\text{K}_2\text{CO}_3$  were added to 1 mL of Au@PtNPs and mixed, then 20  $\mu\text{g}$  mAb1 were added and incubated for 1h. After that, 100  $\mu\text{L}$  of 10% BSA were added and blocked for 1h. Finally, the mixed solution was centrifuged at 10000 rpm for 15 min, and the Au@PtNPs-mAb1 were resuspended into 100  $\mu\text{L}$  of preservation solution (0.1 mol/L PBS, 10% sucrose, 0.5% BSA, pH 7.4) and stored at 4°C.

## 2.5 Detection of NP standards by NLICS

The different concentration of SARS-CoV-2 NP standard samples (0, 0.0125, 0.025, 0.5, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2 ng/mL) were prepared in 100  $\mu\text{L}$  of buffer solution (0.1 mol/L PBS, 2% Tween 20), and added onto the sample pad of strip in the sample well of ICA device on the supporting plate for reacting with Au@PtNPs-mAb1 and mAb2 for 10 min at room temperature. The unbound Au@PtNPs-mAb1 on the NC membrane were removed by washing with 200  $\mu\text{L}$  of PBST buffer. In 5 min later, the customized insertion plate was inserted into the gap between the supporting plate and the U-shaped ICA device, then 200  $\mu\text{L}$  of TMB substrate solution were added for catalyzing reaction of color development. After 10 min, 50  $\mu\text{L}$  of 2 mol/L  $\text{H}_2\text{SO}_4$  was added to terminate the reaction. After pushing the supporting plate into the reading device, the absorbance was read by a hand-held photometer and the results were transmitted to a smartphone for record and analysis of the changes in the intensity of transmitted light. Meanwhile, the traditional ICA and ELISA with the same mAb pair were used as control tests.



## 2.6 Detection of clinical serum samples

A total of 21 clinical serum samples of COVID-19 patients were provided by Shenzhen Center for Disease Control and Prevention (CDC). All patients were diagnosed as SARS-CoV-2 infection by nucleic acid detection as well as comprehensive diagnosis of clinical symptoms. A total of 80 plasma samples from healthy blood donors were obtained from Guangzhou Blood Center, which were collected in June 2019 before the COVID-19 outbreak at the end of 2019 in Wuhan, China, as the negative control. Taking 50  $\mu$ L of individual blood samples were diluted in PBST buffer (0.1 mol/L PBS, 2% Tween-20, pH7.4) for detection by NLICS and commercial ELISA kit.

The use of human blood samples in the study was approved by the Medical Ethics Committees of Shenzhen CDC and Southern Medical University, and followed the ethical guidelines of the 1975 Declaration of Helsinki.

## 2.7 Statistical analysis

Statistical analysis was carried out by SPSS 18.0 software. The results were presented as mean  $\pm$  standard deviation. The difference in detection sensitivity among two assays was calculated by Pearson's chi-squared test.  $P < 0.05$  was considered statistically significant.

## 3. Results and discussion

### 3.1 Operation process of NLICS

The NLICS was designed ingeniously by combining ICA and smartphone devices. The user only needs to complete the following operations: (1) to add the sample dilutions into the sample well of chromatographic device (Fig. 1A); (2) to push the chromatographic device into the supporting plate and to add TMB substrate solution for 10 min incubation, and then to add  $H_2SO_4$  solution for terminating reaction (Fig. 1B); (3) to turn on the power of laser in 10 min advance, to push the supporting plate into the optical channel, and to initiate the photometer and smartphone to read the illumination (Fig. 1C, D). The sensor directly presents the data in digital form, and

synchronously transmits it to smartphone via Bluetooth for result analysis and reporting (Fig. 1E). In comparison with conventional ELISA, the NLICS is simple and rapid, it doesn't need multiple washing steps and additional equipment.

