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Paper-based netlike rolling circle amplification (NRCA) for ultrasensitive and visual detection of SARS-CoV-2

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\textsuperscript{1} COVID-19 is a highly diffuse respiratory infection caused by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2). Currently, quantitative real-time polymerase chain reaction (qRT-PCR) technology is commonly used in clinical diagnosis of COVID-19. However, this method is time-consuming and labor-intensive, which is limited in clinical application. Here, we propose a new method for the ultrasensitive and visual detection of SARS-CoV-2 viral nucleic acid. The assay integrates with a paper device and highly efficient isothermal amplification technology - Netlike rolling circle amplification (NRCA), which can reach a limit of detection of 4.12 aM. The paper-based NRCA owns advantages of specificity, portability, visualization and low-cost. Therefore, this method can effectively meet the requirements of point-of-care testing, providing a novel molecular detection technology for clinical diagnosis of COVID-19 and promoting the development of NRCA devices.

1. Introduction

COVID-19 is a highly diffuse respiratory infection caused by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), which has spread around the world since 2019, seriously threatening the public safety [1–4]. The virus has been confirmed to spread from person to person through droplets, contacts, parasites, etc [5]. The virus genome includes a 5’ untranslated region, replicase complex (ORF1ab), Spike gene (S gene), E gene, M gene, N gene, 3’ untranslated region and several non-structural open reading frames [6]. For the prevention and control of the spread of COVID-19, effective and accurate nucleic acid detection technology has played a huge role. Currently, quantitative real-time polymerase chain reaction (qRT-PCR) technology is commonly used clinically in clinical diagnosis and screening of suspected cases [7,8]. However, this method is time-consuming and labor-intensive, required highly skilled operators, and needs expensive thermal cycling equipment, which is limited in clinical application [9]. In the case of a large number of suspected cases, asymptomatic infection cases and close contacts for testing and screening, as well as nucleic acid testing with limited laboratory capacity areas, there is an urgent need to develop a rapid, sensitive, low-cost and easy-to-operate detection method [10,11].

In recent years, isothermal nucleic acid amplification technology has been widely used for rapid detection of virus-specific genes and other nucleic acid molecules [12–16]. At present, loop-mediated isothermal amplification (LAMP) [17,18], DNA strand displacement amplification (SDA), clustered regularly interspaced short palindromic repeats (CRISPR)-mediated isothermal amplification technology [19] and nick endonuclease amplification reaction (NEAR) [20] have been successfully used in SARS-CoV-2 RNA detection [21]. Compared with other isothermal amplification techniques, NEAR is more convenient in primer design and has a high-speed reaction [22]. Based on the existing NEAR methods, we have developed netlike rolling circle amplification (NRCA), which avoids non-specific amplification and has a lower cost comparing with other NEAR technologies [23]. Furthermore, as a derivative technology of rolling circle amplification (RCA) [24,25] and hyperbranched rolling circle amplification (HRCA), NRCA retains the advantages of simplicity, sensitivity and low-cost, realizing further signal amplification based on the exponential amplification, which has been successfully used to detect tumor biomarker (protein and micro-RNA) [26,27].
Here, in order to meet the needs of rapid and effective point-of-care (POC) diagnostics, we propose a new method for the ultrasensitive and visual detection of SARS-CoV-2 viral nucleic acid (RNA-dependent RNA polymerase-RdRp gene) through the integration with a paper device and NRCA. By adopting padlock probe and nucleic acid amplification, this method can realize specific and sensitive detection of SARS-CoV-2 nucleic acid. Also, paper sensors do not require expensive large-scale equipment and manufacturing costs, which can easily immobilize protein complexes such as enzymes or antibodies [28,29]. Therefore, based on the advantages of efficient amplification in NRCA, the paper sensor can effectively meet the requirements of POC testing (POCT), providing a novel molecular detection technology for SARS-COV-2 nucleic acid, and promoting the development of NRCA devices.

