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Translating daily COVID-19 screening into a simple glucose test: a proof of concept study

“COVID-19 glucose test”. Home testing is an attractive emerging strategy to combat the COVID-19 pandemic. We herein translate SARS-CoV-2 detection into a glucose test by incorporating target-responsive rolling circle amplification and a CRISPR-based collateral cleavage module with a portable glucose meter. Given the facile integration of various bioreceptors into the CRISPR system, the proposed method provides a starting point to provide patients with a single-device solution that can quantitatively monitor multiple COVID-19 biomarkers at home.
Introduction

The recent COVID-19 pandemic has highlighted the significance of accurate and reliable diagnostic technologies for control and prevention of virus spread. Current diagnostic testing of SARS-CoV-2 mainly includes the detection of nucleic acids, antigens, and antibodies using different approaches, such as real-time quantitative polymerase chain reaction (RT-qPCR), optical immunoassay, lateral flow assays, automated microfluidic devices, and clustered regularly interspaced short palindromic repeats (CRISPR)-associated methods. Despite the progress, most of these systems still suffer from some drawbacks because of the tradeoffs between sensitivity, usability and costs, making the test unaffordable to most users at home. Moreover, a most recent investigation demonstrated that effective COVID-19 screening is mainly dominated by the test frequency and speed and is only marginally improved by high test sensitivity. Thus, in addition to sensitivity and specificity, the focus on developing new COVID-19 detection methods has shifted to cost, turnaround time and ease of use by unskilled operators in a resource-limited setting.

Home testing is an attractive emerging strategy to combat the COVID-19 pandemic and prevent overloading of healthcare resources through at-home isolation, COVID-19 screening, and monitoring of symptoms. However, current diagnostic technologies of SARS-CoV-2 still suffer from some drawbacks because of the tradeoffs between sensitivity, usability and costs, making the test unaffordable to most users at home. To address these limitations, taking advantage of clustered regularly interspaced short palindromic repeats (CRISPRs) and a portable glucose meter (PGM), we present a proof-of-concept demonstration of a target-responsive CRISPR-PGM system for translating SARS-CoV-2 detection into a glucose test. Using this system, a specific N gene, N protein, and pseudo-viruses of SARS-CoV-2 have been detected quantitatively with a PGM. Given the facile integration of various bioreceptors into the CRISPR-PGM system, the proposed method provides a starting point to provide patients with a single-device solution that can quantitatively monitor multiple COVID-19 biomarkers at home.
Finally, we applied this system to quantitatively detect the N sensitivity of 30.3 fM for N gene within 3 hours and high POC screening of SARS-CoV-2. The assay achieved a high development to promising alternative methods for low-cost POC screening of SARS-CoV-2. The assay achieved a high sensitivity of 30.3 fM for N gene within 3 hours and high specificity to accurately discriminate N gene mutations. We further validated the performance and reliability of the CRISPR-PGM system with clinical throat swab samples. Finally, we applied this system to quantitatively detect the N protein of SARS-CoV-2 by incorporating an additional antibody-assisted proximity ligation module with the CRISPR-PGM. Although the CRISPR-PGM system is developed in the context of COVID-19, we anticipate that this new method can be expanded to diagnose other pathogens and build preparedness for future potential infectious disease outbreaks.

