Absorption-Modulated SiO$_2$@Au Core–Satellite Nanoparticles for Highly Sensitive Detection of SARS-CoV-2 Nucleocapsid Protein in Lateral Flow Immunosensors

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ABSTRACT: The worldwide spread of coronavirus disease 2019 (COVID-19) highlights the need for rapid, simple, and accurate tests to detect various variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The antigen test, based on the lateral flow immunoassay (LFI), is a suitable “first line of defense” test that enables early identification and timely isolation of patients to minimize viral transmission among communities. However, it is generally less accurate than nucleic acid testing, and its sensitivity needs improvement. Here, a novel rapid detection method is designed to sensitively detect SARS-CoV-2 using isolated gold nanoparticle (AuNP)-assembled SiO$_2$ core–satellite nanoparticles (SiO$_2$@Au CSNPs). Well-grown AuNP satellites in the synthesis of SiO$_2$@Au CSNPs significantly enhanced their light absorption, increased the detection sensitivity, and lowered the detection limit by 2 orders of magnitude relative to conventional gold colloids. The proposed system enabled highly sensitive detection of the SARS-CoV-2 nucleocapsid protein with a detection limit of 0.24 pg mL$^{-1}$ within 20 min. This is the first study to develop a highly sensitive antigen test using the absorption-modulated SiO$_2$@Au CSNPs. Our findings demonstrate the capacity of this platform to serve as an effective sensing strategy for managing pandemic conditions and preventing the spread of viral infections.

KEYWORDS: core–satellite nanoparticles, absorption modulation, lateral flow immunoassay, COVID-19, SARS-CoV-2, nucleocapsid protein, high sensitivity

1. INTRODUCTION

Since the 18th century, the spread of viruses has posed one of the greatest threats to human life worldwide. Enhanced global connectivity and urbanization have accelerated the range and speed of viral spread. The ongoing coronavirus disease 2019 (COVID-19) pandemic has resulted from not effectively preventing the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the early stages after the outbreak. The pandemic led to a dramatic loss of human life and presented significant challenges to public health and the world economy. A total of 2.51 hundred million cases and 5 million deaths have been reported since the outbreak of COVID-19. Additionally, a 4.5% loss of global gross domestic product was estimated in 2020. The scale of social and economic loss has highlighted the need for affordable, sensitive, simple, and rapid methods of viral detection in the early stages of outbreaks.

Early diagnosis is essential during an ongoing pandemic that requires swift action to contain the rapid spread of viral infections. Although rapid nucleic acid amplification tests including reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and isothermal amplification are powerful and highly sensitive tools for viral detection, they are unsuitable for controlling ongoing pandemic conditions because of the relatively long turnaround times (hours to days) and the need for expensive equipment and expert operatives and interpretation of the results. In contrast, paper-based lateral flow immunoassay (LFI) platforms, which typically use gold nanoparticles (AuNPs) as a colorimetric readout material, are effective tools for early diagnosis and are widely used as rapid point-of-care tests (POCTs) due to significant cost advantage, simple interpretation with minimal training or infrastructure, and short turnaround time (in 20 min or less). Antigen tests, a type of paper-based LFI, which detect a specific viral antigen in nasopharyngeal or nasal swab specimens, provide evidence of viral infection at an early stage. Although positive results obtained from antigen tests are considered highly accurate, additional confirmation using a

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molecular test is recommended, often showing negative results. The relatively low sensitivity of antigen tests, a typical limitation of the paper-based LFI, presents a continuing hurdle for testing and highlights the need for further research to increase their sensitivity and accuracy.\textsuperscript{10,11}

Recently, various signal enhancement strategies for a AuNP-based LFI system have been studied to improve the sensing performance. These strategies often include silver enhancement,\textsuperscript{12} dual gold conjugation,\textsuperscript{13} and gold aggregation.\textsuperscript{14} These enhancements increase the size of the AuNPs in the test line of LFIs and improve the absorbance of AuNPs for higher colorimetric contrast. This is a simple method to obtain an amplified signal in the colorimetric LFI system. However, these methods have drawbacks, such as the instability of chemical reagents and the requirement of unavoidable additional steps for chemically enhancing the signal of the test line, which restrict their widespread use. Various AuNP assembly techniques that increase the size of AuNPs, resulting in higher light absorption without cumbersome steps, have been reported. Numerous isolated AuNPs, including nanoaggregates,\textsuperscript{15,16} nanoclusters,\textsuperscript{17,18} and nanocarriers,\textsuperscript{19,20} show unexpected optical properties different from those of individual AuNPs and can be used to establish sensitive, simple, and rapid detection systems. Isolated AuNP-assembled SiO\textsubscript{2} (SiO\textsubscript{2}@Au) core–satellite nanoparticles (SiO\textsubscript{2}@Au CSNPs), with excellent colloidal stability and functionality and size tunability, have been studied for biomedical and biosensing applications over the past 2 decades.\textsuperscript{21,22} These applications include cancer cell targeting and treatment, surface-enhanced Raman spectroscopy (SERS) for the detection of molecules and chemicals, or electrochemical sensing for the detection of glycoproteins.\textsuperscript{23–25} Especially, the SERS-based LFI system has expanded the application of SiO\textsubscript{2}@Au CSNPs to the POCT.\textsuperscript{26,27} However, the requirement of an expensive analytical device and insufficient sensitivity of these applications are drawbacks that prevent the use of these as effective virus detection systems that require sensitive performance in emergencies. For this reason, new strategies to reduce testing costs for the improvement of end-user accessibility and increase detection sensitivity are needed.