2. Experimental section

2.1. Materials and reagents

All oligonucleotides used in this research (Table S1) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) with HPLC purification. Bst DNA polymerase, DNA polymerase I (Klenow fragment exo-), Nicking endonuclease (N.BsrDI and N.BbvCI) containing Cutsmart buffer, T4 DNA ligase (containing 10 × T4 DNA ligase reaction buffer) were all purchased from New England Biolabs (USA). Phi29-DNA polymerase (containing 10 × phi29 DNA polymerase buffer) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Agarose was purchased from Baygene Biotech Co., Ltd. (Beijing, China). 5 × loading buffer was purchased from Generay Biotech (Shanghai, China) Co., Ltd. Deoxynucleotide triphosphates solution mixture (dNTP) mixture and DiaSpin DNA Gel Extraction Kit were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). 100 bp and 10 bp DNA marker were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). SYBR Green I (10,000 × ) was purchased from Solarbio (Beijing, China). Hieff qPCR SYBR Green Master Mix was purchased from YEASEN (Shanghai, China). SARS-CoV-2 RNA transcribed in vitro standard material was obtained from Shanghai institute of metrology and testing technology (the sequence position and concentration are supplied in Table S2). Pullulan was purchased from Sigma-Aldrich. The chemical reagents used in the experiment are all of analytical grade. All solutions were prepared with Milli-Q water (18.2 MΩ·cm<sup>-1</sup>) from a Milli-Q purification system (Millipore, USA).

2.2. Circularization of padlock probe

The ligation reaction was carried out in a 40 μL solution containing 100 nM padlock probe, different concentrations of target, 100 U of T4 DNA ligase, 1 × T4 DNA ligase buffer. The reaction was incubated at 25 °C for 60 min and finally incubated at 65 °C for 20 min to terminate the ligation reaction. The ligation reaction solution was ready for use. In the exonuclease treatment, 20 μL of ligation product, 10 U of exonuclease I, and 50 U of exonuclease III were added to the digestion reaction system. The reaction was incubated at 37 °C for 120 mins and finally incubated at 80 °C for another 20 mins to stop the digestion reaction.

2.3. NRCA reaction

40 μL ligation product, 200 nM Primer 1, 5 nM Primer 2, 200 μM dNTPs, 2.5 U of DNA polymerase and 10 U of Nicking endonuclease were added to the NRCA reaction system. Double distilled water was added to make up the total volume of the reaction system to 50 μL. After that, the NRCA reaction system was performed on a ThermoStat plus (Eppendorf, Germany) at 30 °C for 4 h and incubated at 95 °C for another 10 min to terminate the reaction.

2.4. Gel electrophoresis analysis

1% agarose gel electrophoresis was conducted for the characterization of the product of NRCA. 1 μL of 5 × loading buffer (containing Gelred nucleic acid dye) was added to 4 μL of each of the NRCA reaction products. The loading volume of the 10 bp DNA marker was 2 μL. The electrophoresis experiments were carried out in 1 × TAE (Tris-acetate-EDTA) at 80 V for 30 min 15% polyacrylamide gel electrophoresis was also performed for the characterization of the circularization of padlock probe. 2 μL of 5 × loading buffer (containing Gelred nucleic acid dye) was added to 10 μL each of the ligation products. The loading volume of the 10 bp DNA marker was 2 μL. The electrophoresis experiments were carried out in TBE (Tris-boric acid-EDTA) at 120 V for 60 min. The Bio-Rad GelDoc XR gel Imaging System was used to take pictures, record and observe the amplified bands for analyzing the electrophoresis results. Subsequently, the electrophoresis results were analyzed by Bio-Rad GelDoc XR gel Imaging System.

2.5. Fluorescent detection

1 μL 1000 × SYBR Green I was added to the 50 μL NRCA products and the mixture was incubated at room temperature for 10 min. Then fluorescence spectra were obtained using a SpectraMax M3 Multi-Mode Microplate Reader. The excitation wavelength was 490 nm and the spectra were measured in the range from 500 nm to 650 nm. The fluorescence intensity was recorded at the emission wavelength of 520 nm.

