


Cite this: *Nanoscale*, 2025, 17, 2469

Received 7th October 2024,
Accepted 28th December 2024

DOI: 10.1039/d4nr04130e

rsc.li/nanoscale

Enhancing chemical signal transformation in lateral flow assays using aptamer-architected plasmonic nanozymes and para-phenylenediamine †

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The widespread adoption and commercialization of lateral flow assays (LFAs) for clinical diagnosis have been hindered by limitations in sensitivity, specificity, and the absence of quantitative data. To address these challenges, we developed aptamer-architected gold nanoparticles as nanozymes that catalytically convert para-phenylenediamine (PPD) into Bandrowski's base (BB), thereby amplifying signal strength and sensitivity. The physicochemical properties of the nanozymes were characterized and their specific binding efficiency was demonstrated using experimental studies. The nanozymes and PPD-based LFA test strips were evaluated for the detection of the COVID-19 spike protein in both test and clinical samples. Notably, we achieved a significant visual detection limit of 168 pg mL⁻¹, with a signal quality enhancement of over 20-fold within 15-minute timeframe. Moreover, we rigorously tested 25 clinical samples to assess the transformative potential of the product, demonstrating a semi-quantitative analysis efficiency exceeding 90%. This performance outstripped commercially available LFA kits (87.5%). Notably, the colorimetric system exhibited an *R*² value of 0.9989, a critical factor for clinical testing and industry integration. The incorporation of nanozymes and PPD in LFAs offers a cost-effective solution with significantly improved sensitivity, enabling the detection of ultra-low concentrations (picograms) of spike protein. By addressing key challenges in LFA-based diagnostics, the current technique underscores the potential of this transformative

biomedical sensor for industry integration. It also highlights its suitability for commercialization, positioning it as a universal platform for diagnostic applications.

1. Introduction

Lateral flow assays (LFAs) have emerged as promising point-of-care diagnostic methods for diagnosing a slew of medical conditions, witnessing the very recent example of COVID-19 during the pandemic.^{1–3} Compared to conventional lab-based techniques, LFAs offer distinct benefits such as self-administration, cost-effectiveness and rapid test results.⁴ These advantages enable individuals to make timely treatment decisions and prevent transmission in cases of infectious diseases. Even though LFAs are widely accepted for their simplicity and rapid diagnosis results, they have been criticized in terms of specificity and sensitivity.⁵ Consequently, LFAs are typically viewed as screening platforms rather than diagnostic ones. To address these criticisms, various advancements have been implemented in LFA development. Notably, the utilization of aptamers as capture molecules has emerged as a promising alternative to antibodies due to the inherent limitations associated with antibodies.^{6,7} Recent investigations highlight the efficacy of aptamers in detecting various infectious pathogens such as dengue virus,⁸ type A influenza virus,⁹ Zika virus,¹⁰ and Ebola virus.¹¹ These remarkable affinity and specificity demonstrate the use of aptamers in real-time diagnosis of pathogens and disease-specific biomarkers.^{12–14}

Constant efforts have been devoted to enhancing the sensitivity of LFAs, particularly through the design and utilization of advanced nanomaterials and signal amplification strategies. Traditionally, gold nanoparticles (GNPs) have served as the predominant labeling agents in LFAs, capitalizing on their ability to generate a visual color signal based on the localized surface plasmon resonance (LSPR) phenomenon.^{3,15,16} The sensitivity of conventional LFAs heavily relies on the plasmonic

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d4nr04130e>

properties of GNPs, which can be tailored by manipulating their size, shape and composition.^{12,17} Plasmonic signal amplification strategies have been explored to augment the visual signal output and thereby enhance the detection limit. Gold and silver enhancement methods enlarge the size of GNPs deposited on the test line, thereby increasing their plasmonic signal and enhancing sensitivity.¹⁸ The integration of spectroscopy techniques, such as fluorescence^{19–21} and Raman spectroscopy,^{22,23} has demonstrated their potential to enhance the sensitivity of LFAs multifold.²⁴ However, these techniques inherently introduce complexity and cost, potentially compromising the inherent advantages of a point-of-care approach.

In recent times, a novel strategy has been developed by using the inherent enzyme mimic characteristics of specific nanomaterials, known as nanozymes, to enhance the color contrast in the test zone of LFAs. The peroxidase-like activity of some nanomaterials enables the oxidation of colorless chromogenic substrates to colored products that leads to enhanced sensitivity and overall performance of the LFAs.^{25,26} In the realm of nanozyme-based LFAs, diverse nanomaterials including various noble metal nanostructures,^{27,28} nanoclusters,²⁹ nanocomposites,³⁰ and magnetic nanomaterials³¹ have been explored to further augment assay sensitivity with high nanozymatic properties. Han *et al.* developed a nanozyme-based LFA using a Pd–Pt nanocomposite as a label for the diagnosis of *Escherichia coli* in milk samples.³² Very recently, gold–iridium nanostructures were developed with ultrahigh nanozymatic properties for LFA applications.³³ Most of these nanozyme-based LFAs commonly use 3,3',5,5'-tetramethylbenzidine (TMB) as a chromogenic substrate to enhance the signal.

The selection of chromogenic substrates is a crucial factor for the development of nanozyme-based LFAs as they are responsible for the enhancement of the signal intensity. TMB, a frequently used chromogenic substrate, possesses limitations such as the soluble nature of its colored product, low signal contrast against the white background of the nitrocellulose membrane, sensitivity to light and signal deterioration over time.^{25,34} These limitations restrict the practical utility of TMB in LFAs, necessitating the exploration of alternative chromogenic substrates tailored for LFA applications. *para*-Phenylenediamine (PPD), an organic compound employed as a catalytic substrate for various enzymes, shows promise in this regard.^{35,36} When oxidized by a peroxidase enzyme, PPD undergoes polymerization, resulting in the formation of a robust brown-colored product known as Bandrowski's base (BB).^{36,37} This colored product exhibits persistence, strong color contrast to the test zone, insensitivity to light, and resistance to fading, all of which collectively enhance the sensitivity of LFAs. However, in-depth examinations of the nanozymatic activity of GNPs with PPD as a chromogenic substrate in LFAs have not been extensively explored.

