Attomolar Sensitive Magnetic Microparticles and a Surface-Enhanced Raman Scattering-Based Assay for Detecting SARS-CoV-2 Nucleic Acid Targets

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Cite This: https://doi.org/10.1021/acsami.1c17028

ABSTRACT: Highly sensitive, reliable assays with strong multiplexing capability for detecting nucleic acid targets are significantly important for diagnosing various diseases, particularly severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The nanomaterial-based assay platforms suffer from several critical issues such as non-specific binding and highly false-positive results. In this paper, to overcome such limitations, we reported sensitive and remarkably reproducible magnetic microparticles (MMPs) and a surface-enhanced Raman scattering (SERS)-based assay using stable silver nanoparticle clusters for detecting viral nucleic acids. The MMP-SERS-based assay exhibited a sensitivity of 1.0 fM, which is superior to the MMP-fluorescence-based assay. In addition, in the presence of anisotropic Ag nanostructures (nanostars and triangular nanoplates), the assay exhibited greatly enhanced sensitivity (10 aM) and excellent signal reproducibility. This assay platform intrinsically eliminated the non-specific binding that occurs in the target detection step, and the controlled formation of stable silver nanoparticle clusters in solution enabled the remarkable reproducibility of the results. These findings indicate that this assay can be employed for future practical bioanalytical applications.

KEYWORDS: SARS-CoV-2, nucleic acid target detection, magnetic microparticles, silver nanoparticles, surface-enhanced Raman scattering, attomolar sensitivity

INTRODUCTION

The outbreak of coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has caused an insidious global pandemic.1–3 Early diagnosis is essential to control the COVID-19 outbreak as it helps in reducing the potential spread of the virus.4 Various assays, such as reverse transcription-polymerase chain reaction (RT-PCR), reverse transcription loop-mediated isothermal amplification,5 and clustered regularly interspaced short palindromic repeats,6 have been utilized for the detection of SARS-CoV-2 viral nucleic acids. Among them, the RT-PCR-based assay is the most commonly used method. RT-PCR involves the conversion of viral RNA into complementary DNA using RNA-dependent DNA polymerase, which is amplified through thermal cycling. The RT-PCR-based assay relies on enzymatic amplification to attain attomolar sensitivity; however, this amplification leads to high false-positive results owing to the presence of artificial signals from sequence interference by oligonucleotides and cross-reactivity.7

Therefore, it is essential to develop a new robust assay platform that does not rely on enzymatic amplification for the diagnosis of COVID-19. There are three different nucleic acid targets required to verify the infection of SARS-CoV-2 for the diagnosis of COVID-19,4,8 which are the RNA-dependent RNA polymerase (RdRp), the envelope protein (E), and the nucleocapsid protein (N) genes of SARS-CoV-2. However, there are many variants of these nucleic acid targets, indicating the importance of accurate and high throughput assay platforms.9

Several assay platforms, which are based on unique signal amplification strategies using nanomaterials, have been developed to achieve highly sensitive and reproducible results. Electrical,10,11 electrochemical,12–14 magnetic,15 and optical16,17 signals have been widely investigated as signal transduction modes for the diagnosis of SARS-CoV-2 infection. Among them, assays based on optical signals, such as fluorescence and Raman scattering, have been extensively utilized. Although fluorescence signals are well-established...
optical signals in various assay platforms, the significant photobleaching, broad emission spectrum, and lack of molecular information of this signal have limited their further application. In contrast, Raman signals have emerged as a promising alternative because they are not susceptible to photobleaching. In addition, owing to their molecular information and signal amplification via plasmonic nanomaterials, the surface-enhanced Raman scattering (SERS) phenomenon and the bioanalytical applications of Raman signals have attracted significant research attention. Although this method characterized the state of a reproducible SERS-based analysis of single-protein structure, Dai et al. utilized an optical tweezer method (4 pM). To address the reproducibility issue of dry sensitivity of this assay is lower than that of the conventional non-specific binding step. Moreover, the required conditions for measuring SERS signals have attracted significant research attention. Highly bright SERS tags with Raman reporters can detect multiple nucleic acids or protein targets at aM sensitivity. However, the poor reproducibility of most SERS-based assay platforms that rely on the use of NPs has limited the further application of these platforms. This is because of the non-specific binding of NPs that occurs in the target detection step. Moreover, the required conditions for measuring SERS signals in the dry state or the formation of NP aggregates in the solution state significantly enhance the Raman signals and background signals. These factors significantly affect the efficiency of SERS-based assay platforms, particularly at a low target concentration (<pM).