2.6. qRT-PCR

qRT-PCR primers were designed according to the sequence of SARS-COV-2 target (RdRp-COV RNA mimic). The qRT-PCR reaction was performed in a 25 μL system containing different concentration of target, 200 nM stem loop primer, 200 nM forward primer, 200 nM reverse primer, 1 × Hieff qPCR SYBR Green Master Mix. The thermal cycle of qRT-PCR was 95 °C for 5 min, 40 cycles of (95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s) on CFX-96 Real-Time System (Bio-Rad, USA).

2.7. Atomic force microscope (AFM) characterization of NRCA products

The NRCA products were firstly separated using gel extraction kit to remove enzymes, dNTP and buffers. Then 10 μL of purified DNA fragments and 10 μL of 20 mM MgCl<sub>2</sub> solution were mixed and placed on the mica slice (Zhongjingkeyi Technology Co., Ltd., Beijing). After incubation for 20 min at room temperature, the mica slice was rinsed with water for 30 s and blown completely dried with nitrogen gas. Morphological analysis of the NRCA products on the mica slice was achieved by using an ex situ Agilent 5500 AFM system. The scan rate was 0.5–1 Hz in a tapping mode and AFM tips with resonant frequency was in a range 160–260 kHz. Images were acquired at a resolution of 512 × 512 pixels.

2.8. Preparation of the paper sensors

The ColorQube 8570N solid wax printer was used to neatly print wax in each test area with a diameter of 4 mm on the nitrocellulose membrane (Millipore HF180). Then the printed paper was heated in the ESCO Forced Convection Laboratory Oven at 120 °C for 2 min to melt the wax. After heating, the obtained paper chip was immersed in 1 × PBS (pH = 7.4, 5% BSA) solution for 15 min and washed with ultrapure water 2–3 times. Next, the pullulan solution (10%, wt/v) was printed on the paper and dried at room temperature to form the blank film. Then the NRCA reaction system (padlock probe, Primer 1, Primer 2, T4 DNA ligase, dNTP, Klenow fragment exo- and Nb.BbvCI), SYBR Green I and 15% pullulan solution were printed on the blank film to form the bioactive film. After drying, the papers were pasted onto glass slides for later use.
3. Results and discussion

3.1. The principle of NRCA for detection of SARS-COV-2 nucleic acid

Firstly, we designed a padlock probe that is complementary to the specific sequence of SARS-COV-2 target (RdRp-COV RNA mimic), which could form a closed circular template under the function of T4 DNA ligase (Scheme 1). Then, with the addition of primer 1 and primer 2 to the system, primer 1 can complement to the circular template and extend by DNA polymerase to generate multiple single-stranded linear copies of the original circular template, the process of which is known as rolling circle amplification (RCA). Reverse primer 2 can bind complementary to the extended products of primer 1, and further undergo branched strand extension and strand replacement reactions by DNA polymerase, namely hyperbranched rolling circle amplification (HRCA). Under the function of the nicking endonuclease, the HRCA products at a specific site are specifically recognized and cut into small fragments, which are combined with the template as a new primer triggering new HRCA reaction. Thus, a cascade of primer extension, strand displacement and nicking reaction can be achieved to obtain higher amplification efficiency, which enable sensitive detection of low abundance of SARS-COV-2 nucleic acid.

