



Ultrasensitive and Simultaneous Detection of Two Specific SARS-CoV-2 Antigens in Human Specimens Using Direct/Enrichment Dual-Mode Fluorescence Lateral Flow Immunoassay

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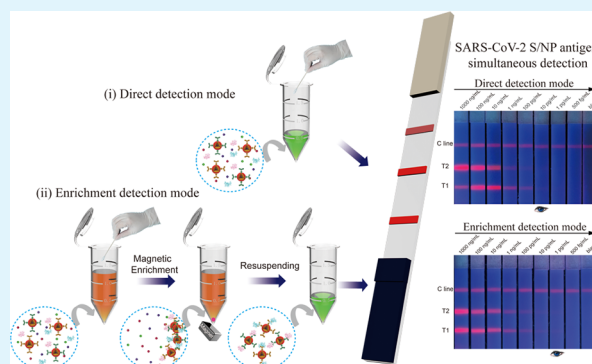
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ABSTRACT: Sensitive point-of-care methods for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigens in clinical specimens are urgently needed to achieve rapid screening of viral infection. We developed a magnetic quantum dot-based dual-mode lateral flow immunoassay (LFIA) biosensor for the high-sensitivity simultaneous detection of SARS-CoV-2 spike (S) and nucleocapsid protein (NP) antigens, which is beneficial for improving the detection accuracy and efficiency of SARS-CoV-2 infection in the point-of-care testing area. A high-performance magnetic quantum dot with a triple-QD shell (MagTQD) nanotag was first fabricated and integrated into the LFIA system to provide superior fluorescence signals, enrichment ability, and detectability for S/NP antigen testing. Two detection modes were provided by the proposed MagTQD-LFIA. The direct mode was used for rapid screening or urgent detection of suspected samples within 10 min, and the enrichment mode was used for the highly sensitive and quantitative analysis of SARS-CoV-2 antigens in biological samples without the interference of the “hook effect.” The simultaneous detection of SARS-CoV-2 S/NP antigens was conducted in one LFIA strip, and the detection limits for two antigens under direct and enrichment modes were 1 and 0.5 pg/mL, respectively. The MagTQD-LFIA showed high accuracy, specificity, and stability in saliva and nasal swab samples and is an efficient tool with flexibility to meet the testing requirements for SARS-CoV-2 antigens in various situations.

KEYWORDS: SARS-CoV-2, lateral flow immunoassay, magnetic-QD tags, dual-mode, antigen simultaneous detection



1. INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel β -coronavirus that causes the severe acute respiratory disease named coronavirus disease 2019 (COVID-19), has rapidly spread to more than 200 countries since December 2019 and posed an unprecedented global public health crisis.¹ SARS-CoV-2 is characterized by high infectiousness (through close person-to-person contact and through air by aerosol) and high mortality.² By January 19, 2021, more than 93,217,287 infected people have been reported by the World Health Organization, resulting in over 2,014,957 deaths.³ The high number of asymptomatic carriers and long incubation period of COVID-19 (0–21 days) contribute to the acceleration of disease spread.⁴ Thus, early detection and early isolation are among the most critical factors that need to be considered to control this pandemic.

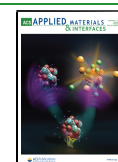
SARS-CoV-2 possesses a single-stranded, positive-sense RNA genome that encodes four structural proteins (spike, S; envelope, E; matrix, M; and nucleocapsid protein, NP).⁵ Two broad categories of diagnostic methods were developed based

on the viral structure. The first category is nucleic-acid-based detection strategies for the SARS-CoV-2 viral genome, such as real-time reverse transcription-polymerase chain reaction (RT-PCR) and sequencing. Despite their excellent selectivity and sensitivity, these nucleic-acid-based methods require a long processing time (>2 h) and dedicated laboratory facilities and have no ability to give an instant result. The second category is immunological detection approaches, which include the serological testing of SARS-CoV-2-specific immunoglobulins (e.g., IgM and IgG) in blood samples and the direct detection of SARS-CoV-2 antigens in biological specimens.⁶ Considering that IgM or IgG to SARS-CoV-2 antigens is usually generated in the late stage of infection (4–15 days after virus exposure),

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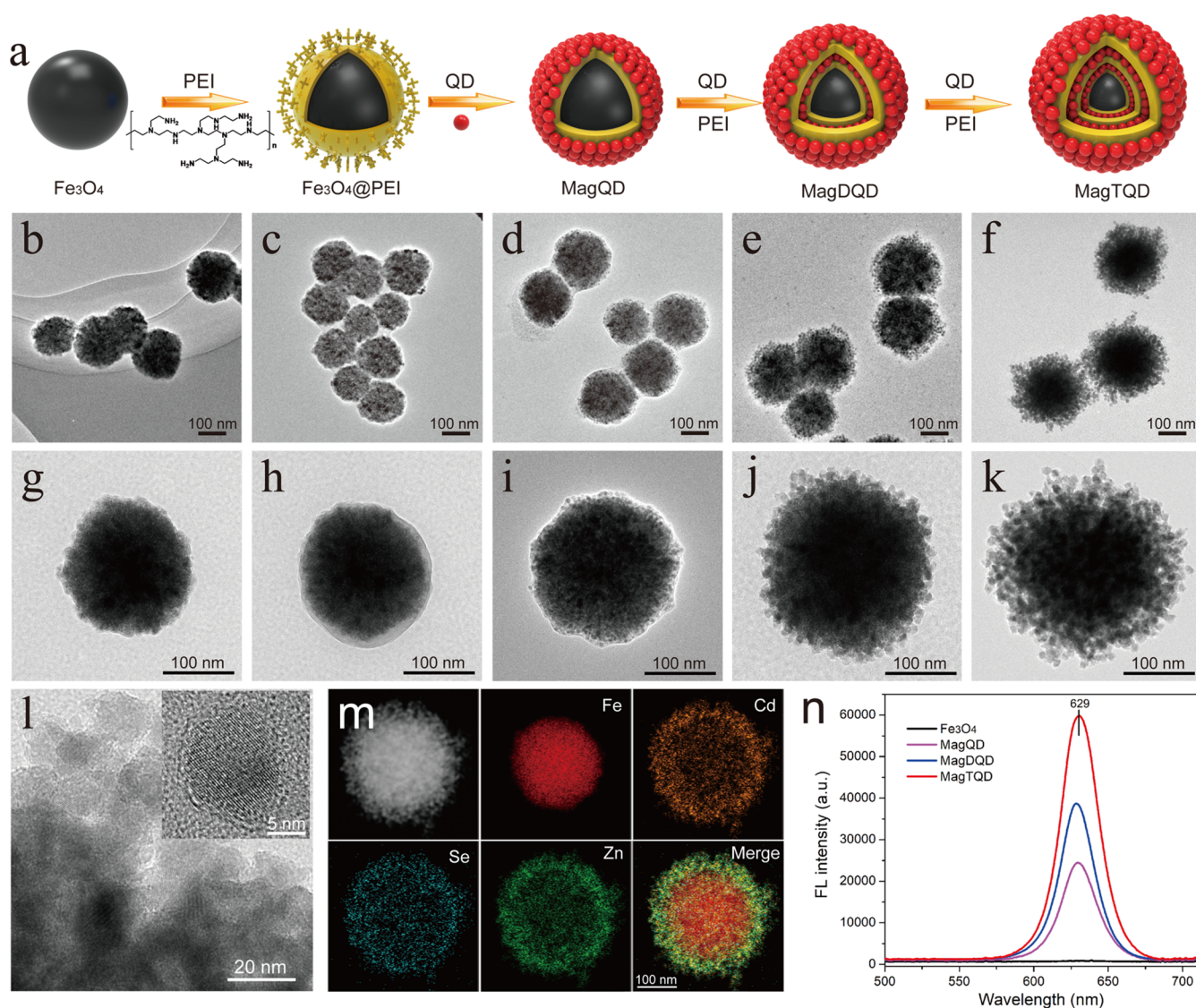


Figure 1. Structural characterization of the MagTQD nanocomposite. (a) Schematic illustration of the synthetic process of MagTQD fluorescent tags. TEM images of the fabricated (b) Fe_3O_4 , (c) $\text{Fe}_3\text{O}_4@\text{PEI}$, (d) MagQD, (e) MagDQD, and (f) MagTQD nanocomposites, with their corresponding magnified TEM images of the single particle in (g)–(k), respectively. (l) Enlarged image of the QD shell of MagTQD. (m) Elemental mapping images of single MagTQD. (n) Fluorescence spectra of the synthesized Fe_3O_4 , MagQD, MagDQD, and MagTQD under UV light.

the serological antibody tests cannot achieve accurate detection at the onset of infection.^{7,8} In comparison, an immunological test for SARS-CoV-2 antigens is a more timely and precise approach for COVID-19 diagnosis because the virus antigens could be detected up to several days before the appearance of clinical symptoms, thereby allowing for the early detection of infection.⁹ The recent WHO guidelines pointed out that to effectively diagnose an active SARS-CoV-2 infection, the diagnostic kit for rapid antigen detection should possess at least 80% sensitivity and 97% specificity.¹⁰ However, the conventional antigen detection methods, such as colloidal gold-based lateral flow immunoassay (LFIA) and enzyme-linked immunosorbent assay (ELISA), commonly suffer from insufficient sensitivity (higher than 0.1 ng/mL) and cannot meet the actual testing demand.