In the present study, we have developed a method wherein aptamer-architected GNPs serve as nanozymes capable of

catalytically transforming *p*-phenylenediamine (PPD) into a colored product, thereby amplifying the signal within the LFA platform. We have demonstrated the efficacy of this approach in the LFA using the COVID-19 spike protein as a model analyte. To the best of our knowledge, this is the first report employing PPD as a chromogenic substrate for the LFA. We have studied the catalytic activity of nanozymes on the PPD chromogenic substrate in detail and the optimum parameters of PPD have been elucidated for the LFA. The PPD-based LFA demonstrated superior selectivity and achieved a limit of detection (LOD) of 168 pg mL^{−1} for the COVID-19 spike protein. Additionally, a mobile application was also developed for semi-quantification of the COVID-19 spike protein. Clinical sample analysis from patients using the PPD-based LFA kit exhibited efficiency over 90%, surpassing that of the commercially available LFA kit of 87.5%. These results highlight the potential of PPD as a chromogenic substrate to enhance the sensitivity and the overall effectiveness of the LFA, positioning it as a universal diagnostic platform.

2. Experimental methods

The detailed information on experimental methods is provided in ESI S1.†

3. Results and discussion

3.1. Fabrication of the nanozyme platform using GNPs

The GNPs prepared using the tri-sodium citrate reduction method were used as a nanozyme platform. The particles exhibited a spherical morphology with monodisperse nature and a uniform size of 15 nm (Fig. 1a). The distinct ring structure of the SAED pattern is indicative of the crystalline nature of GNPs (Fig. 1b). Furthermore, the (111), (200), (220), (311), and (222) planes were consistent with previously reported data.³⁸ DLS analysis confirmed the average hydrodynamic diameter of GNPs as 24 ± 4 nm, which aligns with the results obtained from TEM analysis (Fig. 1c). The UV-vis spectrum further substantiated the generation of GNPs with a distinct LSPR band at 520 nm (Fig. 1d). In the FT-IR study, the citrate-stabilized GNPs showed prominent peaks at 1601, 1397 and 1156 cm^{−1} which are attributed to the COO[−] stretching vibrations of the citrate molecules adsorbed onto the GNPs (Fig. 1e). The –OH stretching vibration and metal oxide interaction (Au–O) peaks are observed at 3324 and between 500 and 600 cm^{−1} respectively.^{39–41} The oxidation state and chemical interaction of the GNPs were confirmed by XPS spectra (Fig. 1f). The deconvoluted XPS spectra of the GNPs showed two distinct peaks at 83.9 and 87.5 eV, corresponding to the Au (0) and Au(I) oxidation states of the GNPs.

3.2. Nanozyme activity of GNPs

The peroxidase mimic nanozyme activity of the GNPs was evaluated by examining their ability to catalyze the oxidation of

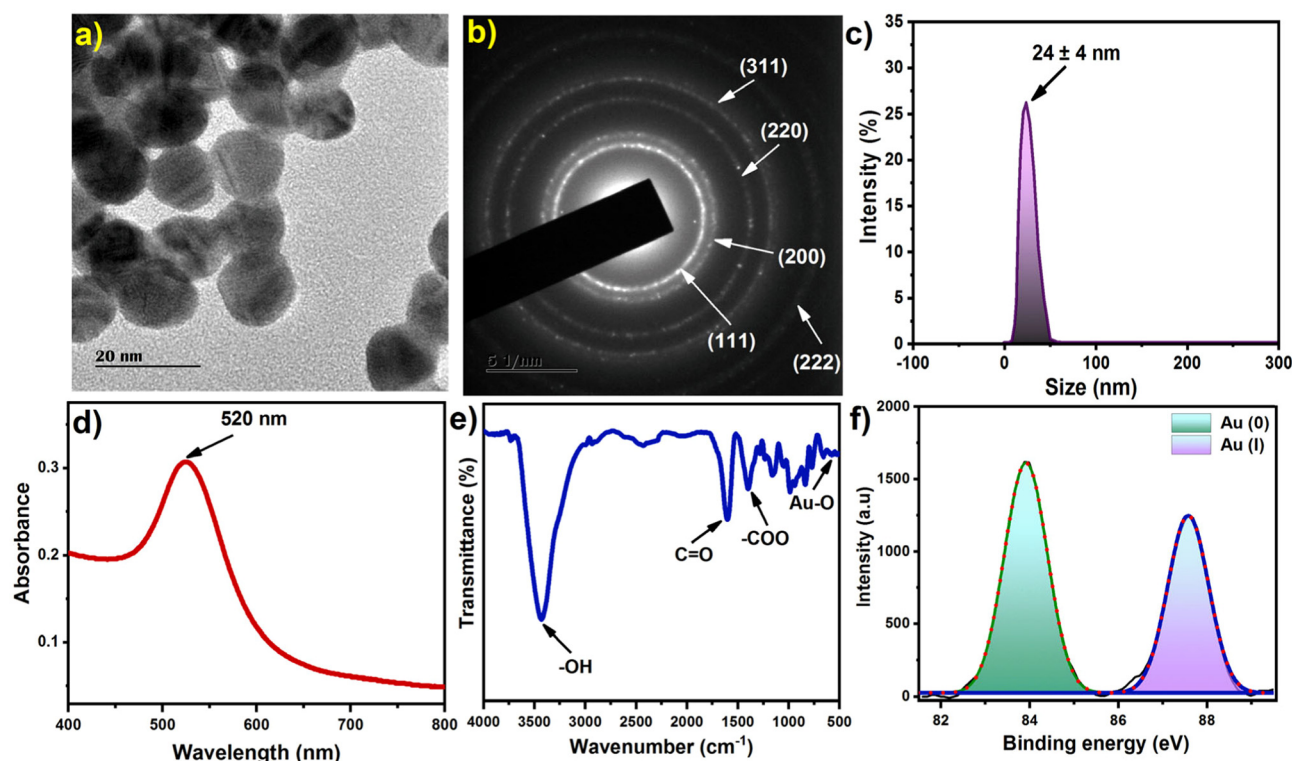


Fig. 1 Physicochemical characteristics of GNPs synthesized by the sodium citrate reduction method. (a) HR-TEM image, (b) SAED pattern, (c) DLS analysis, (d) UV-visible spectrum, (e) FTIR spectrum and (f) XPS analysis of the prepared GNPs.