This study aimed to incorporate SiO\textsubscript{2}@Au CSNPs in an absorbance-based LFI for the sensitive detection of SARS-CoV-2 nucleocapsid protein (NP), which is highly immunogenic and expressed abundantly during infection (Figure 1a).\textsuperscript{28} As sensitivity and selectivity are determined by specific binding (SB) and non-specific binding (NSB) depending on the relationship between the membrane condition and SiO\textsubscript{2}@Au CSNPs, we attempted to maximize the signal-to-noise ratio (SNR) of the test region in the presence and absence of the target analytes by maximizing the SB and minimizing the NSB.
(SNR = SB/NSB) of the LFI system. Therefore, we used SiO$_2$@Au CSNPs which showed strong light absorption to obtain a strong specific signal and reduce the non-specific signal inducing false-positive results in the detection of SARS-CoV-2.

By characterizing the optical and structural changes depending on the growth degree of SiO$_2$@Au CSNPs to investigate optimum assay conditions with a high SNR in the LFI system, we aimed to improve sensitivity and achieve a broad dynamic range that was previously unattainable in conventional colorimetric LFI formats. We achieved sensitive and specific detection of the SARS-CoV-2 NP at low concentrations within 20 min and showed the possibility of the sensitive naked-eye detection, providing improved detection performance.

2. RESULTS AND DISCUSSION

2.1. Synthesis and Characterization of SiO$_2$@Au CSNPs. Figure 1a illustrates the synthetic strategy for SiO$_2$@Au CSNPs that were incorporated into the LFI platform for the highly sensitive detection of the SARS-CoV-2 NP to overcome the sensitivity limitation of the conventional LFI system which uses AuNP colloids. In the first step of the synthesis, gold-seeded SiO$_2$ was prepared by directly seeding Au(OH)$_3$ nanoparticles on amino-functionalized SiO$_2$ using the in situ deposition−precipitation method. During this seeding step, HAuCl$_4$ and NaOH were mixed to form a Au(OH)$_3$ solution through HAuCl$_4$ hydrolysis. The Au(OH)$_3$ solution was added to amino-functionalized SiO$_2$ and then reacted at a high temperature, thereby forming gold-seeded SiO$_2$. In the second step, SiO$_2$@Au CSNPs were synthesized by growing Au(OH)$_3$ nanoparticles (nucleation sites) on the surface of gold-seeded SiO$_2$. The Au(OH)$_3$ solution was added to amino-functionalized SiO$_2$ and then reacted at a high temperature, thereby forming gold-seeded SiO$_2$. The number of gold nanoparticle satellites (AuNP) increases with the growth degree, as shown in Figure 2a. The diameter of SiO$_2$@Au CSNPs as a function of the growth degree is shown in Figure 2c. Gaussian fitting from size distribution histograms of AuNP satellites on the eight different growth degrees of SiO$_2$@Au CSNPs is shown in Figure 2d. Photographs and UV−vis spectra showing the color change and peak shift as a function of the growth degree are shown in Figure 2e.
positively associated with the ratio of gold-seeded SiO$_2$ to the K-gold solution, which affected the concentration of AuNP satellites produced on the SiO$_2$ core surface.

We characterized the synthesis of SiO$_2$@Au CSNPs using transmission electron microscopy (TEM). The smooth surface of amino-functionalized SiO$_2$ (Figure 1b, left) with a diameter of 200 nm changed to a grainy surface with the homogeneous distribution of small Au(OH)$_3$ seeds (Figure 1b, middle) after using the in situ deposition–precipitation method. This indicated the successful synthesis of SiO$_2$@Au CSNPs by final confirmation of the growth of AuNP satellites on the surface of gold-seeded SiO$_2$ via the reduction of complex gold hydroxide anions (Figure 1b, right). Additionally, we analyzed the elemental composition of the SiO$_2$@Au CSNPs using energy-dispersive X-ray spectroscopy (EDS) for elemental mapping. Compared to amino-functionalized SiO$_2$ showing a Si K$_\alpha$ spectral peak at 1.74 keV (Figure S1a), the SiO$_2$@Au CSNPs showed an additional peak at ~2.1 keV, which can be assigned to Au M$_\alpha$ (Figure S1b). EDS mapping (Figures 1c and S1b) showed that predominantly grown Au satellites (64.18 wt %; Figure 1c, center, and Figure S1b) were homogeneously distributed on the entire surface of the SiO$_2$ core (35.82 wt %; Figure 1c, left). We examined the homogeneous size distribution of AuNP satellites (5.89 ± 1.05 nm) using a size distribution histogram obtained through statistical analysis of randomly selected 100 AuNP satellites on single SiO$_2$@Au CSNPs (Figure 1d). We determined the absorbance spectrum for the confirmation of the successful AuNP satellite growth on the SiO$_2$ core. The spectrum of the SiO$_2$@Au CSNPs exhibited a peak at 535 nm attributed to the AuNP satellites on SiO$_2$, whereas amino-functionalized SiO$_2$ and gold-seeded SiO$_2$ displayed a broad absorbance spectrum (Figure 1e).

