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An ultra-sensitive and specific nanoplasmonic-enhanced isothermal amplification platform for the ultrafast point-of-care testing of SARS-CoV-2

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ABSTRACT

The novel mutations attributed by the high mutagenicity of the SARS-CoV-2 makes its prevention and treatment challenging. Developing an ultra-fast, point-of-care-test (POCT) protocol is critical for responding to large-scale spread of SARS-CoV-2 in public places and in resource-poor remote areas. Here, we developed a nanoplasmonic enhanced isothermal amplification (NanoPEIA) strategy that combines a nanoplasmonic sensor with isothermal amplification. The novel strategy provides an ideal easy-to-operate detection platform for obtaining accurate, ultra-fast and high-throughput (96 samples can be tested together) data. For clinical samples with viral detection at Ct value <25, the entire process (including sample preparation, virus lysis, detection, and data analysis) can be completed within six minutes. The method is also appropriate for detection of SARS-CoV-2 γ-coronavirus mutants. The NanoPEIA method was validated using clinical samples from 21 patients with SARS-CoV-2 infection and 31 healthy individuals. The detection result on the 52 clinical samples for SARS-CoV-2 showed that the NanoPEIA platform had a 100% sensitivity for N and orf1ab genes, which was higher than those obtained using RT-qPCR (88.9% and 90.0%, respectively). The specificities of 31 clinical negative samples were 92.3% and 91.7% for the N gene and the orf1ab gene, respectively. The limits of detection (LoD) of the clinical samples were 28.3 copies/mL and 23.3 copies/mL for the N gene and the orf1ab gene, respectively. The efficient NanoPEIA detection strategy facilitates real-time detection and visualization within ultrashort durations and can be applied for POCT diagnosis in resource-poor and highly populated areas.

1. Introduction

The outbreak and spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) posed a major threat to global public health [1–3]. As of April 29, 2022, 510,270,667 COVID-19 cases had been diagnosed globally, including 6,233,526 deaths, according to the WHO [4]. The disease has affected human health considerably, in addition to having major negative impacts on the global economy. The high mutation rate of the novel coronavirus, with the rapid emergence of β, γ, δ, and 28.3 copies/mL for the N gene and 23.3 copies/mL for the orf1ab gene, respectively. The efficient NanoPEIA detection strategy facilitates real-time detection and visualization within ultrashort durations and can be applied for POCT diagnosis in resource-poor and highly populated areas.

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involved repeated heating and cooling, in addition to requiring bulky, expensive tools, as well as specific cryogenic instruments for reagent preservation [5–7]. Consequently, the method has limitations with regard to rapid high-throughput field screening of viruses in public places or rapid detection in remote areas.

Based on the current diagnostic-methods, there is an urgent need to develop affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free, and deliverable platforms. Notably, the isothermal amplification technique has shown great potential in POCT diagnostics owing to its high sensitivity, specificity, and instrument simplification features [9–12]. The recombinase polymerase amplification (RPA) assay is an isothermal amplification system [10]. The amplification of the DNA in the RPA relies on enzymes and proteins to replace the repetitive cycles of three different temperatures (94 °C, DNA denaturation; 50–60 °C, primer annealing; 72 °C extensions) of the PCR technique. The reverse transcription-enzymatic recombinase amplification (RT-ERA) method, developed by GenDx Biotech, is an improved version of the recombinase polymerase amplification (RPA) [10,13,14]. Feng et al. reported that the RT-RPA/Cas12a assay can successfully detect 200 or more copies of the S gene sequence of SARS-CoV-2 RNA within 5–30 min in a one-tube reaction [15]. Sun et al. combined OR-DETECTR technology and the lateral flow assay to achieve a detection limit of 2.5 copies/µL for COVID-19 and a detection time of 50 min in single tube reaction [16]. However, various amplification and detection reagents should be added before each detection. Moreover, the detection reagents (LbCas12a and 0.65 µM crRNA) should be incubated at 37 °C for 30 min, and then stored in a refrigerator. Step-by-step operation is laborious and prone to error. Chen et al. developed a single copy sensitivity assay for the SARS-CoV-2 virus by combining RT-ERA with lateral flow (LF) strips [17]. The opvCRISPR was developed by combining loop-mediated isothermal amplification (LAMP) with the CRISPR reporting system, and requires 45 min for SARS-CoV-2 detection, without accounting for the sample processing time [18]. The commercial ID NOW COVID-19 [19] and Abbott Diagnostics method can be used to detect the RdRP gene using 100–200 copies/µL within 13 min [20]. Such detection methods typically require nucleic acid extraction and pre-amplification. Most workflows still require addition of various amplification reagents separately and expensive and cumbersome equipment that constitute complex procedures, which increase the probability of cross-contamination due to false-negative cases.

Presently, some studies have applied isothermal amplification technologies combined with biosensing for SARS-CoV-2 nucleic acid detection. Nouri et al. developed the solid-state CRISPR-Cas12a-assisted nanopore (SCAN) sensing strategy for the specific detection of SARS-CoV-2. This method may acquire a limit of detection of 13,500 copies/µL of viral RNA at a confidence level of 95% in 30 min [21]. Zhu et al. reported the sensitivity of 240 copies/µL for SARS-CoV-2 using a one-step RT-LAMP-assisted nanoparticle biosensor with a detection time of 1 h [22]. The deCOViD method (microfluidic digital chips) based on CRISPR/Cas technology can achieve qualitative detection in < 15 min and quantitative detection in 30 min with 1 genome equivalent (GE)/µL of SARS-CoV-2 RNA and 20 GE/µL of heat-inactivated SARS-CoV-2 [23]. However, the combination of isothermal amplification and biosensing requires advanced technologies and complex operations that cannot be used widely. The limitations of such approaches include the sample destruction, material scarcity, unstable signals, specific viral strain identification, and semiconductor defects [24]. Moreover, it is challenging to achieve real-time and visual real-time performance simultaneously with such platforms, and they have limited sensitivity and longer detection times. The application of biosensors in nucleic acid detection is currently limited to laboratory settings.