(Insert Fig.1)

### 3.2 Principle and characteristics of NLICS

The NLICS contains two devices of immunochromatographic assay (ICA) and light signal reading. The chromatographic device is used for immunoassay. ICA device includes sample pad, conjugate pad, U-shaped NC membrane strip and absorbent pad (Fig. 2A). The overall size was similar to a RMB coin (Fig. 2B). When the sample solution is added to sample pad, the liquid is sequentially flowed through the conjugate pad, NC membrane and absorbent paper due to capillary action. The sample pad and conjugate pad are pretreated with surfactant buffer solution (maintaining the appropriate ionic strength and pH value) or Au@PtNPs-mAb1 conjugates, respectively. The U-shaped ICA device is fixed and supported by the supporting plate, which can hold 7 U-shaped ICA devices. The NC membrane strip is separated between the conjugate pad and the absorbent pad by insertion plate to prevent TMB substrate and termination solutions being sucked back by the conjugate pad and the absorbent pad.

The reading device is used to read the light signal from immunoreaction in ICA device, which is assembled by a switch, USB port, adjustable resistor and 450 nm laser (Fig. 2C). The laser, U-shaped ICA device, and optical fiber are located in the central axis to form the light sensing channel. The hand-held photometer receives the light signal and converts it into digital data. The total material cost for device manufacturing was \$1.50 (Table S1), of which some parts were fabricated by 3D-printing.

During the NLICS process for detecting SARS-CoV-2 NP antigens in blood, after the sample was added to sample pad, NP antigens flowed to the conjugate pad and reacted with the saturated Au@PtNPs-mAb1, and then migrated and bound to the immobilized mAb2 on T-line of NC membrane strip to form the sandwich structural

complexes (Au@PtNPs-mAb1-NP-mAb2). When TMB substrate was added to U-shaped ICA device, the substrate was catalyzed for color reaction (Fig. 2D). If NP antigens were not present in the sample, Au@PtNPs-mAb1 conjugates would migrate through T-line of NC membrane strip to absorbent pad. When TMB was added, the substrate would not be catalyzed for color development. For measuring the substrate reaction and reporting the detection result, the laser was initiated in 10 min advance. The emission of 450 nm light passed through the filtration of reacted substrate solution and an optical fiber to the photometer (Fig. 2D). The photometer displayed the light intensity and synchronously transmitted the data to smartphone for analysis via Bluetooth (Fig. 2E). The application (app) installed on smartphone is designed specifically for NLICS. The app, after receiving the data from photometer, automatically converts the transmitted light intensity to  $OD_{450}$  value, and then calculates the concentration and reports the detection results for each sample according to the linear equation of the standard curve. The light intensity was inversely proportional to the color intensity of substrate solution, and subsequently inversely proportional to the concentration of Au@PtNPs-mAb1 bound to NP on the NC membrane of strip from blood sample. The NLICS detection process took about 25 min, and was easily performed.

(Insert Fig.2)

The stability of sensing system of NLICS was examined during 60 min working period (Fig. S2). The temperature change of initiated laser was quickly increased during the initial 10 min and stably maintained within 3°C (Fig. S2A), while the light intensity of sensor was quickly decreased during the initial 10 min and then remained stable (less than 3% drift) up to 60 min (Fig. S2B). The results suggested that NLICS had the satisfactory stability of sensing system, particularly after 10 min initiation of reading device.

### 3.3 Preparation of mAb pair binding to NP

In order to obtain the suitable NP antibody pairs, 5 mAbs were selected from 20 mAbs to NP by double-antibody sandwich ELISA. Among five mAbs paired with

each other, 5E4-HRP/4G11 mAb pair had the highest S/CO value (Table S2). The mAbs 5E4 (mAb1) and 4G11 (mAb2) were designated as detection antibody or capture antibody in the assay, of which the titers of mAbs were identified as  $2.44 \times 10^{-7}$  and  $4.88 \times 10^{-7}$ , respectively (Fig. S3). Finally, mAb 5E4 was selected to prepare Au@PtNPs-mAb1 conjugates, and mAb 4G11 was used to be immobilized on T-line of NC membrane strip for reacting with NP in NLICS.

