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Platinum nanoparticles (PtNPs)-based CRISPR/Cas12a platform for detection of nucleic acid and protein in clinical samples

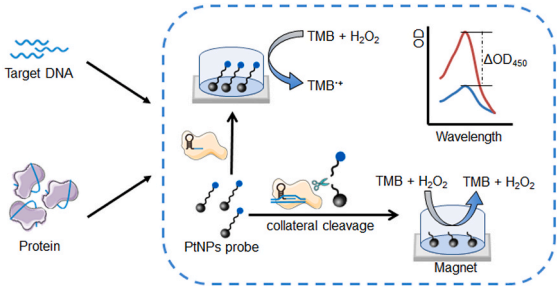
Jiajie Liang [a,b,d,1], Peijun Teng [a,1], Liangshan Hu [c,1], Guanbo He [d], Qifang Song [a], Ying Zhang [a], Bin Peng [a], Gan Li [a], Wei Xiao [c,***], Donglin Cao [c,**], Yong Tang [a,*]

[a] Guangdong Province Engineering Research Center of Antibody Drug and Immunoassay, Department of Bioengineering, College of Life Science and Technology, Jinan University, Guangzhou, 510632, China
[b] Department of Oncology, The First Affiliated Hospital, Jinan University, Guangzhou, 510632, China
[c] Department of Laboratory Medicine, Guangdong Second Provincial General Hospital, Guangzhou, 510317, China
[d] Guangdong Biowings Tech Limited, Foshan, 528000, China

HIGHLIGHTS

- A noble metal nanocatalysts-based CRISPR/Cas12a assay (OD-CRISPR) is reported.
- The OD-CRISPR is used for the detection of nucleic acid in clinical samples.
- The aptamer-based OD-CRISPR is used for the detection of protein in clinical samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Early rapid screening diagnostic assay is essential for the identification, prevention, and evaluation of many contagious or refractory diseases. The optical density transducer created by platinum nanoparticles (PtNPs) (OD-CRISPR) is reported in the present research as a cheap and easy-to-execute CRISPR/Cas12a-based diagnostic platform. The OD-CRISPR uses PtNPs, with ultra-high peroxidase-mimicking activity, to increase the detection sensitivity, thereby enabling the reduction of detection time and cost. The OD-CRISPR can be utilized to identify nucleic acid or protein biomarkers within an incubation time of 30–40min in clinical specimens. In the case of taking severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) N gene as an instance, when compared to a quantitative reverse transcription-polymerase chain reaction (RT-qPCR), the OD-CRISPR test attains a sensitivity of 79.17% and a specificity of 100%. In terms of detecting prostate-specific antigen (PSA), aptamer-based

* Corresponding author. Guangdong Province Engineering Research Center of Antibody Drug and Immunoassay, Jinan University, 601 Huangpu Avenue West, Guangzhou, 510632, China.
** Corresponding author. Department of Laboratory Medicine, Guangdong Second Provincial General Hospital, 466 Central Xingang Road, Guangzhou, 510317, China.
*** Corresponding author. Department of Laboratory Medicine, Guangdong Second Provincial General Hospital, 466 Central Xingang Road, Guangzhou, 510317, China.
E-mail addresses: xkevent@foxmail.com (W. Xiao), caodl@126.com (D. Cao), tyjaq7926@163.com (Y. Tang).
[1] These authors contributed equally to this work.

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OD-CRISPR assay achieves the least discoverable concentration of 0.01 ng mL^{-1}. In general, the OD-CRISPR can detect nucleic acid and protein biomarkers, and is a potential strategy for early rapid screening diagnostic tools.

1. Introduction

Public health and property are endangered by contagious illnesses such as refractory illnesses as well as acute respiratory syndrome including prostatic cancer. Early rapid screening diagnostic assay is vital for the identification, prevention as well as evaluation of these illnesses [1–3]. As a result, in spite of many advancements that have been made in clinical assays, the development of novel diagnostic assay with relatively low cost, rapid, and easy-to-implement is still necessary for early diagnosis of the diseases [4–7].

The clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems are a group of adaptive immune systems that can be observed in archaea and bacteria and have the ability to naturally protect these organisms against infection with invasive genetic components, such as viruses and plasmids [8]. Coupled with one or more Cas endonucleases, CRISPR RNAs (crRNAs) are able to detect and decompose complementary nucleic acid targets [9,10]. Apart from regulating endonuclease activity (targeted cleavage), CRISPR types III, V, and VI RNA-guided nucleases (Cas12, Cas13, and Cas14) also indicate collateral target-activated, nonspecific single-stranded nucleic acid hydrolysis activity (collateral cleavage), which provides new options for the nucleic acid detection [11–14]. Because of their specificity and the simple design of guide RNAs, CRISPR/Cas enzymes are increasingly being used to identify nucleic acids with the aid of lateral-flow strips or fluorescent transduction systems [15–17]. CRISPR/Cas-based nucleic acid diagnostic approaches have been suggested to test a variety of substances in combination with nucleic acid amplification, such as miRNA, pathogens, methyladenosine, and single nucleotide polymorphisms.