At present, two structural proteins S and NP of SARS-CoV-2 are widely used as the target antigens for testing.¹¹ The S antigen is the major surface protein of the virus, and its S1

subunit exhibits amino acid sequence diversity compared with other coronaviruses (including SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV)).¹² Considering its high immunogenicity and specificity to SARS-CoV-2, the S antigen is considered as the best suited for virus particle direct detection. The NP antigen is the phosphoprotein for coronavirus genome RNA packaging and also exhibits strong immunogenicity.¹³ Many studies have revealed that the coronavirus NP is expressed and released abundantly during infection and could be detected in respiratory tract specimens, such as saliva, nasal swab, and throat wash samples in the early stage of infection.^{14–16} Recently, several new methods for SARS-CoV-2 S or NP antigen detection have been reported, such as glycan-based LFIA systems,¹⁷ nanozyme-based paper tests,¹⁸ terahertz plasmonic sensors,¹⁹ field-effect transistor-based biosensors,²⁰ and electrochemical immunosensors.²¹ These strategies significantly improved the sensitivity of conventional antigen detection methods, but none of them

have achieved the simultaneous analysis of S and NP antigens in one test. Many SARS-CoV-2 variants (e.g., B.1.1.7, B.1.351, and P.1) have rapidly emerged around the world, with several mutations in the functional domain of S protein as well as in other proteins (including NP).^{22,23} These variants raise important concerns about their immune evasion potential and the risk of false-negative results, which pose a growing challenge for the accurate and sensitive detection of SARS-CoV-2 via antigens. Some research studies have demonstrated that the combination of multiple target detection has outstanding performance (improving the sensitivity or accuracy) in SARS-CoV-2 and its variant diagnosis.^{24–27} In theory, the simultaneous detection of SARS-CoV-2 S and NP antigens offers tremendous potential for improving the accuracy and detection rate of SARS-CoV-2 infection in the point-of-care testing (POCT) area.

In recent years, our group has developed a series of high-sensitive LFIA biosensors by using bifunctional magnetic nanocomposites as novel signal probes, including magnetic surface-enhanced Raman scattering (SERS) tags ($\text{Fe}_3\text{O}_4/\text{Ag}$ and $\text{Fe}_3\text{O}_4/\text{Au}$)^{28–30} and magnetic quantum dot tags ($\text{Fe}_3\text{O}_4/\text{QD}$).^{31,32} Compared with the traditional colloidal Au-LFIA based on colorimetric signals, these magnetic-LFIA methods provide quantifiable optical signals (SERS/fluorescence) and effectively improve the detection sensitivity of paper-based immunoassay by magnetically enriching the various targets (e.g., influenza A virus (FluA), human adenovirus (HAdV), and protein toxins).^{29,33} Herein, a dual-mode fluorescence LFIA biosensor was proposed for the simultaneous detection of S and NP antigens by using sensitive and flexible magnetic quantum dots with triple-QD shells (MagTQD) to achieve rapid screening and accurate detection of SARS-CoV-2 infection. The innovations and originality of our method can be summarized in the following three aspects. First, the novel MagTQD tag was designed to be composed of three components, as follows: (i) an ~ 160 nm Fe_3O_4 core as a magnetic separation tool and a highly stable supporter; (ii) a multiple-layer QD-shell containing thousands of CdSe/ZnS-COOH QDs to provide superior fluorescence signals; and (iii) surface-modified anti-S antibodies/anti-NP antibodies that can specifically capture SARS-CoV-2 S/NP antigens. The three layers of PEI/carboxylated QDs outside the magnetic core not only significantly increased the maximum load of QDs (from hundreds to thousands) onto the nanostructure but also further improved the stability and dispersibility of MagTQD in the complex solution. The excellent performance of the fabricated MagTQD including monodispersity, superior luminescence, versatility, and excellent magnetic properties was demonstrated, which were better than those of the previously reported magnetic based-QD nanomaterials.^{32–36} Second, the high-performance MagTQD tags were introduced into the LFIA biosensor to provide two detection modes, as follows: the direct mode for the rapid screening or urgent detection of suspected samples within 10 min and the enrichment mode for accurate and quantitative analysis of SARS-CoV-2 antigens in 1 mL of biological samples with a testing time of 35 min. The “dual-mode MagTQD-LFIA” itself is a novel detection technique for POCT, which can meet the testing requirements for viral antigens in various situations. To the best of our knowledge, this is the first report on the use of a direct/enrichment dual mode in the LFIA biosensor. Third, the simultaneous and sensitive detection of SARS-CoV-2 S/NP antigens via LFIA was achieved for the first time. The

limits of detection (LODs) of the proposed method for S/NP antigens under direct and enrichment modes were as low as 1 and 0.5 pg/mL, respectively, which reached the level of ultrasensitive detection.^{37–39} The specificity, accuracy, and stability of the MagTQD-LFIA were further validated in real biological specimens, such as saliva and nasal swab samples. With these advantages, the proposed MagTQD-LFIA could serve as a promising tool to improve the early detection capability for SARS-CoV-2 antigens and helping control the pandemic of COVID-19.