### 3.4 Characterization of Au@PtNPs

With good catalytic activity, low cost, easy coupling and stability, Au@PtNPs were widely used in biosensor application. AuNPs with a diameter of 13 nm were evenly distributed under TEM observation, of which the particle surfaces were smooth (Fig. S4A). By adding chloroplatinic acid to AuNPs with increasing concentration from 0.5% to 10%, the size of Au@PtNPs gradually increased and the color changed from wine red to brown black (Fig. S4A). When AuNPs were saturated with 7% or 10% chlorogold acid, the particle size was about 40 nm, and the surfaces of Au@PtNPs were fully coated with spikes, forming "sea urchin" like protrusions (Fig. S4A). By the full wavelength scanning of spectrum, the peak of AuNPs at 528 nm gradually flattened and finally disappeared with increasing of Au@PtNPs (Fig. S4B). These results indicated that the synthesized AuNPs and Au@PtNPs were successful.

The feasibility of Au@PtNPs catalysis was analyzed after the Au@PtNPs were added to the TMB substrate in microwells. The yellow color of substrate reaction deepened along with the increase of Au@PtNPs concentration (Fig.3A), and accordingly OD<sub>450</sub> values were increased by measurement with a microplate reader (Fig. S4C). In order to verify the catalytic activity, Au@PtNPs' catalytic speed and stability were tested at different concentrations in comparison with HRP in TMB substrate, respectively. The results showed that the Au@PtNPs presented highly catalytic activity in a dose and time dependent manner, even at an extremely low concentration of 0.5 pmol/L, while HRP had lowly detectable catalytic activity at a concentration > 5 nmol/L (Fig. 3B). In addition, Au@PtNPs had good stability at

room temperature remaining 95% activity over 15 days, but HRP lost 80% activity at room temperature after 7 days (Fig. S4D).

### 3.5 Optimization of NLICS

The proper reacting conditions of NLICS were set up by optimizing the concentrations of  $\text{H}_2\text{O}_2$  and Au@PtNPs-mAb1, and reaction time. The absorbance ratio of positive to negative control (P/N) reached to the highest value when  $\text{H}_2\text{O}_2$  concentration was up to 2 mol/L, but decreased when  $\text{H}_2\text{O}_2$  concentration was > 2 mol/L (Fig. 3C). Consequently, 2 mol/L  $\text{H}_2\text{O}_2$  was selected as working concentration in NLICS. Since the amount of Au@PtNPs-mAb1 saturated in the conjugate pad was critical to affect the assay's sensitivity, the eight dilutions of Au@PtNPs-mAb1 were compared (Fig. 3D), in which 1:160 working dilution was determined for having the highest P/N value. The proper reaction time for the faster color development and higher P/N value was selected from 10 min substrate incubation in NLICS (Fig. 3E).

The coefficient variations (CV) of intra-assay and inter-assay were examined for evaluation of NLICS precision or accuracy for detecting three concentrations (0.05, 0.4 and 1.6 ng/mL) of standard NP antigen. The intra-assay CV varied below 10% among 6 replicates in NLICS (Fig. 3F), while the inter-assay CV ranged between 5.2% and 13% among 8 independent tests in NLICS (Fig. 3G). The results suggested that the NLICS had the higher precision in detection of low, middle and high levels of SARS-CoV-2 NP antigen.

(Insert Fig.3)

### 3.6 Quantitative detection of NP standards by NLICS

In this study, 100  $\mu\text{L}$  of sample solutions with 0-51.2 ng/mL standard NP antigen were detected by loading to the sample well of the U-shaped ICA device of NLICS (Fig. 4A). The  $\text{OD}_{450}$  values were calculated by the algorithm (1) after termination of TMB substrate reaction (Fig. 4B). In the algorithm (1) for  $\text{OD}_{450}$  value, the I was the transmitted light intensity (lux) through the TMB substrate solution, and  $I_0$  was the light intensity value measured on the photometer and smartphone when only colorless

water was added. In this experiment, the laser power was controlled by rotating the adjustable resistor, and the I0 value was adjusted to 70,000 lux. The transmitted light intensity was read by the photometer, and the results were synchronously transmitted to the smartphone through Bluetooth for analysis.

