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Self-resetting molecular probes for nucleic acids detection enabled by fuel dissipative systems

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A B S T R A C T
A once-in-a-century global public health crisis, the COVID-19 pandemic has damaged human health and world economy greatly. To help combat the virus, we report a self-resetting molecular probe capable of repeatedly detecting SARS-CoV-2 RNA, developed by orchestrating a fuel dissipative system via DNA nanotechnology. A set of simulation toolkits was utilized to design the probe, permitting highly consistent signal amplitudes across cyclic detections. Uniquely, full width at half maximum regulated by dissipative kinetics exhibits a fingerprint signal suitable for high confidential identifications of single-nucleotide variants. Further examination on multiple human-infectious RNA viruses, including ZIKV, MERS-CoV, and SARS-CoV, demonstrates the generic detection capability and superior orthogonality of the probe. It also correctly classified all the clinical samples from 55 COVID-19 patients and 55 controls. Greatly enhancing the screening capability for COVID-19 and other infectious diseases, this probe could help with disease control and build a broader global public health agenda.

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Introduction

Caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), COVID-19 has spread rapidly with over 1.6 billion reported cases and 3.4 million deaths worldwide as of May 2021. Testing available ad libitum and early diagnosis are essential to identify the disease and provide prompt treatments, and therefore sufficiently large supply of nucleic acid test capacity is critical to screen suspected cases timely [1,2].

Currently, the detection of single-stranded RNA (ssRNA) of SARS-CoV-2 [3] relies on hybridization-based molecular probes such as Taqman probe in reverse-transcription quantitative polymerase chain reaction (RT-qPCR) (Fig. 1A) [4,5]. Alternative molecular probes for PCR-based methods were developed to realize the rapid and portable detection of SARS-CoV-2 RNA [6–8]. However, the signal generation of these probes depends on stable target-probe complexes or target-induced probe degradations dominated by the thermodynamics or enzymatic kinetics which theoretically restrict probe regeneration (Fig. 1B). This has led to a shortage of testing reagents during the COVID-19 pandemic, particularly in countries and regions with poor development of biotechnology and related industries. Consequently, some countries cannot even reach a general benchmark of 10–30 tests per confirmed case suggested by World Health Organization (Fig. S1). In fact, the test number per confirmed case value is positively correlated with Gross domestic product (GDP) per capita, and the control of COVID-19 in some countries is indeed limited by insufficient nucleic acid testing (Fig. S2). Moreover, millions of daily COVID-19 tests worldwide not only require a large amount of materials and labor but also produce enormous biomedical waste (BMW) (Fig. S3).

Recent analyses on the sequence variations of SARS-CoV-2 isolates identified several genomic regions with increased genetic variations [9]. For example, there is the single-nucleotide variant (SNV) D614G in the spike protein which reduces S1 shedding and increases infectivity of the virus [10,11]. Current molecular probes to detect such SNVs rely on the thermodynamic or kinetics differences of single reactions that discriminate perfectly matched (PM) and mismatched (MM) duplex...
the feature dissipative kinetics enables fingerprinting FWHM to guide the operation of fuel dissipation, and highly consistent signal identify SNVs in the viral sequence. In combination with isothermal shortcoming of all current probes in regeneration. More importantly, amplitudes across cyclic detections were achieved overcoming the states, respectively (Fig. 1 C). A set of simulation toolkits were employed to overcome the shortcomings of all current probes in regeneration. More importantly, the featured dissipative kinetics enables fingerprinting FWHM to identify SNVs in the viral sequence. In combination with isothermal amplification, this method could reach a quantification limit of 0.01 fM and is feasible for instrument-free tests. We further employed the self-resetting probes to test multiple human-infectious RNA viruses including ZIKV, MERS-CoV, and SARS-CoV to demonstrate its versatility and superior orthogonality. Self-resetting probes were also deployed for detection of clinical nasopharyngeal swabs and correctly classify all the samples from COVID-19 patients and controls.