Additional confirmation of the successful synthesis of SiO$_2$@Au CSNPs was obtained with the surface characterization techniques using an X-ray diffractometer and an X-ray photoelectron spectrometer. The crystalline structure of the SiO$_2$@Au CSNPs indicated several diffraction peaks with a relatively weakened peak located at 2$\theta$ = 22$^\circ$ (101) attributed to amorphous SiO$_2$ and the others located at 2$\theta$ = 38.55$^\circ$ (111), 44.90$^\circ$ (200), 65.07$^\circ$ (220), 77.86$^\circ$ (311), and 81.86$^\circ$ (222) attributed to AuNP satellites (Figure 1f, red line) unlike amino-functionalized SiO$_2$, for which only a single diffraction peak at 2$\theta$ = 22$^\circ$ (101) was observed (Figure 1f, black line). Elemental composition of SiO$_2$@Au CSNPs obtained from the X-ray photoelectron spectroscopy (XPS) measurement confirmed their successful synthesis. The Au 4f$_{7/2}$ and Au 4f$_{5/2}$ signals in the Au 4f XPS profile of the SiO$_2$@Au CSNPs were visible at 82.35 and 85.95 eV, respectively (Figure 1g).

Structural changes in particles induce changes in their optical properties. To investigate the influence of the structural changes, we grew AuNP satellites by reducing the complex gold hydroxide anions and classified them according to the growth degree (Section 4.4). We characterized the SiO$_2$@Au CSNPs synthesized as the growth degree increased (growth degree 1–8). As it grew in size, the AuNP satellite almost completely covered the SiO$_2$ core; eventually, the spherical shape of the SiO$_2$@Au CSNPs collapsed due to the overgrowth of AuNP satellites (Figure 2a). The Au concentration of the AuNP satellites in SiO$_2$@Au CSNPs was calculated using an inductively coupled plasma mass spectrometer, and the resulting concentration of Au increased as the growth degree increased (Figure 2b). The overall diameter of SiO$_2$@Au CSNPs as a function of the growth degree gradually increased and showed a large standard deviation for the SiO$_2$@Au CSNPs diameter with a growth degree >5 due to the high
size polydispersity of AuNP satellites (Figure 2c). The approximated diameters of the AuNP satellites on SiO\textsubscript{2}@Au CSNPs at 8 different growth degrees were analyzed from TEM images using a size histogram obtained through statistical analysis of randomly selected 100 AuNP satellites. As the growth degree increased, the size of the AuNP satellites on the surface of the SiO\textsubscript{2}@Au CSNPs gradually increased. Meanwhile, the broad size distribution of the AuNP satellites on the SiO\textsubscript{2}@Au CSNPs with a growth degree >5 suggested their irregular structure caused by the overgrown AuNP satellites (Figure 2d). The dependence of the gradual change in the size of SiO\textsubscript{2}@Au CSNPs on the growth degree was also confirmed via a color change of SiO\textsubscript{2}@Au CSNP solution and a peak shift of the UV–vis absorption spectra. The absorbance peak gradually shifted toward higher wavelengths (from 545 to 613 nm), and the intensity increased (from 0.318 to 0.966) as the AuNP satellites grew larger (Figure 2e). These structural characterizations demonstrated that AuNPs were successfully grown over the amorphous SiO\textsubscript{2} core.

The as-synthesized SiO\textsubscript{2}@Au CSNPs showed a significant absorption-dominated spectrum, whereas the AuNP colloids showed a scattering-dominated spectrum (Figure 3a). The results suggested that the increased number of AuNPs on the single SiO\textsubscript{2} particle induced an increase in the absorption ability of SiO\textsubscript{2}@Au CSNPs. We characterized the overall SiO\textsubscript{2}@Au CSNP synthesis, including the conjugation, using zeta potential and Fourier-transform infrared (FTIR) spectroscopy analyses. The zeta potential of bare SiO\textsubscript{2} nanoparticles showed a negative charge due to their silanol group and changed from $-66.59 \pm 3.80$ to $32.33 \pm 0.77$ mV after amination (Figure 3b). The positive charge of amino-functionalized SiO\textsubscript{2} changed to $-2.81 \pm 0.04$ mV by grafting the gold hydroxide [Au(OH)\textsubscript{3}] \textit{via in situ} deposition on the surface of amino-functionalized SiO\textsubscript{2}. This weak negative charge of gold-seeded SiO\textsubscript{2} was shifted toward a more negative state ($-38.86 \pm 0.81$ mV) by the citrate-stabilized AuNPs on the SiO\textsubscript{2} surface \textit{via} SiO\textsubscript{2}@Au CSNP synthesis. After anti-SARS-CoV-2 NP antibodies were adsorbed onto the surface of the SiO\textsubscript{2}@Au CSNPs, the zeta potential slightly shifted toward a neutral state ($-32.35 \pm 0.13$ mV), which was attributed to the electrostatic interaction between AuNP satellites and antibodies, thus indicating the successful immobilization of the antibodies onto the SiO\textsubscript{2}@Au CSNPs during the conjugation of the targeting probe.\textsuperscript{30,31} Additional confirmation of the targeted antibody immobilization was obtained using FTIR spectroscopy. As shown in Figure 3c, peaks at 1537 cm\textsuperscript{-1} (amide II resulting from the C\text{=O} stretching vibration of amide bonds) and 1639 cm\textsuperscript{-1} (amide I arising from the N\text{–}H bond bending vibration of amide bonds) appeared following antibody immobilization. In contrast, SiO\textsubscript{2}@Au CSNPs showed peaks at 797, 958, and 1067 cm\textsuperscript{-1}, which were assigned to the bending vibration of Si–O–Si, stretching vibration of Si–OH, and stretching vibration of Si–O–Si\textsubscript{2}, respectively. These results suggested the successful conjugation of the targeting probe.