Currently, electrochemical as well as gas-volumetric sensing techniques have been used in CRISPR/Cas-based biosensors to improve sensitivity, and they have demonstrated good performance and accomplished amplification-free nucleic acid detection. A noble metallic nanocatalyst, which is another high-sensitivity sensing material, might also be used in CRISPR/Cas-based biosensors. Platinum nanoparticles (PtNPs), a class of noble metal nanocatalysts with ultra-high peroxidase-mimicking activity, have also been widely applied to fabricate colorimetric biosensors [18]. The signal of the colorimetric biosensor through the optical density (OD) transducer generated by PtNPs can be easily measured by a low-cost microplate reader.

In the present research, we performed the CRISPR/Cas12a-based test for the purpose of developing the optical density biosensing systems (OD-CRISPR) because the transduction systems are relatively cost-efficient and high-sensitive. As a validation, we utilized OD-CRISPR in detecting SARS-CoV-2 RNA that was obtained from nasopharyngeal swab specimens. In comparison to the RT-qPCR, the OD-CRISPR attains a sensitivity of 79.17% as well as a specificity of 100%, which suggests that the OD-CRISPR may serve as a nucleic acid screening diagnostic tool. Moreover, a universal biosensing system capable of detecting different types of analytes is important for clinical application. Hence, based on the fabricated OD-CRISPR assay, we evaluated the ability of the aptamer-based OD-CRISPR to detect protein. Take prostate-specific antigen (PSA) as an example, aptamer-based OD-CRISPR was confirmed with the aid of clinical serum samples acquired from prostate cancer patients. Correlation analysis between the aptamer-based OD-CRISPR assay and chemiluminescent immunoassay (CLIA) showed a correlation. Additionally, in the measured amounts of PSA, the differences observed were not significant, demonstrating that the aptamer-based OD-CRISPR may be used as a protein diagnostic tool.

2. Experimental

2.1. Materials and reagents

Amresco (USA) provided Tween-20 and TMB (3,3′,5,5′-tetramethylbenzidine). Furthermore, we obtained Chloroplatinic acid, trisodium citrate, and PSA from Sigma-Aldrich. We obtained Hydrogen peroxide (H_2O_2, 30 wt%) and H_2SO_4 from the GZ chemical reagent (Guangzhou, China). BEAVER (Suzhou, China) supplied magnetic beads (MBs)@Streptavidin. New England Biolabs (USA) supplied RNase Inhibitor and NEBuffer 2.1. We obtained a Microwell plate from CORNING (USA). We utilized the ultrapure water (Milli-Q grade, Millipore) having a resistivity of 18.2 MΩ cm in the present research.

2.2. Nucleic acid preparation

Sangon Biotech (Shanghai, China) produced the N gene fragment from SARS-CoV-2 (Wuhan-1 strain, GenBank: MN908947), after which it was cloned into the pUC57 vector. In accordance with the Manufacturer's Certificate of Analysis (COA) report, the identification of the plasmid concentration was achieved with the aid of Ultraviolet Absorption Spectrometry (A260) while A260/A280 was utilized in identifying its purity. CHOPCHOP and Benchling were utilized for the purpose of designing the crRNA of target locations on the SARS-CoV-2 genome. The aptamer refers to the previous literature and CHOPCHOP was used in designing the crRNA. GenScript (Nanjing, China) generated all additional DNA and RNA utilized in the present research and their sequences are represented in Table S3. RNAs were aliquoted, after which they were stored until further use at −80 ℃.

2.3. The procedure of OD-CRISPR for nucleic acid detection

To acquire the PtNPs probe, we first produced PtNPs as detailed earlier [19–29]. A mixture of 250 μL 100 mM of chloroplatinic acid with 25 mL of ultrapure water was prepared, followed by heating the solution until it was boiling. Rapid addition of 2.7 mL 100 mM sodium citrate aqueous solution was performed to the boiling chloroplatinic acid solution and then vigorously stirred. This mixture was kept for an additional 30 min while boiling, after which it generated stable brown PtNPs. Afterward, the washing of 100 μL MBs@Streptavidin was conducted three times using a magnetic shelf and resuspended with the aid of 500 μL NEBuffer 2.1. An addition of 30 μL 4 mM SH-ssDNA-biotin was introduced into the MBs, followed by incubation for 30min. The magnetic shelf was utilized in washing MB-ssDNA-SH three times, followed by reaction for 20 min using 2.5 mL 50 mM PtNPs to form PtNPs probe (MB-ssDNA-PtNPs). The washing of the PtNPs probe was performed three times, after which the PtNPs probe was put under storage till further use at 4 °C.