Results

Fuel dissipation and self-resetting probe system

The design of self-resetting probes in homogeneous solutions follows such a principle: detection targets should cause a significant conformational change to generate signals and then be sufficiently consumed in the solution. The key to self-resetting probe systems is the design of an out-of-equilibrium system that regulates fuel dissipation. The dissipative process is commonly found in biological processes such as cell division and signal transduction and is typically driven by chemical energy stored in kinetically stable, high-energy molecules [19,20]. As shown in Fig. 1C, the target acts as a fuel to shift the equilibrium to a thermodynamically disfavored high-energy state. The fuel molecule is subsequently consumed, and the system returns to the equilibrium state, allowing for the autonomous probe resetting. The entire process can be simplified as a fuel-to-waste conversion. The high-energy state can be transiently stabilized for a certain amount of time by regulating the forward and the backward reactions. DNA nanotechnology can orchestrate strand self-assembly in a highly programmable way and enables the design of molecular logic systems [21]. For example, fuel dissipative processes such as molecular cargo loading [22], nanostructure dynamic steady assembly [23], and constitutional dynamic networks [24] have been programmed by dynamic DNA nanotechnology. Since the nucleic acids acted as the fuel in our case, we chose the toehold-mediated strand displacement (TMSD) [25] and the enzymatic digestion as the forward and backward reaction, respectively (Fig. 1C). The kinetics of these two reactions could be easily tuned in a programmable manner to fulfill the fuel dissipation requirement. Simulations is also performed to guide the system, achieving effective signals and probe reuses.

DNA probe design guided by oxDNA simulation

Here, we first performed MD simulations to guide the design of the basic form of DNA probes. In the forward reaction TMSD, the target strand binds to the toehold region and initiates branch migration, resulting in reporter strand release. If the fluorophore and quencher are labeled at appropriate positions of the probe, target recognition can be visualized by fluorescence quenching. In principle, the probe can be designed into linear form or hairpin form (Fig. S4). In the linear design, intermolecular TMSD results in a complete separation of fluorophore and quencher, whereas in the hairpin design, intramolecular TMSD only leads to an increase in the
distance between the two functional groups. With the coarse-grained MD simulation program oxDNA [26], the molecular behavior and structural flexibility of the hairpin probe were evaluated to study the distance distribution of the fluorophore and quencher upon target recognition. The simulation conditions and schematic drawings are noted in Table S2 and Fig. S5A, respectively. The distance increased as a function of loop length, but all the average distances were less than 10 nm, which is within the range of fluorescence resonance energy transfer (FRET) quenching (Fig. S5B). Moreover, due to the flexibility of single-stranded DNA (ssDNA) in hairpin probe, the distance exhibited a broad distribution, increasing the intramolecular collision frequency in the DNA nanostructure and resulting in unexpected quenching. Experimentally, the fluorescence enhancement of all hairpin probes was weaker than that of the linear probe, consistent with the theoretical simulation (Fig. S5C). Considering the above evidence, we chose the linear design for self-resetting probe construction.

**Kinetics simulation of the fuel dissipation-based self-resetting probe**

The implementation of the self-resetting probe based on energy dissipation is shown in Fig. 2A. Target binding induced reporter strand release through TMSD, resulting in fluorescence enhancement. Exonuclease III (Exo III), the enzyme used for dissipation, is a double-stranded DNA (dsDNA)-specific exonuclease that catalyzes the removal of single nucleotides from linear or nicked dsDNA in the 3' to 5' direction [27,28]. The TMSD product allows the formation of Exo III substrate. Phosphonothioate (PS) modification at the 3' ends of the probes was designed to prevent digestion of the probe strands (Table S1) and ensure that degradation of the target is the only enzymatic reaction. The reporter strand rehybridizes with the receptor to realize autonomous probe resetting with fluorescence quenching. The process is realized by increasing hydrogen bonds and phosphodiester bonds in the DNA structures through TMSD and then breaking these bonds via hydrolysis catalyzed by exonuclease. This dynamic network is a typical dissipative out-of-equilibrium system: the external fuel via self-assembly drives the equilibrium state to a high-energy state, and the consumption of the fuel activates the return process to the equilibrium state. The forward and backward reactions are chemically independent, structurally selective, and kinetically tunable. Moreover, the structure is completely reversible on the molecular level, which is a critical feature for the construction of self-resetting probes. Therefore, this target oligonucleotide-fueled dynamic network constitutes a general strategy to establish dissipative self-resetting DNA probes.