$$OD = \lg[(I_0 - I)/I] \quad (1)$$

The light intensity values (lux) were converted to OD<sub>450</sub> values by formula calculation (Table S3). The results showed that OD<sub>450</sub> values were positively correlated with NP concentrations, the linear detection range (LDR) of NLICS for NP was 0.05-1.6 ng/mL (Fig. 4C). The following line equation was used to calculate NP concentration in serum samples.

$$y = 0.52271 + 0.86087x, \text{ and the fitting degree } R^2 \text{ was } 0.9954.$$

The limit of detection (LOD) of NLICS was determined as 0.026 ng/mL, calculated by substituting the mean + 3SD value of negative samples (n = 40) into the linear equation. Meanwhile, the traditional ELISA and ICA were used as controls to detect the standard NP dilutions. In traditional ELISA, the LDR was 0.05-12.8 ng/mL NP, with a LOD of 0.025 ng/mL (Fig. S5A). In the traditional ICA, the T-line was clearly visible only when the NP concentration was greater than 0.1 ng/mL (Fig. S5B). The results suggested that NLICS was simpler and faster assay, which had similar sensitivity with ELISA but higher sensitivity than traditional ICA for detecting the NP standard in artificial samples.

(Insert Fig.4)

### 3.7 Specificity of the NLICS

In order to verify the specificity of NLICS, several proteins including SARS-CoV-2 NP, S and RBD proteins, H1NI hemagglutinin (HA) and neuraminidase (NA) proteins, hepatitis B surface antigen (HBsAg), and hepatitis C virus core protein (HCV core) were tested in triplicate (Fig. 5). The results showed that only NP (12.8 ng/mL) was reactive, while other proteins were not cross-reactive at concentration of 100 ng/mL in NLICS, suggesting the assay had high specificity to SARS-CoV-2 NP antigen.

(Insert Fig.5)

### 3.8 Detection of clinical serum samples from COVID-19 patients

By using the smartphone based NLICS, 21 serum samples from COVID-19 patients were tested in comparison with 80 plasma samples from healthy blood donors. To match NP within the LDR of NLICS, 100  $\mu$ L of two-fold diluted serum samples were measured in triplicate. The transmitted light intensity (lux) from filtration of color developed substrate correlating to NP concentration was counted by the photometer and converted to OD<sub>450</sub> value by the app in-stored smartphone (Table S4). In the meanwhile, the COVID-19 patients' samples were measured for NP by a commercial ELISA, of which the individual results were compared with NLICS' measurements (Table S4).

In an overall, among 21 COVID-19 patients' serum samples, 16 (76.2%) were tested reactive by NLICS, while 10 (47.6%) were positive by ELISA ( $P=0.057$ ; Table 1). Among 80 healthy blood donor samples, none was positive by these two assays, suggesting 100 % specificity for both NLICS and ELISA. The accuracy of detection in clinical samples was 95.1% for NLICS and 89.1% for ELISA, respectively (Table 1). Notably, the sensitivity and accuracy of NLICS were higher than ELISA. In aspect of quantification of NP in clinical samples between NLICS and ELISA (Fig. 6A), a strong correlation was observed with no significant difference (Fig. 6B;  $P < 0.01$ ,  $r = 0.990$ ). These results suggested that the NLICS was reliable, which was potential to be used for the point of care testing of SARS-CoV-2 infection in terms of high specificity, sensitivity and accuracy in clinical quantitative detection.