the model substrate TMB using H_2O_2 .³³ This chemical reaction leads to the transformation of TMB from a colorless state to a blue-colored compound, Ox-TMB, which is characterized by a maximum absorbance wavelength at 652 nm, and the quantification of this reaction was performed using UV-visible spectroscopy. Through steady state kinetics, the catalytic efficiency of the GNPs on TMB and H_2O_2 was determined and subsequently compared with that of a standard enzyme, horseradish peroxidase (HRP). The optimum conditions for the catalytic reaction were ascertained by performing the reaction at varying pH values and time intervals. Under varying pH conditions, the catalytic activity steadily increased at acidic pH ranging from 6.5 to 4 and reached its maximum at pH 4. However, a reduction in catalytic activity was observed upon lowering the pH value beyond 4 (see Fig. S1†). As the reaction time increases, the catalytic activity of the GNPs exhibited an increasing trend and reached equilibrium after 15 min (Fig. S2a†). As a diamine compound, TMB exhibits stability at neutral pH, rendering it relatively less reactive with the GNPs. However, as the pH value decreases, the protonation of the amino group makes the TMB more reactive and increases the catalytic activity of the GNPs. Further lowering the pH value below 4 causes TMB to become more protonated, thereby reducing its solubility and reactivity.⁴² The optimum reaction time and pH for the GNPs to exhibit peroxidase-like activity were fixed to be 15 min and 4, respectively. The entire catalytic activity analysis was carried out at room temperature (27 °C).

To quantify the peroxidase mimic activity of the GNPs, the concentration of TMB was varied by fixing that of the GNPs (3.58×10^{-12} M) and H_2O_2 (5 mM). The resulting data were used to construct Michaelis-Menten (MM) kinetics and Lineweaver-Burk reciprocal plots (Fig. S2b-d†). The results showed that the GNPs exhibit higher catalytic activity towards TMB which was characterized by higher V_{max} and lower K_{m} values when compared to those of HRP (Table S2†). The K_{cat} value of the GNPs was calculated to be 1.041×10^6 , which surpasses most of the reported catalysts.

In the context of the lateral flow application, the suitability of the augmented nanozyme activity was further evaluated on a nitrocellulose (NC) membrane. Initially, varying concentrations of GNPs were deposited on the NC membrane and left to air dry. Subsequently, the optimized chromogenic substrate (TMB) concentration was applied to the GNP spot on the NC membrane. Following 10 min of incubation, a colored product was formed, and it was noted that the color intensity of the product changed in accordance with the concentration of GNPs. The signal enhancement (colored product) of the chromogenic substrate (TMB) was clearly observed only at higher GNP concentrations (Fig. S3†). At lower concentrations of GNPs, the formed colored product readily diffused in the membrane due to its soluble nature and resulted in a low signal intensity which cannot be visually observed. Moreover, the blue color of the oxidized product of TMB (ox-TMB) showed low contrast against the white background of the NC

membrane. Also, the susceptibility to color loss due to oxidation further restricts the effectiveness of TMB as a signal enhancer of LFA. To overcome the limitation of TMB, we used PPD as a chromogenic substrate to enhance the color signal intensity of LFA. Unlike TMB, the chromogenic form of PPD generates a dark brown color with significant contrast against the white background of the NC membrane. In the presence of H_2O_2 , gold nanozymes facilitate the catalytic conversion of monomeric PPD into diimine radical cations. Subsequently, the nucleophilic attack of PPD on one of the dimeric radical species leads to the formation of a trimeric product known as Bandrowski's base (BB). It has been reported that the oxidized diamine radical is capable of undergoing a coupling reaction with a coupler (aromatic compounds featuring electron-donating groups arranged in a *meta* position to each other, and they include *m*-phenylenediamines, resorcinol and naphthols). Interestingly, in the absence of couplers, the oxidation of PPD generates only BB (Fig. 2a).^{37,43} The resulting BB forms a stable dark brown precipitate with a maximum absorbance at 460 nm, making it a valuable signal enhancement substrate for LFA applications.^{36,44} The ideal parameters for the catalytic reaction of PPD were determined by conducting the reaction at different pH values and varying time intervals. The optimum time and concentration of PPD have been evaluated to be 10 min and 0.8 mM, respectively (Fig. 2b and c). The catalytic activity of the GNPs against PPD was evaluated using MM kinetics. The catalytic activity was higher at pH 4 with K_m and K_{cat} values of 0.6 mM and 2.905×10^{-4} , respectively (Fig. 2d and e). When compared with HRP, the GNPs showed higher K_m and K_{cat} values for PPD, making it an efficient chromogenic substrate for LFAs. When the optimized PPD concentration (0.8 mM) was added to the GNP spots on the NC membrane, it formed a brown colored product after 10 min with a more intense signal than TMB even at a low GNP concentration (Fig. S3†).

3.3. Optimization of PPD in the NC membrane

The behavior of the chromogenic substrate varies depending on the specific matrix, such as the nitrocellulose membrane commonly used in the LFA. Factors like membrane porosity, surface characteristics and interaction with PPD could influence the optimal concentration required for the LFA. Hence, the concentrations of PPD and H_2O_2 were systematically evaluated in the NC membrane and optimized (Fig. S4†). Initially, the PPD concentration was varied from 0.5 to 12.5 mM with a fixed concentration of the GNPs (0.1 nM). The result indicates a gradual increase in the dark brown signal intensity with 0.5–5 mM concentration of PPD. Furthermore, increasing the concentration beyond 5 mM did not exhibit a notable increase in the signal intensity. Then, the concentration of H_2O_2 was varied (1–100 mM) and optimized. Like PPD, the signal intensity gradually increased within the range of 1–50 mM H_2O_2 . However, increasing the concentration beyond 50 mM shows no significant change in the signal intensity. Thus, the optimized chromogenic substrate was taken as 5 mM and 50 mM of PPD and H_2O_2 , respectively.