The cooperation between TMSD and the enzymatic reaction creates a dynamic system allowing for autonomous probe resetting. As discussed above, the concentration of the reporter was expected to follow a single oscillation pattern (Fig. 2B). The maximum concentration of the reporter represents the probe sensitivity, and the lifetime of the reporter stands for the detection efficiency. As long as the enzymatic reaction is slowed down, we can increase the maximum concentration of the reporter, but this will also increase the lifetime of the reporter. The long lifetime of the reporter not only leads to low detection efficiency but also reduces the number of cycles the system can undergo because the enzyme activity may decay over time. Therefore, the simulation of dissipative kinetics is
critical for optimizing the height and full width at half maximum (FWHM) of the trace in Fig. 2B. We defined the ratio of height and FWHM (height/FWHM) which reflects the capability for fuel dissipation per unit time as a key factor for the simulation and optimization. Briefly, a high height/FWHM is desired (Fig. 2C, Table S3).

To achieve such a value, we established kinetics differential equations to guide the optimization of the toehold configuration, enzyme concentration, and catalytic rate constant. The forward TMSD reaction could be irreversible (Fig. 2A) or reversible (Fig. S6) (see Supporting note 1 for the corresponding differential equations). The kinetics of TMSD is understood, and according to the established model, the toehold length influences the kinetics greatly. The rate constant increased exponentially from 10 to 10⁶ M⁻¹ s⁻¹ as a function of toehold length ranging from 0 to 6. The rate is saturated when the toehold length is more than 6. Therefore, we chose toehold lengths from 2 to 6 for simulation, and their rate constant k_{TMSD} was predicted with the established model. To simulate the scenario with reversible TMSD, we predicted the rate constants k_{TMSD}⁻ and k_{TMSD}⁺ for reverse toehold lengths of 2 or 4 when the forward toehold length was 6. For the backward reaction of the dissipative system, we obtained a Michaelis–Menten constant (k_{cat}) of 120 nM and rate constant (k_{cat}⁻) of 0.44 s⁻¹ for Exo III with a dsDNA substrate and a blunt 3' terminus (see Supporting note 2 for more details of the simulation parameter setup).

Based on the above parameters, we used the Runge–Kutta method to solve the differential equations, and all the obtained concentration-vs-time curves were consistent with expectations. The concentration of reporter followed a single oscillation pattern (Fig. S7A), and the concentration of Exo III agreed with the Michaelis–Menten kinetics model (Fig. S7B). To guide the setup of experimental conditions, different conditions are simulated to obtain the highest value of height/FWHM. For the forward reaction, the height increased as a function of toehold length, and FWHM showed a reverse trend (Fig. S8). Highest height/FWHM values were achieved when the toehold length was six (Fig. 2D). When reverse TMSD was present, an increasing reverse toehold length slightly reduced the height and enhanced the FWHM (Fig. S9A and B). For the backward reaction, the enhancement of enzymatic digestion (e.g., increasing k_{cat} or Exo III concentration) decreased the FWHM and height simultaneously (Fig. 2E). Therefore, appropriate k_{cat} and Exo III concentrations yielded the highest height/FWHM value (Fig. 2E). Collectively, in order to obtain the highest height/FWHM experimentally, the forward and reverse toehold lengths were set up to six and zero, respectively, and enzymatic reaction condition was chosen as the region in the black circle in Fig. 2E.

