MnO$_2$ Nanozyme-Mediated CRISPR-Cas12a System for the Detection of SARS-CoV-2

Lina Wu,$^\text{⊥}$ Xinjie Wang,$^\text{⊥}$ Xiangchuan Wu, Shiqi Xu, Ming Liu, Xizhong Cao, Taishan Tang, Xingxu Huang,* and He Huang*

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ABSTRACT: The CRISPR-Cas system was developed into a molecular diagnostic tool with high sensitivity, low cost, and high specificity in recent years. Colorimetric assays based on nanozymes offer an attractive point-of-care testing method for their low cost of use and user-friendly operation. Here, a MnO$_2$ nanozyme-mediated CRISPR-Cas12a system was instituted to detect SARS-CoV-2. MnO$_2$ nanorods linked to magnetic beads via a single-stranded DNA (ssDNA) linker used as an oxidase-like nanozyme inducing the color change of 3,3',5,5'-tetramethylbenzidine, which can be distinguished by the naked eye. The detection buffer color will change when the Cas12a is activated by SARS-CoV-2 and indiscriminately cleave the linker ssDNA. The detection limit was 10 copies per microliter and showed no cross-reaction with other coronaviruses. The nanozyme-mediated CRISPR-Cas12a system shows high selectivity and facile operation, with great potential for molecular diagnosis in point-of-care testing applications.

KEYWORDS: MnO$_2$ nanorods, CRISPR-Cas12a, SARS-CoV-2, Nanozyme, Detection

1. INTRODUCTION

Nanozymes have been applied in many fields, including disease diagnosis,$^1$ cancer therapy,$^2$, pollutant degradation,$^3$, and biosensing.$^4$ They offer advantages of inexpensiveness, high stability under harsh conditions, and easy preparation.$^5$ Nanozyme-mediated colorimetric assays offer an attractive approach for point-of-care detection based on their operational simplicity and easy adaptability for microplate-based high-throughput assays.$^6$ Peroxidase-like nanozymes are commonly used for colorimetric assays.$^7$ Peroxidase-catalyzed oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) requires H$_2$O$_2$, which is unstable and corrosive.$^8$ By contrast, oxidase-like nanozymes can oxidize TMB without H$_2$O$_2$, which makes the assay simpler and more convenient. Mn oxides have been successfully used as an efficient oxidase-like nanozyme.$^9$ Zhu et al. established a single-atom enzyme based on pegylated manganese that could catalyze the O$_2$ to cytotoxic $\bullet$O$_2^{-}$ through oxidase-mimic activity.$^{10}$ Wu et al. used a MnO$_2$ sheet with oxidase-like activity to establish an electrochemical sensor to detect organophosphate pesticides without the interference of H$_2$O$_2$.$^{11}$

Recently, CRISPR-Cas-based detection systems began to be used for the detection of nucleic acids,$^{12}$ proteins,$^{13}$ and small molecules.$^{14}$ The CRISPR-Cas system provided an ultrasensitive, inexpensive, and specific diagnostics platform for COVID-19.$^{15}$ Fluorescence detection is the most commonly used method united with the CRISPR-Cas system. Zhang et al. reported the clinical validation of SHERLOCK for SARS-CoV-2 using Cas13a enzyme. The specificity and sensitivity of SHERLOCK with fluorescence readout reached 100%.$^{16}$ Doudna et al. reported a CRISPR-Cas13a assay of SARS-CoV-2 in which the fluorescence signals were measured using a

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mobile phone microscope.\(^\text{42}\) In addition to fluorescence detection, colorimetric assays are also an excellent choice due to their low cost and easy operation. Moon et al. reported a CRISPR/Cas9 involved colorimetric method in which streptavidin-horseradish peroxidase reacted with a biotin-PA_Mmr, inducing the color change of TMB.\(^\text{43}\) Zhang et al. developed a gold nanoparticle-based colorimetric assay of SARS-CoV-2 that combines with CRISPR/Cas12a.\(^\text{44}\) Here, we report a colorimetric method on the strength of a MnO\(^2\) nanozyme-mediated CRISPR-Cas12a system. The synthesized MnO\(_2\) nanorods exhibited excellent oxidase-like activity, resulting in efficient catalytic reaction of TMB with a clearly visible color change from pale yellow to blue. MnO\(_2\) nanorods were used as nanoyzmes to label magnetic beads (MBs) via single-stranded DNA (ssDNA). When SARS-CoV-2 was present, the ssDNA linker was cleaved by the activated Cas12a and induced the color change for colorimetric detection. The MnO\(_2\) nanoyzme-mediated CRISPR-Cas system is simpler than the HRP catalyzed TMB-H\(_2\)O\(_2\) system since it does not need H\(_2\)O\(_2\) and the nanoyzme is highly robust.