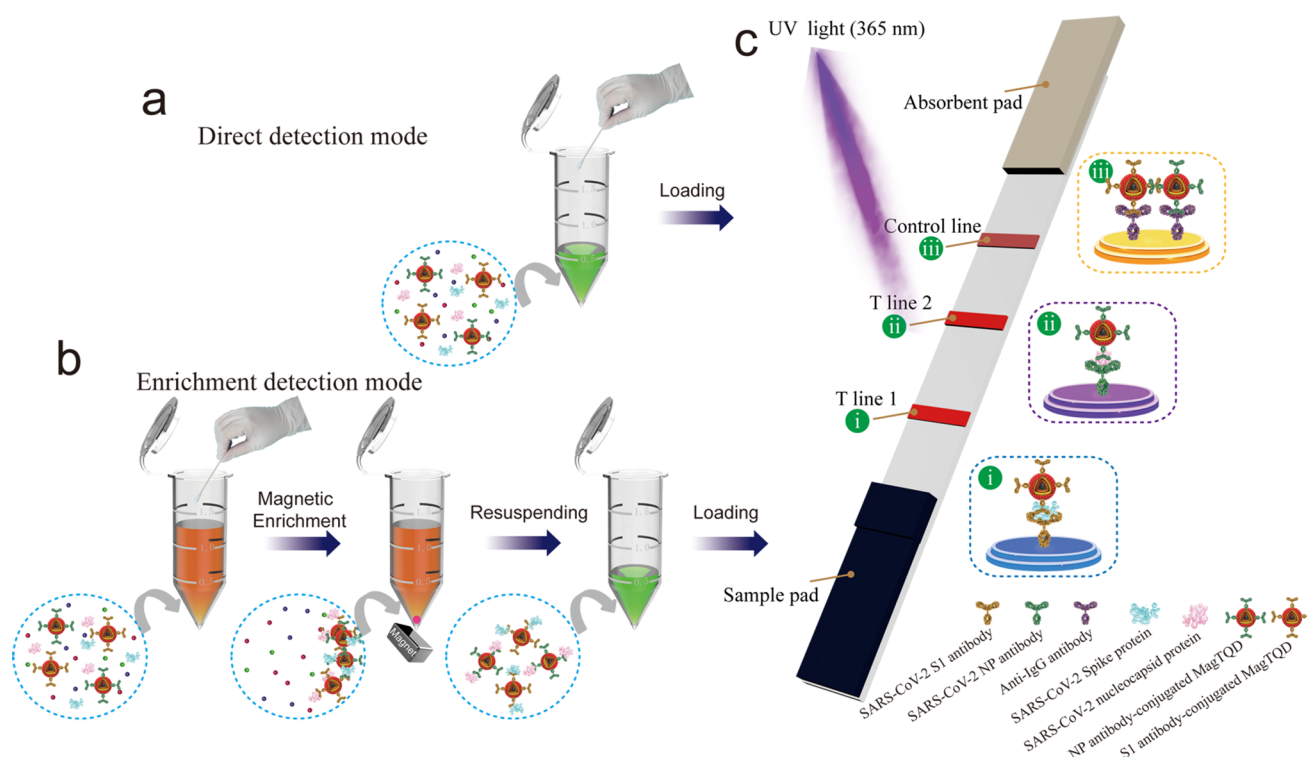
2. EXPERIMENTAL SECTION

2.1. Preparation of MagTQD Nanocomposites with a Multilayer QD-Shell. The novel MagTQD nanocomposite with a 160 nm Fe_3O_4 superparamagnetic core and three layers of QD-shell was fabricated via cationic polymer-mediated self-assembly, as illustrated in Figure 1a. First, we synthesized Fe_3O_4 beads with a diameter of 160 nm through a typical solvothermal reaction.⁴⁰ Then, 1 mL of the fabricated Fe_3O_4 MNPs (10 mg/mL) was reacted with 50 mL of aqueous PEI solution (0.2 mg/mL) under sonication for 30 min. During this process, PEI could quickly self-assemble onto the surface of Fe_3O_4 MNPs and formed a positively charged PEI shell.⁴¹ The $\text{Fe}_3\text{O}_4/\text{PEI}$ MNPs were magnetically collected and rinsed twice with deionized water to remove excess PEI. Third, the purified $\text{Fe}_3\text{O}_4/\text{PEI}$ MNPs were mixed with 50 mL of CdSe/ZnS-MPA QDs (1 nM), and the mixture was vigorously sonicated for 40 min; during this process, the negatively charged CdSe/ZnS-MPA QDs were densely adsorbed on the surface of $\text{Fe}_3\text{O}_4/\text{PEI}$ MNPs via strong electrostatic attraction of PEI. The obtained $\text{Fe}_3\text{O}_4/\text{QD}$ core-shell nanocomposites (MagQD) were magnetically collected and then redispersed in 5 mL of deionized water. Fourth, the MagQD NPs were reacted with PEI and CdSe/ZnS-MPA QDs sequentially, and the $\text{Fe}_3\text{O}_4/\text{dual QD-shell}$ nanocomposites (MagDQD) were fabricated through the PEI-intermediated LBL assembly. After coating of the third layer of PEI and adsorption of the third layer of QD, the $\text{Fe}_3\text{O}_4/\text{triple QD-shell}$ nanocomposites (MagTQD) were formed and collected by an external magnet. The MagTQD NPs were dried under vacuum at 60 °C. Finally, the obtained MagTQD powder was dispersed in ethanol to form standard solution (1 mg/mL) and stored out of light for future use.

2.2. Preparation of Antibody-Conjugated MagTQD Tags. The corresponding monoclonal antibodies against the SARS-CoV-2 N antigen and S antigen were conjugated with MagTQD NPs via EDC/NHS chemistry. In brief, 1 mL of MagTQD solution (1 mg/mL) was added into a 2 mL tube. The MagTQD NPs were magnetically separated and resuspended in 0.5 mL of MES buffer (0.1 M, pH of 5.5), and then the mixture was mixed with 5 μL of EDC (0.1 M) and 10 μL of sulfo-NHS (0.1 M). After 15 min of incubation, the activated MagTQD NPs were collected by a magnet and resuspended in 200 μL of PBS buffer (10 mM, pH 7.4). Subsequently, the solution was mixed with 10 μg of antibody and shaken for 2 h at 800 rpm. The loading amount of antibody onto the MagTQD tags was investigated by using a BCA Protein Assay Kit (Thermo Scientific). After the MagTQD tags were magnetically collected, the concentration of excess antibody in the supernatant was determined using a microplate reader. The unreacted sites on the MagTQD surface were then blocked with 200 μL of 5% BSA (w/v) for another 1 h. Finally, the prepared immune-MagTQD tags were collected by magnetic separation, rinsed once with PBS solution (10 mM, pH 7.4), and stored in 500 μL of preserving solution (10 mM PBS containing 10% sucrose (w/v), 1% BSA (w/v), and 0.02% sodium azide) for future use.

2.3. Fabrication of a Two-Channel LFIA Biosensor. For the premodification of the sample pad, the glass fiber sample pads were soaked in 10 mM PBST solution (pH 7.4 containing 0.5% Tween 20) and then dried at 45 °C for 2 h. Next, we separately set the two test lines (T line 1 and T line 2) and one control line (C line) on the nitrocellulose (NC) membrane by coating SARS-CoV-2 S1 detection

Scheme 1. Schematic Illustration of the Dual-Mode LFIA for Simultaneous Detection of SARS-CoV-2 S and NP by MagTQD Fluorescent Tags: (a) Direct Detection Mode and (b) Enrichment Detection Mode of (c) Two-Channel LFIA Strip



antibody, SARS-CoV-2 NP detection antibody, and goat-anti-mouse IgG antibody, respectively. Detection antibody solution (30 μ L; 1.5 mg/mL) and goat-anti-mouse IgG solution (30 μ L; 0.5 mg/mL) were dispensed onto the T lines and C line by using the XYZ spraying platform (Biodot, USA) at an application volume of 0.1 μ L/mm. Subsequently, the NC membrane was placed into a 37 $^{\circ}$ C drying oven for 2 h. Finally, the fully assembled card, which consists of the treated sample pad, NC membrane, and absorbent pad, was cut to individual LFIA strips (3 mm width) for subsequent use.

2.4. Simultaneous Detection of SARS-CoV-2 S and NP Antigens in Biological Samples. The human biological samples including saliva and nasal swab from healthy volunteers were collected from Affiliated Hospital of Xuzhou Medical University (Xuzhou, China). Our study is approved by the Ethics Committee of the hospital and Beijing Institute of Radiation Medicine. Biological samples (nasal swab and saliva) were spiked with different concentrations of SARS-CoV-2 S and NP antigens to simulate the actual clinical samples. Analysis of the S/NP antigen by the direct detection mode of MagTQD-based LFIA was performed as follows. Immuno-MagTQD tags (2 μ L) were first mixed with 60 μ L of each sample in a 2 mL tube. Then, the solution was mixed with 10 μ L of 5 $\times$ running buffer (50 mM PBS containing 5% Tween 20, 5% Triton X-100, and 5% BSA), vigorously vortexed for 15 s, and then pipetted onto the sample pad of a prepared LFIA strip. After 10 min of chromatography, the fluorescence signal on the two test lines was recorded by using a portable fluorescence strip reader at 365 nm excitation.

Analysis of the S/NP antigen by the enrichment detection mode of MagTQD-based LFIA was performed as follows. In a typical procedure, 1 mL of the test sample was added in a 2 mL tube, mixed with 2 μ L of immuno-MagTQD tags, and incubated for 20 min with vigorous shaking (800 rpm). Then, the MagTQD immunocomplexes were magnetically collected and resuspended in 70 μ L of 1 $\times$ running buffer (10 mM PBS containing 1% Tween 20, 1% Triton X-100, and 1% BSA). The mixture was fiercely vortexed for 15 s and pipetted onto the sample pad of an LFIA strip for chromatographic assay. After 10 min of reaction, the LFIA strip was placed on a

portable fluorescence strip reader, and the fluorescence signal on the two test lines was recorded at 365 nm excitation.

3. RESULTS AND DISCUSSION

3.1. Fabrication of Multifunctional MagTQD Tags. We aimed to develop a flexible LFIA biosensor for the early and accurate identification of SARS-CoV-2 infection, which can support the simultaneous detection of the virus S/NP antigens on a strip with high sensitivity and provide a direct/enrichment dual-detection mode to meet the testing requirements for different purposes. As illustrated in Scheme 1a,b, the direct detection mode of our biosensor is similar to that of the common QD-based LFIA for rapid screening of suspicious samples, whereas the enrichment mode is used to provide more sensitive and precision results based on magnetic enrichment of target antigens from large volume samples. To achieve the goal, a high-performance magnetic quantum dot nanomaterial should be utilized as a multifunctional fluorescent tag of the LFIA system instead of common quantum dot materials, which must possess the following characteristics: (i) magnetic enrichment ability to enrich the target antigen, thus improving the detection sensitivity; (ii) high luminescence to support the sensitive fluorescence detection; (iii) excellent dispersibility to ensure the good transport of immunocomplexes on the NC membrane of strip; and (iv) good stability in complex biological samples to guarantee testing reliability.