3.2. Detection of SARS-COV-2 nucleic acid by NRCA in tube

Polyacrylamide gel electrophoresis was also performed for the characterization of the circularization of padlock probe. In the presence of the target RNA, the circular DNA template can hybridize to the target RNA, causing the migration rate of the ligation product bands to be significantly lower than that of the single padlock probe and target RNA (Fig. 1A). After exonuclease treatment, it can be observed that the hybridized band of padlock and target RNA still exist and any non-circularized probe as well as the target RNA is removed. The results showed that the circular padlock probe obtained by ligation reaction has stable structure and is not easily digested by exonuclease, which could be used as specific template for subsequent amplification reaction. The performance of NRCA for the analysis of SARS-COV-2 nucleic acid is verified by fluorescent detection, as shown in Fig. 1B. It can be seen significant fluorescent signal when padlock probe, target, primer 1 and primer 2 are present. Additionally, the performance of NRCA and primer generation-rolling circle amplification (PG-RCA) are also compared here [30]. The result shows that the fluorescent intensity of NRCA is higher than PG-RCA when the primer 2 is absent, indicating NRCA owns higher amplification efficiency that can realize detection of SARS-COV-2 nucleic acid. The morphological analysis of the NRCA products was performed using AFM, which exhibits dense and uniform network of structure in Fig. 1C. After diluting the NRCA products, the branch-like configuration of different lengths can be seen more clearly (Fig. 1D). We have also found that the height of the products of NRCA is about 2 nm by measuring the height of the DNA (the under side of Fig. 1C and D), which is characteristic of dsDNA. The AFM results further confirm the massive amplification with ultrahigh efficiency of NRCA.

3.3. Dynamic analysis of SARS-COV-2 nucleic acid by NRCA

In order to be able to better distinguish between the specific amplification products and non-specific amplification products, the reaction time of NRCA was investigated as shown in Fig. 2. It can be observed that the fluorescence value of the amplification products gradually increases as the reaction time increasing. At the same time, the fluorescent signal of negative control also increases with the time extending (Fig. 2A). Fig. 2B shows that when the reaction time reaches 240 min, the ratio of fluorescent intensity between amplification products of target and negative control is the highest, indicating that the optimal reaction time is 240 min, which can distinguish the specific amplification products from the negative control.

3.4. Optimization of NRCA reaction conditions for detection of SARS-COV-2 nucleic acid

In order to obtain better detection effect, we have screened different combinations of nucleic acid tool enzyme and buffer systems for the NRCA. Three sets of nucleic acid tool enzyme combinations with close reaction temperatures were selected: Bst. DNA polymerase and Nb.BsrDI, Klenow fragment exo- and Nb.BbvCI, phi29 DNA polymerase and Nb.BbvCI. At the same time, six kinds of buffer systems suitable for

**Scheme 1.** Schematic diagram of the principle of NRCA detection of SARS-CoV-2 nucleic acid. HRCA products are specifically scissored into branches (B1, B2…Bn), which are combined with the template as a new primer triggering new HRCA reaction.
constant temperature amplification reactions are set up, including Low-buffer solution (26 μM Tris), T4 buffer or CutSmart buffer for the ligation and amplification reaction. As shown in Fig. 3A–C, the combination of Klenow fragment exo-/Nb.BbvCI in T4 buffer and phi29 DNA polymerase/Nb.BbvCI in low-buffer have a higher fluorescent signal-to-noise ratio. In addition, NRCA was performed adopting Klenow/Nb.BbvCI (T4 buffer) and phi29 DNA polymerase/Nb.BbvCI (low-buffer) at different concentration of target (Fig. S1). We found that the fluorescence signal-to-noise ratio of the amplification products adopting Klenow/Nb.BbvCI (T4 buffer) has better discrimination than that of phi29 DNA polymerase/Nb.BbvCI (low-buffer) at low concentration of target (20 fM-200 pM), which is larger than 1. When adopting phi29 DNA polymerase/Nb.BbvCI (low-buffer), there is almost no difference when the target concentration is less than or equal to 200 pM. In summary, the

![Fig. 1. Detection of SARS-COV-2 nucleic acid by NRCA in tube. (A) Electrophoretic characterization of the ligation product (left). Lane 1: marker, lanes 2: hybridization product of target and padlock probe, lane 3–4: padlock probe and target alone. Electrophoretic characterization of the ligation product after exonuclease treatment (right). Lane 1: marker, lanes 2: hybridization product of target and padlock probe, lane 3–4: padlock probe and target alone. (B) Fluorescent intensity of NRCA in the different combination of target, padlock probe, primer 1 and primer 2. (C–D) AFM images of NRCA products in a concentrated and diluted state, respectively. The height of the positions marked with blue lines is shown under the corresponding AFM images.](image-url)
further improve the detection effect. With the increase of concentration of padlock probe, the fluorescent signal-to-noise ratio gradually decreases. When the concentration of the nicking endonuclease is 2.5 U (0.25 μL), which cannot easily distinguish based on clinical symptoms alone. Therefore, in order to avoid misdiagnosis, a diagnostic test that can distinguish COVID-19 from influenza viruses is necessary. In the specificity testing, the combination of Klenow fragment exo- and Nb.BbvCI in T4 buffer has better discrimination and lower background signal at low concentration of target. Thus, T4 buffer was selected as the buffer system in the NRCA.