(Insert Fig.6, Table 1)

## 4. Conclusions

The viral nucleic acid testing (NAT) and antibody detection have been commonly used for diagnosis of SARS-CoV-2 infection, while the NAT requires special facility and antibody is not presence in the window period of early infection. Antigen detection is effective like NAT for detecting virus in the early stage of infection with low operational requirements. In this study, a novel POCT, namely smartphone-based

NLICS, was developed and validated for detecting SARS-CoV-2 NP antigen in blood samples. NLICS successfully combined the specially designed lateral flow immunochromatography and nanozyme (Au@PtNPs) catalyzed substrate reaction into a smartphone based light signal transmission, capture and process system. NLICS showed an LDR of 0.05-1.6 ng/mL and a LOD of 0.026 ng/mL, respectively, and the latter was similar to that of ELISA and much lower than conventional ICA. By using NLICS to detect SARS-CoV-2 NP in blood samples, 76.2% COVID-19 patients were found positive in 25 min, of which the measured individual samples' NP concentrations were significantly correlated with commercial ELISA. The smartphone based NLICS is a reliable, simple and sensitive POCT assay, it can be potentially used for diagnosis or quick detection of SARS-CoV-2 infection in clinical practice.

#### **CRedit authorship contribution statement**

Chaolan Liang: Conceptualization, Methodology, Formal analysis, Writing- original draft. Bochao Liu: Methodology, Investigation, Formal analysis. Jinhui Lu: Methodology, Investigation, Validation. Enhui Zhang: Investigation, Software. Qitao Deng: Investigation, Software. Jinfeng Li: Resources. Ling Zhang: Formal analysis. Ruiai Chen: Resources. Yongshui Fu: Resources. Chengyao Li: Supervision, Project administration, Funding acquisition, Writing - review & editing. Tingting Li: Supervision, Project administration, Funding acquisition, Writing - review & editing.

#### **Declaration of Competing Interest**

The authors report no declarations of interest.



## Acknowledgments

The authors thank Mr. Ze Wu for his technical assistance in the laboratory, Shenzhen CDC for providing COVID-19 patients' serum samples, and Guangzhou Blood Centers for providing healthy blood donor samples.

This work was supported by grants from National Natural Science Foundation of China (No. 32070929), the China Postdoctoral Science Foundation (No. 2021M691474), and Guangzhou Bai Rui Kang (BRK) Biological Science and Technology Limited Company.

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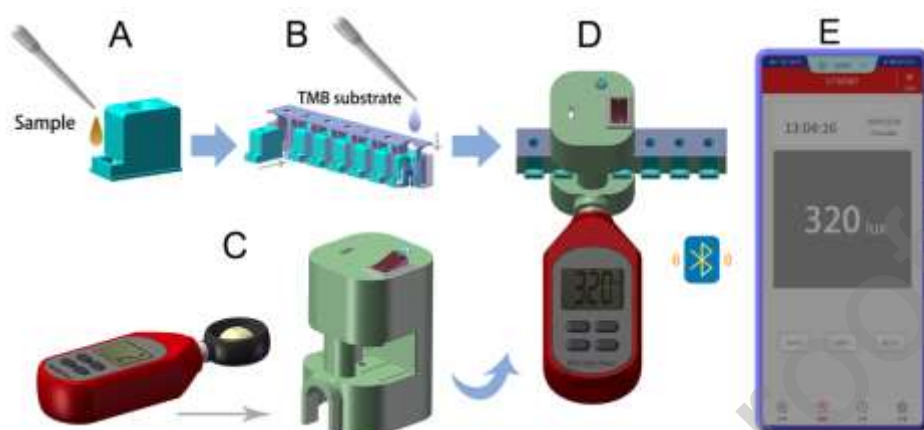
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## Legends