2.4. Clinical samples and statement of ethics

Clinical samples derived from patients with prostate cancer and COVID-19 (the features of patients are shown in Tables S1 and 2) were acquired and processed in strict compliance with WHO standards. We obtained approval from the Guangdong Second Provincial General Hospital's Scientific Research Ethics Review Committee (Ethical Approval No.20200915-01-01-YXKXYJ-CRB). The patients or their legal representatives' written informed consent was obtained. Nasopharyngeal swabs of patients who were potentially infected with SARS-CoV-2 were treated at the Department of Laboratory Medicine Guangdong Second Provincial General Hospital. The MAGPURE RNA Kit (Hybribio,

Guangdong, China) was used to extract RNA from the samples in accordance with the instructions of the manufacturer. Firstly, RNA extracts were utilized in the molecular diagnosis of SARS-CoV-2 with the aid of the 2019-nCoV RT-qPCR Kit (DAAN Gene) in which RT-qPCR targeted N and Orf1a genes. Excess RNA extracts obtained from these samples were aliquoted and stored for further verification of SARS-CoV-2 by performing OD-CRISPR detection at −80 °C. The samples were provided to study staff after randomization. During validation experiments of OD-CRISPR assays, they were kept blind to the diagnostic results of the SARS-CoV-2 RT-qPCR samples.

2.5. Detection of SARS-CoV-2 in clinical samples by OD-CRISPR

RNA extracts from nasopharyngeal swabs were reverse transcribe by ReverTra Ace®qPCR RT Kit (TOYOBO, FSQ-101) in accordance with the instructions of the manufacturer. The RT products were detected by OD-CRISPR assay respectively according to the procedure of OD-CRISPR.

2.6. SARS-CoV-2 detection in clinical samples by OD-CRISPR

ReverTra Ace®qPCR RT Kit (TOYOBO, FSQ-101) was used to reverse transcribe RNA extracts from nasopharyngeal swabs in accordance with the instructions of the manufacturer. OD-CRISPR assay was performed in RT product detection in accordance with the OD-CRISPR procedure.

2.7. Detection of PSA in serum samples by aptamer-based OD-CRISPR

The serum samples from definite prostate cancer patients were incubated with the 8 μL aptamer in binding buffer (2 mM $MgCl_2$ mixed with 0.02% Tween-20 in PBS, pH 7.4) for 30 min at 37 °C. After binding reactions, the aptamer was subjected to incubation for 20 min at 37 °C with 50 nM LbCas12a, 62.5 nM crRNA, 40U RNase Inhibitor, and PtNPs probe in 1 × NEBuffer 2.1. The MBs were rinsed using a magnetic shelf three times, after which 100 μL TMB solution and H_2O_2 were added, followed by incubation for a total of 10 min. Then, the reaction was discontinued through an addition of a 50 μL stop solution (containing 2 M H_2SO_4). A SYNERGY microplate reader (BioTek Instruments, H1) was used to measure the absorbance at 450 nm.

2.8. Statistical analysis

GraphPad Prism 8 was used for data analysis. Unless stated otherwise, all the experimental outcomes were expressed as mean ± standard error of the mean. The Clopper-Pearson approach was utilized in computing the two-sided confidence intervals of sensitivity, specificity, negative predictive agreement (NPA), as well as positive predictive agreement (PPA).

3. Results and discussion

3.1. Establishment of OD-CRISPR for the nucleic acid detection

OD signal is easier to measure using a low-cost microplate reader, which has been widely applied to fabricate colorimetric biosensors. Here, we speculated that PtNPs are suitable for developing the OD-CRISPR assay. We designed a PtNP probe (MB-ssDNA-PtNPs), in which PtNPs were coupled with MBs and an ssDNA for the collateral cleavage of Cas12a. Cas12a collateral cleavage activity is activated by the presence of the target DNA, thereby cleaving the PtNP probe and isolating the PtNPs from the MBs. This results in a decreased OD value of the oxidized-TMB (ΔOD) (Fig. 1a).