Because the sensing performance of the LFI system is directly attributed to the optical properties of the targeting probe, we conducted a comparative analysis of the minimum detectable concentration on nitrocellulose (NC) membranes using SiO\textsubscript{2}@Au CSNPs and 40 nm AuNP conjugates, which are typically used in colorimetric LFI systems. To confirm the effect of absorption-dominated SiO\textsubscript{2}@Au CSNPs on the sensing performance of the LFI, two types of particles were compared by measuring the colorimetric intensity of dilutions on an NC membrane after immobilization of the targeting antibody under the same conjugation process (Section 4.5).
SiO₂@Au CSNPs showed high sensitivity and were detectable at up to 4.56 × 10⁸ particles (40 nm gold colloids: 6.83 × 10⁶ particles) (Figure 3d,e). Uniformly grown AuNP satellites on the SiO₂ surface allowed ~150-fold more sensitive results than those obtained using conventional gold colloids due to the strong light absorption caused by the increase in the number of AuNPs on a single SiO₂ particle (Figure 3f). We also investigated the influence of the AuNP satellite growth degree on the SiO₂ core and the concentration of SiO₂@Au CSNPs on the LFI sensing performance by comparing the SNRs of various SiO₂@Au CSNPs. To obtain the best SNR, eight SiO₂@Au CSNPs (growth 1–8) were prepared by immobilizing the targeting antibody, and then, the SNRs were compared. The SNR of 100 to 0 pg mL⁻¹ SARS-CoV-2 NP increased with the growth degree until the fourth degree and then decreased. The low growth degrees of SiO₂@Au CSNPs presented a low colorimetric intensity caused by the low amount of AuNPs participating in the immunoreaction on the NC membrane owing to the low level of reduction of AuNPs satellites on the SiO₂ surface. Further reduced AuNP satellites showed strong SB signals because their light absorption characteristics improved as SiO₂@Au CSNPs grew; however, the SNR decreased owing to an increase in the NSB signal on the test line of the NC membrane. This result indicated that the strongest SB signal was induced by the irregular structure formed in the overgrown SiO₂@Au CSNPs (Figure S2). The fourth degree with the highest SNR was chosen as the optimal condition for the targeting probe in the proposed LFI system (Figure 4a).

2.2. Preparation of the SARS-CoV-2 Targeting Probe. The SARS-CoV-2 targeting probe was prepared by conjugating antibodies to the surface of the AuNP satellites via electrostatic adsorption. Subsequently, the blocking reagent was immobilized on the remainder of the surface to increase particle stability and prevent self-aggregation, which is strongly related to the sensing performance. Comparison tests were conducted to optimize the blocking conditions using bovine serum albumin (BSA), skim milk, or protein saver. The results showed a high NSB signal and low SNR with BSA and protein saver, which suggested that skim milk played an effective role in protecting the free surface of the SiO₂@Au CSNPs after the antibody immobilization (Figure S3a). The skim milk concentration was also investigated as an effective blocking condition. The SNR corresponding to 1 ng mL⁻¹ versus 0 ng mL⁻¹ increased with the skim milk concentration and plateaued at 2 wt % skim milk (Figure S3b). The SB signal was constant at all skim milk concentrations; however, the NSB signal decreased with the increasing concentration. Thus, 2 wt % skim milk was selected as the optimal blocking condition because 4 wt % skim milk was too dense to remove unblocked skim milk during the preparation of the SARS-CoV-2 targeting probe.

2.3. NC Membrane Preparation. The NC membrane is the main component and the globally preferred substrate for the test strip design of the diagnostic LFI system, wherein the majority of events occur, including the flow of the loaded sample and binding of the antigen and the antibody, which should be optimized for the proposed system. The NSB in the test region was caused by the analytes or conjugates nonspecifically bound to the NC membrane via hydrophobic and electrostatic interactions or by the conjugates physically trapped in the membrane owing to their pore size. Therefore, we attempted to reduce the NSB caused by the trapped conjugates and physical adsorption by screening the NC membranes and blocking the test region, respectively. First, we optimized the NC membranes by conducting a comparison test among five membranes with different pore sizes. We investigated the SNR corresponding to 10 ng mL⁻¹ versus 0 ng mL⁻¹ SARS-CoV-2 NP on five different NC membranes. The SNR of each membrane decreased with the increasing capillary flow time (Figure 4b). Smaller-pore-size NC membranes induced an increase in flow time owing to the relationship between membrane porosity and particle size in the flowing fluid, indicating increased SB and NSB signals with decreasing pore size (based on pore size information provided by Sartorius). However, the SNR of the smaller-pore-size NC membranes showed a lower value because the increase rate in the NSB signal was much greater than that in the SB signal (Figures 4c and S4a). Scanning electron microscopy (SEM) images of five different NC membranes obtained after an immunoassay were performed showed trapped SiO₂@Au CSNPs in each NC membrane. The number of trapped particles increased with the decreasing pore size (Figure S4b). In addition, the smaller the pore size, the greater the number of undesirably trapped particles observed in the SEM image of the strip detecting 0 ng mL⁻¹. Eventually, strong SB and NSB signals were presented in CN180 having the smallest pore size among the compared membrane groups (Figure S4c). Although CN95 showed the lowest SB signal, it was associated with the highest SNR due to the low NSB signal. Thus, the lowest-pore-size CN95 NC membrane was selected as the NC membrane for this proposed LFI system. The test region on the CN95 NC membrane was blocked with a blocking reagent to maximize the SNR by reducing the NSB signal. Test region blocking was performed by fully covering the immobilized capture antibody region on the NC membrane with three different blocking reagents (BSA, skim milk, and protein saver). To determine the optimal blocking condition, we investigated the SNRs corresponding to 1 ng mL⁻¹ versus 0 ng mL⁻¹ SARS-CoV-2 NP. The NSB signal was reduced by all blocking reagents compared to that observed on the non-blocked NC membranes. The protein saver presented the highest SNR resulting from the lowest NSB compared to that associated with BSA and skim milk (Figure S5a). The optimal concentration was determined using a comparison test for four different concentrations (1, 2, 4, and 6 wt %), among which 2 wt % protein saver showed the highest SNR for all antigen concentrations and thus was selected as the optimal concentration (Figure S5b).