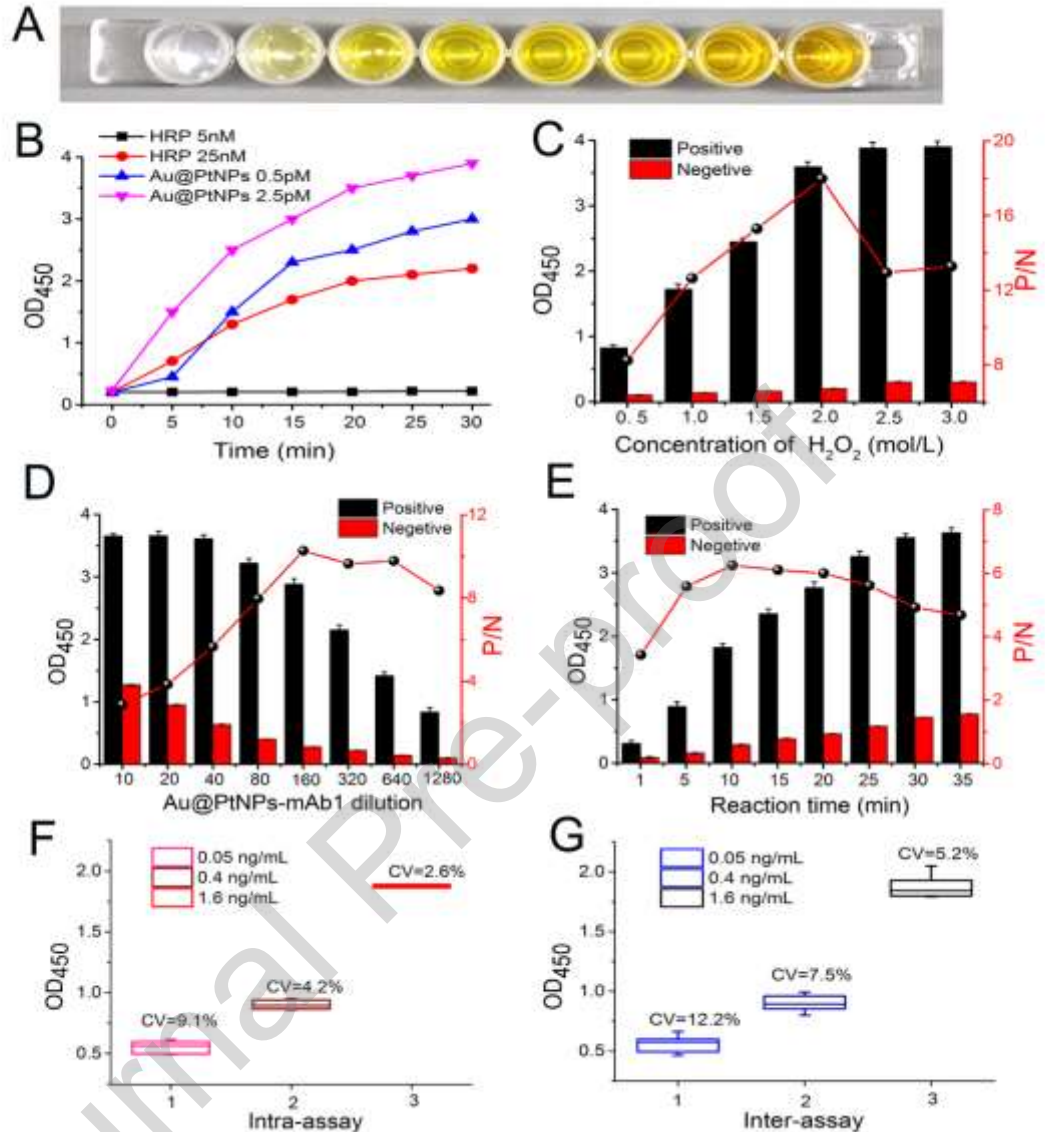


**Fig. 1.** Procedure of smartphone-based NLICS. (A) Adding the sample solution to the sample well of U-shaped ICA device and starting immunochromatography for 10 min and washing for 5 min; (B) Pushing the ICA device into the supporting plate and adding TMB substrate solution for 10 min and then stopping reaction; (C) Connecting the optical system to the photometer in 10 min advance; (D) Inserting the supporting plate into the optical accessory and starting the photometer to read the light intensity; (E) Analyzing results by the app in-stored smartphone.