POCT diagnosis requires only simple materials to prepare miniaturized instruments, and simple operation steps replace large, expensive, and cumbersome laboratory equipment. Such a tool is necessary for nucleic acid testing in response to emergencies in large public facilities and in remote areas where resources are scarce. Previous reports have described several advanced POCT strategies for SARS-CoV-2 diagnosis. For example, the SHERLOCK was combined with miniaturized instruments to construct a low-cost sHERLOCK platform for fluorescent visual POCT diagnosis [25]. Tang et al. reported a new CLIP-ON strategy using CRISPR and large DNA assembly-induced pregnancy bands to detect HPV and SARS-CoV-2 nucleic acid [26]. To date, there have been many reports on POCT diagnosis; however, there are few homemade pre-use nucleic acid detection platforms that can integrate reagent pre-freeze drying and simplify nucleic acid extraction operations, simultaneously achieving real-time and visual multi-flux detection through portable instruments. To achieve portable and simple viral nucleic acid detection with simple sample addition operations, and combining visualization and real-time POCT diagnosis of SARS-CoV-2 virus nucleic acid for home “pre-use”, we developed a nanoplasmmonic-enhanced isothermal amplification (NanoPEIA) viral nucleic acid detection platform based on an asymmetric ERA reaction. The platform is constructed by thiolated forward primers (F-SH primers) and a modified nanoplasmonic array chip sensor (NanoACS) surface, which is an ultrastable, ultrasensitive, specific, and high-throughput POCT for real-time and visual detection of SARS-CoV-2. The workflow of a direct SARS-CoV-2 nucleic acid testing assay is illustrated in Scheme 1. It integrates high-temperature viral lysate and lyophilized reagents in a high throughput closed tube amplification device to establish a preliminary portable POCT diagnosis platform. The NanoPEIA method has been validated by testing 52 clinical nasopharyngeal swab samples and results consistent with that of the RT-qPCR method were obtained. Based on traditional POCT, the NanoPEIA platform can address the issue of low-temperature preservation of reagents via pre-freeze-drying of reagents. The portability of a simple nucleic acid extraction device may provide the basis of development of a novel visual or real-time nucleic acid detection platform for home use.

2. Experimental section

2.1. Materials

Tris (2-carboxyethyl) phosphine hydrochloride (TECP, 98.0%) and 6-Mercapto-1-hexanol (MCH, 98.0%) were purchased from Sigma-Aldrich. The fluorescence ERA (No. KS103) and RT-ERA (No. ZK009) reaction kits were purchased from GenDx Biotech (Suzhou, China). The Mag-MK Virus RNA Extraction Kit (NO. B518767), magnetic separation device (NO. B518800), One-Step RT-qPCR Probe Kit (NO. B6392778), DEPC-treated water (DEPC, NO. B501005), 1 × TE buffer (NO. B548106), and RNase inhibitor (RNaseI) (NO. B600478) were purchased from Sangon Biotech (Shanghai, China). Nuclease-free water was purchased from TINGEN (RT-121, Beijing, China). SARS-CoV-2 RNA standard (NO.HZ2101262/004) was purchased from SIMT (Shanghai, China). SARS-CoV-2 𝜌-activated virus strain (ZK009) and 𝜌-activated virus strain (ZK009), human SARS, and human influenza A viruses (H1N1, H3N2, H5N1, and H7N9 subtypes) were purchased from Bio come (Nanjing, China). Conventional primer, thiolated primer, fluorescence resonance energy transfer (FRET), probe, and plasmid DNA (orf1ab and N gene) associated with the SARS-CoV-2 virus were synthesized by Sangon Biotech (Shanghai, China).

2.2. Fabrication of nanoplasmonic arrays chip sensor (NanoACS) and NanoACS characterization analysis

The nanoplasmonic arrays chip sensor (NanoACS) used in this study adopts our previous preparation method [18]. The NanoACS was prepared by depositing 9 nm Titanium (Ti) and 70 nm gold (Au) onto nanoplasmonic arrays. The NanoACS film was prepared by nanoimprinting. The NanoACS comprised a nanoplasmonic array chip with a height of 500 nm, an upper diameter of 200 nm, and a lower diameter of 180 nm. The original mold uses laser interference lithography and ion etching to form conical nanoplasmonic arrays on silicon oxide thin sheets. Polyethylene
terephthalate (PET) was used as solid-phase support material and the special structure of the original mold was copied onto the PET film by UV irradiation for 3 min, after which the PET sheet was carefully peeled off. This was followed by the deposition of 9 nm Ti and 70 nm Au onto the nanocrystalline array by RF plasma sputtering [27]. Scanning electron microscopy (SEM; Quanta 3D FEG SEM/FIB, FEI, USA) was employed to examine the morphologies of the nanoplasmonic arrays.