3.4. Molecular dynamics simulation and *in vitro* binding efficacy of the COVID-19 spike protein with aptamers

On establishing the suitability of PPD as a better chromogenic substrate to enhance the color signal intensity, we have chosen COVID-19 spike protein as a model analyte to check the new substrate for the LFA. Aptamer 51 and aptamer 67 were selected for the detection of the COVID-19 spike protein. *In silico* analysis was conducted to assess the binding affinity and specificity of selected aptamers towards the spike protein. The stability profile of these aptamers was evaluated by predicting their secondary structure and sequence motif using the UNA fold web server at a temperature of 25 °C (Fig. S5†). The result showed high stability of aptamer 51 and aptamer 67 with the Gibbs free energy (ΔG) of $-7.28 \text{ kcal mol}^{-1}$ and $-5.21 \text{ kcal mol}^{-1}$ respectively. The tertiary structures of the aptamers were modelled and analyzed using 'RNA Composer' and 'Discovery Studio Visualizer' respectively (Fig. 3a and b). The three-dimensional conformation of the COVID-19 spike protein was retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (ID: 6VXX) (Fig. S6†). The molecular docking of the spike protein with the tertiary structure of aptamers was performed through multiple runs on the PatchDock server and the result showed the most favorable conformation of the aptamers based on scoring energy and the number of frequent occurrences (Fig. 3c and d). Also, the more negative ΔG confirms the stability of the aptamer and COVID-19 spike protein complex.⁴⁵

The effectiveness and translational potential of the selected aptamer for diagnosing the COVID-19 spike protein were assessed through *in vitro* techniques. A sandwich-type dot blot assay was performed to evaluate the binding efficacy of the aptamer to the COVID-19 spike protein under static conditions. Initially, aptamer 51 was immobilized on the NC membrane and the free sites were blocked using BSA. Then the COVID-19 spike protein was added to the immobilized aptamer 51. Then different concentrations of biotinylated aptamer 67 (0.1–0.01 μM) were added to form a sandwich type model. The addition of streptavidin-HRP to the sample resulted in the binding of biotin and streptavidin. Furthermore, the addition of a chromogenic substrate (TMB/ H_2O_2) to the sample resulted in the formation of a blue color due to the catalytic activity of HRP (Fig. 3e). This result is in accordance with our *in silico* analysis and confirms the binding efficacy of the aptamer to COVID-19 spike protein. The intensity of the blue color increases with an increase in the concentration of aptamer 67 which signifies the specific interaction between the aptamer and the spike protein. Furthermore, the binding efficacy of the aptamer and COVID-19 spike protein was evaluated in the LFA strips under non-static conditions. Then, aptamer 67 was immobilized on a NC membrane with a pore size of 10 μm at the test line. The spike protein conjugated with the GNPs (5–50 ng mL^{-1}) was added to the conjugation pad and the LFA strips were assembled accordingly. Upon the addition of 200 μL of running buffer to the conjugation pad, a distinct red color-

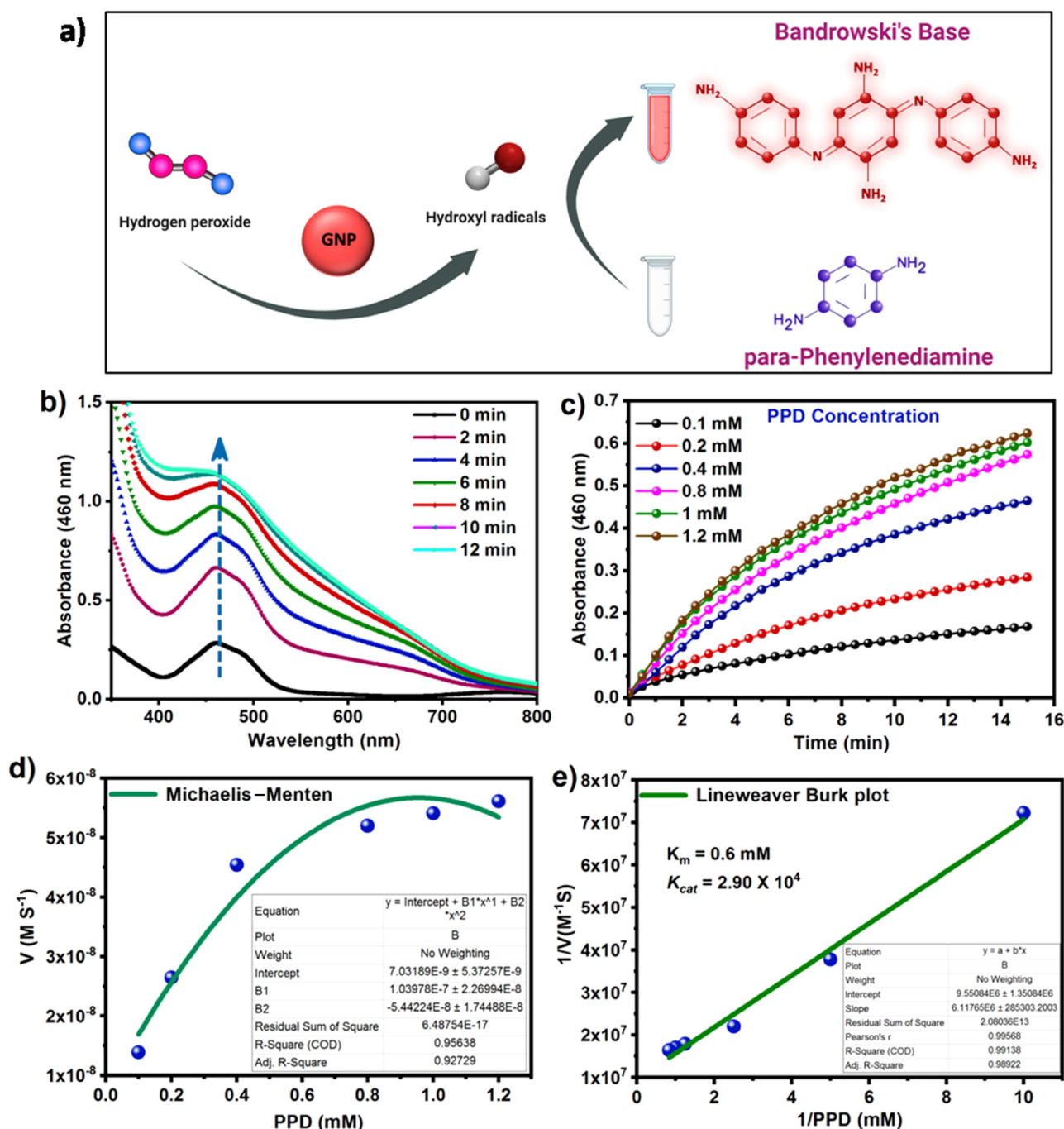


Fig. 2 Catalytic activity of GNPs on PPD as a chromogenic substrate. (a) Schematic representation of GNP-catalyzed oxidation of PPD with H₂O₂ to Bandrowski's base. Optimization of (b) time and (c) PPD concentration. (d) Plot of the initial velocity (V) against the PPD concentration and (e) double-reciprocal plot generated from (d).

ation was observed at the test line after 10 min. This red color signifies the strong binding affinity between the COVID-19 spike protein-conjugated GNPs and aptamer 51 (Fig. 3f). The intensity of red color varies with the concentration of spike protein conjugated to the GNPs. Furthermore, it was observed that the flow from the membrane did not wash off the aptamer from the test line. These results showed the translational approach for the real-time detection of COVID-19.