Results and discussion

Detection strategy and workflow of the CRISPR-PGM system for COVID-19 screening

The detection strategy and workflow of the CRISPR-PGM system that incorporates RCA and CRISPR-mediated amplification with a PGM-based glucose test is shown in Scheme 1. The whole workflow is composed of three components: the input of viral RNA by extraction from SARS-CoV-2 infected patients, the CRISPR-based signal transduction, and the output glucose test using a PGM. Among them, the CRISPR-based signal transduction is the key process, in which the specific gene region of interest is amplified using RCA. Briefly, a padlock probe N (PPN) is designed to be capable of hybridizing with the target N gene, facilitating its cyclization by T4 DNA ligase. After addition of phi29 DNA polymerase, the RCA reaction occurs, producing a long single stranded DNA product with a large amount of tandem sequence repeats, each of which contains one recognition site of restriction endonuclease EcoRI. Then, in the presence of EcoRI endonuclease, the RCA product is cut into many small DNA fragments (activator), each of which is complementary to 3' and 5' terminals of crRNA. Then, the resulting DNA fragment is recognized by a pre-assembled Cas12a/crRNA ribonucleoprotein complex, activating the collateral activity of Cas12a. The active Cas12a then cleaves the MNPs-poly-invertase to release a number of DNA–invertase conjugates and subsequently catalyzes the hydrolysis of sucrose.
to glucose. Finally, the glucose produced was measured using a PGM. Since the output glucose signal is directly related to the presence and concentration of the released poly-invertase in solution, which in turn depends on the collateral cleavage activity of Cas12a triggered by the target viral RNA, the presence and concentration of the target can be determined by monitoring the glucose signal in the system. Using this strategy, we can transform the viral RNA detection into a simple glucose test for SARS-CoV-2 diagnosis. In addition, the combination of RCA and CRISPR-Cas12a with a glucose test takes advantage of the isothermal amplification ability of RCA, the high specificity to single-base variation of CRISPR technology, the significant turnover enzyme activity of both Cas12a and the poly-invertase system, and more importantly the user-friendly, portability, and cost-effective properties of the PGM. Therefore, we anticipate that our CRISPR-PGM system will be useful for rapid COVID-19 screening with high sensitivity and specificity in a POC format.

**Design and validation of the CRISPR-RCA assay for SARS-CoV-2 N gene**

As a proof of concept for detecting SARS-CoV-2, we initially designed a padlock probe to target an already validated N (nucleoprotein) gene in the SARS-CoV-2 genome (Fig. 1A). All oligo sequences are specified in Table S1.† The padlock probe N (PPN) consists of two recognition regions with complementary DNA sequences to the N gene target; one, a region with complementary DNA sequences to the phi29 primer, and two, specific EcoRI recognition sites for subsequent endonuclease cleavage. Such a construct could efficiently prevent the nonspecific RCA reaction in the absence of N gene. Specifically, when N gene was present, it hybridizes with the padlock probe and induces the circularization of PPN with the assistance of DNA ligase. The N gene-mediated ligation process was confirmed by denatured polyacrylamide gel electrophoresis (PAGE), which is shown in Fig. S2.† The subsequent RCA reaction is then triggered upon the addition of phi29 DNA polymerase (phi29 DP), resulting in multiple repeated hairpin DNA regions that could be recognized and cleaved by EcoRI. This enzymatic cleavage reaction produces thousands of ssDNA activators for subsequent CRISPR-Cas12a activation. Thus, a small amount of the N gene target could be converted to a large number of Cas12a activators to initiate the collateral cleavage amplification reaction, achieving CRISPR-RCA-mediated signal amplification.