2.4. Conjugate Pad Preparation. The concentration of SiO₂@Au CSNP conjugates dried on the conjugate pad strongly influences the detection sensitivity of the LFI system because of the variations in the colorimetric signal, which is dependent on the number of particles participating in the immunoreaction. Thus, we increased the concentration of the fourth-grown SiO₂@Au CSNP conjugates and measured the SNRs corresponding to 1 ng mL⁻¹ versus 0 ng mL⁻¹ SARS-CoV-2 NP at six different concentrations of SiO₂@Au CSNP conjugates under the same experimental conditions (Figure S6a) (Section 4.7). Large amounts of conjugates induced an increase in the intensity of the test line during the immunoreaction. The SB signals gradually increased with the increasing conjugate concentrations, while the NSB signals were first constant and then started to increase at a conjugate concentration of 7× (1× denoted 7 × 10⁶ SiO₂@Au CSNPs mL⁻¹) (Figure S6b). As a result, the 7× conjugate with the
highest SNR was selected as the optimal concentration to prepare the conjugate pad (Figure 4d).

2.5. Evaluation of the SAR-CoV-2 Detection System.

The buffer pH can greatly influence the NSB. Therefore, we investigated the optimal assay buffer conditions within a pH range of 7.2—9.0. The buffer condition was optimized using phosphate buffered saline (PBS) (pH 7.2), which induced the maximum SNR obtained by detecting 1 ng mL$^{-1}$ SARS-CoV-2 NP (Figure S7a,b). The optimized conditions, including SiO$_2$@Au CSNP conjugates and membrane blocking, were used in all subsequent experiments. Finally, we evaluated the SiO$_2$@Au CSNPs to determine their detection sensitivity for the SARS-CoV-2 NP (Figure 5a). To this end, a sandwich immunoassay was performed using SiO$_2$@Au CSNPs against various concentrations of NP. The resulting test line signal was distinguishable up to 10 pg mL$^{-1}$ by the naked eye (marked with an asterisk on the test strip image). Colorimetric images were obtained 20 min after sample addition. The results

Figure 5. Detection of SARS-CoV-2 in the optimized LFI test strip. (a) Schematic representation of the sandwich immunoassay on a lateral flow immunosensor strip. (b) Colorimetric images showing detection of serially diluted SARS-CoV-2 NP. Colorimetric intensity of the test lines shown in images corresponding to antigen concentrations of 0.0001—10 ng mL$^{-1}$, showing sensitive antigen detection at concentrations as low as 1 pg mL$^{-1}$. Error bars represent the standard deviation for five independent experiments. (c) Evaluation of selectivity for SARS-CoV-2 (1 ng mL$^{-1}$) and five other viruses (interferences; 10 ng mL$^{-1}$). (d) Colorimetric images showing detection of serially diluted heat-inactivated SARS-CoV-2 viral culture fluid. Colorimetric intensity of the test lines shown in images corresponding to cultured viral sample concentrations of $3.8 \times 10^{0}$—$3.8 \times 10^{4}$ TCID$_{50}$ mL$^{-1}$, showing an LOD of 4.9 TCID$_{50}$ mL$^{-1}$ with a coefficient of determination of 0.9962. All asterisks indicate the lowest concentrations of samples distinguishable by the naked eye. Error bars represent the standard deviation for three independent experiments.
showed that this system enabled highly sensitive and quantitative SARS-CoV-2 NP detection at concentrations as low as 1 pg mL$^{-1}$ with a wide dynamic linear range (0.001–10 ng mL$^{-1}$; Figure 5b). Moreover, the limit of detection (LOD) provided by the IUPAC guidelines [LOD = blank signal + 3 standard deviations] was calculated as 0.24 pg mL$^{-1}$ with a correlation coefficient ($R^2$) of 0.9946. The detection sensitivity of SiO$_2$@Au CSNPs for the SARS-CoV-2 NP increased by 100-fold compared to that of AuNP colloids, which was in line with the results of the colorimetric intensity of dilutions on the NC membrane (Figure S8).

2.6. Selectivity and Stability. Selectivity was evaluated using NPs from various viruses including influenza A, influenza B, Middle East respiratory syndrome coronavirus (MERS-CoV), Ebola, and COV-229E. The NPs from these five viruses were prepared at a concentration of 10 ng mL$^{-1}$. The colorimetric intensity of SARS-CoV-2 NP at a concentration of 1 ng mL$^{-1}$ was much higher than that of the other five viruses. Moreover, the colorimetric intensity of a mixture containing SARS-CoV-2 NP (1 ng mL$^{-1}$) and NPs of other five viruses showed negligible interference with the SARS-CoV-2 NP detection (Figure 5c). These results demonstrate that this system exhibits excellent selectivity for the detection of SARS-CoV-2 NPs. Stability was investigated by confirming the maintenance of a constant SNR obtained by detecting 1 ng mL$^{-1}$ SARS-CoV-2 NP using preliminarily prepared test strips fabricated simultaneously for 15 days. The strips provided fairly stable results, with a relative standard deviation of 4.31%, indicating the stability of the platform (Figure S9).