**Experimental validation of the self-resetting probe**

Based on the simulation results, fluorescence assays for the sequences shown in Fig. S10 with 200 nM DNA strands and 8.6 nM Exo III were performed to validate the dissipative process. The toehold length was 6, and the enzyme activity k_{cat} (0.44 s⁻¹) was not tuned. The fluorescence intensity F_t was con-verted to the reporter concentration C_t via the formula C_t = (F_0 - F_{t0})/(F_{max} - F_{t0}) * C_{max}, where F_{max} and F_{t0} are the reporter and the equilibrate probe, respectively, and C_{max} is the initial probe concentration. The kinetics of the basic reaction modules was characterized, and target-initiated TMSD allowed for up to 90% reporter release within 1.6 min (blue line, Fig. 3A). The rehybridization process in the absence of the target rapidly recycled the released reporter, enabling 90% probe resetting within 1.16 min (green line, Fig. 3A). If the enzyme was added after TMSD, the target was digested, and the probe was reset via rehybridization (brown line, Fig. 3A). Under dissipative conditions, the addition of DNA fuel resulted in a rapid increase in reporter concentration followed by a gradual decrease, indicating autonomous probe resetting (Fig. 3B). The reporter concentration reached a maximum of 177 nM (88.5%), and 95% of probe resetting within 10 min. There was no increase in the reporter concentration in the absence of the target, suggesting that PS modifications effectively protect the probe from degradation. The probe was rapidly degraded by Exo III if no PS was appended at the 3' termini of the probe strands (Fig. S11). Gel electrophoresis results also verified the dissipative process (Fig. 3C) as the band of the reporter appeared due to TMSD (lane 2) and disappeared under dissipative conditions (lane 3). The reversibility of the probe was validated by performing multiple cycles through repetitive additions of a constant amount of target (Fig. 3D), rendering highly consistent signal amplitudes across cyclic detections (Fig. 3E). Owing to the simulation-guided parameter setup for the dissipation system, stable signals were realized for each cycle of detection by achieving a high released reporter concentration and short single cycle time simultaneously. Notably, FWHM increased slightly with cycling, which likely originated from the decreased stability of the enzyme over time (Fig. S12).

It is necessary to study which parameter can be used for target quantification because the thermodynamic and kinetics of the dissipative probe are distinct from that of equilibrium probes. We simulated reporter kinetic curves with different target concentrations (Fig. 3F, S13A), and found that the maximum concentration of released reporters, shown as the peak height, was proportional to the target concentration with an R² value 0.995 (Fig. 3G, S13B). Consistent with the simulation, the target concentration showed a linear relation with peak height at the coefficient of 0.985 (Fig. 3H and I). The high linearity could be attributed to the fact that the yield of the TMSD reaction is proportional to the target strand amount. These results suggest that this dissipative self-resetting probe in combination with suitable amplification steps, such as PCR or recombinase polymerase amplification (RPA), would allow the quantification of low-concentration targets.

We performed dissipative reactions under different conditions to validate the rationality of the simulation. First, the toehold length was varied to adjust the flux of the target (Fig. S14A). Typical TMSD kinetics was observed when the toehold length was longer than 2 (Fig. S14B). Fuel dissipation with toehold lengths of 6 and 8 could be achieved at least five times (Fig. S14C), and their kinetics is almost identical (Fig. S14D). However, a toehold length of 4 did not lead to a significant signal owing to the slow forward reaction rate under dissipative conditions. Interestingly, a toehold length of 8 yielded a broader FWHM, probably because the degradation of longer targets by Exo III needed more time (Fig. S14E). Next, the reverse toeholds with length 2 or 4 were investigated to explore its effect (Fig. S15A). Its presence of the reverse toehold slightly decreased the TMSD rate (Fig. S15B) and thereby the peak height (Fig. S15C and D), but did not affect the FWHM (Fig. S15E).

We changed the Exo III concentration from 2.15 nM to 17.2 nM (Fig. S16A), and the increase had two main effects: a lower peak height (Fig. S16B) and a narrower FWHM (Fig. S16C) in the kinetic trace. Both effects are generated by the selective acceleration of enzyme digestion which shifts the kinetic balance. The dissipation system could also be tuned by changing the temperature, which is particularly crucial to understand the performance of instrument-free detection at room temperature (25 °C) (Fig. S17A). TMSD yielded the faster kinetics at 37 °C than at 25 °C, but according to the prediction model, the difference was negligible when the toehold length was longer than six. Exo III showed the highest activity at 37 °C, and the lower temperature (25 °C) disfavored the digestion, shifting the reaction balance to the TMSD side. The slightly reduced enzymatic reaction rates at room temperature and slower fuel dissipation had negligible effects on the peak height (Fig. S17B). Moreover, the FWHM at 25 °C exceeded that at 37 °C during the first few cycles but showed the opposite trend after the third cycle (Fig. S17C), suggesting that the decay rate of enzyme activity was slower at lower temperatures. The FWHM at room temperature (25 °C) was less than
5 min for five cycles, which is beneficial for the instrument-free detection. Overall, all the single-cycle dissipation results from the optimization experiments agree with the simulation, highlighting the prediction accuracy of the kinetics simulation.