3.5. Functionalization of GNPs with aptamer 51

For the functionalization of GNPs with aptamer 51, the GNPs were initially functionalized with the carboxyl group using 16-mercaptohexadecanoic acid (16-MHDA).⁴⁶ The thiol group in 16-MHDA reacts with the GNPs and forms a covalent Au-S bond. The 5' amine-terminated aptamer 51 was further covalently linked to GNP-COOH using EDC and NHS chemistry.

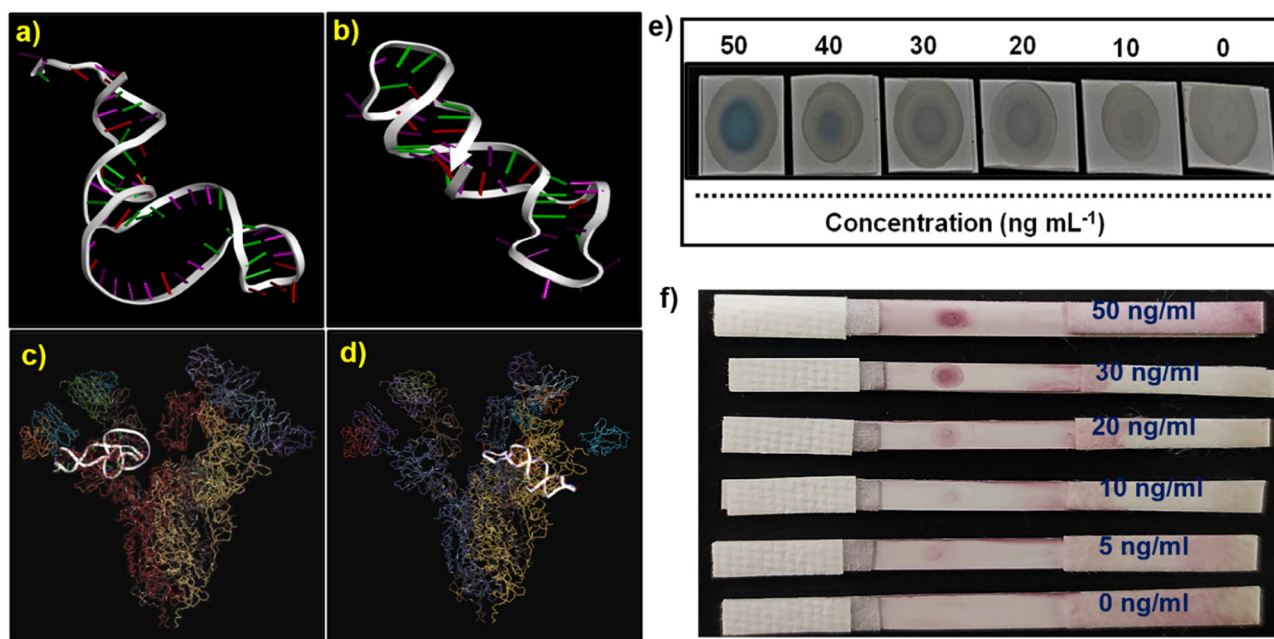


Fig. 3 Molecular simulation of aptamers with the COVID-19 spike protein. Tertiary structures of (a) aptamer 51 and (b) aptamer 67. Tertiary structures of the COVID-19 spike protein complex with (c) aptamer 51 and (d) aptamer 67. (e) Dot-blot assay of aptamers with the COVID-19 spike protein and (f) lateral flow assay of aptamers with the COVID-19 spike protein.

The amine group in the aptamer and the carboxyl group in the GNPs form a carbodiimide bond. The schematic representation of the GNPs functionalized with aptamer 51 is illustrated in Fig. 4a. The successful functionalization of the GNPs with aptamer 51 was confirmed *via* agarose gel electrophoresis (Fig. 4b and c). Under ambient light, the GNP fraction was visually identified as a distinct red band (lanes 3 and 4), while visualization of aptamer 51 was facilitated by ethidium bromide staining and UV light. A single band of free aptamer 51 was clearly observed in the UV light (Fig. 4c). Upon functionalization of aptamer 51 with the GNPs (lane 3), the band became invisible under UV light due to the well-known fluorescence quenching behavior of the GNPs. This functionalization resulted in reduced mobility of aptamer 51 through the gel matrix when compared to the free GNPs, indicating the successful functionalization. DLS analysis demonstrated an increase in the average particle size of the aptamer-architected GNPs from 24 nm to 32 nm (Fig. S7†). In the UV-visible spectra, the GNPs exhibited a prominent LSPR band at 520 nm. Subsequent functionalization with a COOH group led to a shift in the LSPR band of the GNPs from 520 to 527 nm. Upon functionalizing with the aptamer, the LSPR peak further red-shifted to 531 nm (Fig. 4d). The red shift observed during each functionalization step is ascribed to the alteration in the surface chemistry of the GNPs.