To overcome the non-specific binding problem of SERS-based assay platforms, Chuong et al. reported a dual-reporter SERS-based biomolecular assay that reduced false-positive signals, and the reported assay detected 86 pM of the thrombin target and eliminated false-positive results. However, the sensitivity of this assay is lower than that of the conventional method (4 pM). To address the reproducibility issue of dry-state SERS-based analysis, Dai et al. utilized an optical tweezer to control the hot spots in solution to achieve a sensitive and reproducible SERS-based analysis of single-protein structures. Although this method characterized the state of a single protein, it is not a feasible method for a practical assay. To address these issues, we designed new strategies with the hypothesis, which is excluding the use of NPs in the target detection step and measuring the SERS signals in a stable NP cluster solution. In a recent study, we demonstrated a two-step assay platform, which utilizes magnetic microparticles (MMPs) and amplifies SERS signals using gold nanoparticles (AuNPs) to detect bacterial nucleic acid targets, and the platform exhibited a high sensitivity of up to 30 fM and excellent reproducibility. However, its sensitivity is significantly lower than that of the PCR-based assay.

In this study, we designed an MMP–SERS-based platform to detect three nucleic acid targets (i.e., RdRp, E, and N genes) in SARS-CoV-2. A highly stable and controlled AgNP cluster was prepared, and its ensemble-averaged responses were measured to examine the reproducibility of the platform. In addition, the sensitivity and selectivity of the platform for the detection of the targets using different optical signals (i.e., fluorescence and Raman signals) were rigorously compared. Further investigation revealed that the use of anisotropic Ag nanostructures (i.e., nanostars (AgNSs) and triangular nanoplates (AgTPs)) greatly improved the sensitivity of the assay for the detection of the RdRp gene target (10 aM). These results indicate that the MMP–SERS-based assay platform can effectively address the long-standing issues of NPs and SERS-based assay platforms. In addition, the results indicate that the MMP–SERS-based assay is a promising platform, which does not rely on enzymatic amplification, for future practical bioanalytical applications.

**EXPERIMENTAL SECTION**

**Chemicals.** The oligonucleotides used in this study were purchased from Integrated DNA Technologies (IDT Inc., Coralville, IA, USA). Dynabeads (M-270, carboxylic acid) were obtained from BBI Solutions (Madison, Waltham, Massachusetts WI, USA). Silver nitrate (AgNO3), KB, PVP (MW 55,000), sodium borohydride (NaBH4), hydroxylamine, trisodium citrate dihydrate, l-ascorbic acid, sodium hydroxide (NaOH), ethylene glycol (EG), AgNPs (40 nm), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N′-hydroxysuccinimide (NHS), and 2-(N-morpholino)ethanesulfonic acid (MES) hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphate monobasic dihydrate (NaH2PO4·2H2O), sodium phosphate dibasic anhydride (Na2HPO4), sodium chloride (NaCl), sodium hydroxide (NaOH), and hydrogen peroxide (H2O2; 30%) were obtained from Daejung Chemicals & Metals Co., Ltd. (Siheung, South Korea).

**Instruments.** Extinction spectra were obtained using ultraviolet–visible (UV–vis) spectrometry (SCINCO, South Korea). The NPs were characterized using TEM (H-7100, Hitachi, Tokyo, Japan). Bright-field and fluorescence images were recorded using a microscope equipped with a 40X air objective and 100X oil immersion objective lens (1.3 N.A.; Olympus, DP80, Tokyo, Japan) and EScanner software (NOST, South Korea). A Cytation 3 cell imaging multimode reader (BioTek Inc., Winooski, USA) was used to measure the fluorescence intensity.

**DNA Information.** Target-specific probe sequences were designed by modifying SARS-CoV-2 sequences (25 base pairs). The target sequence of the RdRp, E, and N genes were S′-GCATTCCTTGTAGGGTGTCACCTG-3′ (melting temperature (Tm): 61.0 °C), S′-CGAAGGCGCATAGGAATGTGCTG-3′ (Tm: 62.3 °C), and S′-TGCAATGTTGCTTCCCTTGGAGAAGT-3′ (Tm: 59.0 °C), respectively. The capture probe sequence, which was half complementary to the target sequence, was modified using an amine group, an A20 spacer (i.e., 10 adenine bases), and a short polyethylene group on the 3′ end of the sequence. The RdRp, E, and N gene capture probes were S′-NH2-A10-PEG-CAGTTGGAACCT-3′, S′-NH2-A10-PEG-ACACTAGGCTATCC-3′, and S′-NH2-A10-PEG-ACCTTCTCAGG-3′, respectively.

The signal probe sequences were designed and used for both fluorescence- and SERS-based detection. The RdRp, E, and N gene signal probe sequences were S′-CATCAGAGATGTC-ATTO 488 dye-3′, S′-TTACCTGGGTCCG-ATTO 565 dye-3′, and S′-AACACATTGGCA-ATT0 647N dye-3′, respectively. Three different fully complementary DNA sequence probes with fluorescent dyes, i.e., the ATTO 488-modified RdRp gene signal probe (S′-AGTTTCCACCTG-ATTO 488 dye-3′), ATTO 565-modified E gene signal probe (S′-GGATGGCTAGTGTGATT0 565 dye-3′), and the ATTO 647N-modified N gene signal probe (S′-CTTGGAGAAGT-ATTO 647N dye-3′), were used to measure the number of the capture probes attached to the MMPs.