The N gene-dependent CRISPR-RCA process was first verified by electrophoresis analysis (Fig. 1B). A very bright band in lane 4 with high molecular weight is observed in the presence of N gene, but no band is observed in the same region for PPN (lane 1), N gene (lane 2), or phi29-free control (lane 3). In addition, adding EcoRI results in multiple bright bands with lower molecular weight, indicating the enzymatic digestion of RCA products. To further confirm that the digestion of RCA products could trigger the collateral activity of Cas12a, we performed a fluorescence assay by introducing a 5 nt ssDNA reporter as the substrate for Cas12a-crRNA (Fig. 1C), with a fluorophore (FAM) and quencher (BHQ1) conjugated at the 5’ and 3’ ends, respectively (denoted as ssDNA-FQ). As illustrated in Fig. 1D, a dramatic increase of the fluorescence enhancement rate (FER) was observed for the RCA-mediated CRISPR-Cas12a assay, as compared to that of the RCA-free CRISPR-Cas12a assay. The FEA value was calculated to be 1.57 s⁻¹ and 0.066 s⁻¹, respectively, indicating that a dramatically amplified fluorescence signal was achieved by the RCA reaction. To better evaluate the capability of signal enhancement through the RCA reaction, we further compared the fluorescence responses of CRISPR-Cas12a assay for different concentrations of the N gene target with and without the RCA reaction (Fig. S3†). Compared with the fluorescence signal of blank samples (F₀), no significant difference of the fluorescence signal (F₁) was obtained for 100 pM N gene using the RCA-free CRISPR-Cas12a assay, while 340% of the fluorescence enhancement ratio (FER, defined as (F₁ - F₀)/F₀) was observed for 100 pM N gene using CRISPR-RCA assay (Fig. 1E). In addition, the FER further increases to 13.2 for 1000 pM N gene using the CRISPR-RCA assay. Considering that the FER is only 0.4 for 10³ pM N gene using the RCA-free CRISPR-Cas12a assay, an approximately 33-fold increase of signal amplification is therefore obtained by coupling RCA with CRISPR-Cas12a assay. Taken together, these experimental results indicated successful fluorescence amplification for N gene recognition, further demonstrating the feasibility of our CRISPR-RCA system regarding the overall improved sensitivity.
Programmable assembly and characterization of the poly-invertase-DNA immobilized magnetic nanoparticles

To improve the sensitivity and portability of the CRISPR-Cas12a sensor, we further integrated the CRISPR-RCA assay with a PGM and developed a CRISPR-PGM system by introducing MNPs-poly-invertase as the substrate for Cas12a-crRNA. The programmable assembly of the MNPs-poly-invertase by RCA, as illustrated in Fig. 2A (the DNA sequences are shown in Table S1 in the ESI†), includes three steps. First, the RCA primer was immobilized on the streptavidin-coated MNPs through the streptavidin–biotin interaction. Then, a circular padlock probe 2 (cPP2) was prepared (Fig. S4A†) and served as the template to initiate the in situ RCA, producing a large amount of RCA product-immobilized MNPs (MNPs-RP). We employed native PAGE (10%) to evaluate the successful assembly of these DNA-RCA products (Fig. S4B†). The distinct band observed in the high molecular weight (MW) region in lane 3 confirms the successful synthesis of the DNA-RCA products. Finally, a DNA-invertase conjugate containing the complementary DNA to the tandem DNA sequence of RCA products was synthesized (Fig. S4C†) and added for hybridization, which enables the assembly of numerous invertases on the MNPs-RP to obtain MNPs-poly-invertase.

To verify the assembly process of MNPs-poly-invertase, the dynamic light scattering (DLS) analysis was conducted, and the average hydrodynamic diameter ($D_h$) of streptavidin-MNPs, MNPs-primers, MNPs-RP, and MNPs-poly-invertase increased gradually from 421.0 nm to 725.0 nm (Fig. 2B), indicating the successful step-by-step assembly of poly-invertase on MNPs. It is to be noted that, compared with MNPs-RP, the $D_h$ value of MNPs-poly-invertase, from the DLS and zeta potential measurements, showed a 25% increase which could be due to the enhanced rigidity of the DNA-RCA products after hybridization with DNA-invertase conjugates.41 In addition, to corroborate the results from DLS, zeta potential measurements were further performed in 10 mM PBS, and the ζ-potential value gradually decreased during the assembly process (Fig. 2C), revealing the increasing amount of negatively charged DNA on the MNP surface. Next, we investigate the catalytic activity of MNPs-poly-invertase by mixing 10 μL of MNPs-poly-invertase (0.5 mg mL$^{-1}$) with 40 μL of sucrose reporter solution. A 14°C incubation at 37°C for 30 min, the production of glucose was measured using a PGM. As shown in Fig. 2D, the MNPs-poly-invertase could yield nearly 101-fold more glucose from sucrose than from a blank sample. Taken together, these results confirmed the formation of highly active MNPs-poly-invertase through the RCA-mediated DNA assembly. In addition, to enable the sensitive detection of glucose, an ultra-sensitive PGM (GSI, USA) was applied with a limit of detection (LOD) of 26.0 μM (Fig. S5†), which offers more than 20-fold sensitivity enhancement over the majority of commercially available PGMs (~0.6 mM).