2.3. NanoACS surface functionation with thiolated primers and synthesis of NanoPEIA.

The NanoACS functionalization was investigated according to our previous research; for the microplate type-NanoPEIA device, thiolated primers (Table S1) were activated with 10 μM TCEP, and 100 μL of 1 μM activated primers were added to NanoACS place in 12 h at 4 °C, thiolated primer was fixed on NanoACS by Au-S bond. Free thiolated primers were removed before the plates were cleaned thrice with nuclease-free water. After thiolated primer modification, NanoACS functionalized microplates were prepared by blocking the unbound site with 100 μL of 10 μM MCH, the uncombined MCH was removed before the plates were cleaned thrice with nuclease-free water for later use. For tube cap or bottom-type device, 2 μL of 132 nM thiolated primers (activated using 10 μM TCEP) were fixed on the central position of the NanoACS using the 10 μL pipettor and place in 12 h at 4 °C. The free thiolated primers were washed thrice using nuclease-free water. Finally, 100 μL of 10 μM MCH was used to keep in dark place in 1 h at 25 °C. Free MCH was cleaned thrice using nuclease-free water.

To further verify the feasibility of the NanoPEIA detection platform, we synthesized SARS-CoV-2 plasmid DNA (3.54 × 10^3 copies/mL and 3.54 × 10^2 copies/mL) as a template and compared the results with those of the F-SH primers (Group1) (See the text for detailed steps). DNA and RNA templates revealed that the F-SH primer-modified NanoACS (Group1) had the optimal sensitivity for low concentration detection (Figure S3, 4). Consequently, we selected Group1 for use in the construction of the NanoPEIA platform in the follow-up experiment. The amplification efficiency of the synthesized plasmid (from 1.94 × 10^5 copies/mL to 1.94 × 10^3 copies/mL for the N gene; from 3.50 × 10^5 copies/mL to 3.50 × 10^2 copies/mL for orf1ab gene) was thoroughly tested based on the microwell plate type-NanoPEIA device.

2.4. Sensitivity, universality, and specificity evaluation of the NanoPEIA

The sensitivity of the NanoPEIA method was evaluated using RNA extracted from β- and γ- inactivated viruses. Under the above optimization conditions, 10-fold serial dilutions of β- and γ- inactivated SARS-CoV-2 virus were diluted using nuclease-free water (3.8 × 10^3 copies/mL to 3.8 × 10^2, and 76 copies/mL). According to the manufacturer’s instructions, the total RNA of β- and γ- inactivated SARS-CoV-2 virus was...
extracted from 200 μL of the above diluents using the Mag-MK Virus RNA Extraction Kit, respectively. The extraction method of cross-inactivated specimens of the human SARS virus and human influenza A viruses (H1N1, H3N2, H5N1, and H7N9 subtypes) were the same as above. The RNA was stored at −80 °C until further use. The RNA was enriched in 40 μL nuclease-free water before utilization in evaluating the specificity and effectiveness of the present RT-ERA assay.

2.5. Evaluation of NanoPEIA method using clinical samples

Fifty-two clinical nasopharyngeal swab samples were collected from BIOER Technology (Hangzhou, China) and UNION Hospital Medical.

Fig. 1. Characterization of nanoplasmonic array chip and SARS-CoV-2 nucleic acid testing using NanoPEIA. (a) Asymmetric ERA amplification reaction process on NanoACS by functionalization of the F-SH primer. (b) Scanning electron micrograph (SEM) image of the NanoACS without F-SH primer modified in top view. (c) SEM images of the NanoACS with and without F-SH primer modified in top view. (d) SEM image of the NanoACS without F-SH primer modified in sectional view. (e) Amplification curve of low concentration plasmid DNA template of optimized different testing platforms using different primers process NanoACS, including (1) the F-SH primers modified NanoACS platform, (2) modified without NanoACS platform (forward primer, 420 nM), (3) R-SH primer modified NanoACS platform, (4) F-SH primer modified non-nanostructured gold film platform, (5) F-SH primers and R-SH primers modified NanoACS platform, (6) ordinary 96-well plate amplification platform, and (7) without primers modified NanoACS platform (F-SH primer, 132 nM), for orf1ab plasmid target detection at low concentrations of $3.54 \times 10^3$ copies/mL and $3.54 \times 10^2$ copies/mL. (f) Comparing the detection sensitivity of NanoPEIA and RT-ERA for SARS-CoV-2 RNA standard orf1ab gene, (1) represents NanoPEIA and (2) represents RT-ERA. (g) Relative fluorescence intensity values (RFU (×10^5)) of different target concentrations for the SARS-CoV-2 RNA standard orf1ab gene at 20 min, NanoPEIA (red), and RT-ERA (black). (h) The detection sensitivity of the NanoPEIA platform based on the microwell plate device for SARS-CoV-2 N gene RNA standard following 10-fold dilution.
nucleic acid was eluted in 50 μL of nuclelease-free water and stored at –80 °C until use. RT-qPCR was performed using a One-Step RT-qPCR Probe Kit (NO. B639278) according to the manufacturer’s instructions. Clinical samples were positive when PCR gave rise to reliable signals (Ct value < 38) for either or both genes [28].

A series of 10 positive samples (S4-S13) were prepared in dilution solution and tested for the dynamic range and detection limit of the NanoPEIA. Besides, we compared the specificity of the NanoPEIA with the RT-qPCR. To examine the validity and reliability of the proposed NanoPEIA, three positive samples with different concentrations of the virus were diluted to varying degrees.