In addition, the concentration of padlock probe was also optimized to further improve the detection effect. With the increase of concentration of padlock probe, the fluorescent signal-to-noise ratio gradually increases. When the concentration of the padlock probe reaches 1 nM, the highest fluorescent signal-to-noise ratio can be obtained (Fig. 3D). Therefore, the padlock probe concentration of 1 nM was selected as the optimal reaction concentration for the NRCA.

Since the concentration of nicking enzyme has a greater impact on the efficiency of product digestion and amplification in the reaction system, the concentration of nicking enzyme in the NRCA reaction system was optimized in this experiment. The reaction mediated by nicking endonuclease is relatively easier to cause non-specific amplification. As shown in Fig. 3E, with the concentration of nicking endonuclease increasing, the fluorescent signal-to-noise ratio increases and reaches the highest value when the concentration of the nicking endonuclease is 2.5 U (0.25 μL). Then, the fluorescent signal-to-noise ratio begins to decrease and reaches plateau gradually when the concentration of nicking endonuclease is 5 U (0.5 μL), which cannot easily distinguish specific amplification. Therefore, considering the avoidance of non-specific amplification on the detection signal, 2.5 U was selected as the optimal concentration of nicking endonuclease for NRCA reaction system.

Finally, we explored the influence of different concentrations of primer 1 and primer 2 on the NRCA. As shown in Fig. 3F, the fluorescent signal-to-noise ratio gradually increases with the increase of the concentration of primer 1 and the decrease of the concentration of primer 2. When the concentration of primer 1 and primer 2 is 200 nM and 5 nM, respectively, the signal-to-noise ratio reaches the highest value. It can be observed when the concentrations of primer 2 and primer 1 are the same, the amplification of negative control also significantly happened, which ascribes the high concentration of primer 2 easily to trigger non-specific amplification. Therefore, 200 nM primer 1 and 5 nM primer 2 was finally used as the optimal concentration for the NRCA.

### 3.5. Ultrasensitive and specific detection of SARS-CoV-2 nucleic acid by NRCA

The sensitivity of NRCA reaction to detect SARS-CoV-2 virus nucleic acid was studied under optimal experimental conditions. We measured the fluorescence spectra after NRCA at different concentration of target. It can be observed that the characteristic peak appears at 500 nm and disappears at 650 nm. By measuring the peak value at 520 nm, it can be known that in the range of 0–20 nM, the fluorescent value gradually increases with the increase of the concentration of target (Fig. 4A). The inset graph shows that when the concentration of target varies from 20 aM to 200 pM (Fig. 4B), the fluorescent value shows a log-linear relationship with the concentration of target. The linear equation is $Y = 6442 + 1786X$ ($R^2 = 0.991$), and the detection limit is 4.12 aM (LOD = 3 SD/k; LOD is the detection limit; SD is the standard deviation of the blank sample; k is the slope of the fitted curve). Furthermore, we also compared NRCA with the standard method (qRT-PCR). As shown in Table S3 and Fig. S2, NRCA can achieve ultrasensitive detection of the SARS-CoV-2, which is comparable to the qRT-PCR (the detection limit is 2 aM). This detection limit is also similar to the qRT-PCR reported in the literature for the detection of SARS-CoV-2 (1.66 aM) [31]. In addition to its high sensitivity, our method does not require expensive thermal cycling equipment and additional reverse transcription process.