Based on our recently proposed PEI-mediated electrostatic adsorption,^{31–33,42} herein, a novel MagTQD nanocomposite with a 160 nm Fe₃O₄ core and a triple-layer QD-formed shell was designed and synthesized, which integrated thousands of QDs onto the surface of one magnetic particle. Theoretically speaking, the sensitivity of QD-fluorescent LFIA highly depended on the performance of QD materials used.^{34,43,44}

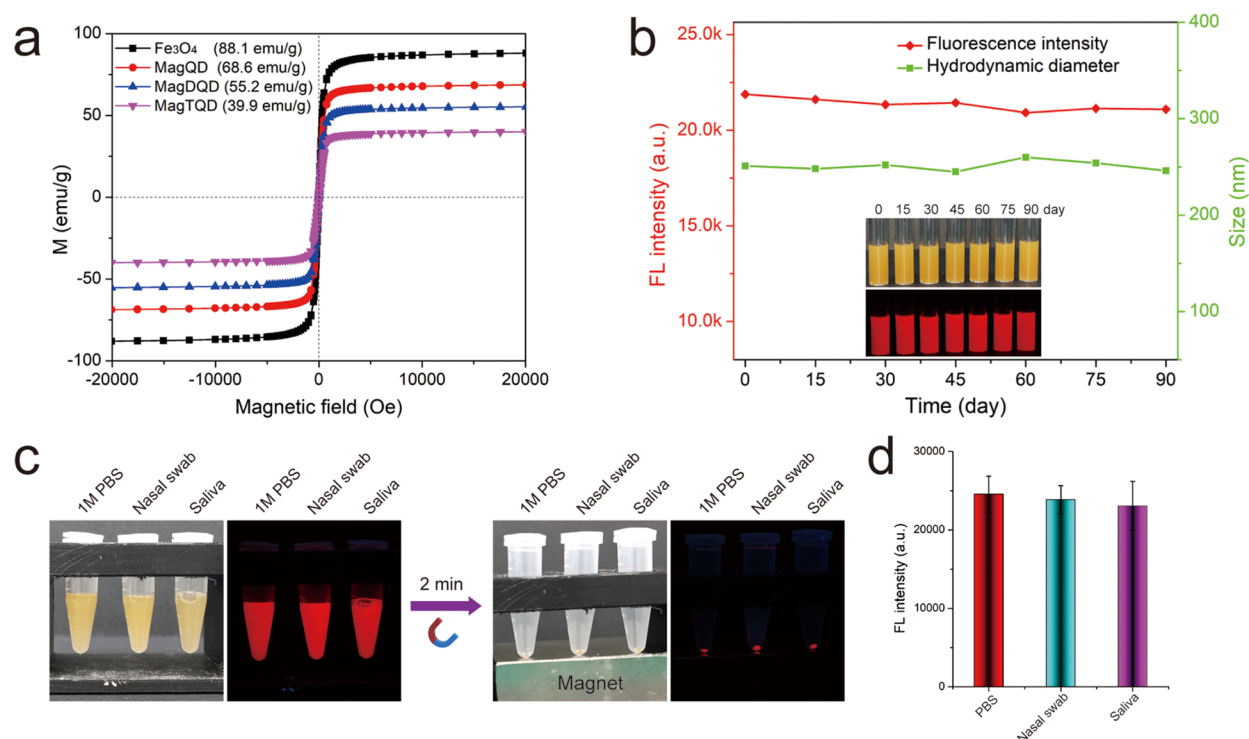


Figure 2. Property characterization of the MagTQD nanocomposite. (a) Magnetic property of the fabricated Fe₃O₄ and MagTQD. (b) Fluorescence property (red line) and hydrodynamic diameter (green line) of MagTQD stored in ethanol for different times. (c) Magnetic separation behavior and (d) fluorescence intensity of MagTQD in different samples (PBS, nasal swab, and saliva).

Thus, coating or embedding more QDs into one fluorescent probe is conducive to improving the LFIA sensitivity.

The synthetic route of MagTQD is illustrated in Figure 1a, which is a typical layer-by-layer (LbL) assembly strategy that involves the adsorption of PEI and CdSe/ZnS-MPA QDs repeatedly on the surface of the Fe₃O₄ core. We first fabricated 160 nm superparamagnetic Fe₃O₄ nanobeads as the magnetic supporter by the classical solvothermal method.⁴⁵ Figure 1b displays the transmission electron microscopy (TEM) image of fabricated Fe₃O₄ MNPs. Our previous studies demonstrated that branched PEI could self-assemble on the surface of negatively charged Fe₃O₄ MNPs quickly under sonication and form a positively charged shell in 30 min.⁴¹ As shown in Figures 1c and S1, the PEI-coated Fe₃O₄ (Fe₃O₄@PEI) MNPs exhibited good dispersibility in solution. Due to the plentiful amino groups of the cationic PEI shell, the Fe₃O₄@PEI MNPs allowed the rapid and effective adsorption of dense carboxylated CdSe/ZnS QDs with the action of ultrasonic waves, thereby forming the Fe₃O₄-QD core-satellite nanocomposites (MagQD) (Figure 1d). The zeta potential results (Figure S2) demonstrated that the LbL assembly of PEI and QDs on the Fe₃O₄ surface was driven by electrostatic interactions between the negatively charged nanoparticles and the positively charged polymer. After the first layer of QD coating, the zeta potential of MagQD decreased to 8.7 mV, which offered a foundation for the self-assembly of a second layer of PEI. Thus, the MagQD could repeatedly react with PEI and QDs to form multiple-layer QD-coated Fe₃O₄ nanocomposites. With the successive adsorption of second and third layers of QD, the resulting MagQD with a double-QD shell (MagDQD) and MagQD with a triple-QD shell (MagTQD) were successfully prepared, as shown in Figure 1e,f, respectively. Displayed in Figure 1g–k are high-resolution

TEM images for single Fe₃O₄ core, Fe₃O₄@PEI, MagQD, MagDQD, and MagTQD, respectively. A magnified TEM image in Figure 1l shows that the multiple QD-shell consisted of numerous 15 nm CdSe/ZnS QDs, which improved the dispersibility and stability of the MagTQD and provided vast surface sites for further bioconjugation. EDS elemental mapping analysis (Figure 1m) clearly shows the elemental distributions of the Fe₃O₄ core (Fe) and the multiple-layer QD-shell (Cd, Se, and Zn), which further demonstrated the hierarchical structure of MagTQD. The maximum numbers of QDs adsorbed onto one MagQD, MagDQD, and MagTQD surface were calculated to be approximately 490, 1163, and 2049, respectively. (The calculation details are provided in Section S3 and Figure S3.) The fluorescence spectra of the as-obtained three kinds of MagQD probes are shown in Figure 1n. All MagQD probes exhibited narrow emissions with the major emission peak at ~629 nm. The fluorescence intensity of MagTQD was 2.44 and 1.56 times higher than those of MagQD and MagDQD, respectively. Obviously, the higher luminescence of MagTQD was due to the larger amount of loaded QDs.