2.6. Visual detection and POCT application scenarios with NanoPEIA

Conditions for virus lysis and lyophilization and the reagent details are listed in Supporting Information (S1). The reaction device (tube cap or bottom type–NanoPEIA device) was placed in a constant temperature water bath at 39 °C and incubated for 30 min. For the tube cap type–NanoPEIA device, it was briefly centrifuged after the amplification, and the whole reaction system was centrifuged to the bottom of the tube as far as possible for the convenience of color comparison. The reaction of the tube-bottom amplification device can be directly colorimetric under LED blue light and UV light (details see Supporting Information).

To meet the needs of POCT diagnosis, we made the following improvements based on the above experiments: (1) instrument miniaturization, (2) optimization of the freeze-drying conditions of reagents, (3) simplification of sample adding operation, (4) replacement of traditional nucleic acid extraction methods with virus lysis using the high-simplification of sample adding operation, (4) replacement of traditional nucleic acid extraction methods with virus lysis using the high-simplification of sample adding operation, (5) construction of a closed tube NanoPEIA amplification device. Details are provided in Supporting Information.

3. Results and discussion

3.1. Construction of NanoPEIA sensor platform for SARS-CoV-2 nucleic acid detection

To better explain the working principle of the system, we preliminarily clarified NanoPEIA performance from an asymmetric ERA reactions perspective. A previous study reported that upon fixing the positive primers on the chip surface, the amplification efficiency in asymmetric RPA reaction can be enhanced [29]. As shown in Fig. 1a, we used F-SH primers to modify the NanoACS via Au-S bond to perform an asymmetric ERA reaction. In the present study, the ERA reaction system in which the concentration of forwarding F-SH primer (132 nM) was lower than that of reverse primer (420 nM) (Figure S2, Table S2), was referred to as an asymmetric ERA reaction. According to the asymmetric ERA reaction we designed, subsequent asymmetric ERA reactions almost occur almost on the F-SH functionalized NanoACS surface. As the reaction proceeds, the number of amplicons on the surface of the functionalized NanoACS increase exponentially. Fluorescence Resonance Energy Transfer (FRET) probes (design principle of FRET probe was shown in Figure S3) were more likely to find complementary DNA sequences. In addition, under the action of Exo III, FAM fluorescence groups were released, leading to emission and detection of the green fluorescence signal. The intensity of the fluorescence signal was correlated with template concentration in the reaction system, and the template concentration can be evaluated based on the fluorescence intensity. The asymmetric ERA amplification process based on NanoACS proceeded in the following manner. First, the entire asymmetric ERA amplification was carried out on the NanoACS functionalized with F-SH primers. Secondly, in the initial stage of the reaction, the recombinant enzyme formed a recombinant enzyme/primer complex with reverse primers and F-SH primers modified on the NanoACS, which was targeted to identify homologous dsDNA, wherein SSB protein binds to the replaced DNA strand making the established D-loop environment stable. Third, after activation of the strand displacement reaction, strand displacement polymerase initiates synthesis followed by mother chain separation and continuation of the synthesis process. Two F-SH primer-complemented double chains were assembled on the NanoACS and released a fluorescent group into the solution. With the continuous occurrence of the reaction, the NanoACS gathered numerous dsDNA, and the same mass of fluorescent groups appeared in the solution. Finally, after the termination reaction, high amounts of dsDNA molecules gathered on the NanoACS, and numerous FAM fluorescent groups were released into the solution, which enabled the detection of the fluorescence signals on a detection instrument. The concentration of target DNA in the reaction system was quantitatively estimated based on the strength of the fluorescence signals.

The nanoplasmonic array chip on a polymer substrate was prepared using the periodic Au NanoACS structure (diameter: 200 nm, depth: 500 nm, periodicity: 400 nm), which contained 9nm Ti and 70nm Au [27]. The scanning electron microscope (SEM) images of NanoACS without F-SH primers modified is shown in Fig. 1 b (top view) and Fig. 1 d (sectional view), and the SEM image of NanoACS with 132 nm F-SH primers modified showed that the lower part of the NanoACS became more blurred after the F-SH primer modification, when compared with the upper part of the NanoACS without F-SH primer modification (Fig. 1 c). This is due to the negative charge on the primers. After the primers were modified to NanoACS, the negatively charged F-SH primers were adsorbed on the gold surface (similar to formation of an insulating layer), which weakened the ability of electrons to move toward the gold atoms and weakened the conductivity of NanoACS. The weaker charge resulted in blurred image, which indirectly indicated that the F-SH primer had successfully modified the surface of NanoACS. As shown in Fig. 1 b-d, the SEM images of NanoACS all showed the NanoACS had a distinct cup array structure. According to the above chip morphology characterization, the NanoACS structure is similar to the simulation results, and the chip preparation process we used is consistent with the previous report [27,30].

To achieve the optimal amplification efficiency for the asymmetric ERA reaction, we further optimized the material thickness of NanoACS. As shown in Figure S3, upon experimenting with different materials at different thicknesses, 70 nm Au and 9 nm Ti provided the most ideal amplification effect. According to the metal characteristics, we inferred that Au and Ag thickness were the major factors affecting the amplification efficiency of the experiment, wherein with the higher thickness of Au, higher the amplification efficiency was achieved. This may be related to the easier heat transfer of Au compared to Ag. Based on the objective of achieving high amplification efficiency at the lowest cost, we finally chose 9 nm Ti + 70 nM Au as our final specification for developing the plasma-thermal sensing chip.