Performance of the CRISPR-PGM system for N gene detection

Once the MNPs-poly-invertase was synthesized, we then assessed its capability to amplify the PGM signals for N gene detection. As a control, the nonpolymeric invertase immobilized magnetic beads (MNPs-invertase) were prepared by direct
hybridization of DNA–invertase conjugates on the MNPs-primer surface (Fig. S6A†). Assay performance was compared at an N gene concentration of 10 pM using MNPs-invertase and MNPs-poly-invertase (Fig. S6B†) as the substrate, respectively. A 6.4-fold increase of the PGM signal was observed for MNPs-poly-invertase over MNPs-invertase (Fig. S6C†), suggesting a good signal amplification capability of MNPs-poly-invertase because of the advantage of release of multiple invertase molecules with one DNA cleavage event. It should be noted that N gene-DNA, the DNA mimic of N gene-RNA from the N1 region of SARS-CoV-2, was used as the model target for CRISPR-PGM experiments. Given the highly sensitive and selective detection of N gene, the CRISPR-PGM system was further applied to detect SARS-CoV-2 from throat swab samples. We evaluated a total of 48 viral RNA samples extracted from two COVID-19 positive and four COVID-19 negative people, which were validated by RT-qPCR in the hospital laboratory. Based on the typical CRISPR-PGM workflow, the PGM signal for each sample was collected and the corresponding heat map is shown in Fig. 3C. By targeting the N gene of SARS-CoV-2, the average PGM values for the healthy people and two patients were obtained as 228 ± 29.3 (n = 32), 371 ± 23.1 (n = 8), and 342 ± 22.7 (n = 8), respectively. Based on the calibration curve of N gene detection in Fig. 3A, the correlated concentration of N gene was thus calculated to be 74.1 ± 14.6 fM, 202 ± 32.4 fM, and 165 ± 27.1 fM, respectively. Although a detectable N gene signal in healthy people was obtained due to the non-specific hydrolysis of sucrose to glucose during the CRISPR-PGM assay, a significantly higher level of N gene was observed in SARS-CoV-2 infected patients (P < 0.00001, Fig. S8†). Thus, the CRISPR-PGM system demonstrated the required sensitivity and specificity for COVID-19 screening, with 100% concordance with the results obtained by standard RT-qPCR from the clinical laboratory.