The magnetic properties and stability of MagTQD tags were investigated. The superparamagnetic properties of MagTQD were confirmed by the magnetic hysteresis curves (Figure 2a). Moreover, the saturation magnetization (M_S) value of MagTQD decreased with the times of LbL assembly from 88.1 to 39.9 emu/g because the proportion of the hydrophilic substance (PEI and carboxylated QDs) gradually increased. Notably, the M_S value of MagTQD was still higher than those of most of the reported magnetic based-QD nanomaterials.^{34–36} The long-term stability of MagTQD tags in ethanol was next tested, and their fluorescence intensity and dispersibility remained stable for 90 days (Figure 2b). The

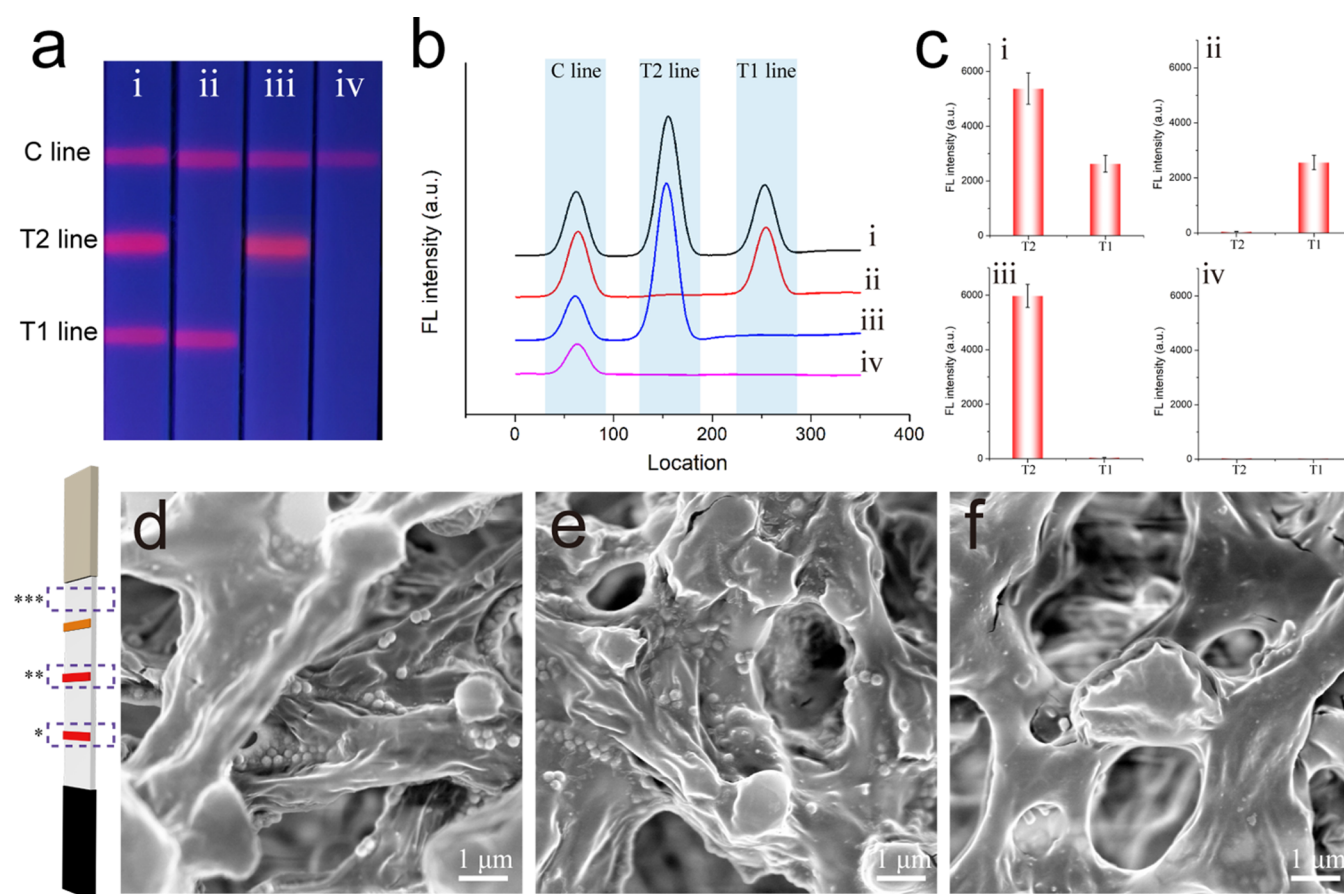


Figure 3. (a) Photographic images, (b) corresponding fluorescence signal, and (c) fluorescence intensity of MagTQD-based LFIA with different concentrations of SARS-CoV-2 S and NP antigens: (i) 10 and 10 ng/mL, (ii) 10 and 0 ng/mL, (iii) 0 and 10 ng/mL, and (iv) 0 and 0 ng/mL. SEM images of test line 1 (d), test line 2 (e), and NC membrane (f) of MagTQD-LFIA for 10 ng/mL of SARS-CoV-2 S and NP antigens.

superior magnetic properties and stability of MagTQD tags ensured their actual use in the rapid separation of targets in biological specimens. As shown in Figure 2c, the MagTQD tags were dispersed well in the three complex samples (1 M PBS, nasal swab, and saliva) and could be completely separated from these samples within 2 min by using an external magnet. Moreover, the fluorescence intensity of MagTQD tags remained stable in these complex biological samples (Figure 2d). To further investigate the stability of MagTQD nanocomposites in different environments (pH and high-salt), two parallel experiments were performed. As demonstrated in Figure S4a, the MagTQD nanocomposites showed remarkable stability in aqueous solution with a wide range of pH from 4 to 12. Meanwhile, the fluorescence intensity of MagTQD was unaffected by the high-salt environment (0–1000 mM NaCl) (Figure S4b). All of these results confirmed the excellent chemical and optical stabilities of MagTQD nanocomposites in the complex solution. Notably, the size of the magnetic core has a major impact on the performance (magnetic response ability and liquidity) of MagTQD nanocomposites, which need to be well-optimized. The detailed optimization content of the Fe_3O_4 core is given in Section S4 and Figure S5, and 160 nm Fe_3O_4 MNP was finally chosen to build the MagTQD tag.

3.2. Construction of Two-Channel MagTQD-Based LFIA. For simultaneous detection of S/NP antigens, the LFIA strip is made up of five parts as illustrated in Scheme 1c, including a sample pad for MagTQD mixture loading, an absorbent pad to provide capillary force, two separate test lines

(T1 and T2) to coat the SARS-CoV-2 S and NP antigen detection antibodies, and a control line to coat the goat anti-mouse IgG antibody. When the test samples contain SARS-CoV-2 S/NP antigens, these target antigens bind tightly to immuno-MagTQD tags by the antibody–antigen reaction and form MagTQD-S/NP antigen immunocomplexes immediately. The formed immunocomplexes migrated along the NC membrane under the capillary forces of the absorbent pad and were caught by the corresponding test lines that coated S and NP antigen detection antibodies. Free immuno-MagTQD tags will not bind on the test lines but will be captured by the anti-mouse IgG of the control line. When the chromatography was complete, the two test lines exhibited red fluorescence bands in the presence of the two viral antigens, whereas only the T1 line or T2 line showed visible fluorescence signals in the presence of one target (S or NP antigen). The fluorescence intensities of the two test zones were derived from the amount of MagTQD immunocomplexes caught, which were proportional to the concentrations of the target S/NP antigen in the clinical samples; thus, they could be used for quantitative analysis.

The detection performance of the LFIA biosensor highly depended on the affinity and specificity of antibodies used. For the two-channel LFIA strip construction, we needed to select pairs of specific antibodies with the best activity for SARS-CoV-2 S and NP antigens. We proposed a simple method for antibody rapid screening, as illustrated in Figure S6a. First, the SARS-CoV-2 S and NP antigens were separately conjugated