Under the optimal reaction conditions and in the microwell plate type-NanoPEIA device based on a 70-μL reaction system (Fig. 1e), seven primers treated groups were designed to determine the optimal asymmetric ERA reaction conditions using low concentrations of plasmid DNA as a template (3.54 × 10^5 copies/mL and 3.54 × 10^6 copies/mL). As shown in Fig. 1e, Group 1 was used for subsequent experiments, after being established as having the optimal performance detection during assay development (see Figure S5 (plasmid DNA) and Figure S6 (RNA standard as template)). The platform generated was named NanoPEIA. Subsequent experiments were carried out under the following optimal conditions: (1) reaction buffer, distilled deionized water (DDW), and 1 × TE (containing 0.5 U RNase Inhibitor) for DNA and RNA, respectively; (2) amplification temperature, 37 °C for DNA and 39 °C for RNA. The details are provided in Figure S7 in Supporting Information.
To systematically evaluate the role of NanoACS, orf1ab gene and N gene of RNA standard (10^5 to 10^2 copies/mL) at 10-fold dilution were used as the amplification templates, and the detection sensitivity was compared between the NanoPEIA and ordinary RT-ERA in a 96-microwell plate. The results showed that the amplification curve of the NanoPEIA group was significantly higher than that of the common 96-microwell plate group for the same concentration template. (1) Fig. 1f is based on the microwell plate type-NanoPEIA (NanoPEIA) group and (2) the ordinary 96-well microwell plate (RT-ERA) group (Fig. 1f). As shown in Fig. 1g, the RFU (%×10^5) values of the NanoPEIA group are significantly higher than that of the ordinary 96-microwell plate group (RT-ERA) based on the same concentration of target RNA standard (orf1ab gene) at 20 min. It further demonstrates that the NanoPEIA can indeed significantly improve the amplification performance of RT-ERA. To further confirm the feasibility of the NanoPEIA method, we designed the same experimental validation for N gene of RNA standard at different target concentrations (from 10^5 copies/mL to 10^2 copies/mL). The result shows that the amplification effect has a similar performance via NanoPEIA for N gene of RNA standard (Fig. 1h), indicating that the established NanoPEIA method is feasible for use in the detection of SARS-CoV-2 virus nucleic acid.

3.2. The sensitivity, universality, and specificity evaluation of NanoPEIA

To validate the sensitivity of the NanoPEIA platform for inactivated virus samples, the NanoPEIA was applied for the detection of β-inactivated SARS-CoV-2 virus, and β-activated SARS-CoV-2 virus was used as the positive control. As shown in Fig. 2a and 2d, the detection sensitivity of the N gene and orf1ab gene was 76 copies/mL. This is consistent with the results obtained for the plasmid DNA (Figure S8), the overall trend of the real-time amplification curve shows the required detection time sharply decreases with an increase in sample concentration.

To further verify the universality of the NanoPEIA platform for the detection of SARS-CoV-2, the N and orf1ab genes in SARS-CoV-2 γ-variant (initial concentration from 10^5 to 10^6, 76 copies/mL) was analyzed. As shown in Fig. 2b and 3e, the lowest concentration of N and orf1ab genes (SARS-CoV-2 γ-variant) at a detection limit of 76 copies/mL was obtained by NanoPEIA, suggesting that NanoPEIA could be used for the direct detection of SARS-CoV-2 mutant strains.

We subsequently investigated the selectivity of NanoPEIA detection, which is important for improving detection accuracy. Total RNA was extracted from other cross-inactivated virus (H3N2, H1N1, H5N1, H7N9, and SARS) samples were used and their N and orf1ab genes were detected to demonstrate the specificity of the NanoPEIA system. As shown in Fig. 2c and 2f, the fluorescence curves of these viruses are horizontal lines and closed to the negative sample lines. In contrast, the fluorescence curves of NanoPEIA in the β-inactivated SARS-CoV-2 group increased with time, indicating that the NanoPEIA had high specificity for SARS-CoV-2 detection.

To further verify the detection accuracy of the NanoPEIA platform assay for different types of SARS-CoV-2 samples, traditional RT-qPCR testing was carried out using RNA extracted from SARS-CoV-2 β-inactivated virus and γ-mutated strain, as shown in Figure S9. The NanoPEIA showed results comparable with traditional RT-qPCR in the aspects of accuracy, specificity and sensitivity, whereas the NanoPEIA platform provided a rapid, efficient and cost-effective method for SARS-CoV-2 detection.

3.3. The performance validation of NanoPEIA in clinical samples

To confirm the appropriate cutoff value for the NanoPEIA platform, the detection accuracy for 52 clinical samples was investigated to evaluate the performance of NanoPEIA. The 52 clinical samples were nine N and 10 orf1ab gene samples were strong positive, 12 N gene and...
11 orf1ab gene samples were suspected positive, and 31 genes samples were negative, when RT-qPCR was applied. The cutoff value of RFU \((\times10^5)\) (the mean of negative samples plus thrice the standard deviation) was calculated as 1.683 for the N gene and 1.6001 for the orf1ab gene. All nine N gene and 10 orf1ab gene samples that were strong positive samples based on RT-qPCR had RFU \((\times10^5)\) values > 1.683 and 1.6001 (Fig. 3a and 3b), respectively. All negative samples via the NanoPEIA detection were below the cutoff line, indicating that the developed NanoPEIA can detect SARS-CoV-2 N gene and orf1ab gene-positive samples.