2. EXPERIMENTAL SECTION

2.1. Reagents. The LbCas12a protein was obtained as previously reported.\(^\text{45}\) The crRNAs, primers for the polymerase chain reaction (PCR), RT-RPA, and other DNA probes are listed in Table S1 and were synthesized by GenScript (Nanjing, China). Streptavidin-coated magnetic beads (MBs, 300 nm, 10 mg/mL) were purchased from Beaver for Life Sciences (Suzhou, China). The synthetic target gene fragment or RPA product, 1 \(\mu\)L of Tris-HCl buffer (pH 7.4), after which 10 \(\mu\)L of ssDNA-MBs were incubated with 10 \(\mu\)L of EDC and 10 \(\mu\)L of NHS (10 mM) at 37 °C to activate the carboxyl group of dsDNA. After magnetic separation, ssDNA-MBs were dispersed in 10 \(\mu\)L of Tris-HCl, NaCl, MgCl\(_2\), and Tween-20 were purchased from Aladdin (Shanghai, China). RT-RPA was performed using a commercial kit (GenDx Biotech Co., Ltd., Canada). Pseudoviruses corresponding to four coronavirus species (SARS-CoV-2-E, hCov-HKU1-E, MERS-uPE-E, and SARS-E) were synthesized by Cobbio Biosciences Co. Ltd. (Nanjing, China). The clinical samples were provided by Guangzhou Institute of Respiratory Health, The First Affiliated Hospital, Guangzhou Medical University.

2.2. Instruments. Transmission electron microscopy (TEM) photographs were obtained on a HT7700 transmission electron microscope (Hitachi High-Technologies Co., Ltd., Japan). The absorption spectrum was obtained on a UV-1800 spectrophotometer (AoYi Instruments Shanghai Co., Ltd., China). The enzyme-labeled immunosorbent assay (ELISA) instrument was from Gene Co., Ltd. The MnO\(_2\) nanorods were synthesized in an electrically heated constant temperature air-blast drying oven (Jinghong Shanghai Co., Ltd., China).

2.3. Synthesis of Amino-Functionalized MnO\(_2\) Nanorods (MnO\(_2\)–NH\(_2\) NRs). MnO\(_2\) nanorods were prepared according to the method provided by Padhi et al.\(^\text{46}\) MnO\(_2\)–NH\(_2\) NRs were synthesized using organosilane as follows: 50 mg of the prepared MnO\(_2\) nanorods were dispersed in 20 mL of ethanol and stirred for 1 h in a nitrogen atmosphere, after which 0.2 mL of 3-aminopropyl triethoxysilane was added for the functionalization of MnO\(_2\). After stirring for 12 h, the material was obtained by centrifugation, washed with ethanol and double distilled water three to five times, and dried in an oven at 60 °C for 12 h to obtain amino-functionalized MnO\(_2\) nanorods.

2.4. Synthesis of ssDNA-MnO\(_2\)-MBs. A sample comprising 10 \(\mu\)L of 2 \(\mu\)mol/L ssDNA modified with biotin and carboxyl was complexed with 10 \(\mu\)L of 10 mg/L MBs modified with streptavidin and incubated at 37 °C for 1 h. Then, ssDNA and MBs were conjugated via the interaction of streptavidin and biotin. After that, the ssDNA-MBs were magnetically separated and cleaned twice to remove the excess ssDNA. Finally, the ssDNA-MBs were dissolved in 10 \(\mu\)L of Tris-HCl buffer (pH 7.4).

Then, 10 \(\mu\)L of ssDNA-MBs were incubated with 10 \(\mu\)L of EDC and 10 \(\mu\)L of NHS (10 mM) at 37 °C to activate the carboxyl group of ssDNA. After magnetic separation, ssDNA-MBs were dispersed in 10 \(\mu\)L of Tris-HCl, and 10 \(\mu\)L of MnO\(_2\)–NH\(_2\) NRs were added and reacted for 12 h at 4 °C. The mixture was magnetically separated and washed to remove the excess reagent to obtain ssDNA-MnO\(_2\)-MBs. Finally, the product was redispersed in 10 \(\mu\)L of Tris-HCl buffer (pH 7.4) and stored at 4 °C.