**Preparation of the Capture Probe-Modified MMPs.** Capture probe-modified MMPs were prepared using the EDC coupling

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ACS Applied Materials & Interfaces

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Scheme 1. Schematic of the Magnetic Microparticle (MMP)-Based Assay for the Detection of Virus DNA Targets

(a) First, the three capture probes for the RNA-dependent RNA polymerase (RdRp), the envelope protein (E), and the nucleocapsid protein (N) genes of SARS-CoV-2 were attached to the MMPs in solution, after which the sample was added. Subsequently, the MMPs hybridized with the capture probes and target DNA were separated by applying a magnetic field. (b) Fluorescence intensity and surface-enhanced Raman scattering (SERS) signals from the MMPs and released signal probes were measured from the capture probes and Ag nanoparticles (AgNPs). T, assay temperature; T_m, melting temperature.

Method. First, carboxylic acid-modified Dynabeads (MMPs, 100 µL, 30 mg/mL) were washed using 25 mM MES buffer (100 µL) for 10 min. Subsequently, the MMPs were separated by applying a magnetic field for 2 min, after which the supernatant was removed. This step was repeated two to three times. Thereafter, amine-modified DNA oligonucleotides (i.e., RdRp, E, and N gene capture probes, 10^{-4} M, 20 µL each) were added to the MMP solution (100 µL, 30 mg/mL). After incubating the solution using slow-tilt rotation at room temperature for 30 min, EDC solution (100 mg/mL in 100 mM MES buffer, 30 µL) was added to the MMP solution, after which the resulting solution was incubated overnight using slow-tilt rotation. Last, the MMPs were washed with 50 mM ethanolamine in PBS (pH 8.0) for 60 min using slow-tilt rotation at room temperature. The washing step with PBS was repeated four times. The obtained capture probe-modified MMPs were redispersed in distilled water (DW, 10 mL).

Measurement of the Number of the Capture DNA on the MMPs. The binding and quantity of the capture probe sequences on the MMPs were confirmed by hybridizing the MMPs with fully complementary dye-modified DNA sequences. After hybridizing the MMPs (100 µL) with the dye-modified DNA sequences (50 µL, 10^{-6} M) for 3 h in 0.3 M PBS, the sequences were washed five times using 0.15 M PBS, after which they were separated from the MMPs by heating the solutions to 95 °C. The number of released DNA sequences was determined based on the fluorescence intensity and calibration curves. The numbers of the capture DNA sequences of RdRp, E, and N genes per MMP were approximately 2.1 × 10^4, 4.7 × 10^4, and 1.6 × 10^4, respectively.

Fluorescence Measurement Conditions. The fluorescence images of the MMPs hybridized with signal probes were obtained using a microscope equipped with a filter set. The ATTO 488, ATTO 565, and ATTO 647N signals on the MMPs were detected using green fluorescent protein (518–559 nm), tetramethylrhodamine (572–638 nm), and cyanine 5 (665–715 nm) filters exposed for 0.5, 0.15, and 2 s, respectively. The fluorescence intensities of the signal probe solutions were measured by exciting them at specific wavelengths of 500 nm (ATTO 488), 570 nm (ATTO 565), and 650 nm (ATTO 647N) and recording the fluorescence intensity at wavelengths of 530, 600, and 680 nm, respectively.

SERS Measurement Conditions. SERS spectra were collected using an inverted Raman microscope (NOST, South Korea) with a 40× objective lens (0.6 N.A.) (Olympus, Tokyo, Japan) from a solution by illuminating the solution with a 532 nm laser (21 mW at the sample). The scattered Raman signal was detected using a confocal motorized pinhole (100 µm) directed toward a spectrometer (FEX-MD, NOST, South Korea; 1200 g mm^{-1} grating) and finally to a spectroscopy charge-modified device camera (Andor (DV401A-BVF), Belfast, Northern Ireland).

Solution-State Raman Analysis. For each SERS spectral analysis, AgNPs (optical density: 10, 3 µL) were mixed with 3.0 µL of the signal probe solution released from the MMPs. Immediately after the addition of 3.0 µL of PBS (0.15 M) to the mixture, the solution (9.0 µL) was transferred to the well of a silicone isolator (diameter: 2.5 mm) on a cover glass and the Raman spectrum was measured (exposure time: 1 s).

Conditions for Raman Mapping. The dried spots on the glass surface of AgNP solution with signal probe sequences were imaged with Raman mapping analysis. About a 1.5–2.0 mm spot on the glass was scanned with a 40× objective for the 25 × 25 pixels (1 s exposure time per pixel; it takes about 12 min for single-spot imaging). The Raman mapping images were based on the intensity of the Raman
shift at 1348 cm$^{-1}$ for ATTO 488 (RdRp gene), 1502 cm$^{-1}$ for ATTO 565 (E gene), and 1426 cm$^{-1}$ for ATTO 647N (E gene).