Functional group analysis of the aptamer-architected GNPs was carried out using FTIR spectroscopy. As shown in Fig. 4e, the GNPs showed prominent peaks at 1601, 1397 and 1156 cm^{-1} corresponding to COO^- stretching vibrations.³⁹ The

band at 780 cm^{-1} is ascribed to the metal-sulfur interaction (Au-S).^{40,47} The peak at 2550 cm^{-1} corresponds to the stretching vibration of the S-H band. After functionalizing the GNPs with 16-MHDA, the peak intensity of the carboxyl groups showed a marked increase. Moreover, the absence of the band corresponding to the S-H group suggests the effective surface functionalization of 16-MHDA over the surface of the GNPs.^{19,40} The FTIR spectrum of the aptamer-architected GNPs exhibits much sharper peaks at 1656 and 1541 cm^{-1} , which are attributed to the amine and heterocyclic moieties of nucleotide units in the aptamer structure, respectively. The multitude of peaks observed between 1200 and 800 cm^{-1} corresponds to the ribose and anti-symmetric stretching vibration of the phosphate groups present in the aptamer.⁴⁸ All these results clearly suggest the successful functionalization of the aptamer over the surface of the GNPs.

3.6. Detection of COVID-19 spike protein using LFA

LFA test strips were assembled as shown in Fig. S8† and tested for their efficiency to detect COVID-19 spike proteins. The signal amplification principle of the PPD chromogenic substrate on LFA strips is schematically depicted in Fig. 5a. A standard of the COVID-19 spike protein with concentrations ranging from 0.1 to 50 ng mL^{-1} was prepared and analyzed using LFA strips.

Before and after the addition of the chromogenic substrate (PPD), the strips were photographed with a digital camera and the color intensity of the test spot was quantified. The intensity of the test line increased with the concentration of COVID-19 spike protein. Before signal amplification by PPD, a

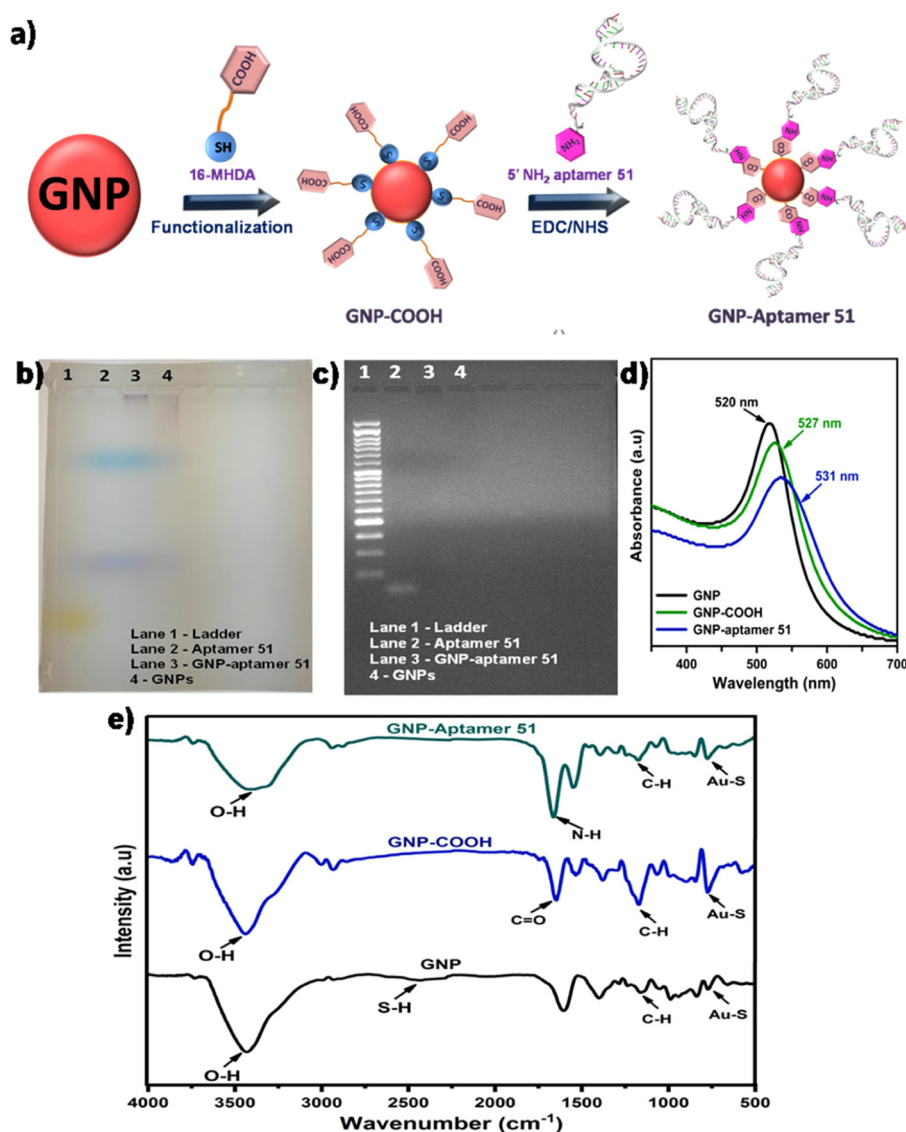


Fig. 4 Functionalization of aptamer 51 with GNPs. (a) Schematic representation of GNPs functionalized with aptamer 51. (b) and (c) Agarose gel images of GNP-functionalized aptamer 51. (d) UV-visible spectra and (e) FTIR spectra of GNP-functionalized aptamer 51.

red spot on the test line (GNP plasmonics) could be visualized up to 4 ng mL^{-1} (Fig. 5b). After the addition of PPD, the signal was significantly enhanced, and a dark brown spot could be visualized up to a concentration of 200 pg mL^{-1} (Fig. 5c). This result demonstrates that the addition of PPD enhanced the visual detection limit by 20-fold when compared to the conventional GNP-based LFA. Subsequently, a calibration curve was plotted by measuring the color intensity of each strip at different concentrations of COVID-19 spike protein before and after signal amplification (Fig. 5d and e). After the addition of the PPD chromogenic substrate, the signal intensity of the strips increased rapidly at low concentrations, reaching equilibrium at 10 ng mL^{-1} . The developed LFA kit showed good linearity between 200 and 2000 pg mL^{-1} ($R^2 = 0.9855$) with an LOD of $168.39 \text{ pg mL}^{-1}$. It is noteworthy that the sensitivity of the LFA strip with GNP-functionalized aptamer 51 rivals the detec-

tion sensitivity of some laboratory instrument-based assays, such as ELISA. This finding indicates that incorporating a chromogenic substrate improves the LOD of the LFA test. Table S3† compares the LOD of the prepared PPD-based LFA for the SARS-CoV-2 spike protein with previously reported values. The current PPD-based LFA sensor showed a lower LOD than most of the previous reports. Although the fluorescence⁴⁹ and SERS-based LFA⁵⁰ methods showed lower LOD (0.1 ng mL^{-1}) than the current PPD-based LFA (0.16 ng mL^{-1}), the high cost of operation and the need for specialized equipment made them less user-friendly. The selectivity of the PPD-based sensor was analyzed using different interferences like BSA, HSA, MERS-S1 and rhinovirus. The prepared LFA strips effectively distinguish the COVID-19 spike protein among other interferences at a concentration of 5 ng mL^{-1} (Fig. 5f). This high selectivity of the LFA is due to the specificity of the