2.5. Detection of SARS-CoV-2. The reaction mixture for the detection of SARS-CoV-2 using the ssDNA-MnO\(_2\)-MBs reporter consisted of 1 \(\mu\)L of Cas12a (200 ng/\(\mu\)L), 1 \(\mu\)L of crRNA (1 nM), 1 \(\mu\)L of the synthetic target gene fragment or RPA product, 1 \(\mu\)L of RNase inhibitors (40 U/\(\mu\)L), 2 \(\mu\)L of reaction buffer, and double distilled water to 20 \(\mu\)L. The reaction incubated for 12 h at 37 °C for 1 h. After the reaction was completed, the magnetic beads were collected through magnetic separation, and then TMB (200 \(\mu\)L) was added to initiate the chromogenic reaction. The solution was gently mixed for quantitative analysis by a UV–vis spectrometer.

2.6. RT-RPA Preamplification and RT-qPCR Assay. RT-RPA reactions were processed as previously reported by us.\(^\text{47}\) The RT-qPCR detection was done according to the WHO recommended procedure.

3. RESULTS AND DISCUSSION

3.1. Working Principle. The MnO\(_2\) nanoyzme-mediated CRISPR-Cas12a detection was performed as shown in Figure 1. To establish a visual colorimetric assay, the ssDNA-MnO\(_2\)-MBs reporter system was developed by ligating MnO\(_2\)–NH\(_2\) NRs to streptavidin-coated magnetic beads via an ssDNA linker. The ssDNA has a biotin group at one end and a carboxyl group at the other, which can be used to immobilize the MnO\(_2\) nanorods via streptavidin-

Figure 1. Scheme of the CRISPR-Cas12a system based on the ssDNA-MnO\(_2\)-MBs reporter for detecting SARS-CoV-2.
carboxyl group at the other. Biotin binds to streptavidin with a powerful affinity, while the carboxyl group binds to the amino groups functionalized onto the MnO$_2$ nanorods. For the detection of SARS-CoV-2, the target RNA was preamplified with RT-RPA and then used for the Cas12a mediated reaction. When SARS-CoV-2 target nucleic acid is added, Cas12a is activated and begins to cleave the ssDNA connector between the MnO$_2$ nanorods and MBs. The MnO$_2$ nanorods collected through magnetic separation will catalyze the oxidation of TMB. When the amount of MnO$_2$ nanorods bound to the MBs was reduced, the catalytic reaction decreased, inducing the visible color change of TMB solution. As a consequence, the SARS-CoV-2 detection result was observed visually or quantified spectrophotometrically.

3.2. Characterization of the MnO$_2$−NH$_2$ NRs. The synthesis of MnO$_2$−NH$_2$ NRs is a critical part of the formation of signal probes to use in the CRISPR-Cas system. MnO$_2$−NH$_2$ NRs were characterized by TEM, XRD, and FTIR. The TEM (Figure 2A and B) showed that MnO$_2$ NRs and MnO$_2$−NH$_2$ NRs possessed similar morphologies. They both have a length of about 1 μm and a diameter of 6–8 nm, indicating that the amino-functionalization of MnO$_2$ nanorods did not cause morphological changes. The Mn, O, N elements are homogeneously distributed in the elemental mapping (Figure 2C, D, and E) that verified successful synthesis of MnO$_2$−NH$_2$ NRs. The crystalline nature of MnO$_2$−NH$_2$ NRs was investigated by XRD. The XRD patterns of MnO$_2$−NH$_2$ NRs (Figure 2F) showed distinct diffraction peaks at 12.80°, 18.10°, 28.60°, 37.50°, 42.70°, 49.80°, 56.00°, 62.70°, and 69.2°, which were indexed to the different planes of the pure tetragonal phase of MnO$_2$ (JCPDS File No. 44-0141). For the MnO$_2$ NRs, a broad band of about 60° is assigned to amination. Figure 2G showed the FTIR spectrum of MnO$_2$ NRs and MnO$_2$−NH$_2$ NRs. The peak at 821 cm$^{-1}$ manifests Mn−O. The peak at 1404 cm$^{-1}$ manifests O−H bending vibrations combined with a manganese molecule. The peak at

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**Figure 2.** (A) TEM image of MnO$_2$ NRs. (B) TEM image of MnO$_2$−NH$_2$ NRs. (C, D, E) Elemental mapping of MnO$_2$−NH$_2$ NRs. (G) FTIR of MnO$_2$ NRs and MnO$_2$−NH$_2$ NRs.

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3412 cm\(^{-1}\) in the spectrum of MnO\(_2\)–NH\(_2\) NRs which contributed to the N–H group suggests that MnO\(_2\) has completely been aminated.