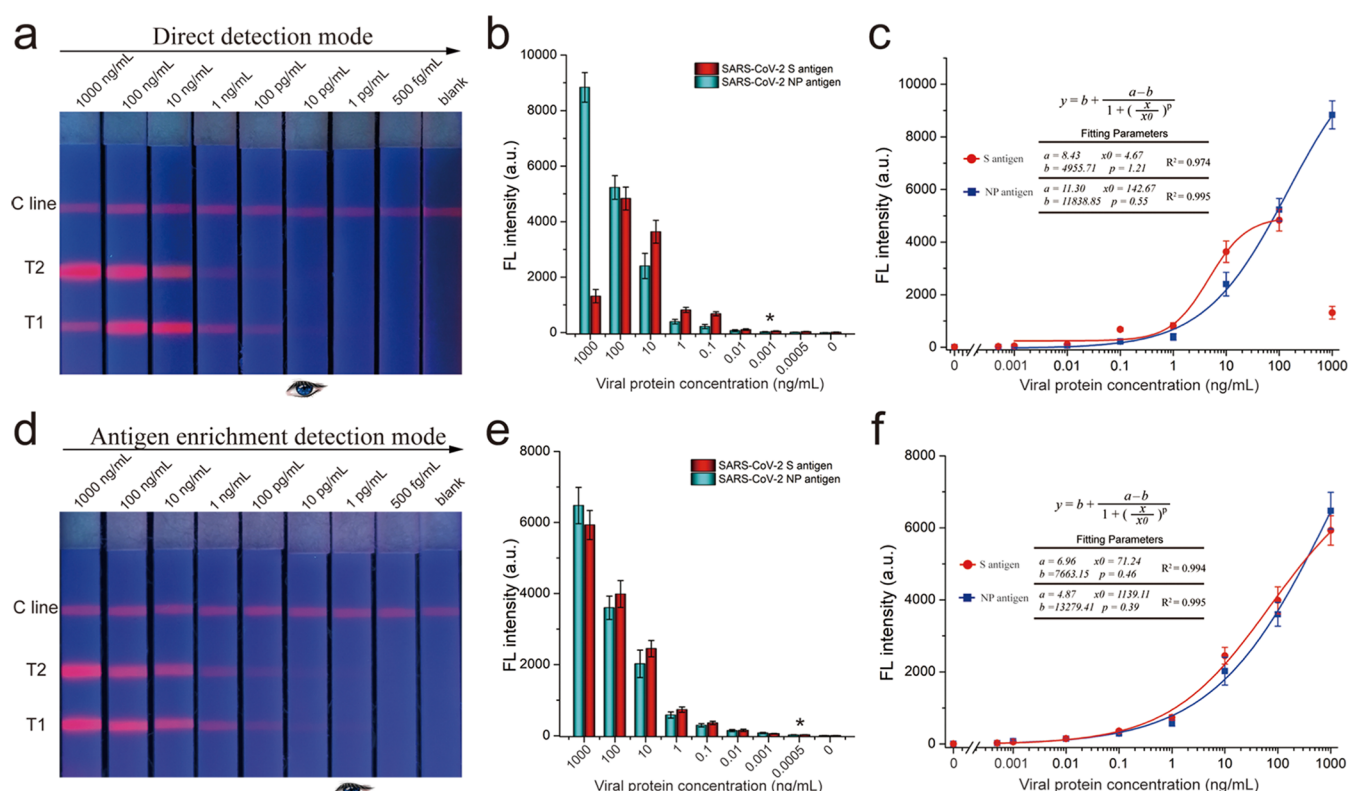


Figure 4. Analytical performance of the dual-mode fluorescence LFIA based on MagTQD tags. (a and d) Fluorescence images of MagTQD-LFIA for SARS-CoV-2 S and NP antigen detection in nasal swab samples by using direct detection mode (a) and enrichment detection mode (d). Corresponding test line intensities of direct detection mode (b) and enrichment detection mode (e) for different concentrations of SARS-CoV-2 S and NP antigens. Corresponding calibration curves for SARS-CoV-2 S and NP antigens by direct detection mode (c) and enrichment detection mode (f). Error bars are calculated from five experiments.

onto the carboxyl groups of the MagTQD surface by EDC/NHS coupling chemistry to prepare MagTQD-S antigen and MagTQD-NP antigen tags. Meanwhile, all of the tested commercial monoclonal antibodies (mAbs) were sprayed on the NC membrane of strips at a concentration of 1 mg/mL. Then, the prepared MagTQD-S/NP antigen tags were dispersed in the running buffer and loaded on the LFIA strip to detect the activity of immobilized antibodies. As shown in Figure S6b,c, the monoclonal antibodies S2-mAb (Catalog #40 150-D002) and S4-mAb (Catalog #40 150-D004), which had the highest affinity toward the SARS-CoV-2 S1 antigen, and the antibodies N2 (Catalog #40 143-R040) and N6 (Catalog #40 143-MM08), which had the highest affinity toward the SARS-CoV-2 NP antigen, were selected. We further tested the match performance of these mAbs as capture antibodies on the MagTQD and detection antibodies on the NC membrane and finally determined the best matching antibody pairs. As shown in Figure S6d–g, the S2-mAb and N6-mAb as capture antibodies and the S4-mAb and N2-mAb as detection antibodies can achieve the best signal-to-noise ratio (SNR) for the detection of SARS-CoV-2 S/NP antigens. The loading capacity of S2-mAb and N6-mAb onto the MagTQD surface was determined to be 0.75% and 0.72%, respectively, via the BCA method.

After the screening of antibodies, the multiplex detection ability of MagTQD-based LFIA was assessed. The detection mAbs for the S antigen and NP antigen were sprayed on NC membranes to set the two test lines (T1 for the S antigen and T2 for the NP antigen), thereby ensuring the simultaneous detection of two target antigens in one test. The samples

containing 10 ng/mL S, NP, and S/NP antigens were prepared and tested by using the MagTQD-based LFIA. The fluorescence images in Figure 3a clearly show two bright fluorescence lines, which appeared for the two target antigens that existed together, whereas only one fluorescence band was observed for the S or NP antigen that existed alone. No cross reaction was found between the two test lines, which suggested the good selectivity of the two-channel LFIA. By utilizing a commercial fluorescent instrument, the fluorescence spectra and detailed fluorescence intensity of the two test lines could be easily measured, as displayed in Figure 3b,c, respectively. In addition, the scanning electron microscopy (SEM) images of T1/T2 zones and the blank area of the NC membrane revealed that many MagTQD tags were caught on the test zones by immune binding (Figure 3d–e), whereas no nonspecific binding of tags was observed on the blank area (Figure 3f). When a low concentration (0.1 ng/mL) of target S/NP antigens was applied on the LFIA strip, some aggregated MagTQD tags could also be observed on the test zone (Figure S7). These results confirmed the fluorescence signals of test lines from the MagTQD attached and formed in proportion to the amount of MagTQD immunocomplexes, thereby providing the foundation for quantitative analysis. Many recent studies indicated that the QD microspheres with a multiple-layer structure could work well in the LFIA system.^{46–48} We have systemically studied the important parameters of paper-chromatography strip with large size nanotags (>200 nm) in our previous works.^{28,29} Based on these foundations, the NC membrane type, running buffer ingredient, and detection

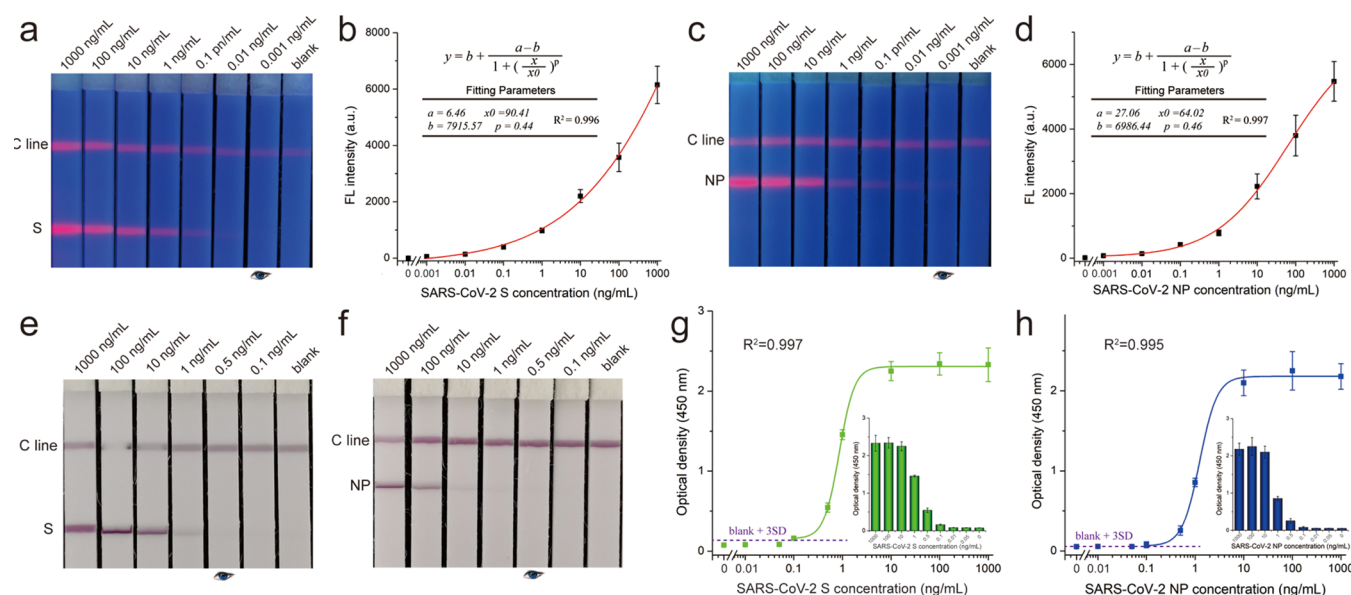


Figure 5. Detection performance comparison between MagTQD-LFIA with enrichment mode, AuNP-based LIFA, and ELISA for SARS-CoV-2 S and NP antigens in saliva samples. Fluorescence images of the enrichment detection results of MagTQD-LFIA for the S antigen (a) and NP antigen (c). Calibration curves (b) and (d) of the MagTQD-based method for S and NP antigens, respectively. Photographs of AuNP-based LIFA strips for the detection of the SARS-CoV-2 S antigen (e) and NP antigen (f). ELISA results for different concentrations of the SARS-CoV-2 S antigen (g) and NP antigen (h). Error bars are calculated from three experiments.

antibody dosage were well-optimized (Section S5 and Figure S8).