We first linked PtNPs to MBs@streptavidin through SH-ssDNA-biotin to synthesize the PtNP probes, and the concentration levels of PtNPs and SH-ssDNA-biotin were then determined (Fig. 1b). Here, the magnetic beads, coated by silicon dioxide, were purchased from BEAVER. To ensure unaffected, we tested the color response of magnetic beads and the result indicated that the magnetic bead we used could not oxidize TMB (Fig. S1). SEM images indicated that the PtNP probes were successfully prepared (Figs. S2a and b). Afterward, we utilized the Target DNA with the aim of verifying whether Cas12a could cleave the PtNP probe to change the OD value. SEM image indicated the PtNP probes were cleaved by Cas12a (Fig. S2c). The OD value decreased following an increase in the concentration level of the Target DNA indicating the capacity of the PtNP-based OD-CRISPR assay in detecting nucleic acids (Fig. 1c).

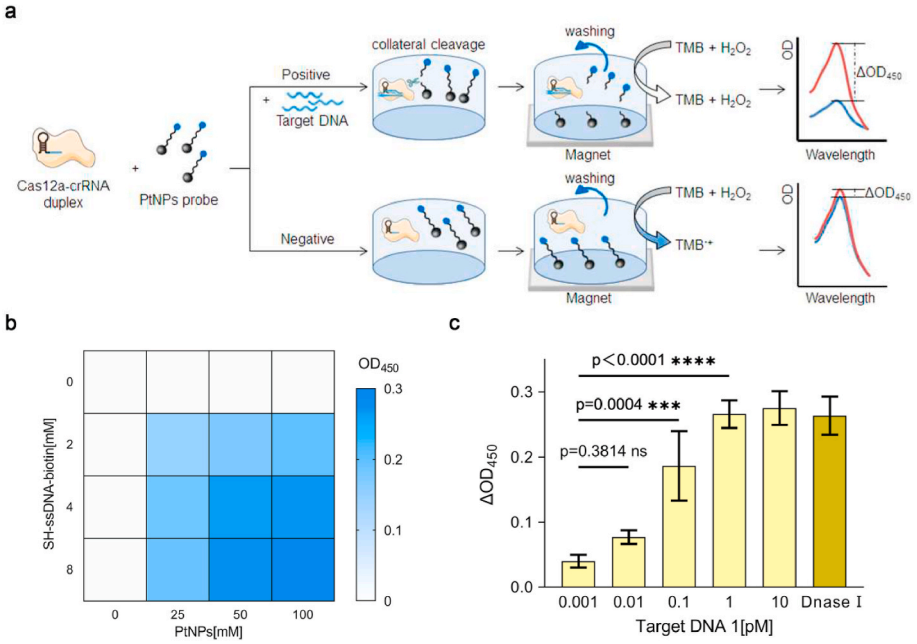

Fig. 1. Establishment of OD-CRISPR for nucleic acid detection. a) Representation of the OD-CRISPR assay. b) Optimization of the SH-ssDNA-biotin and PtNPs concentrations. c) Target DNA 1 was used for the purpose of confirming the PtNP-based OD-CRISPR assay. The concentration of DNase I is 100 U. All the error bars are obtained from three experiments that were carried out independently.

3.2. Verification of the OD-CRISPR for SARS-CoV-2 RNA detection

In early January 2020, a cluster of pneumonia known as Coronavirus disease 2019 (COVID-19) triggered by SARS-CoV-2, was reported for the first time. After a short period, due to the long incubation period, this novel coronavirus paralyzed the world. In this study, we utilized OD-CRISPR in detecting SARS-CoV-2 RNA. In order to achieve accurate and fast detection of SARS-CoV-2 (accession NC_045512) in the N gene, we designed crRNAs in this study (Fig. S3) in accordance with the guidelines provided by World Health Organization (WHO) (January 17, 2020). Subsequently, we assessed the OD-CRISPR assay for the SARS-CoV-2 detection following the conditions of the optimum *trans*-cleavage. Five dilutions of an N gene-positive and -negative plasmid were utilized in plotting a performance curve, each with three replicates. The assay's capacity of generating a substantial variation of ΔOD450 at the lowest concentration of N gene-positive plasmid was confirmed, and the lowest detection limit was 0.02 pM, demonstrating the viability of the OD-CRISPR assay in detecting the cDNA reverse transcribed from SARS-CoV-2 RNA (Fig. 2a). A quantitation standard curve was created based on five dilutions of a control IVT viral nucleoprotein RNA using an in vitro-transcribed (IVT) SARS-CoV-2 RNA gene target that was manufactured (Fig. 2b). The analytic limit of detection (LoD) determined by a remarkable difference of ΔOD_{450} at the lowest concentration was determined to be 0.01 pM. The specific experiments were also performed and the result indicated that the nucleic acid of SARS-like CoV had no reaction with SARS-CoV-2 crRNA (Fig. 2c). Furthermore, the preliminary verification results of the consistency of the PtNP probe and the Cas12a-crRNA duplex at 37 °C and room temperature indicated that there were no significant changes up to day 7 in the OD-CRISPR assay performance in detecting SARS-CoV-2 RNA (Fig. 2d). Therefore, we concluded that PtNP-based OD-CRISPR assay has the ability in detecting SARS-CoV-2 RNA.