Design and performance of the CRISPR-PGM system for SARS-CoV-2 protein detection

Another challenge in current COVID-19 screening is the potential false positive due to unintended amplification of DNA/RNA contaminants. Therefore, serological assays for SARS-CoV-2 proteins or antibodies have been developed for complementary diagnosis. However, the detection of minute amounts of these proteins poses a considerable challenge because proteins cannot be directly amplified. To address this issue, motivated by the above success in detecting the SARS-CoV-2 N
gene, we sought to adapt our CRISPR-PGM system for SARS-CoV-2 N protein detection. To do this, an antibody-assisted proximity ligation assay (PLA) was first designed\(^{47,48}\) and combined with CRISPR-RCA for signal amplification (Fig. 4A). Such a construct consists of two proximity probes (PPA and PPB) to target N protein, which were synthesized by conjugating two non-competitive N antibodies with specific DNA sequences via the maleimide–thiol reaction using a heterobifunctional linker, sulfo-SMCC. The synthesis process was characterized using 10% native PAGE (Fig. S9\(^\ddagger\)). When the paired proximity probes bind to the same N protein in close proximity to each other, they could hybridize with two linear oligonucleotides and guide the formation of circular DNA via enzymatic DNA ligation. Then, the circular DNA could serve as the template for subsequent CRISPR-RCA reactions, producing the amplified Cas12a activators. To examine whether CRISPR-Cas12a can be activated by the N protein-mediated PLA-RCA reaction, we first performed a fluorescence assay for N protein using ssDNA-FQ as the substrate for Cas12a-crRNA (Fig. S10A\(^\ddagger\)). In the absence of N protein, minimal fluorescence signal was observed (Fig. S10B\(^\ddagger\)), indicating a negligible cleavage of ssDNA-FQ by the Cas12a-crRNA complex. However, a considerable fluorescence signal was observed in the presence of 50 pM N protein and more importantly showed a further increase for 200 pM N protein (Fig. S10B\(^\ddagger\)), indicating the efficient collateral cleavage of ssDNA-FQ by the Cas12a-crRNA complex in response to N protein. These results suggest that only when the target N protein was present was the Cas12a activator generated from the PLA-RCA reaction, thereby confirming both target-dependent proximity ligation and subsequent activator amplification.

Then, we further conducted similar tests and tried to detect N protein using a PGM by introducing the MNPs-poly-invertase as the substrate for Cas12a-crRNA (Fig. 4A). Compared with blank samples, the presence of N protein results in a significantly higher PGM signal in a dose-dependent manner (Fig. 4B). This observation is in agreement with the fluorescence results, which indicated that the N protein target could effectively trigger the proximity ligation assisted RCA reaction to activate the Cas12a-crRNA complex, followed by an efficient collateral cleavage of MNPs-poly-invertase that could finally catalyze the generation of a large amount of glucose. Moreover, this PLAGRISPR-PGM system exhibited a wide dynamic range from 10 to 5000 pM and showed a limit of detection of 11.4 pM based on 3\(\sigma\)/slope (Fig. 4B). This LOD correlates to approximately 2.0 \(\times 10^5\) VPs per \(\mu\)L because of an average of 30–35 viral ribonucleoprotein complexes per virion.\(^{49}\) This sensitivity is comparable to that of several commercial ELISA kits and graphene-based electrochemical sensors\(^{49}\) but much higher than that of previous lateral flow sensors.\(^{50,52}\) The high sensitivity was due to the cooperative signal amplification by PLA-RCA, CRISPR, and poly-invertase labels. Finally, to demonstrate the selectivity of the PLA-CRISPR-PGM system for N protein detection, we performed the PGM test using different competing proteins and the glucose signals were recorded, including spike S1, cardiac troponin I (cTnI), interferon-gamma (IFN-\(\gamma\)), and bovine serum albumin (BSA). As shown in Fig. 4C, compared with the blank samples, no significant difference of the glucose...
signal was observed for competing proteins at a concentration of 50 pM, while the glucose signal in response to 50 pM N protein of SARS-CoV-2 showed a more than 3-fold increase ($p < 0.0001$), suggesting good specificity arising from the proximity ligation assisted CRISPR-RCA reaction. Taken together, we demonstrated that the CRISPR-PGM system could be easily reconfigured to detect a broad range of COVID-19 related biomarkers using suitable recognition probes, with high sensitivity and specificity in a POC format.

To demonstrate the potential clinical application of the PLA-CRISPR-PGM system, we further conducted a similar protein assay for SARS-CoV-2 spike protein on our PLA-CRISPR-PGM system using SARS-CoV-2 spike pseudo viruses. As shown in Fig. S11A† a series of concentrations of pseudo virus from 0 to $2 \times 10^5$ VPs per μL were lysed with NE-PER buffer and measured with the established PLA-CRISPR-PGM system as described above, except that two non-competitive spike protein antibody-DNA conjugates were applied. Fig. S11B† shows that the PGM signal increased with increasing concentrations of pseudo virus from 0 to $2 \times 10^5$ VPs per μL, with a LOD of $9.7 \times 10^4$ VPs per μL. This LOD correlates to approximately 4.2 ± 2.4 pm spike protein because of an average of 26 ± 15 spike proteins per virion.19 These results not only verify the feasibility of our PLA-CRISPR-PGM system for the detection of SARS-CoV-2 pseudo-viruses, but also demonstrate the generality of the protein assay.