Although COVID-19 and influenza are infectious respiratory diseases caused by different viruses, their symptoms are so similar which can be difficult to distinguish based on clinical symptoms alone. Therefore, in order to avoid misdiagnosis, a diagnostic test that can distinguish COVID-19 from influenza viruses is necessary. In the specificity testing, three-bases mismatch (mT-3), five-bases mismatch (mT-5) and totally unmatched variant (uT) were used as negative controls. As shown in Fig. 4C, hybridization bands of ligation product (padlock probe and target) were observed in the polyacrylamide gel electrophoresis and the migration rate was lower than that of other negative controls. After
exonuclease treatment, the closed circular product still exists stably, while the other bands in the control group are completely digested (Fig. 4C). Furthermore, the results using agarose gel electrophoresis and fluorescent detection also show that significant amplification bands and fluorescent signal can be observe in the presence of target, indicating that the assay has good specificity and can detect specific sites of SARS-CoV-2 nucleic acid through sequence design (Fig. 4D–E).

3.6. Detection of SARS-COV-2 nucleic acid by NRCA on paper device

Finally, a paper device was designed and fabricated to detect SARS-CoV-2 nucleic acid by NRCA as shown in Fig. 5A–B. The paper device contains 6 × 3-microzone and the diameter of each test zone is 4 mm. The nitrocellulose membrane supporting NRCA reaction system can be well penetrated by ultraviolet light and the background fluorescence is very low, which enable the NRCA reaction on paper. As shown in Fig. 5C, significant green fluorescence can be observed on the microzone when the target is present. The result of fluorescence value using ImageJ analysis also shows that NRCA products can form in the presence of target. It is worth mentioning that the paper-based NRCA was also performed for analysis of SARS-CoV-2 RNA (transcribed in vitro standard material). As shown in Fig. 5C, significant fluorescent signal is also observed even in a mixture of 10 RNA transcribed in vitro containing target RNA. These results show that the paper has excellent specificity and is consistent with previous experimental results in centrifuge tubes. In addition, the fluorescent signal of different concentration of target is analyzed on the paper device. It can be seen that with the increase of target RNA, the fluorescence in the microzone increases gradually as shown in Fig. 5D. The results indicate that our paper-based NRCA can achieve POC diagnosis of SARS-COV-2 RNA. Furthermore, the long-term stability of the paper device was also investigated as shown in Fig. 5E. The NRCA reagents after pullulan encapsulation on the paper can retained 62% of initial activity after storage at room temperature for 15 days. In contrast, the activity of NRCA reagents in solution decreased by 57% within 3 days and became completely inactivated within 15 days at room temperature. The results indicate that due to the pullulan encapsulation, the biomolecules in NRCA reagents can avoid thermal denaturation of chemical modification, which enables the paper device room-temperature transportation and storage.

4. Conclusion

In summary, we have developed a new method for ultrasensitive and visual detection of SARS-CoV-2 nucleic acid based on NRCA. This method combines a paper device with highly efficient isothermal amplification, which avoids the use of expensive thermal cycling equipment in clinical nucleic acid detection. Through optimizing the experimental conditions of the method from many aspects, good specific and ultrasensitive detection of SARS-CoV-2 nucleic acid can be realized. The detection limit is 4.12 aM. Furthermore, NRCA reaction can be performed on paper device, which owns advantages of portability, visualization and low-cost, and effectively meet the requirements of
POCT. Therefore, the paper-based NRCA can promote the detection of SARS-CoV-2 nucleic acid in some harsh circumstances and resource-poor areas like rigorous military medicine and the third world, that is still no proper method to realize the detection of COVID-19 with favorable cost-efficiency and conveniences. On the other hand, our method also provides new ideas for the application and development of NRCA and promotes the development of NRCA devices.

CRediT authorship contribution statement

Yuchen Song: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Data curation. Yuqing Chao: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Data curation. Yi Guo: Validation, Formal analysis, Methodology. Fan Zhang: Validation, Formal analysis, Methodology. Changqing Mao: Data curation, Formal analysis. Guifang Chen: Writing – review & editing, Resources, Funding acquisition, Project administration. Chang Feng: Writing – review & editing, Resources, Funding acquisition, Project administration.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.131460.

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