3.3. Evaluation of the Dual-Mode MagTQD-LFIA. After the optimization of the chromatographic system, the analytical performance of MagTQD-LFIA was evaluated by detecting SARS-CoV-2 S/NP antigen-spiked nasal swab samples (1000–0 ng/mL). Considering the combination of superior fluorescence properties of thousands of QD-formed shells and superparamagnetic properties of the Fe_3O_4 core, the MagTQD tags can not only act as high luminescent probes for direct LFIA detection of analytes but also serve as multi-function magnetic fluorescent tools for ultrasensitive target capture/enrichment detection. To evaluate the direct detection ability of the MagTQD-LFIA, the prepared samples (50 μL) with different concentrations of S/NP antigens were mixed with immuno-MagTQD tags and were directly loaded onto the sample pad of two-channel strips for rapid testing. Figure 4a shows the fluorescence image of MagTQD-LFIA strips used for the direct detection of various concentrations of the target S/NP antigen under UV light. The visual limit of the fluorescence test lines of both SARS-CoV-2 antigens was 10 pg/mL. However, an obvious “hook effect”^{49,50} was observed at a high concentration of the S antigen (1000 ng/mL), which led to a contradictory fluorescence signal decrease in the T1 line at antigen concentrations higher than 1000 ng/mL. The corresponding fluorescence intensity of two test lines was measured as shown in Figure 4b. Based on these fluorescence LFIA values, the calibration curves for SARS-CoV-2 S and NP antigens were plotted as displayed in Figure 4c. The fluorescence intensities gradually decreased with decreasing concentrations of target S and NP antigens in the wide detection ranges of 100 ng/mL–1 pg/mL and 1000 ng/mL–1 pg/mL, respectively. The LODs for the S antigen and NP antigen were estimated to be 1 pg/mL in the direct mode of MagTQD-LFIA, which was defined as $y_{\text{blank}} + 3SD_{\text{blank}}$ (y_{blank} is the mean of fluorescence intensity of the blank groups; SD_{blank} is the standard deviation). Moreover, an assay time of 10 min

was sufficient for MagTQD-LFIA direct detection (Figure S9a).

We next assessed the enrichment detection ability of MagTQD-LFIA by testing the same viral antigen-spiked samples. As illustrated in Scheme 1b, a sample enrichment step was added to the detection process of LFIA. Through this step, the process can selectively enrich and magnetically separate SARS-CoV-2 S/NP antigens from 1 mL of nasal swab samples to 70 μL of running buffer for concentrating the target antigens, thereby further improving the detection sensitivity and avoiding interferences caused by complex components in real samples. The reaction time for the enrichment step was optimized, and the best SNR can be obtained with an incubation time of 20 min (Figure S9b). The detection result of MagTQD-LFIA strips by enrichment mode is displayed in Figure 4d. The red fluorescence band of two test lines was clearly visible at high concentrations of S/NP antigens under 365 nm UV light, and their intensities concomitantly weakened with the decreasing viral antigen concentration. The visual LODs of the fluorescence band of test lines were as low as 1 pg/mL. Next, the corresponding fluorescence intensities of T1/T2 lines were analyzed using a portable fluorescence reader (Figure 4e). The fluorescence image and intensity results indicated that the enrichment detection mode of MagTQD-LFIA can overcome the high dose “hook effect” in LFIA-based SARS-CoV-2 antigen detection. The fluorescence intensity values of two test lines of enrichment mode were lower than that of direct detection mode at high concentrations of S/NP antigens (100–10 ng/mL) (Figure 4b,e), which may be attributed to the slight loss of MagTQD tags during the magnetic enrichment process. Notably, the overall fluorescence intensity of enrichment mode was higher than that of direct detection mode to detect low concentrations of S/NP antigens (<0.1 ng/mL), which demonstrated the amplification effect of fluorescence signals by antigen enrichment using MagTQD. The regression curves were fitted between the logarithm of the concentrations of the two target

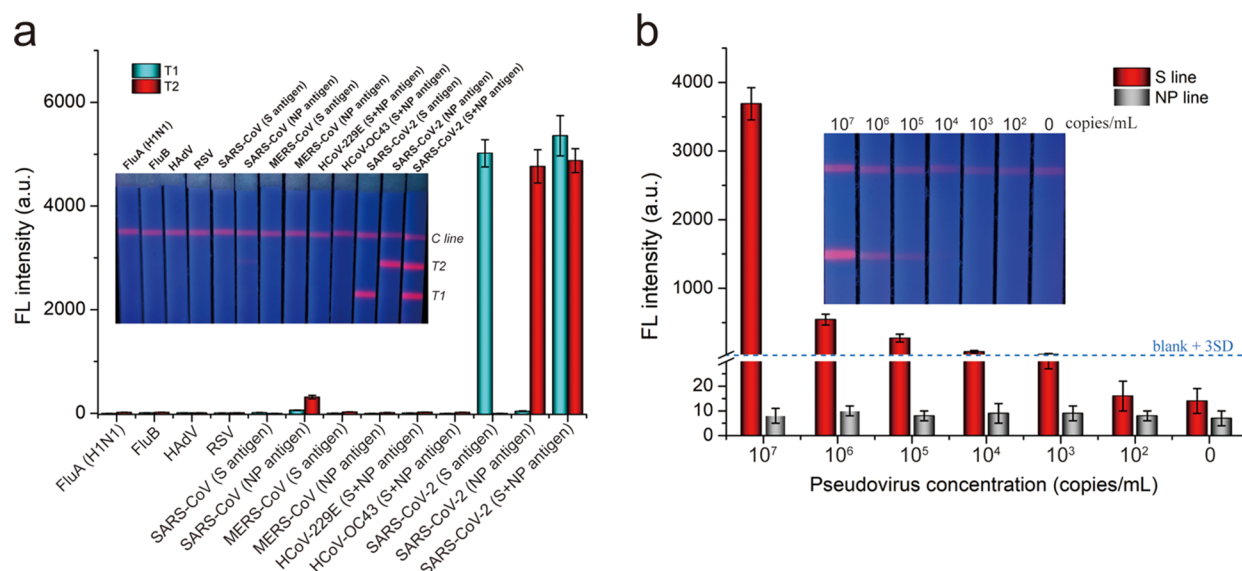


Figure 6. (a) Specificity of the MagTQD-LFIA biosensor. (b) Detection results of MagTQD-LFIA strips for SARS-CoV-2 S pseudovirus. The insets show the photographs of the tested strips. Error bars are calculated from three experiments.

antigens and the fluorescence intensity of the two test zones (Figure 4f). Their corresponding four-parameter sigmoid functions are provided in the inset of Figure 4f. The LODs by fluorescence intensity for S and NP antigens by enrichment detection mode were determined to be 0.5 pg/mL at an SNR of 3. Hence, the enrichment mode of MagTQD-LFIA resulted in 10 and 2 times improvement in visual sensitivity and detection limit, respectively, compared with the direct detection mode. Moreover, the enrichment mode of MagTQD-LFIA can provide a wider detection range and more accurate results because it eliminates the “hook effect” for samples with a high antigen concentration.