Preparation of Silver Nanostars (AgNSs). AgNSs were synthesized based on a previously reported method. Typically, 0.5 mL of 6.44 mM hydroxylamine and 0.5 mL of 0.05 M NaOH were mixed and agitated for 2 min. Subsequently, 9.0 mL of 0.98 mM AgNO$_3$ solution was added into the mixture immediately and the solution was further agitated for 5 min. Last, 100 μL of 0.29 mM trisodium citrate was added and the solution was shaken for 15 min. After the solution turned dark gray, it was centrifuged (2600 relative centrifugal force (RCF), 15 min) and the residue was redispersed in DW to adjust the optical density to 1.0.

Preparation of Silver Triangular Nanoplates (AgTPs). Citrate-stabilized AgTPs were synthesized as follows. Typically, a solution consisting of 39.3 mL of DW, 2.0 mL of trisodium citrate (75 mM), 256 μL of H$_2$O$_2$ (0.6%), and 186 μL of AgNO$_3$ (10 mM) was prepared. Subsequently, the solution was subjected to vigorous stirring, after which 192 μL of sodium borohydride (NaBH$_4$, 100 mM) was rapidly added to initiate reduction, which was accompanied by a change in the color of the solution to a pale yellow color. After 5 min, the color shifted to golden yellow. Thereafter, the solution was stored overnight at room temperature. Following this aging period, 2.1 mL of the seed stock was added to a clean 8 mL vial and stirred vigorously. Subsequently, 200 μL of l-ascorbic acid (5.0 mM) was added to this mixture, after which AgNO$_3$ (10 mM) was added to the solution dropwise until a yellow-orange color was obtained (typically 100 μL). The resulting solution was centrifuged (2600 RCF, 15 min) three times, after which the residue was redispersed in 5.0 mL of water and stored at 4 °C.

Preparation of Silver Nanocubes (AgNCs). AgNCs were synthesized based on a previously reported method. Typically, 6.0 mL of EG was pre-heated (150 °C) under stirring. Next, 100 μL of 3.0 mM NaHS solution in EG was injected. After heating the reaction mixture for 10 min, 1.5 mL of PVP (20 mg/mL, MW ~55,000) and 0.5 mL of AgNO$_3$ (48 mg/mL) in EG were sequentially injected into the mixture. After the addition of AgNO$_3$, the clear and colorless solution turned purple-black and changed to a transparent bright yellow color. Finally, the solution changed to a whitish brown color and remained opaque. The reaction mixture was cooled down, diluted with acetone, and centrifuged at 2600 RCF for 15 min. The obtained residue was washed with water, centrifuged (2600 RCF, 15 min, 3 times), and finally suspended in 4.0 mL of DW.

### RESULTS AND DISCUSSION

Design of the MMP-Based Assay for the Detection of Target Viral DNA. MMPs showed excellent dispersibility in solutions owing to their superparamagnetic property. Consequently, MMPs can efficiently bind with target molecules and can be easily separated by applying an external magnetic field. In addition, a high throughput assay can be achieved using MMPs owing to the multiple binding sites of MMPs. In this study, as a proof of concept, MMPs (2.8 μm, COOH surface functionality) were modified using the
three amine-modified capture probe sequences of RdRp, E, and N gene targets via EDC coupling chemistry. The probe sequences were partly complementary to their specific target DNA sequence (Scheme 1). The numbers of the capture probe sequences of the RdRp, E, and N gene targets per MMP were approximately $2.1 \times 10^4$, $4.7 \times 10^4$, and $1.6 \times 10^4$, respectively (Figure S1). In the first step, the MMPs were hybridized with both the target and signal probe sequences (fluorescence-tagged), which were partly complementary to their specific target DNA sequences. In the second step, the fluorescence intensity of the MMPs, which is proportional to the quantity of the target DNA, was measured. The fluorescence signal intensity could also be measured after releasing the signal probes into distilled water by increasing the temperature of the MMP solution to $95 \, ^\circ C$ ($> T_m$) (Figure S2). The SERS-based detection was enabled by the fluorescence molecules in the released signal probes and the PBS-induced stable AgNP clusters in the solution (Figure S3). Consequently, the performances of the assay based on two different optical signals (i.e., fluorescence and Raman scattering) could be accurately compared. As the NPs were not involved in the target detection step (first step), the possibility of non-specific binding between the NPs and the target was eliminated. In the second step, the detection of optical signals (i.e., fluorescence or Raman responses) from the MMPs or AgNP clusters in solution was performed to compare.

Figure 2. Magnetic microparticle (MMP)-based assay for the detection of a single-nucleic acid target. (a) Assay procedures for three different targets (RNA-dependent RNA polymerase (RdRp), the envelope protein (E), and the nucleocapsid protein (N) genes of SARS-CoV-2). (b) Measured fluorescence intensity and images of the MMPs. (c) Fluorescence intensities of the released signal probes from the MMPs. (d) Surface-enhanced Raman scattering (SERS) spectra of the released signal probes (ATTO 488 for the RdRp gene, ATTO 565 for the E gene, and ATTO 647N for the N gene). Scale bar: 10 \( \mu m \). $T$, assay temperature; $T_m$, melting temperature.
the assay performances of both platforms (Scheme 1). For Raman analysis, two different experimental setups such as solution-state analysis or dry-state analysis (Raman mapping) were performed (Figure S3).