Finally, to realize the full potential of the CRISPR-PGM system and broaden its application for daily COVID-19 screening, we challenged the CRISPR-PGM system with the saliva specimens spiked with different concentrations of either N gene (Fig. S12A†) or N protein (Fig. S13A†). Fig. S12B† shows that the PGM signal increased with increasing N gene concentration from 0 to 200 pM, and a significant difference in PGM signals between 0.2 pM N gene and the blank sample was observed ($p < 0.05$). The LOD was calculated to be 604 fm, correlating to $3.6 \times 10^3$ VPs per μL. Similarly, when the detection of N protein in saliva samples was performed using the established PLA-CRISPR-PGM system, the PGM signal increased with increasing N protein concentration from 0 to 300 pM (Fig. S13B†), and a significant difference in PGM signals between 40 pM N protein and the blank sample was observed ($p < 0.05$). The LOD was calculated to be 47 pM, correlating to $8.2 \times 10^2$ VPs per μL. Since saliva is easier to collect than either blood or nasopharyngeal swab, its accessibility makes it an ideal specimen for daily COVID-19 screening.24 In addition, previous studies have showed the presence of high viral loads in the saliva specimens of infected patients.24 Therefore, our CRISPR-PGM system could provide a potential method for daily COVID-19 screening of saliva specimens.

**Conclusion**

In summary, we have demonstrated a general and versatile CRISPR-PGM system for the quantitative detection of COVID-19 related biomarkers. Using this system, a specific N gene and N protein of SARS-CoV-2 have been detected quantitatively with a single portable device. Taking advantage of both target-dependent RCA and sequence-specific recognition of CRISPR, the CRISPR-PGM system offers significant improvements in both analytical sensitivity and specificity. Moreover, integrated with a glucose meter, this method has the potential to enable rapid, low-cost, and point-of-care screening for SARS-CoV-2. These features would allow for earlier detection and more accurate screening for asymptomatic infections at home through telemedicine care. In addition, given the facile integration of various bioreceptors into the CRISPR-PGM system, the proposed method holds great promise to provide patients with a single-device solution that can quantitatively monitor multiple COVID-19 biomarkers at home. Although the CRISPR-PGM system was not fully integrated into a single step detection, as a proof-of-concept, this platform paves the way to further broaden the POC applications of PGM-based sensors. In addition, one major limitation of our study is that our experiments have thus far been conducted only with a small clinical sample size; however, our data provide a window into what a larger clinical screening might look like. It should be noted that the CRISPR-PGM system offers a sample-to-result time of approximately 3 hours; however, we expect improvements in the turnaround time with optimization of RNA extraction, Cas12a-crRNA engineering,35 and additional integration of the PGM with a lateral flow device.26 Overall, in addition to highlighting new strategies for SARS-CoV-2 detection, the results provide a starting point for the development of cost-effective home self-testing devices with the ultimate goal of daily screening and reducing the burden of the ongoing COVID-19 pandemic.

**Data availability**

All the data supporting this article have been included in the main text and the supplementary material.

**Author contributions**

R. Liu and J. J. Zhang designed the experiments. R. Liu performed the synthesis, characterization and assays. Y. S. Hu assisted to test the clinical samples. Y. He helped to perform part of the additional experiment. All authors contributed to the analysis and discussion of the results. R. Liu and J. J. Zhang wrote the manuscript. J. J. Zhang conceived and supervised this research.

**Conflicts of interest**

There are no conflicts to declare.

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Notes and references