The oxidase-like activity of nanozymes was evaluated by UV–vis with TMB as a substrate. The Michaelis–Menten and the Lineweaver–Burk curves are shown in Figure S1. The kinetic parameters are displayed in Table S2. The results indicate the excellent oxidase-like activity of MnO\(_2\) NRs and MnO\(_2\)–NH\(_2\) NRs. So, MnO\(_2\)–NH\(_2\) NRs could be used to label ssDNA instead of the natural enzyme. The \(V_{\text{max}}\) and \(K_m\) of MnO\(_2\)–NH\(_2\) NRs was a litter higher than that of MnO\(_2\)–NRs, which proved that amino-functionalization did not influence the catalytic activity of MnO\(_2\)–NRs.

3.3. Characterization of the ssDNA-MnO\(_2\)-MBs. For a proof-of-concept demonstration that the ssDNA-MnO\(_2\)-MBs reporter was valid in the Cas12a detection system, the labeling of magic beads, oxidase-like activity, and ssDNA cleavage were characterized.

Figure 3 showed TEM images of MBs, as-prepared ssDNA-MnO\(_2\)-MBs, and ssDNA-MnO\(_2\)-MBs cleaved by the activated CRISPR-Cas12a system. As shown in Figure 3A and D, the MBs are presented as nanospheres with a diameter of 700–800 nm. As shown in Figure 3B and E, numerous MnO\(_2\) nanorods were successfully bound to the MBs. Figure 3C and F showed ssDNA-MnO\(_2\)-MBs cleaved by the activated CRISPR-Cas12a system. The amount of the MnO\(_2\) nanorods on the MBs was...
obviously decreased as the ssDNA connecting the MBs and MnO₂ nanorods was cleaved by activated Cas12a. The TEM image demonstrated the successful cleavage, releasing MnO₂ nanorods from the MBs.

The quenched fluorescent ssDNA reporter confirmed the feasibility of the CRISPR-Cas12a detection system (Figure S2). When the CRISPR system identified the activation chain, Cas12a protein was activated and indiscriminately cleaved the quenched fluorescent ssDNA reporter generating the fluorescent signal. The result demonstrated that this strategy was feasible.

The oxidase-like activity of the MnO₂ nanorods, the as-prepared ssDNA-MnO₂-MBs, as well as the ssDNA-MnO₂-nanorods cleaved by the activated CRISPR-Cas12a system were assayed via the oxidation of TMB. As shown in Figure 4A, the MnO₂ nanorods induced an obvious increase of absorption at 650 nm due to the oxidation of TMB, indicating excellent oxidase-like activity. The as-prepared ssDNA-MnO₂-MBs also had a similar adsorption peak due to the MnO₂ nanorods immobilized on their surface via the ssDNA. When the ssDNA-MnO₂-MBs were incubated with an activated Cas12a system, the adsorption peak was reduced significantly due to the decrease of MnO₂ nanorods attached to the MBs, indicating the successful cleavage of the ssDNA link between the MnO₂ nanorods and MBs.

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Polyacrylamide gel electrophoresis (PAGE; Figure 4C) was used to demonstrate the feasibility of the CRISPR-Cas12a system. Line 2 shows the bands of ssDNA and the synthetic target E gene fragment, respectively. The ssDNA and the synthetic target gene fragment bands did not change when Cas12a was added to the system (line 3). A new band appeared when crRNA was added to the system (line 4). Independent bands of crRNA and ssDNA were visible (line 5) when the Cas12a/crRNA/ss DNA mixture was incubated without the synthetic target gene fragment, which indicated that Cas12a was inactive. When the synthetic target gene fragment was added to the system, the bands of crRNA, the synthetic target gene fragment, and ssDNA disappeared because the activated Cas12a could indiscriminately cleave ssDNA (line 6). The PAGE results (line 6) indicated that when the solution contained Cas12a, the synthetic target gene fragment, and crRNA, then trans-cleavage happened.

3.4. Optimization of Reaction Conditions. The experimental conditions such as the length of ssDNA, ssDNA concentration, incubation temperature, and incubation time were optimized. Three different lengths of ssDNA (20, 40, and 60 nt) were used as a linker to connect MnO₂-NH₂ NRs to the MBs. As shown in Figure 5C, the length would affect the trans-cleavage efficiency of activated Cas12a. A longer ssDNA could reduce the steric hindrance of Cas12a and improve the trans-cleavage efficiency. Accordingly, an ssDNA of 60 nt was selected as the linker between the MnO₂-NH₂ NRs and MBs.