2.7. Detection of SARS-CoV-2 in Viral Culture Fluid. We analyzed SARS-CoV-2 in viral culture fluid with SiO$_2$@Au CSNPs conjugates using the LFI platform designed in this study. Heat-inactivated viral culture fluid of SARS-CoV-2 at a concentration of 3.8 × 10$^6$ tissue culture infective dose-50% (TCID$_{50}$) per milliliter was prepared using 10-fold serial dilutions in universal transport medium (UTM). Three replicates with five different concentrations of the viral sample were diluted 10-fold with the optimized assay buffer (final concentrations ranging from 3.8 × 10$^4$ to 3.8 × 10$^0$ TCID$_{50}$ mL$^{-1}$). The test line signal was distinguishable up to 3.8 × 10$^4$ TCID$_{50}$ mL$^{-1}$ by the naked eye (marked with an asterisk). The resulting colorimetric signal indicated a highly sensitive viral detection with an LOD of 4.9 TCID$_{50}$ mL$^{-1}$ and a coefficient of determination of 0.9962 (Figure 5d). The results were compared with those of the gold-standard RT-qPCR to analyze the SARS-CoV-2 viral culture fluid. RT-qPCR could detect 3.8 × 10$^3$ TCID$_{50}$ mL$^{-1}$ of the viral sample, which corresponded to an averaged cycle threshold (C$_T$) value of 33.86 (Figure S10a,b). This suggests that the proposed system is comparable to the standard method. RT-qPCR results for the SARS-CoV-2 in viral culture fluid were positively correlated with the colorimetric intensity obtained using our LFI platform with SiO$_2$@Au CSNP conjugates (3.8 × 10$^4$ to 3.8 × 10$^0$ TCID$_{50}$ mL$^{-1}$). This high correlation curve ($R^2 = 0.9960$) suggested that the sensor developed in this study can be successfully used for quantitative and qualitative measurement of SARS-CoV-2 levels in patient samples (Figure S11). Moreover, our system showed superior performance with high sensitivity and a wide detection range compared to the other approaches that use the LFI format (Table 1) and electrochemical strategies (Table S1) for SARS-CoV-2 antigen detection, thus overcoming the limitations of the current methods and showing great potential as an alternative virus detection tool for molecular analysis.
diagnosis. Therefore, our system has great potential for detecting SARS-CoV-2 NP in patients with COVID-19.

3. CONCLUSIONS
The enhanced sensing performance of the LFI system was achieved by improving the optical properties of the signal probe. Successful synthesis and optimization of SiO$_2$@Au CSNPs resulted in strong absorption with relatively weak scattering suitable for the antigen test, which is required for improved detection sensitivity. Here, we studied the optical and structural changes of SiO$_2$@Au CSNPs depending on the growth degree of AuNP satellites and examined the effect of synthesized SiO$_2$@Au CSNPs on the performance of the LFI system in detecting SARS-CoV-2 NPs. SARS-CoV-2 NP could be sensitively detected using SiO$_2$@Au CSNPs, which showed strong light absorption characteristics, in the optimized LFI system. The proposed system detected 1 pg mL$^{-1}$ SARS-CoV-2 NP (LOD: 0.24 pg mL$^{-1}$), which was 2 orders of magnitude lower than that detected using the conventional LFI platform utilizing 40 nm gold colloids, and showed no cross-reactivity with influenza A, influenza B, MERS-CoV, Ebola, and HCoV-229E NPs. SARS-CoV-2 in the viral culture fluid was successfully detected at 4.9 TCID$_{50}$ mL$^{-1}$, suggesting that our system exhibits comparable sensitivity to that of the standard RT-qPCR analysis (38 TCID$_{50}$ mL$^{-1}$). This is the first study to use the absorption-modulated SiO$_2$@Au CSNPs in an LFI system to sensitively detect SARS-CoV-2. Our findings show the potential of this method as an effective alternative tool for molecular diagnosis under emergency conditions.

4. EXPERIMENTAL SECTION

4.1. Materials. For synthesis and modification of the SiO$_2$@Au CSNPs, tetraethyl orthosilicate (TEOS), ammonium hydroxide solution (NH$_4$OH; 28–30%), acetone, (3-aminopropyl)trimethoxysilane (APMTMS), potassium carbonate (K$_2$CO$_3$), sodium hydroxide (NaOH), gold(III) chloride hydrate (HAuCl$_4$·3H$_2$O), trisodium citrate dehydrate [HOC(COO)Na$_2$·CH$_2$OH], and sodium borohydride (NaBH$_4$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gold colloid solutions of 40 and 200 nm were purchased from BBI Solutions (EM, GC20, Crumlin, UK) and Sigma-Aldrich, respectively. Ethyl alcohol was obtained from Duksan (Seoul, South Korea). To design the LFI strip for SARS-CoV-2 NP detection, NC membranes (Unisart CN95, 110, 140, 150, and 180) were purchased from Sartorius (Boehmen, NY, USA). Absorbent pads (grade 222), conjugate pads (grade 6613), and sample pads (grade 8964) were purchased from Ahlstrom-Munksjö Oyj (Helsinki, Finland). BSA (BSA30-AB74) and the surfactant 10G (95R-103) were purchased from Fitzgerald (Acton, MA, USA). PBS (1× PBS; pH 7.2) (PR2004) and Tris–HCl (pH 7.4, 8.0, 8.5, and 9.0) were purchased from Biosesang Co. (Seongnam, Korea), and phosphate buffer solution (PB; pH 7.4; P3619) was purchased from Sigma-Aldrich. Anti-SARS-CoV-2 NP antibodies were purchased from MEDILAB (Guri, Gyeonggi-do, South Korea). The COVID-19 NP (BHAG-N1) was purchased from Bore Da Biotech Co., Ltd. (Seongnam-si, Gyeonggi-do, South Korea). Other NPs of influenza A (11675-V08B), influenza B (40438-V08B), MERS-CoV (40068-V08B), the Ebola virus (40443-V07E1), and human coronavirus 229E (HCoV-229E; P1506) were purchased from Sino Biological (Wayne, PA, USA) and BioVision (Milpitas, CA, USA). Neo protein saver (NPS-301) was purchased from Toyobo Co., Ltd. (Osaka, Japan), and the goat anti-mouse IgG antibody (M8642), skim milk powder (70166), polyanlypyrrolidone (29 K) (234257), D-(-)-trehalose dehydrate (TS251), Tween 10 (P1379), and Triton X-100 (Triton X-100) were purchased from Sigma-Aldrich.