To further evaluate the convergence-positive accuracy of the NanoPEIA method, eight N gene-positive and nine orf1ab gene-positive samples, 13 N gene and 12 orf1ab gene negative but clinically suspected samples (tested by RT-qPCR) were revalidated by NanoPEIA. The \(\chi^2\) test showed that the P-value of the McNemar test was 1.0 for the N gene and orf1ab gene, and the value obtained with the kappa test was 0.901 \((P < 0.001)\) for the N gene and 0.904 \((P < 0.001)\) for orf1ab gene (Fig. 3c). Therefore, there was no statistically significant difference between the results of NanoPEIA assay and hospital RT-qPCR tests. The results from NanoPEIA were highly consistent with those of RT-qPCR. Besides, one of the 13 N genes clinically suspicious positive Samples was negative in the RT-qPCR test; conversely, it was SARS-CoV-2 N gene-positive in the NanoPEIA assay. The 12 orf1ab gene suspected positive samples detected by RT-qPCR were tested to be SARS-CoV-2 orf1ab gene-positive by the NanoPEIA assay. The results suggested that the sensitivity of the NanoPEIA method is higher than that of the RT-qPCR; however, the specificity is slightly lower than that of RT-qPCR.

The \(\chi^2\) test analysis was used to calculate the sensitivity and specificity of the NanoPEIA and RT-qPCR methods (Table S3). In the NanoPEIA, all 21 positive samples showed 100% sensitivity (N and orf1ab gene); however, the sensitivity of RT-qPCR to the 21 clinical samples...
were 88.9% for the N gene and 90.0% for the orf1ab gene. The specificity of NanoPEIA for the 31 clinical negative samples was 92.3% for the N gene and 91.7% for orf1ab gene; in the RT-qPCR test results, all 31 clinical negative samples had 100% specificity. Hence, the sensitivity of the NanoPEIA method was higher than that of RT-qPCR, whereas the specificity of the NanoPEIA method was lower than that of RT-qPCR. According to the receiver operating characteristic (ROC) curves of N and orf1ab genes (Fig. 3d and 3e), the sensitivity and specificity were comprehensively considered. The results showed that the areas under the curve (AUC) of RT-qPCR and NanoPEIA for N gene amplification were 0.9985 and 0.9585, respectively. There was no significant difference in the area under the curve (AUC) of N gene amplification between the two methods. Similarly, the orf1ab gene amplification by RT-qPCR test (AUC = 0.9846) and NanoPEIA test (AUC = 0.9601) were not significantly different. The sensitivity across studies of the NanoPEIA for identifying SARS-CoV-2 was 1.00 (95% CI, 0.60–1.00) and 1.00 (95% CI, 0.63–1.00), respectively (Table S4). A p-value of the sensitivities of the NanoPEIA was calculated as 0.4286 for the N gene and 0.4762 for the orf1ab gene. The specificities were 0.92 (95% CI, 0.62–1.00) for N gene and 0.91 (95% CI, 0.60–1.00) for orf1ab gene (Table S4); the corresponding p values were 0.5714 for N gene and 0.5238 for orf1ab gene. In other words, in terms of sensitivity and specificity, the NanoPEIA method is comparable to the performance of traditional gold standard RT-qPCR. To accurately calculate the LoD value of clinical samples, a series of different concentrations (from $10^5$ to 10 copies/mL) of SARS-CoV-2 virus RNA were determined via the NanoPEIA method (Fig. 3f and 3g). The LoD was 28.3 copies/mL for N gene and 23.3 copies/mL for the orf1ab gene. Compared with the RT-qPCR analysis, the proposed NanoPEIA exhibited good performance for SARS-CoV-2 virus RNA detection with higher sensitivity and broader applications, meeting clinical requirements.

To further investigate the diagnostic sensitivity and reliability of the
NanoPEIA platform, we randomly selected three positive samples with different Ct values. Depending on the Ct values, the SARS-CoV-2 virus was diluted on a 10-fold gradient with nuclease-free water, and the RNA extracted from the above-diluted virus was tested on the NanoPEIA platform. As shown in Fig. 4a–f, the amplification efficiency of the orf1ab gene was significantly higher than that of the N gene in all three samples. This was consistent with the previous validation with RNA extracted from inactivated viruses. Moreover, we quantified respiratory swab samples from strong positive samples (9 N gene and 10 orf1ab gene) (S4–S13) based on the Ct values and the RT-qPCR standard curve (Fig. 4g and 4h), and diluted them serially using nuclease-free water. We extracted the SARS-CoV-2 viral nucleic acids from the diluted samples and then measured them using RT-qPCR (NO. B639278). The signal samples, which remained undetected by RT-qPCR, were captured using our method at low concentrations. As a comparison, RUF ($10^3$) responses to the addition of the diluted samples occurred even when the concentration decreased to nearly 30 copies/mL in nuclease-free water (Fig. 4g and 4h). The results showed no statistically significant difference in sensitivity between the NanoPEIA and RT-qPCR groups; however, the results of the NanoPEIA method are more reliable.