**Formation of Stable Silver Nanoparticle Clusters in Solution.** The formation of hot spots using plasmonic NPs is essential to obtain a significantly enhanced Raman scattering signal intensity.22,23 However, the SERS measurement in the dry state showed a high background signal and poor reproducibility in the assay results. The solution-state analysis also showed a low signal reproducibility because of the random aggregation of NPs and rapidly changing aggregated states in the solution.22,23 Therefore, in this study, we focused on solution-state Raman analysis and investigated the electrolyte conditions that enable the production of uniform and stable AgNP clusters in solution for more than 30 min at room temperature (Figure 1). The four representative electrolyte compositions such as phosphate buffer (PB), NaCl, 0.3 M PBS, and 0.15 M PBS were investigated. As shown in Figure 1a, the addition of PB alone (10, 1.0, and 0.1 mM) did not induce the formation of AgNP clusters in the AgNP mixture and signal probe sequence, which could be attributed to the weak adsorption affinity of PB on the AgNPs.41 In contrast, the addition of NaCl solution (0.3 and 0.15 M) into the mixture induced an instant change in the color of the mixture to gray and the complete aggregation of NPs within 0.5 h. Furthermore, strong and uniform Raman responses were obtained from the AgNP cluster solutions induced by 0.3 and 0.15 M PBS (Figure 1e, 3, and 4). The results indicate that the instant formation of aggregates and low colloidal stability is not a desirable condition to obtain a reproducible Raman

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**Figure 3.** Comparison of the sensitivity of the fluorescence signal intensity and surface-enhanced Raman scattering (SERS)-based signal for three targets of SARS-CoV-2. (a) Fluorescence intensity of the magnetic microparticles (MMPs) and signal probes. (b) Fluorescence images of the magnetic microparticles (MMPs) (ATTO 488). (c) SERS spectra and (d) Raman mapping images of the RdRp gene target at various concentrations (10⁻⁹ to 10⁻¹⁵ M). (e) Fluorescence intensities of the MMPs and signal probes (ATTO 565). (f) Fluorescence images of the MMPs. (g) SERS spectra and (h) Raman mapping images of the E gene target at various concentrations (10⁻⁹ to 10⁻¹⁵ M). (i) Fluorescence intensities of MMPs and signal probes (ATTO 647N). (j) Fluorescence images of the MMPs. (k) SERS spectra and (l) Raman mapping images of the N gene target at various concentrations (10⁻⁹ to 10⁻¹⁵ M). Scale bar = 10 μm.
The mild electrolyte condition that can maintain the state of aggregates in solution for a long time is a more desirable condition for a practical SERS-based assay platform. In this regard, it is concluded that the formation of AgNP clusters induced by 0.15 M PBS is the most preferable electrolyte condition for the formation of stable AgNP clusters with dye-modified signal probe sequences.

**Figure 4.** Performance of the magnetic microparticle (MMP)-based assay with fluorescence- and surface-enhanced Raman scattering (SERS)-based detection for multiple binding of the signal probes of the RNA-dependent RNA polymerase (RdRp), the envelope protein (E), and the nucleocapsid protein (N) genes of SARS-CoV-2. (a) Schematic overview of the MMP-based assay for the detection of multiple DNA targets. (b) Fluorescence intensities and images of MMPs hybridized with multiple signal probes (ATTO 488 for the RdRp gene, ATTO 565 for the E gene, and ATTO 647N for the N gene). (c) Fluorescence intensities of the multiple released signal probes. (d) SERS spectra of the multiple DNA targets. Scale bar = 10 μm. T, assay temperature; Tm, melting temperature.

MMP-Based Assay for Single-Target Detection. Next, we performed a single-target detection using the MMP-based assay (Figure 2a). First, the MMPs modified with the three capture probes were hybridized with a single target (i.e., RdRp, E, or N gene targets at a concentration of 10^{-9} M) and signal probes (10^{-6} M) in 0.3 M PBS for 3 h. Subsequently, the MMPs were washed in 0.15 M PBS five times, after which the fluorescence intensities of the MMP solution and the released
signal probes from the MMPs were measured and the former was imaged. Last, the SERS spectra were obtained from a mixture containing 3.0 μL of the released signal probes, 3.0 μL of AgNPs, and 3.0 μL of 0.15 M PBS solution (Figure 2a). The color of the AgNP solution (yellowish green) readily changed to gray, indicating the slight aggregation of AgNPs. In addition, a strong fluorescence intensity could only be obtained from the MMP solutions in the presence of a specific target gene sequence, such as RdRp, E, and N, whereas a low fluorescence intensity was observed in the absence of a target (Figure 2b). Moreover, similar results were observed in the fluorescence images of the MMPs. In addition, the released signal probe solution exhibited a strong fluorescence intensity in the presence of targets (Figure 2c).