Figure 5. Relative activity (A), absorption spectra (B), and color change (E) of different crRNAs targeting the SARS-CoV-2 E gene. The relative activities (C), absorption spectra (D), and color change (F) of systems with ssDNA linkers of different lengths.
A highly efficient crRNA is the key to sensitive and specific detection. Six crRNAs (crRNA1−crRNA6, Table S1) were designed and screened. The spacer sequence and the secondary structure affected the targeting cleavage efficiency of the CRISPR detection. As shown in Figure 5A−E, all six crRNAs were effective, although crRNA2, crRNA3, and
RT-RPA products were detected by the MnO reverse-transcribed and amplified using RT-RPA. Then, the crRNA6 were better than the three remaining crRNAs. The extracted RNA from samples containing different pseudovirus samples were used instead of actual clinical samples. The incubation time was varied from 20 to 150 min. As shown in Figure 6C, the color change increased from 20 to 37 °C and decreased at higher temperatures. Thus, the optimal incubation temperature was 37 °C.

3.5. The Detection of SARS-CoV-2. Different concentrations of the synthetic target gene fragment were used to evaluate the analytical performance (Figure 7A). The color change increased with the concentration of the target gene fragment. The system had good linearity in the concentration range from 1 ng/L to 1 μg/L (Figure 7B). The detection limit of the method was 0.32 ng/L, which was calculated according to the 3σ rule.

However, the detection limit was not sensitive enough for SARS-CoV-2 detection without further amplification. So, the RT-RPA step was included to amplify the RNA sequence of SARS-CoV-2. Due to biosafety concerns, SARS-CoV-2-E pseudovirus samples were used instead of actual clinical samples. The extracted RNA from samples containing different concentrations of the SARS-CoV-2-E pseudovirus were reverse-transcribed and amplified using RT-RPA. Then, the RT-RPA products were detected by the MnO2 nanorods reporter; the selectivity of the proposed system; stability of the ssDNA-MnO2-MBs reporter; nucleic acids sequences; the performance of the system for SARS-CoV-2 detection compared with real-time PCR (PDF).

4. CONCLUSIONS

In this study, we established a nanzyme-mediated colorimetric assay to detect SARS-CoV-2 based on the CRISPR-Cas12a system. MnO2 nanorods were used as a reporter based on the oxidase-like activity. In the presence of SARS-CoV-2, activated Cas12a mediates the trans-cleavage of the ssDNA linker between MnO2 nanorods and MBs, which decreased the oxidation of TMB, inducing the color change. The use of MnO2 nanozymes instead of natural enzymes avoids the use of H2O2, resulting in low cost and operational simplicity. Moreover, the colorimetric analysis based on nanozymes and the CRISPR-Cas12a system makes the assay easy to operate and applicable to microplate-based high-throughput analysis. The MnO2 nanozyme-mediated CRISPR-Cas12a system can be used as an excellent tool for detecting emerging pathogens in the future.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.2c14497. Steady-state kinetics of MnO2 NRs and MnO2-NH2 NRs; the feasibility of the CRISPR-Cas12a detection system confirmed by the quenched fluorescent ssDNA reporter; the selectivity of the proposed system; stability of the ssDNA-MnO2-MBs reporter; nucleic acids sequences; the performance of the system for SARS-CoV-2 detection compared with real-time PCR (PDF).

AUTHOR INFORMATION

Corresponding Authors
Xingxu Huang — Zhejiang Laboratory, Hangzhou 311100, People’s Republic of China; Email: xingxuhuang@njnu.edu.cn
He Huang — School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Nanjing 210023, People’s Republic of China; Email: huangh@njnu.edu.cn

Authors
Lina Wu — School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Nanjing 210023, People’s Republic of China; Zhejiang Laboratory, Hangzhou 311100, People’s Republic of China; https://orcid.org/0000-0001-8640-6279
Xinjie Wang — Shenzhen Branch, Guangdong Laboratory of Lingnan Modern Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture and Rural Affairs, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen 518120, People’s Republic of China
Xiangchuan Wu — School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Nanjing 210023, People’s Republic of China
Shiqi Xu — School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Nanjing 210023, People’s Republic of China
Ming Liu — State Key Laboratory of Respiratory Disease, National Clinical Research Center for Respiratory Disease, Guangzhou Institute of Respiratory Health, The First

Guangzhou Institute of Respiratory Health, The First
Author Contributions

L.W. and X.W. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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