Rapid assays for SARS-CoV-2 diagnosis have been developed recently. Nucleic acid testing is regarded as the gold standard for SARS-CoV-2 diagnosis, owing to its high accuracy [31,32]. The NanoPEIA method has been compared with isothermal amplification and biosensor detection methods (Table S5) and SARS-CoV-2 assays (Fig. 4i), including RT-qPCR [31,33–35], China National Medical Products Administration (NMPA) [36] and US Centers for Disease Control (CDC)-approved RT-qPCR [37], clustered regularly interspaced short palindromic repeats (CRISPR) [18,38–42], recombinase polymerase amplification (RPA) [12,43,44], reverse transcription loop-mediated isothermal amplification (RT-LAMP) [12,45–48], biosensor [49–51], and SHERLOCK [25,41,52]. As in Table S5 displays, our work is significantly superior to the current isothermal amplification method for SARS-CoV-2 nucleic acid in sensitivity, specificity, detection time, and high throughput performance. The NanoPEIA method may be advantageous in that it offers rapid detection of SARS-CoV-2 nucleic acids, ultrafast detection, ultra-sensitivity, specificity, high throughput, easy operation, portability, real-time detection, and visual POCT diagnosis.

3.4. POCT diagnosis and visual detection

POCT is a simple, scalable, and time-saving alternative for testing viruses in low-income regions, as well as a promising tool for at-home testing. Consequently, the POCT diagnostics device can facilitate SARS-CoV-2 detection as well as in the surveillance of asymptomatic individuals in such localities, which could further facilitate the prevention of the spread of the virus from. Although we have demonstrated the performance of real-time detection of SARS-CoV-2 nucleic acid based on a high-throughput-based device, a visual or real-time POCT diagnosis platform for SARS-CoV-2 virus nucleic acid diagnosis based on portable instruments is yet to be developed.

To effectively apply the real time and visual detection using the NanoPEIA platform (Fig. 5b) in POCT diagnosis, we integrated the mini chip into a disposable PCR tube. We further optimized the lyophilization conditions of the reagent to avoid freezing during storage and allow portability of the reagent in the short term, and finally simplified the viral nucleic acid extraction operation. Firstly, a sample nucleic acid extraction device with a closed tube can be developed by combining lysis virus (95 °C, 5 min) (Fig. 5a). Secondly, to meet different detection requirements of users, we integrated the mini chip into the cap (named the tube cap type-NanoPEIA device) and the bottom of the PCR tube (named the tube bottom type-NanoPEIA device) (Fig. 5c and 5d). The tube cap type-NanoPEIA amplification device is mainly used for colorimetric analysis, the bottom-tube NanoPEIA amplification device is mainly used with portable real-time detection instruments to meet the real-time detection requirements of POCT diagnosis. Finally, all reagents of the NanoPEIA assay can be lyophilized and stored in a disposable PCR tube (Fig. 5e and Figure S10), which eliminates the need for cold chains and tedious multiple open tube operations, and enables rapid detection outside a laboratory setting. The detailed operation is outlined in supporting information.

At present, the POCT diagnostic platform based on NanoPEIA requires some portable devices, such as heat block, centrifuge, a vortex, and a detection device; however, the devices are generally small instruments. The small instruments are easy to carry in the field (we plan to integrate all the equipment into a portable suitcase for outdoor use). Conversely, conventional qPCR platforms require some expensive and professional large-scale instruments, such as qPCR instruments, large centrifuges, and refrigeration equipment, which cannot be taken out for POCT diagnosis at any time, and can only be used in a laboratory. It has previously been reported that a SHERLOCK test can be redesigned and synthesized for as low as $0.61 per test [54]. Therefore, in terms of cost, CRISPR-based diagnostic tests are valuable candidates as point-of-care diagnostic tests. However, equipment such as vortexes, heaters, and mini centrifuges required by our POCT diagnostic platform based on the NanoPEIA assay basically cost tens to thousands of dollars (the most expensive portable fluorescent thermostatic amplifiers only cost approximately $2,000). With reagents and other costs, the POCT diagnostic platform based on NanoPEIA can be performed at the rate of about $1 per sample. Therefore, our study preliminarily evaluated the NanoPEIA method and simulated POCT for detecting of the SARS-CoV-2 virus (Scheme S1).

To understand the visualization and real-time detection of the results based on the above optimization conditions, we further simplified the detection equipment and instruments and carried out experimental verification in a sequential manner. The orf1ab gene RNA standard was amplified in the PCR tube without NanoACS (RT-ERA) and the tube cap type-NanoPEIA device with the NanoACS via functionalization of F-SH primer. We compared the detection sensitivity of RNA standard amplification based on the tube without NanoACS (RT-ERA) and the tube cap type-NanoPEIA device, using visualizing readout under UV and LED blue light (Fig. 5f and 5g). The group without NanoACS showed no significant difference between the low concentration ($10^3$ and $10^2$ copies/mL) and the NTC group (Fig. 5f). The low concentration RNA standard ($10^3$ and $10^2$ copies/mL) was amplified with the tube cap type-NanoPEIA device based on NanoPEIA amplification to produce a distinct green fluorescent signal (Fig. 5g). To evaluate the validity of the NanoPEIA platform, we adapted the above reaction device to detect viral RNA extracted from the SARS-CoV-2 virus in β-inactivated virus (orf1ab gene) (Fig. 5h) and γ-inactivated mutant strain (N and orf1ab gene) (Fig. 5i and 5j). The evaluation results from visual detection were consistent with that of the high throughput real-time NanoPEIA. Crucially, we successfully demonstrated an instrument-portable NanoPEIA assay for SARS-CoV-2 detection in β- and γ-inactivated mutant strains with a simple heater.