3.3. SARS-CoV-2 detection in clinical samples utilizing OD-CRISPR

We confirmed the viability of the OD-CRISPR in detecting SARS-CoV-2 in clinical samples. A total of 112 nasopharyngeal swab samples that were acquired from patients in Guangdong Second Provincial General Hospital were utilized for the purpose of validating the OD-CRISPR work-flows (Fig. 3a). 32 of the 112 patients were found to be COVID-19-positive on the basis of the clinical diagnosis whereas 80 patients were found to be negative (patient characteristics are shown in Table S1). To reduce the rate of degradation, we placed the RNA samples at −80 °C as RT-qPCR was conducted before the proposed CRISPR-based assays for all the samples. To prevent samples' degradation on transportation, all the CRISPR-based validation tests were carried out in the Guangdong Second Provincial General Hospital.

Among a total of 112 nasopharyngeal swab samples, N gene OD-CRISPR identified 19 samples as positive and 93 samples as negative (Fig. 3b). In comparison with the RT-qPCR (Table 1), the OD-CRISPR had a sensitivity of 79.17% and a specificity of 100%. These values were in line with 100% PPA and 94.62% NPA for the OD-CRISPR (Table 2). Compared with RT-qPCR, the OD-CRISPR assay was rapid (30–40 min) and easy-to-use, which are desirable properties for early rapid screening. In addition, the OD-CRISPR used similar sample collection procedures and RNA extraction methods as that in RT-qPCR, which are easy to operate. The short time spent in the development and validation of the assays (<1 weeks) during the detection of SARS-CoV-2 shows that these assays can offer rapid diagnosis of infections due to emerging viruses.

3.4. Establishment of aptamer-based OD-CRISPR for detection of protein

In addition to dsDNA, the collateral cleavage activity of Cas12a can be activated with the aid of target ssDNA. Therefore, we explored whether the OD-CRISPR can be used to detect protein. An ssDNA aptamer was utilized as the recognition component for the target protein, which made direct analysis of complex samples possible without the need for time-consuming processes. The Cas12a-crRNA duplex was designed with the purpose to identify the aptamer specifically. The aptamer was obtained without the target protein, and the OD-CRISPR assay was then used to identify its residual concentration in the sample (Fig. 4a). Here, the designed aptamer-based OD-CRISPR was assessed for PSA detection in serum. The fluorescence assay was used to validate the collateral cleavage activity of the Cas12a-crRNA duplex (Fig. S4a). Subsequently, to determine the appropriate concentration of