All reagent solutions were prepared using deionized water (18.2 MΩ) generated in an ELGA water purification system (Lane End, UK). To validate the applicability of the viral sample and evaluate the correlation of the colorimetric signal obtained from the proposed LFI platform with the $C_t$ value obtained from RT-qPCR, a QiAamp viral RNA extraction kit was purchased from Qiagen Inc. (Valencia, CA, USA). A TOPreal one-step RT-qPCR kit was purchased from Enzymics (Daejeon, Korea). Heat-inactivated SARS-CoV-2 viral culture fluid (USA-WA1/2020, 0810587CFHI) was purchased from Zeptometrix (Buffalo, NY, USA). Oligonucleotides were synthesized by Zenotech Corp. (Daejeon, Korea).

4.2. Synthesis of the SiO$_2$ Core and Surface Modifications. A bare SiO$_2$ core was used as a scaffold and prepared based on the Stöber process of the sol–gel method, with minor modifications. First, 0.9 mL of ammonium hydroxide solution was added to a mixture containing 10.5 mL of ethanol and 4.5 mL of deionized water, and the mixture was stirred for 20 min to obtain a transparent solution. Next, 0.45 mL of TEOS was added dropwise to the mixture, and the hydrolysis and condensation reactions were allowed to continue for 4 h at 25 °C with vigorous stirring. After the reaction, the final SiO$_2$ core was precipitated by adding acetone, washed with ethanol, isolated via centrifugation twice at 6448×g at 25 °C for 20 min, and then stored in ethanol. Amino-functionalized SiO$_2$ was prepared as follows: 500 μL of APTMS was added to 100 mg of the SiO$_2$ core in 14.5 mL of ethanol, and the resulting solution was incubated at 25 °C for 2 h. Amino-functionalized SiO$_2$ was separated using centrifugation, washed with ethanol three times, and stored in ethanol to a final concentration of 20 mg mL$^{-1}$.

4.3. Gold Seeding on Amino-Functionalized SiO$_2$. Gold-seeded SiO$_2$ was prepared according to the literature describing the deposition–precipitation method with modifications. First, 0.45 mL of 0.1 M NaOH was added to 2 mL of a 6.35 mM HAuCl$_4$ solution and stirred at 25 °C for 15 min to form a light-yellowish gold hydroxide [Au(OH)$_3$] solution via the hydrolysis of HAuCl$_4$. Next, 0.4 mL of 20 mg mL$^{-1}$ amino-functionalized SiO$_2$ was added to the Au(OH)$_3$ solution, heated to 70 °C, and maintained at that temperature for 1 h under vigorous stirring to initiate gold nucleation. After successfully grafting the Au(OH)$_3$ nanoparticles via in situ deposition on the surface of amino-functionalized SiO$_2$, final gold-seeded SiO$_2$ changed from milky white to a dark-orange color, was washed twice with ethanol and then three times with deionized water, centrifuged at 5751xg at 25 °C for 20 min, and stored in 1 mL of deionized water. The concentration of gold-seeded SiO$_2$ was denoted "1X".

4.4. Synthesis of SiO$_2$@Au CSNPs. To grow Au(OH)$_3$ nanoparticles deposited on the surface of gold-seeded SiO$_2$, a potassium–gold solution (K-gold) was prepared by adding 30 mg of K$_2$CO$_3$ and 0.375 mL of 0.05 M HAuCl$_4$ stock solution dissolved in a final volume of 50 mL of deionized water. The K-gold solution was stirred and aged overnight in the dark at 25 °C. SiO$_2$@Au CSNPs (growth degree 1) were first formed by adding 0.15 mL of 1X gold-seeded SiO$_2$ into 1 mL of K-gold solution and stirring the solution for 15 min at 25 °C. Next, 100 μL of freshly prepared and ice-cold 6.6 mM NaBH$_4$, which was used as a reducing agent, was added to the mixture and stirred for 20 min to reduce the complex gold hydroxide anions ([Au(OH)$_3$]$_{3-}$) on the Au(OH)$_3$ seeds. Eight growth degrees of gold were achieved by varying the K-gold as follows: The gold-seeded SiO$_2$ volume ratio was changed from 20 to 160:3. For the increase in the growth degree from 2 to 8, K-gold solution and NaBH$_4$ were added to the reaction at the previously identified growth degree. Additionally, 100 μL of 10 mM trisodium citrate dehydrate was used as a capping agent per 2 mL of K-gold and added to the reaction solution at even-numbered growth degrees (2, 4, 6, and 8).