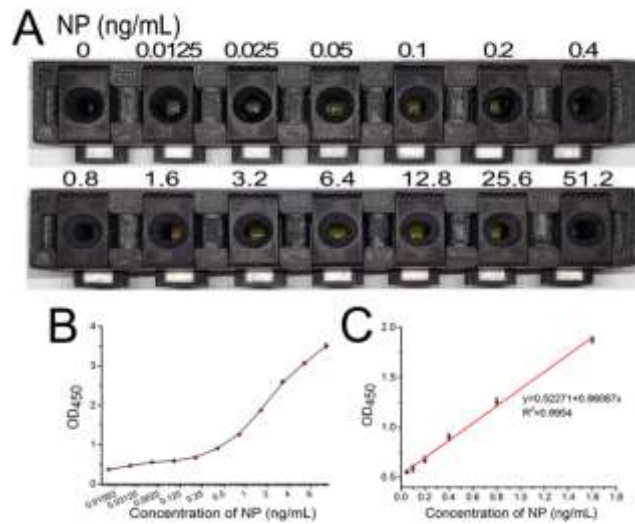
**Fig. 2.** Schematic illustration of disposable immunochromatography device and smartphone-based optical system. (A) The U-shaped ICA device was composed of functional components; (B) An actual size of U-shaped ICA device closer to a RMB coin; (C) Composition of entire NLICS system; (D) Principle of immunoreaction, optical signal transmission and photometer sensing; (E) Calculating the transmitted light intensity and reporting the results by smartphone.



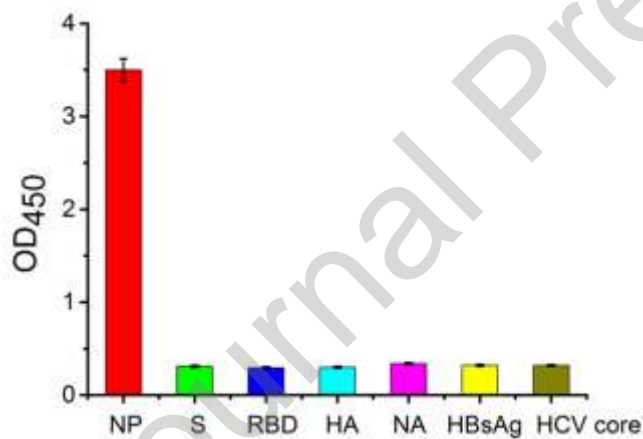


**Fig. 3.** Optimization of NLICS. (A) TMB substrate reaction catalyzed by different concentration of Au@PtNPs; (B) Comparison of substrate reaction sensitivity between Au@PtNPs and horseradish peroxidase (HRP) with various concentrations; (C) The catalytic efficiency of Au@PtNPs-mAb1 at different concentration of H<sub>2</sub>O<sub>2</sub>; (D) The substrate reaction of Au@PtNPs-mAb1 with different dilution; (E) The substrate reaction with different reaction time in NLICS; (F) Intra-assay coefficient variation (CV) of NLICS for three levels of NP; (I) Inter-assay coefficient variation (CV) of NLICS for three levels of NP.

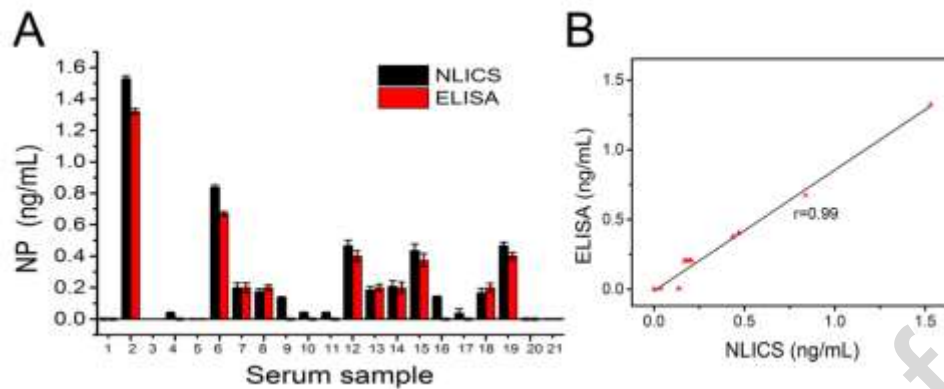




**Fig. 4.** Detection of NP standard by NLICS. (A) The detection of different concentration (0- 51.2 ng/mL) of NP by NLICS; (B) The calculated OD<sub>450</sub> values correlating with different concentration (0.0125- 12.8 ng/mL) of NP by NLICS; (C) The linear curve for detection of NP standard by NLICS.