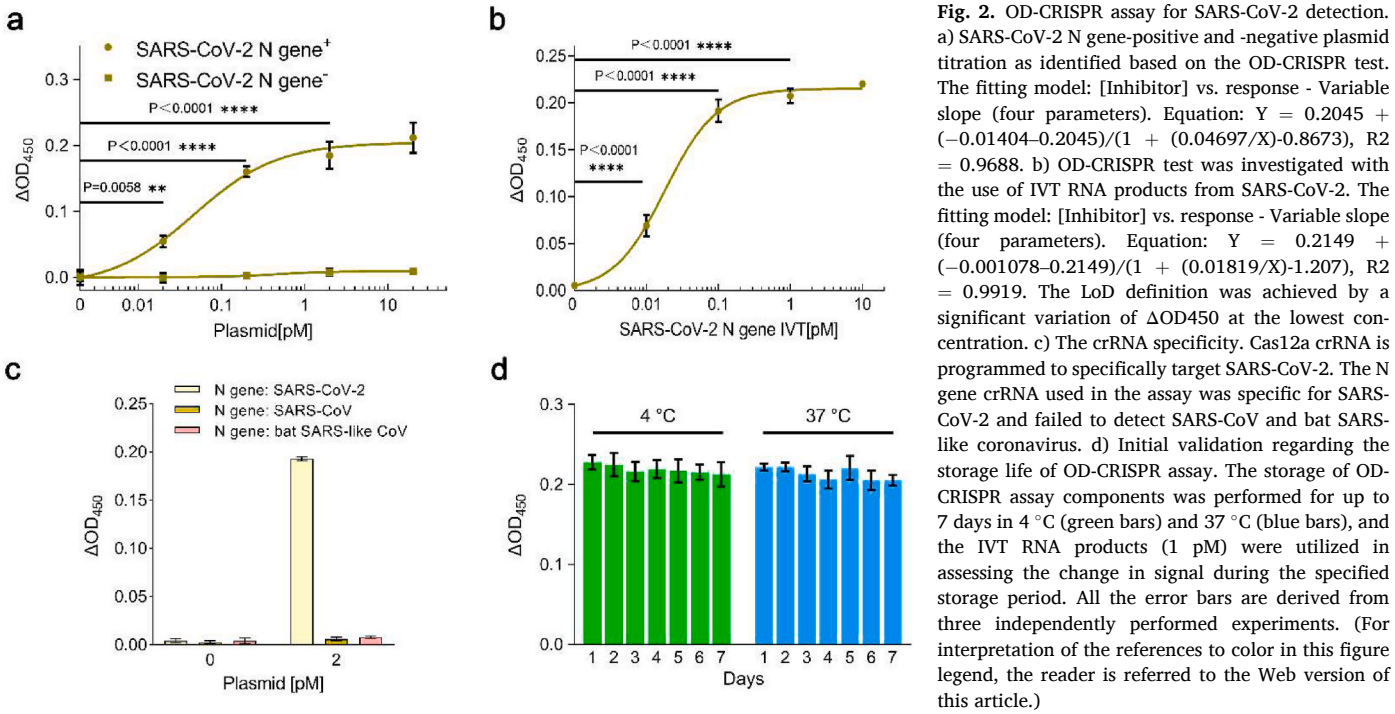

Fig. 2. OD-CRISPR assay for SARS-CoV-2 detection. a) SARS-CoV-2 N gene-positive and -negative plasmid titration as identified based on the OD-CRISPR test. The fitting model: [Inhibitor] vs. response - Variable slope (four parameters). Equation: Y = 0.2045 + (−0.01404–0.2045)/(1 + (0.04697/X)-0.8673), R2 = 0.9688. b) OD-CRISPR test was investigated with the use of IVT RNA products from SARS-CoV-2. The fitting model: [Inhibitor] vs. response - Variable slope (four parameters). Equation: Y = 0.2149 + (−0.001078–0.2149)/(1 + (0.01819/X)-1.207), R2 = 0.9919. The LoD definition was achieved by a significant variation of ΔOD450 at the lowest concentration. c) The crRNA specificity. Cas12a crRNA is programmed to specifically target SARS-CoV-2. The N gene crRNA used in the assay was specific for SARS-CoV-2 and failed to detect SARS-CoV and bat SARS-like coronavirus. d) Initial validation regarding the storage life of OD-CRISPR assay. The storage of OD-CRISPR assay components was performed for up to 7 days in 4 °C (green bars) and 37 °C (blue bars), and the IVT RNA products (1 pM) were utilized in assessing the change in signal during the specified storage period. All the error bars are derived from three independently performed experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