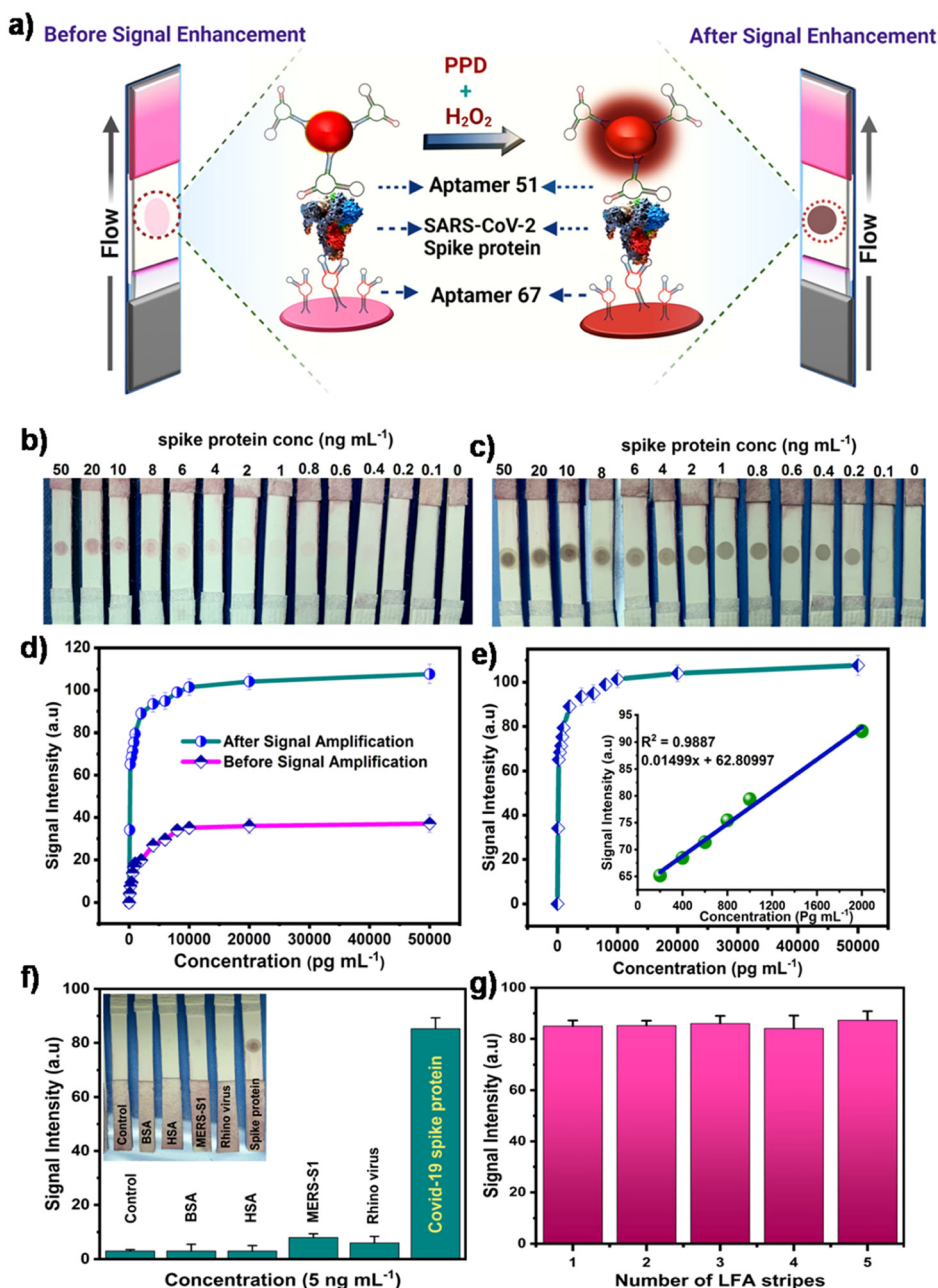


Fig. 5 Diagnosis of the COVID-19 spike protein using the PPD-based LFA kit. (a) Schematic representation of the principle of the developed PPD-based LFA kit in detecting the COVID-19 spike protein and its signal enhancement. Photographs taken from the LFA strips (b) before and (c) after amplification using PPD as a chromogenic substrate and its respective signal intensity profiles (d) before and (e) after amplification. The inset graph depicts the linearity range of the PPD-based LFA kit against the COVID-19 spike protein. (f) Selectivity analysis of the PPD-based LFA kit using different interference agents (5 ng mL⁻¹). (g) Repeatability performance of 5 individual test strips against the COVID-19 spike protein (5 ng mL⁻¹). The error bar represents the SD of measurement from 5 independent strips.

employed aptamers. The repeatability of the PPD-based LFA strips was studied with 6 independent experiments (Fig. 5g). The result showed an SD of 1.56%, suggesting excellent repeatability of the prepared LFA kit for the COVID-19 spike protein.

The traditional interpretation of the LFA results relies on visual inspection of the color intensity, which can be subjective and prone to errors, particularly for individuals without a

scientific background. So, we have developed a mobile application that offers an ingenious solution to this limitation by providing a simple, user-friendly interface that quantifies the color intensity of the LFA test lines into numerical values, thereby empowering ordinary individuals to perform reliable COVID-19 testing in a self-administered manner. The detailed information of mobile application development and sample

analysis using the developed mobile application are given in ESI S2.† By incorporating this semi-quantitative approach, our method offers a more accurate and reliable means of assessing the viral load in patients, facilitating improved disease management and treatment decisions. The analysis of the COVID-19 spike protein was conducted utilizing both the mobile applications and ImageJ software. The obtained R^2 value of 0.9989 demonstrates consistency between the two methods, indicating non-significant discrepancies between the measurements obtained from ImageJ and the mobile application (Fig. S10†). These findings underscore the potential of the PPD-based LFA for clinical applications, particularly for the semi-quantitative detection of the COVID-19 spike protein. The subsequent section of this manuscript leverages this mobile app-based semi-quantitative analysis.