Furthermore, the SERS spectra of the solution containing the signal probes and AgNPs were consistent with the reference Raman spectra (dotted line) of ATTO 488, ATTO 565, and ATTO 647N dyes obtained at a laser excitation of 532 nm (Figure 2d). The two characteristic peaks at 1348, 1643 cm⁻¹ for ATTO 488 (RdRp gene), 1502, 1651 cm⁻¹ for ATTO 565 (E gene), and 1426, 1631 cm⁻¹ for ATTO 647N (E gene) were clearly identified. As a higher local electromagnetic field was obtained with the excitation of AgNPs at a shorter wavelength (532 vs 633 nm), a wavelength of 532 nm was considered as a more preferable laser source for the Raman field was obtained with the excitation of AgNPs at a shorter wavelength (532 vs 633 nm), a wavelength of 532 nm was considered as a more preferable laser source for the Raman analysis (Figure S3). These results indicate that the MMPs exhibited a sequence-specific binding capability toward their single DNA target.

Comparison of the Sensitivity of the Fluorescence and SERS Signals. The sensitivity performances of the MMP-based assay for two different optical signals (i.e., fluorescence and Raman signals) were compared. To this end, the binding of MMPs with various concentrations of the target (10⁻⁹, 10⁻¹⁰, 10⁻¹², and 10⁻¹⁵ M) at a fixed signal probe concentration (10⁻⁶ M; 0.15 M PBS, 3.0 h hybridization time) was performed. After the washing step, the fluorescence intensities of the MMPs and released solutions as well as the SERS spectra of the solution mixtures and dried spots of the solution mixtures were obtained. For the RdRp gene target, the fluorescence intensity (blue square) of the MMPs and released solution decreased significantly at concentrations below 10⁻¹⁰ M (Figure 3a). This could be attributed to the fact that the signal-to-noise ratio was not sufficiently large at ranges between 10⁻¹⁰ and 0 M. Moreover, the MMPs were not visible in the fluorescence images at target concentrations below 10⁻¹⁰ M (Figure 3b). However, distinguishable Raman spectra were observed in the SERS spectra from the mixture of released solution at a target concentration of 10⁻¹⁵ M (Figure 3c) and AgNPs. The SERS mapping images for the dried spot of the mixture, which was based on the Raman shift at 1348 cm⁻¹, could be observed at a target concentration of 10⁻¹⁵ M (Figure 3d).

For the E gene target, the limit of detection (LOD) of the fluorescence intensity of the MMPs and the released signal probes was observed at 10⁻¹⁰ M (Figure 3e,f), whereas a signal intensity was observed in the SERS spectrum and SERS mapping image (based on the Raman shift at 1502 cm⁻¹) at a target concentration of 10⁻¹⁵ M (Figure 3g,h). For the N gene target, the LOD values of the fluorescence intensity of the MMPs and released signal probes were observed at 10⁻¹⁰ M (Figure 3i,j), whereas a signal intensity was observed in the SERS spectra and SERS mapping images (based on 1426 cm⁻¹) at a target concentration of 10⁻¹² M (Figure 3k,l), which was lower than those of ATTO 488 and ATTO 565. This could be attributed to the non-resonant effect of ATTO 647N dyes at an excitation wavelength of 532 nm.

In summary, the LOD of the MMP–SERS-based system for the detection of RdRp and E gene targets was 1.0 μM and that of the N gene target was 1.0 pM with detection ranges from 10⁻⁹ to 10⁻¹⁵ M and 10⁻⁹ to 10⁻¹² M, respectively, in the solution and dry-state analysis, thereby demonstrating its excellent performance as a highly sensitive assay platform.

In addition to the high sensitivity, the signal reproducibility of the SERS-based assay is also a critical parameter for practical application. As shown in the Raman spectrum (Figure 3c,g,k), the Raman spectral patterns were consistent with a decreasing target concentration from 10⁻⁹ to 10⁻¹² M. However, small new peaks could be observed in the case of the 10⁻¹⁵ M target concentration and no target. Since the Raman signal is obtained from the solution state, these fluctuating signals are expected to be the background noise signal from the random orientation of Raman reporters in the silver nanoparticle clusters. This kind of transiently fluctuating signal becomes more clearly visible in the case of the low target concentration because of a decreased intensity of characteristic Raman peaks. This is the reason why the small unusual peak could be seen in the 10⁻¹² M target concentration. However, the small transient peak is not greatly problematic in determining the assay results because of the observed two characteristic peaks of Raman reporters (Figure 3c,g,k). To further examine the signal reproducibility of the SERS-based assay platform, three independent assays were performed for the targets at varying concentrations (10⁻⁹, 10⁻¹⁰, 10⁻¹², and 10⁻¹⁵ M and no target) using MMP, and the Raman responses were measured. No significant difference in the SERS spectral patterns and intensities in the three independently performed assays demonstrates the excellent signal reproducibility of the MMP–SERS assay platform (Figure S4).