4.5. Conjugation of Antibodies to SiO$_2$@Au CSNPs. Antibody conjugation to the surface of AuNP satellites was performed via electrostatic adsorption. First, 20 μL of the 1 mg mL$^{-1}$ anti-SARS-CoV-2 NP detection antibody was added to 1 mL of 1X SiO$_2$@Au CSNPs solution and incubated at 25 °C for 1 h. Then, 200 μL of 10 wt % skim milk dissolved in 1X PBS was added as a blocking agent. After 1 h of incubation at 25 °C, the mixture was centrifuged at 3635×g for 18 min at 10 °C. The supernatant was discarded, the product
obtained was resuspended in 10 mM borate buffer (pH 8.5), and the centrifugation and resuspension steps were repeated twice. Finally, the SiO$_2$@Au CSNP conjugates were concentrated 20-fold in storage buffer [0.5 wt % protein saver, 5 wt % trehalose, 1% Triton X-100, and 0.2% Tween-20 in 0.1 M Tris–HCl (pH 8.0)]. The concentration of the SiO$_2$@Au CSNP conjugates was denoted “20x” (1x indicated 7 × 10$^9$ particles mL$^{-1}$).

4.6. Characterization. The shape and size of the SiO$_2$@Au CSNPs were characterized using TEM (Tecnai G2 F30 S-Twin, FEI, Hillsboro, OR, USA), and elemental analysis was performed using high-resolution field-emission SEM (Verios 5 XHR, Thermo Fisher Scientific, Waltham, MA, USA) equipped with an X-ray EDS system (Oxford Ultim Max EDS detector, Oxford Instruments, Buckinghamshire, UK). The chemical compositions of the SiO$_2$@Au CSNPs were analyzed using XPS (Sigma Probe, Thermo VG Scientific, West Sussex, UK), and the crystal structures of the SiO$_2$@Au CSNPs were determined using a high-resolution X-ray diffractometer (Smartlab, Rigaku, Tokyo, Japan). High-angle annular dark-field scanning TEM (HAADF STEM) and elemental mapping of a single SiO$_2$@Au CSNP were performed using image-side aberration-corrected TEM (Titan 3 G2 60-300, FEI, Hillsboro, OR, USA). The absorbance spectra of the SiO$_2$@Au CSNPs were recorded using a microplate reader (Cytation 5 Imaging Reader, Biotek; Winooski, VT, USA), and the surface charges of the dispersed nanoparticles following each synthesis were determined using a particle size and zeta potential analyzer (ELSZ-1000, Otsuka Electronics, Osaka, Japan). The contents of elemental Au in SiO$_2$@Au CSNPs were measured with an inductively coupled plasma mass spectrometer (NexION 350D, PerkinElmer, Waltham, MA, USA). The surface functional groups of the SiO$_2$@Au CSNPs and conjugates following each synthesis were analyzed using the absorbance peaks and an FTIR spectrometer (TENSOR27, Bruker, Ely, Leipzig, Germany) with a platinum-attenuated total reflection accessory. Absorption and light scattering spectra for the comparison of the optical performance of SiO$_2$@Au CSNPs and gold colloids were measured using a UV–vis spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan) and a fluorescence spectrometer (FluoRoMate FS-2; SCINCO, Seoul, Korea), respectively.

4.7. Preparation of the Test Strip. The test strip was prepared by immobilizing the SARS-CoV-2 NP capture antibody (test line) and anti-mouse IgG secondary antibody (control line) onto the NC membrane, which was stuck with the conjugate pad (containing pre-dried antibody-labeled SiO$_2$@Au CSNPs), sample pad, and absorbance pad on a plastic card. The NC membrane, which is the main substrate of the LFI system, was optimized through a comparison test against five different NC membranes. During the optimization of the NC membrane, the capillary flow time of each membrane was preliminarily measured by applying assay buffer to the NC membrane and obtaining the time to flow at a 25 mm distance. Antibodies were spotted on each NC membrane with 0.7 μL of the 0.25 mg mL$^{-1}$ anti-mouse IgG and 1 mg mL$^{-1}$ SARS-CoV-2 NP capture antibody. The optimal substrate condition was obtained by choosing a membrane with the best SNR obtained after an incubation at 37 °C for 20 min. The optimal substrate condition was considered an additional condition to be optimized. The test strip was prepared through the binding between the capture antibody immobilized on the test region of the NC membrane and the detection antibody immobilized on the NC membrane (25 × 3.9 mm), which was pre-blocked with 6 mL of blocking solution containing 1% BSA, 0.1% Tween 20, and 5 mg mL$^{-1}$ skim milk in 1× PBS (pH 7.4) for the complete release of the SiO$_2$@Au CSNP conjugates from the pad during the immunomassay. After drying at 37 °C for 15 min, 5 μL of the as-prepared antibody-functionalized SiO$_2$@Au CSNPs (7x) was dried on a conjugate pad using 1% Triton X-100 and incubated at 37 °C for 20 min. The sample pad (20 × 3.9 mm), NC membrane (25 × 3.9 mm), and absorbent pad (20 × 3.9 mm) were constructed on a plastic adhesive supporting card (60 × 3.9 mm) with a 2 mm overlap between every two adjacent pads. The fabricated strips were stored in a desiccator before use.

4.8. SARS-CoV-2 Detection. The sandwich immunomassay was performed through the binding between the capture antibody immobilized on the test region of the NC membrane and the detection antibody immobilized on the NC membrane (1× 3.9 mm × 1000, Otsuka Electronics, Osaka, Japan). The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.2c13303. The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.2c13303.

Supporting information, figures, particle characterization, and optimization data for demonstrating sensing performance (PDF)

ASSOCIATED CONTENT

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