We further selected saliva as another target test sample for the MagTQD-LFIA. Saliva reportedly has high SARS-CoV-2 viral loads, similar to nasal swab samples.^{51,52} A series of saliva samples containing SARS-CoV-2 S and NP antigens were prepared and then detected by MagTQD-LFIA with enrichment mode. As shown in Figure 5a,c, the visual sensitivity of the MagTQD-LFIA for S and NP antigen-spiked saliva samples can reach 1 pg/mL, which was almost equal to those for nasal swab samples. The fluorescence intensities of the corresponding test lines for S and NP antigens also showed the same wide dynamic range as shown in Figure 4f, with $R^2 = 0.996$ for the S antigen and $R^2 = 0.997$ for the NP antigen (Figure 5b,d). Moreover, to investigate the accuracy and precision of the proposed MagTQD-LFIA, recovery tests were conducted by using the enrichment mode. As shown in Table S1, the average recovery values for the two antigens in nasal swab and saliva samples varied from 85.8% to 97.4%, and the coefficients of variability (CV) ranged from 2.7% to 12.3%, indicating the accuracy of our method. These results confirmed that the MagTQD-LFIA worked well for the main respiratory tract samples. In addition, the repeatability of the proposed MagTQD-LFIA strips was measured at different concentrations of SARS-CoV-2 S/NP antigens (100 and 1 ng/mL) on ten separate tests in unprocessed nasal swab. As shown in Figure S10, the relative standard deviation (RSD) values of the fluorescence signal on the two test lines of the tested strips in both direct detection mode and enrichment detection mode

were less than 8.2%, indicating the high repeatability and reliability of our method.

To verify the superiority of MagTQD with a triple-QD shell for fluorescence signal amplification, a comparison between the proposed MagTQD-LFIA and two other kinds of magnetic QD (MagQD and MagDQD)-based LFIA was performed. Figure S11a shows the fluorescence images of MagQD-LFIA, MagDQD-LFIA, and MagTQD-LFIA for testing the SARS-CoV-2 NP antigen in different concentrations (100–1 ng/mL). Clearly, the tested strips of MagTQD-LFIA displayed the highest detection sensitivity for viral antigen by eye observation. Moreover, the fluorescence intensity values of T lines of MagTQD-LFIA were much higher than those of MagQD-LFIA and MagDQD-LFIA strips (Figure S11b). These results confirm the enhancement effect of sensitivity by using MagTQD tags. It should be noted that too many layers of QD outside the magnetic core will further increase the particle size of the nanocomposite and severely weaken the magnetic responsiveness of the magnetic QD tags. As shown in Figure S12a, the average diameter of magnetic QD with four-layer QD increased to approximately 320 nm. The too big size of nanocomposites easily blocked the pores of the NC membrane and consequently resulted in a high nonspecific signal on the strips. Figure S12b demonstrates that the fluorescence background of magnetic QD with four-layer QD-based LFIA strips was rather high, which affects the quantification of the fluorescence signal and detection sensitivity. By comparison, the MagTQD-LFIA strips achieved the higher SNR of the fluorescence signal on the T lines than that of magnetic QD with four-layer QD-based LFIA (Figure S12c). These results indicate that the MagTQD tag is more suitable for our LFIA system.

As a newly developed emergency POCT product for COVID-19, the sensitivity of the MagTQD-LFIA needs to be compared with that of traditional immunoanalytical methods. The standard colloidal gold (AuNP)-LFIA method and commercially available ELISA kits were used for SARS-CoV-2 S and NP antigens. The experimental details of AuNP-LFIA preparation are given in Section S2. Figure 5e,f shows that the visual sensitivity of AuNP-LFIA strips for two target

viral antigens was 0.5 ng/mL with the naked eye. The ELISA kits for the SARS-CoV-2 S antigen (Catalog #KIT40591) and NP antigen (Catalog #KIT40588) detection were purchased from Sino Biological, Inc. (Beijing, China). As shown in Figure 5g,h, the LODs determined by the ELISA kits for viral S and NP antigens were 0.1 ng/mL. By comparison, the detection sensitivity of MagTQD-LFIA was at least 500 and 100 times higher than those of AuNP-LFIA and ELISA, respectively.

The specificity of MagTQD-LFIA was assessed by testing other two highly pathogenic coronaviruses (SARS-CoV and MERS-CoV), two human coronaviruses (HCoV-229E and HCoV-OC43), and four common respiratory viruses, namely, FluA, influenza B virus (FluB), HAdV, and respiratory syncytial virus (RSV). High concentrations of four common respiratory virus samples (approximately 10^6 pfu/mL) and four coronavirus S/NP antigen samples (1000 ng/mL) were prepared and detected by our proposed method. As displayed in Figure 6a, most of the control virus and antigen groups exhibited relatively low fluorescence signals except the NP antigen of SARS-CoV, whereas the three positive groups (100 ng/mL of SARS-CoV-2 S, NP, and S/NP antigens) exhibited a distinct fluorescence band on the corresponding test lines. These results indicated that our method had a weak cross-reaction with the SARS-CoV NP antigen at a high concentration and can specifically distinguish the SARS-CoV-2 from other common respiratory viruses and coronaviruses by the simultaneous detection of S/NP antigens.

The SARS-CoV-2 S pseudovirus at a concentration of 10^{10} copies/mL (Catalog #PSV001) was obtained from Sino Biological, Inc. (Beijing, China) and used to simulate the inactivated virus. The pseudovirus samples were prepared using gradient dilution and detected by MagTQD-LFIA. As displayed in Figure 6b, the LOD of MagTQD-LFIA for SARS-CoV-2 S pseudovirus was as low as 10^3 copies/mL. No obvious fluorescence signal of NP test lines was found because the constructed pseudovirus contains no NP antigen. Previous studies have revealed that the SARS-CoV-2 viral loads on the respiratory tract specimen shortly after onset were generally higher than 1×10^6 copies/mL.^{53,54} Thus, the proposed MagTQD-LFIA is sensitive enough for respiratory tract specimen analysis. Besides remarkable advantages such as short testing time (10–35 min), ease of operation, low cost, good stability, and reproducibility, our method showed great potential as an efficient immunoanalytical tool for POCT of SARS-CoV-2.

4. CONCLUSIONS

In summary, we proposed a dual-mode fluorescence LFIA biosensor for the sensitive and simultaneous detection of SARS-CoV-2 S/NP antigens by using multifunctional MagTQD tags. The MagTQD was fabricated by using a PEI-mediated LBL self-assembly strategy. The 160 nm Fe_3O_4 core was coated with a three-layer QD-formed shell, which possessed monodispersity, high luminescence, and strong magnetic responsiveness. By combining the MagTQD tags and LFIA strip, our proposed method successfully detected viral S/NP antigens in biological samples with flexibility (dual detection mode), fast detection speed (10–35 min), high sensitivity (0.5–1 pg/mL), good reproducibility (CV < 12.3%), excellent specificity, and high stability. This is the first report on the use of a direct/enrichment dual mode-LFIA biosensor, and we are the first to simultaneously detect SARS-CoV-2 S/NP antigens by LFIA strip. In the future, the

accuracy of the MagTQD-LFIA will be verified by testing clinical samples of patients with COVID-19. We believe that our proposed method will exert a positive effect on the rapid and accurate detection of virus infections.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsami.1c11461>.

Additional experimental description (S1: materials, reagents, and instrumentation; S2: preparation of AuNP-LFIA; S3: calculation of the maximum number of QDs on the MagTQD; S4: optimization of the Fe_3O_4 core for MagTQD tags; and S5: optimization of assay conditions for MagTQD-LFIA strip); SEM image of Fe_3O_4 @PEI MNPs; zeta potentials of the products from each step; stability results of MagTQD nanocomposites; optimization of the size of MagTQD NPs; screening of suitable SARS-CoV-2 S and NP antibodies for the LFIA system; SEM images of the test lines for 0.1 ng/mL SARS-CoV-2 S and NP antigens; optimization of the NC membrane, running buffer, and detection antibody concentration on the test lines for the MagTQD-LFIA strip; TEM images of MagQDs; optimization of the testing time for the dual-mode MagTQD-LFIA; recovery results for SARS-CoV-2 S/NP antigens spiked in human saliva and nasal swab samples; reproducibility results of the dual-mode MagTQD-LFIA strips; and optimization of QD layers of magnetic QD tags for the LFIA system (PDF)

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Author Contributions

*C.W.W., X.D.C., L.Y.L., and X.C.Z. contributed equally to this work. C.W.W. and S.Q.W. designed and managed the study. C.W.W., X.D.C., L.Y.L., X.C.Z., X.S.Y., and S.Z. performed all the experiments. X.C.Z. and Z.R. provided guidance on data analysis. C.W.W., X.D.C., L.Y.L., and X.C.Z. wrote the manuscript. All authors reviewed the manuscript.

Notes

The authors declare no competing financial interest.

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