Selectivity of the MMP-Based Assay. The capability of an assay to selectively detect a specific target sequence in the presence of multiple sequences in an analyte is an essential parameter for the practical application of an assay. To this end, the selectivity of the MMP–SERS-based assay was evaluated in the presence of dual-target sequences (Figure S5) and triple-target sequences (Figure 4). First, in the presence of dual-target sequences, the assay was performed by adding the MMPs to binary mixtures of RdRp and E gene target sequences (concentration: 10⁻⁹ M), where the ATTO 488-modified sequence was used for the RdRp gene, the ATTO 565-modified sequence was used for the E gene, and the ATTO 647N-modified sequence was used for the N gene. The binding of the ATTO 488 and ATTO 565 signal probe sequences to their target sequences was observed; however, the binding of ATTO 647N was not observed owing to the absence of its target sequence in the mixture (Figure S5).

Similar results were observed in the fluorescence images of the MMPs, released signal probe solution, and SERS spectra, indicating the excellent selectivity of the signal probe sequences with no interference in the presence of dual-target sequences (Figure S5).

The selectivity of the MMP-based assay was further investigated in the presence of triple-target sequences (Figure 4a). For this, MMPs were added to tertiary mixtures of the RdRp, E, and N gene target sequences (concentration: 10⁻⁹ M) containing the same three signal probe sequences. Strong and discriminable fluorescence intensity was observed for the
specific target only (Figure 4b,c). In addition, a high background fluorescence intensity was observed for the RdRp gene target, which could be attributed to the relatively high fluorescence signals of the MMPs. The results in Figure 4b−d indicate the absence of mutual sequence interference and the excellent selectivity of the designed assay.

Improved Sensitivity Using Anisotropic Ag Nanostructures. In this study, the SERS-based assay using AgNPs achieved a sensitivity of 1.0 fM for RdRp and E gene targets and a sensitivity of 1.0 pM for the N gene target (Figure 3). However, to enhance the possibility of replacing the PCR-based assay with the SERS-based assay, it is essential to further improve the sensitivity of the SERS-based assay to the attomolar range. In our recent study, we reported an MMP−SERS assay, which exhibited a sensitivity of 10 fM for the detection of an Enterococcus faecalis target using AuNPs (Cys-AuNPs, 30 nm). In this study, the sensitivity of SERS-based detection was only slightly improved using AgNPs instead of AuNPs. Accordingly, we anticipated that the sensitivity of SERS-based detection can be further improved by utilizing anisotropic Ag nanostructures because of the enhanced localized electromagnetic field in their tips and edges.

Therefore, AgNSs, AgTPs, and AgNCs were synthesized to compare the effects of various anisotropic Ag nanostructures on the sensitivity of the SERS-based assay (Figure S6). The sizes of the AgNSs, AgTPs, and AgNCs were 45 ± 5 nm with 5−6 nm tips, 65 ± 5 nm, and 55 ± 5 nm, respectively, with peak extinctions at 390, 477, and 432 nm, respectively (Figure S6a−d). These sizes were selected to accurately compare the effect of the shape of the nanostructures on the signal enhancement compared to that of spherical AgNPs (40−45 nm). Since all silver nanostructures in this study are negatively charged, the salt conditions to produce Ag nanoclusters in solution could be applied for all structures. The changes of AgNPs as a representative were investigated in detail for the
behavior in PB, NaCl, and PBS (Figure 1). AgTPs also showed the same trends of cluster formation with that of AgNPs as observed in solution color changes, TEM, and D/F images as well as SERS responses (Figure S7).

To compare the effects of anisotropic Ag nanostructures on the sensitivity of the SERS-based assay, the SERS intensity performances of the three signal probe sequences (i.e., ATTO 488, ATTO 565, and ATTO 647N) in the presence of the various Ag nanostructures were compared by obtaining their SERS mapping images. Spots of the mixtures on glass were imaged using SERS mapping at 1348 cm\(^{-1}\) for ATTO 488, 1502 cm\(^{-1}\) for ATTO 565, and 1426 cm\(^{-1}\) for ATTO 647N with 532 nm excitation. When AgNPs were used, signals were observed in the SERS mapping images of the ATTO 488 and ATTO 565 signal probe sequences at a concentration of 10\(^{-15}\) M (Figure S6e–g), whereas signals were observed in the SERS mapping image of the ATTO 647N probe sequence at 10\(^{-12}\) M (Figure S6g). In addition, the SERS mapping results revealed that, when AgNSs were used, the mapping images of ATTO 488, ATTO 565, and ATTO 647N signal probe sequences were observed at 10\(^{-18}\) (Figure S6h), 10\(^{-13}\) (Figure S6i), and 10\(^{-12}\) M (Figure S6j), respectively. Furthermore, when AgTPs were used, the mapping images of ATTO 488, ATTO 565, and ATTO 647N signal probe sequences were observed at 10\(^{-18}\) (Figure S6k), 10\(^{-15}\) (Figure S6l), and 10\(^{-12}\) M (Figure S6m), respectively. Additionally, when AgNCs were used, the mapping images of ATTO 488 and ATTO 565 probe sequences were observed at 10\(^{-12}\) M (Figure S6n–o), whereas the mapping image of the ATTO 647N probe sequence was not possible to obtain at 10\(^{-12}\) M (Figure S6p). These results demonstrate that the attomolar sensitivity of the MMP–SERS-based assay is possible in the case of SERS with AgNSs or AgTPs and the ATTO 488 dye as the Raman reporter molecule.