To meet the need for POCT diagnosis, the feasibility of the NanoPEIA platform using a miniaturized portable real-time instrument was tested. The same concentration of target RNA from β-inactivated virus lysis (95 °C, 5 min) (Fig. 5a) was used to verify the reliability of the instrument in tube bottom type-NanoPEIA device with lyophilized reagents (Fig. 5e). By comparing the amplification efficiency of RNA extracted by magnetic beads and RNA was released by direct high-temperature lysis, we found that the detection time of RNA released by high-temperature lysis was shorter than that of RNA extracted by magnetic beads (Table S6). This may be because some chemical reagents still affected RT-ERA amplification in the magnetic beads extraction process. The details of the lyophilized reagents and operation are provided in Supporting Information (Figure S10). In Fig. 5k and 5l, the lysis virus releases RNA (95 °C, 5 min) that binds the lyophilized reagents, with green fluorescence still being observed for low concentrations of RNA ($10^3$ and $10^2$ copies/mL) through visual detection protocols. The results preliminarily demonstrate the feasibility of high-temperature lysate viral and
Fig. 5. POCT diagnosis and visual detection. (a) RNA was released by a simple nucleic acid extraction device (at 95 °C for 5 min). (b)-(d) Schematic diagram of three NanoPEIA amplification devices. (b) Microwell plate type-NanoPEIA device. (c) Tube cap type-NanoPEIA device. (d) Tube bottom type-NanoPEIA device. (e) Three NanoPEIA sample addition methods are as follows: microwell plate type-NanoPEIA amplification platform, tube cap type-NanoPEIA amplification platform, and tube bottom type-NanoPEIA amplification platform, respectively. (f) Visual detection for RNA standard orf1ab gene without NanoACS (RT-ERA). (g) Visual detection for RNA standard orf1ab gene based on tube cap type-NanoPEIA device. (h) Visual detection for RNA orf1ab extracted from β-inactivated virus, γ-inactivated virus (I) N, and (j) orf1ab gene based on tube cap type-NanoPEIA device. (k) POCT visual detection of the SARS-CoV-2 β-inactivated virus orf1ab was using tube cap type-NanoPEIA device and (l) based on tube bottom type-NanoPEIA device. (m) The feasibility of POCT detection suitcases was verified through real-time detection of high-throughput microwell plate type-NanoPEIA device by Multimode Plate Reader. (n) POCT real-time detection of the SARS-CoV-2 β-inactivated virus orf1ab was using tube bottom type-NanoPEIA device by portable real-time instrument.
lyophilized reagents. Subsequently, we compared the real-time detection performance of the multimode plate reader and portable isothermal instrument. The RNA obtained from high-temperature lysates of SARS-CoV-2 β-actin-inactivated virus was used as a template, 3.80 × 10^7 to 76 copies/mL templates were amplified for real-time detection using a multimode plate reader at 39°C in the microwell plate type-NanoPEIA device with lyophilized reagents. According to the results, the 76 copies/mL orf1ab gene group is slightly higher than the NTC group (Fig. 5m and 5n). This result is consistent with that of the magnetic beads RNA extraction group and that of the group without the lyophilized reagents. The NanoPEIA platform can pre-freeze all amplification reagents (except template and supplementary buffer) in advance, and all reagents are added at one time and then pre-freeze dried. This pre-freeze-drying of powder can be stored at 37°C for a week without deterioration. In the case of the NanoPEIA platform, only the template and supplementary buffer need to be added during detection. The whole system can be supplemented to 50 μL with supplementary buffer for on-machine detection. This is very essential for the construction of POCT diagnostic platform. The above optimization results demonstrate that the NanoPEIA is a potential POCT tool for SARS-CoV-2 virus nucleic acid detection.

4. Conclusions

In the present study, we have successfully developed a NanoPEIA platform, which exhibits highly sensitive, ultrafast, high throughput, and reliable diagnostic capacity for SARS-CoV-2 virus detection. The NanoPEIA biosensing system integrated a nanoplasmonic sensor with asymmetric isothermal amplification via thiolated forward primer functionalization on the NanoACS surface to enhance ERA amplification efficiency. The NanoPEIA platform’s superior performance is mainly manifested in sensitivity, stability, reliability, high throughput, and short readout time. It has the universality to cross virus specificity and γ-inactivated mutant strains. This platform has a sensitivity of 100% for N and orf1ab genes when compared with those of hospital qPCR tests, and has limits of detection of 28.3 copies/mL for the N gene and 23.3 copies/mL for the orf1ab gene. The NanoPEIA exhibits ultrafast detection performance, which generates faster results in 6 min from sample preparation to analysis in clinical samples with Ct < 25. Moreover, even with a single copy of viral RNA, results can be obtained in <30 min, highlighting its impressive testing speed. We preliminarily explored a potential POCT detection suitecase, which provides a strong foundation for POCT layout and nucleic acid detection in remote areas with scarce resources in future. Overall, this POCT platform can be used for large-scale field testing and could facilitate the prevention and management of the spread of COVID-19 or other infectious diseases.

Author contributions

J.X. L. performed all the experimental data collection. Gang L. L. conceived the study, supervised the study, and revised the manuscript. X.H. X. and P. C. conducted statistical analysis. P. W., L. P. H., and W.J. H. revised the manuscript. H. X., Z.G., and L.W. assisted with collection of clinical samples. All authors participated in manuscript editing and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supplementary data

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