**Fig. 5.** The specificity of NLICS for NP detection.



**Fig. 6.** Quantitative detection of NP in clinical serum samples by NLICS and commercial ELISA. (A) Quantitative measurement of NP was obtained from 21 individual serum samples from COVID-19 patients and data was represented as a mean of triplicate for each sample; (B) The correlation of quantification for NP in blood samples between NLICS and commercial ELISA.

**Table 1** Detection of NP in clinical blood samples by NLICS and commercial ELISA.

clinical samples	NLICS		ELISA	
	Negative	Positive	Negative	Positive
Negative group (n=80)	80	0	80	0
Positive group (n=21)	5	16	11	10
Total	85	16	91	10
Specificity (%)	100% (80/80)		100% (80/80)	
Sensitivity (%)	76.2% (16/21)		47.6% (10/21)	
Accuracy (%)	95.1% (96/101)		89.1% (90/101)	

Note: The difference in sensitivity between two assays was calculated by Pearson's chi-squared test, of which  $P$  value was 0.057.

## Graphical abstract



## Credit Author Statement

**Chaolan Liang:** Conceptualization, Methodology, Formal analysis, Writing- original draft.

**Bochao Liu:** Methodology, Investigation, Formal analysis

**Jinfeng Li:** Resources, Methodology, Investigation.

**Jinhui Lu:** Methodology, Investigation, Validation

**Enhui Zhang:** Investigation, Software.

**Qitao Deng:** Investigation, Software.

**Ling Zhang:** Formal analysis.

**Ruai Chen:** Resources.

**Yongshui Fu:** Resources.

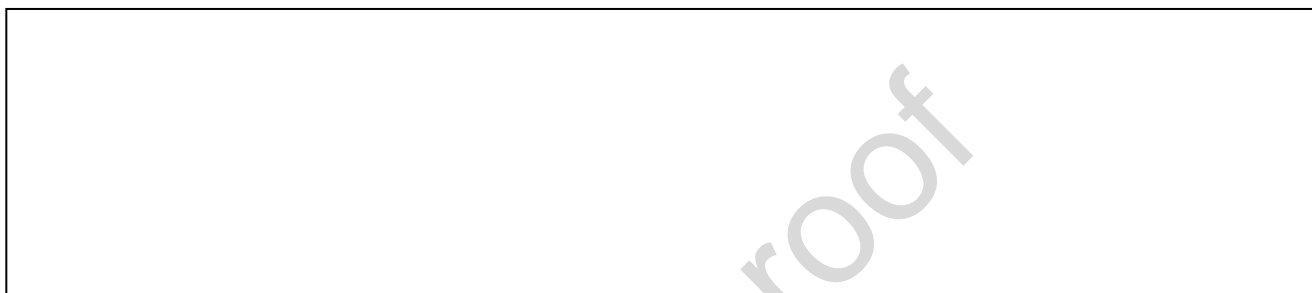
**Chengyao Li:** Supervision, Project administration, Funding acquisition, Writing - review & editing.

**Tingting Li:** Supervision, Project administration, Funding acquisition, Writing - review & editing.

**Declaration of interests**

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

**Highlights**

- SARS-CoV-2 lead to the pandemic of COVID-19 of which needs rapid, reliable, quantitative and easy diagnostic assays for antigen.
- A nanoenzyme linked immunochromatographic sensor (NLICS) was established with a LOD of 0.026 ng/mL SARS-CoV-2 nucleocapsid protein (NP).
- NLICS had 100% specificity, 76.2% sensitivity and 95.1% accuracy for detecting SARS-CoV-2 NP in clinical samples.
- Smartphone App and portable device were applied to quantitative detection.
- NLICS was a sensitive, rapid, portable, easy-operation, low-cost and safe immunoassay for early diagnosis of SARS-CoV-2 infection.