Based on these results, we next performed the MMP–SERS-based assay for the RdRp gene target at varying target concentrations (10\(^{-12}\), 10\(^{-15}\), 10\(^{-16}\), 10\(^{-17}\), 10\(^{-18}\), and 0 M) using AgNPs, AgNSs, AgTPs, and AgNCs (Figure 5a). The difference in the Ag nanostructures did not affect the Raman spectra of the ATTO 488 dye (Figure 5b). The LOD was measured using SERS mapping at a Raman shift of 1348 cm\(^{-1}\). When AgNPs, AgNSs, AgTPs, and AgNCs were used, the target was detected at concentrations of 10\(^{-15}\), 10\(^{-17}\), and 10\(^{-12}\) M, respectively (Figure 5c–f). This indicates that AgNSs or AgTPs were significantly more sensitive than the other structures. The significantly enhanced SERS intensity when AgNSs or AgTPs were used could be attributed to the high degree of anisotropy and a large number of edges in the AgNS and AgTP structures.\(^{58,47}\)

As reproducibility in a low target concentration is crucial for SERS-based assays, three independent assays were performed for the detection of RdRp gene targets using AgNSs and AgTPs to examine their signal reproducibility in both the dry and solution states (Figure 8). The three independent assays performed using AgNSs (Figure 8a–c) or AgTPs (Figure 8d–f) showed a detectable Raman response at a concentration of 10 aM in both analyses. However, the SERS mapping images performed in the dry-state analysis were not consistent in the result (10 aM in Figure 8c) because of the random distribution of hot spots in the dry-state analysis. In contrast, the results obtained from the solution state clearly showed the difference Raman spectrum between 10\(^{-17}\) and 10\(^{-15}\) M. These repeated assay results indicate that the use of anisotropic structures, such as AgNSs and AgTPs, greatly amplified the SERS signal and excellent signal reproducibility from the stable NP clusters in solution.

- **CONCLUSIONS**

In this study, we demonstrated a highly sensitive and reproducible MMP–SERS-based assay platform for nucleic acid target detection. The DNA sequences designed to detect the RdRp, E, and N genes of SARS-CoV-2 exhibited reliable sensitivity and reproducible assay results. The sensitivity of the assay platform using two different optical signals (i.e., fluorescence and SERS) was rigorously compared. The SERS-based detection using AgNPs exhibited significantly higher sensitivity (1.0 fM for the RdRp and E genes and 1.0 pM for the N gene) than the fluorescence-based detection for all the three target genes. In addition, the use of anisotropic Ag nanostructures (AgNSs and AgTPs) for the MMP–SERS-based assay significantly improved the sensitivity of the assay for the detection of the RdRp gene target (10 aM). The number of target sequences in 10 aM LOD is calculated to be 114, which corresponds to ca. 376 copies/mL. Therefore, the sensitivity of the MMP–SERS-based assay is comparable with that of current commercial kits (200–500 copies/mL).\(^{24}\) It is important to note that the possibility of false-positive results from the use of NPs, particularly at low target concentrations, was significantly minimized in this platform owing to exclusion of the NPs in the target detection step. In addition, the ensemble-averaged measurement of the SERS response from the highly stable Ag clusters in solution significantly improved the signal reproducibility of this platform. These results indicate that the method demonstrated in this study can effectively address the two key issues of NPs and the SERS-based assay. Therefore, we expect that the MMP–SERS-based method will be a promising high-throughput platform technology with attomolar sensitivity and robust assay results.

- **ASSOCIATED CONTENT**

- **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.1c17028.

Measurement of the quantity of the capture probes on the magnetic microparticle (MMP); experimental setup of the filter set; surface-enhanced Raman scattering (SERS) spectra of the three different signal probes with ATTO 488, ATTO 565, and ATTO 647N dyes and experimental setups for solution-state or dry-state Raman analysis; Raman spectra of the three independent assays for the detection of RNA-dependent RNA polymerase (RdRp) gene target DNA; performance of the magnetic microparticle (MMP)-based assay with fluorescence- and surface-enhanced Raman scattering (SERS)-based detection of dual DNA targets; characterization of the various Ag nanostructures; results of the salt-induced formation of AgTPs clusters in solutions; and reproducibility of the three independent SERS-based assays for the detection of RdRp gene target DNA using AgNSs and AgTPs (PDF)

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