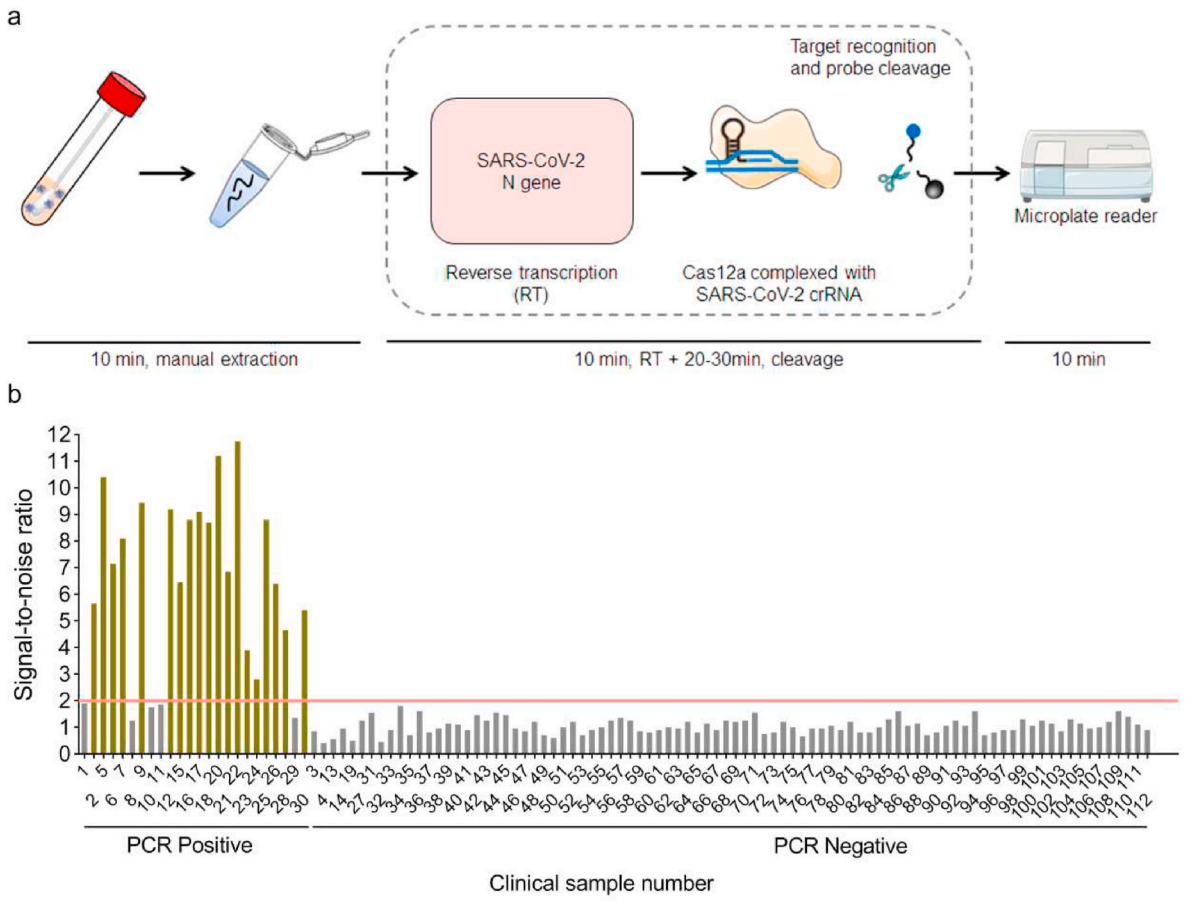

Fig. 3. OD-CRISPR for detecting SARS-CoV-2 in nasopharyngeal swab samples. a) Schematic of the workflow for SARS-CoV-2 OD-CRISPR. Extraction of Standard RNA can be utilized as the first stage in CRISPR-based assays, and measurement can be achieved by a microplate reader. b) OD-CRISPR utilized in SARS-CoV-2 detection in 32 clinically diagnosed COVID-19 positive samples and 80 negative samples. The threshold (pink line) of signal-to-noise ratio (S/N), for the ΔOD readouts, was fixed as follows; with the noise as the signal intensity of a negative sample containing water measure equivalent to a positive result was set at 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Concordance between the RT-qPCR and OD-CRISPR outcomes in the SARS-CoV-2 N gene detection.

		Samples diagnosed by RT-qPCR		
		Positive	Negative	Total
OD-CRISPR	Positive	19	0	19
	Negative	5	88	93
	Total	24	88	112

the aptamer, different concentrations were used to OD-CRISPR (Fig. S4b). For the OD-CRISPR assay, an aptamer concentration of 0.1 pM, which has the largest OD slope, was selected. Next, we attempted to bring aptamer-based OD-CRISPR assay for the detection of PSA. A linear detection range was achieved covering 0.01–10 ng mL-1 and the LoD defined by a signal-to-noise ratio of 3 was determined to be 0.01 ng mL^{-1} (Fig. 4b). Then, HRP-based ELISA, which is the most frequently used method to detect clinical biomarkers, was compared to aptamer-based OD-CRISPR assay. Notably, the sensitivity of aptamer-based OD-CRISPR was higher compared to that of HRP-based ELISA (Fig. S5). In order to assess the selectivity of the aptamer-based OD-CRISPR, specificity experiment was performed using 10 ng mL^{-1} PSA, bovine serum albumin (BSA), carcinoembryonic antigen (CEA), α-fetoprotein (AFP) and carbohydrate antigen 125 (CA125). The result indicated that there was almost no ΔOD using BSA, CEA, AFP and CA125 (Fig. S6), suggesting a high selectivity of the developed aptamer-based OD-CRISPR.

3.5. PSA detection in clinical samples using OD-CRISPR

We lastly examined serum samples from 23 patients with prostate cancer (Table S2) using the aptamer-based OD-CRISPR. These patients had a confirmed diagnosis of prostate cancer and their PSA level was previously determined by CLIA. Correlation analysis between the aptamer-based OD-CRISPR and CLIA showed correlation, and there were no remarkable differences identified in the amounts measured of PSA ($P > 0.05$) (Fig. 4c). These findings suggest that the aptamer-based

Table 2
Specificity, sensitivity, and predictive agreement characterizations[a] of the OD-CRISPR for SARS-CoV-2 N gene detection.

	Sensitivity [95% CI] [b]	Specificity [95% CI]	PPA [95% CI]	NPA [95% CI]
OD-CRISPR	79.17% (61.65–96.68)	100% (94.79–100)	100% (79.08–100)	94.62% (87.32–98.00)

[a] Values were computed from 112 samples for clinical validation and subjected to a comparison with those of RT-qPCR.
[b] CI, confidence interval.