3.7. Protein spiked sample analysis

To validate the diagnostic potential and analytical performance of the developed PPD-based LFA kit, the collected nasopharyngeal (NPh) swab samples from healthy volunteers were immersed in the universal transport medium (UTM). Varying concentrations of the COVID-19 protein (100–2000 pg mL⁻¹) were spiked into the UTM and analyzed using the prepared LFA strip as mentioned earlier. After signal amplification was achieved using PPD, the obtained results were subsequently quantified using a mobile app. In order to evaluate the efficiency and reliability of the LFA kit, the spiked protein in the NPh sample was validated with the standard ELISA test. The results of the LFA test demonstrated a strong agreement with the ELISA results, as evidenced by a correlation coefficient of 0.9949 (Fig. S11†). The obtained results displayed similar values to the ELISA result with minor variation with a recovery percentage of 98% in the spiked UTM (Table 1). These findings provide compelling evidence that the PPD-based LFA results are highly reliable and hold significant promise for potential clinical applications.

3.8. Validation of the technique in clinical samples

In order to showcase the clinical viability, practicality and translation potential of the PPD-based LFA kit, we conducted tests on NPh swab samples collected from COVID-19 patients ($n = 25$) who were confirmed positive through RT-PCR. The results obtained from the PPD-based LFA kit were compared with the cyclic threshold (CT) value of RT-PCR. Among the 25 RT-PCR positive samples, our testing kit effectively identified

23 of them as positive, yielding an overall sensitivity rate of 92% (Table S4†). The false-negative results for the remaining two positive samples could be attributed to the concentration of viral load being lower than the LOD of the present method. Regarding the negative samples ($n = 10$), which included patients with symptoms caused by common seasonal viruses, the LFA test exhibited an overall specificity of 100%. This high specificity indicates its capability to differentiate between infections caused by common seasonal viruses and those caused by SARS-CoV-2. This exceptional specificity is attributed to the selective nature of the aptamers employed in the process. In addition, the results of LFA were compared with the CT value of RT-PCR. The higher CT value represents the lower viral load and the lower CT value represents the higher viral load. From the LFA result, the samples with low CT values exhibit higher signal intensity and the sample with a higher value showed low signal intensity. When the signal intensities of the COVID-19 positive samples were compared, nearly 10 patient samples showed higher signal intensities (<95), indicating that their viral loads were above 2 ng mL⁻¹. Among the other 12 positive samples, the viral load was within our detection limit and quantified (Table S4†). Furthermore, we compared the efficiency of the prepared PPD-based LFA kit with the COVID-19 antigen test kit available in the market. Out of 8 positive samples, the commercial kit detected only three, whereas the current PPD-based LFA kit showed 7 positive results with an overall sensitivity of 87.5%. The enhanced accuracy of the PPD-based LFA kit is attributed to the specificity of the aptamers used. The clinical comparison conducted further validates the sensitivity of our PPD-based LFA kit when compared to the currently available LFA kit in the market. These results strongly support the suitability of our PPD-based LFA kit for commercialization and clinical applications.

4. Conclusion

In conclusion, we have developed an innovative LFA platform that addresses the pressing challenges hindering the widespread adoption and commercialization of LFAs in clinical diagnosis. By integrating PPD as a chromogenic substrate, our engineered LFA platform achieves enhanced sensitivity, specificity, and semi-quantitative detection capabilities. The developed assay demonstrated exceptional sensitivity for COVID-19 spike proteins, achieving a LOD of 168 pg mL⁻¹. The signal amplification strategy using PPD in the LFA exhibited a sensitivity of 92% and a specificity of 100% in detecting COVID-19 spike proteins from clinical samples, emphasizing its significant potential for clinical translation. The entire assay process, inclusive of a 5-minute signal amplification step, can be completed within a short timeframe of 15–20 minutes. To facilitate spike protein quantification, a dedicated mobile application, tailored for this purpose, has been integrated. Our PPD-based signal enhancement approach effectively addresses the primary drawbacks of traditional LFAs, establishing it as a reliable technique for point-of-care detection of COVID-19 and

Table 1 Recovery percentage of the COVID-19 spike protein in protein spiked NPh samples ($n = 3$)

COVID-19 spike protein (pg mL ⁻¹)	Detected spike protein concentration of LFA (pg mL ⁻¹)	Percentage recovery (%) (mean ± RSD) ($n = 3$)
200	206.2	103.1 ± 6.19
500	520.73	104.14 ± 8.03
1000	984.73	98.47 ± 9.04
2000	1954.5	97.72 ± 8.04

other pathogens. By overcoming the key limitations in LFA-based diagnostics, our research contributes to the transformative journey of bio-medical sensors, positioning our innovative platform for industry integration and commercial success.

Author contributions

Elangovan Sarathkumar: methodology, experiments, data analysis, and writing of the original draft. Kunnumpurathu Jibin: experiments and review of the original draft. Subramani Sivaselvam: writing of the original draft. Arumugam Selva Sharma: methodology and review of the original draft. Vincent Alexandar: mobile app design. AN Resmi: experiments. Poornima Velswamy: mobile app and review of the original draft. Ramapurath S. Jayasree: conceptualization, resources, reviewing, editing, and supervision.

Data availability

All data that support the findings of this work are included within the article and its ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors acknowledge the financial assistance received from various government agencies in India. Support was provided by the Science and Engineering Research Board (SERB), Government of India, under the grant DST(CVD/2020/000778), and the Life Science Research Board, Defence Research and Development Organisation, Government of India, under the grant LSRB/01/15001/LSRB-412/SH&DDI2023. ES and AS acknowledge the financial support from the Science and Equity, Empowerment and Development (SEED) Division, Department of Science and Technology, Government of India, through the project SEED/SCSP/2019/117. SS acknowledges the Department of Science and Technology (DST), Science and Engineering Research Board (SERB) and the Government of India for the financial support of the N-PDF fellowship (PDF/2022/002036).

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