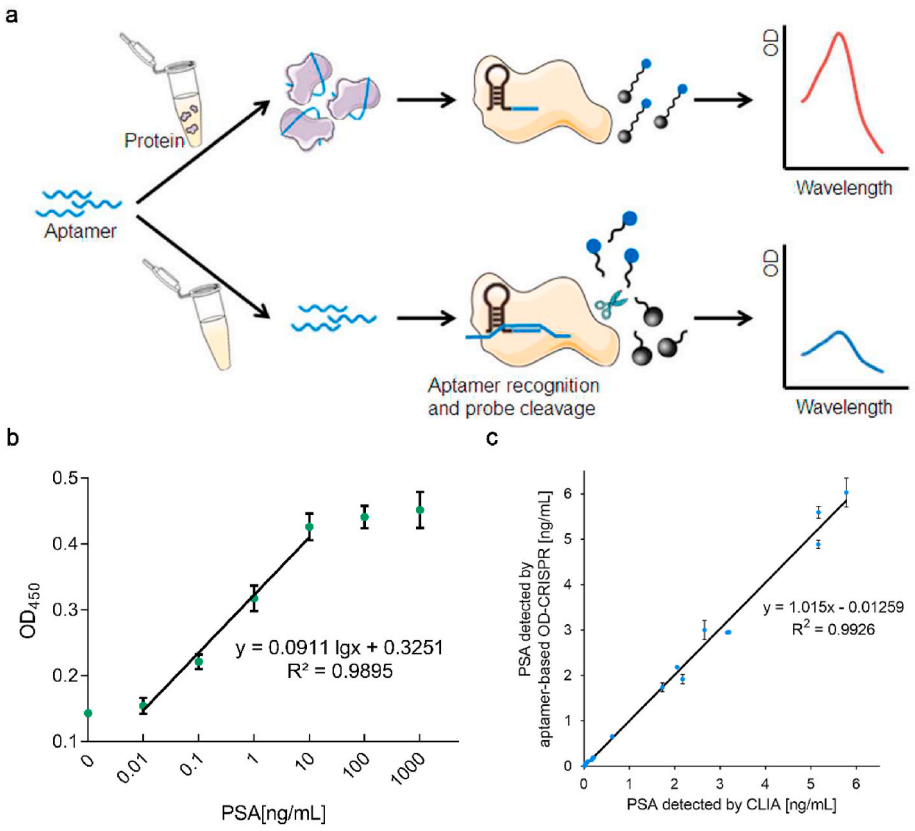

Fig. 4. Aptamer-based OD-CRISPR assay for PSA detection. a) Representation of the aptamer-based OD-CRISPR. b) Titration of the PSA as detected by aptamer-based OD-CRISPR assay. c) Correlation analysis between the aptamer-based OD-CRISPR assay and CLIA using the serums of 23 patients with prostate cancer. All the error bars are obtained from three experiments that were carried out independently.

OD-CRISPR could be utilized for the purpose of identifying protein biomarkers in clinical samples. Due to their efficacy and simplicity, the proposed assays have shown remarkable potential as protein detection platforms in actual clinical settings. However, a greater number of clinical samples need further evaluation so as to confirm the findings of this study. Further research is also needed to detect more significant proteins.

4. Conclusions

Timely and rapid diagnostic assays are required for controlling contagious or refractory diseases. These assays also need to be quick, simple, and easy to use. This study presents a novel approach to developing optical density biosensors utilizing PtNPs in probing the CRISPR cleavage activity (OD-CRISPR). Because of high specificity in the target recognition, we utilized the CRISPR Type V system, Cas12a, which is a coherent biosensing system that exceeds gene-editing tools, to transform the target recognition activity into an identifiable optical density signal via a PtNPs probe with non-specific ssDNA. Different parameters were evaluated to develop a relatively low-cost and easy-to-implement OD-CRISPR-based detection platform. Furthermore, our verification indicates that the OD-CRIPSR system can be implemented in more ways than only detection of nucleic acid. The OD-CRISPR can as well be utilized to perform protein detection through the addition of an aptamer-based sensing cascade, contributing to a cost-effective, robust, and generalizable detection system.

CRediT authorship contribution statement

Jiajie Liang: Conceptualization, Methodology, Investigation, Writing – original draft, Formal analysis. **Peijun Teng:** Validation, Investigation, Validation. **Liangshan Hu:** Conceptualization, Investigation, Resources, Writing – review & editing. **Guanbo He:** Methodology, Software. **Qifang Song:** Investigation. **Ying Zhang:** Investigation. **Bin Peng:** Investigation. **Gan Li:** Investigation. **Wei Xiao:** Resources, Writing – review & editing, Funding acquisition. **Donglin Cao:** Resources, Writing – review & editing, Funding acquisition. **Yong Tang:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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