**FWHM fingerprint signal for identifying SNVs**

The capability of the dissipative probe to identify SNVs was tested by using two targets with mismatches located at the toehold domain (Fig. 4A). Conventional molecular probes such as TMSD probes rely on the kinetics differences to discriminate PM and MM duplex (Fig. 4B). However, the kinetics is concentration dependent, and thus different PM and MM may generate very similar signals, resulting in poor specificity. Because both the TMSD and enzymatic digestion are sensitive to mismatches, [31,32] we speculated that the dissipative probe can identify SNVs. As expected, the peak height of 42 nM for the MM was fourfold lower than that for the PM, and the FWHM of the MM was 17.6 min, which was 7.6 times broader than that of the PM (Fig. 4C). More importantly, we found that FWHM was species specific and did not vary with concentration (Fig. 4D).

The dissipative process of the MM target was simulated. The kinetics of TMSD and enzymatic reaction for the MM were measured for the simulation: $k_{\text{TMSD}}$ for the MM was fit as $4.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, which is about 100 times lower than that for the PM (Fig. 4B); $k_{\text{cat}}$ for the MM was slightly lower than that for the PM; and $K_m$ had no significant difference between the two species (Fig. S18). The simulation results confirm that the FWHM is dependent on species but independent of species concentration (Fig. 4D). Notably, the simulated FWHM value for the PM was very close to the experimental results, but that for the MM was much higher (Fig. 4D), possibly due to the unknown interactions between TMSD and the enzymatic reaction of MM species (see Supporting note 3 for additional details).

Therefore, we speculated that the FWHM in dissipative kinetic traces could be used as fingerprints to identify PM and MM. The dissipative probe creates a novel signal mode for SNV detection. The discrimination between PM and MM is effective through multiple cycles of detection (Fig. 4E), although the differences in peak height and FWHM slightly decreased with cycling due to the decay of Exo III activity (Fig. S19A and B). Effective methods for preventing enzymatic activity loss could further improve the FWHM fingerprinting.

**Feasibility of the dissipative reusable probe for pathogen detection**

To demonstrate the broad adaptability of the self-resetting probe, we performed detections of nucleic acid biomarkers from various pathogens. Three-way junction (TWJ) probes are designed to detect various targets, enabling the economic use of labeled strands because only the unlabeled strands need to be altered if additional targets are detected (Fig. 5A). The fluorescent strand on the TWJ was labeled with the dyes FAM, HEX, ROX, and Cy5 to designate the human-infecting RNA viruses SARS-CoV-2, ZIKV, MERS-CoV, and SARS-CoV, correspondingly. The quencher strand was labeled with BHQ1 for FAM, HEX, and ROX, as well as with BHQ3 for Cy5. MD simulation was performed to study the average distances of the fluorophore and quencher in the TWJ, as shown in Fig. S20. The distances were narrowly distributed with a mean of 2.8 nm, which was within the range of effective quenching.

The digestion depth of Exo III was studied to guarantee effective degradation of the long amplification products. As shown in Fig. S21, duplexes with 20, 26, and 36 bases were completely digested, but duplexes with 46 bases were only partially digested. Therefore, the probes were designed to be complementary to the 3’ terminus of the amplicons to ensure complete digestions. We used synthetic amplicons (35 nt and 119 nt) to validate the feasibility of
Fig. 4. Detection of SNVs by the dissipative self-resetting probe. (A) Strand sequences for testing SNV specificity. SNVs are marked with gray circles. (B) Kinetic traces of TMSD for PM and MM targets. (C) Dissipative kinetic traces for PM and MM species. All strands were present at 200 nM, Exo III was present at 4.3 nM, and the assays were performed at 37 °C. (D) Experimental and simulated FWHMs for the PM and MM originating from 20 concentrations of targets. (E) Multiple cycles of dissipation can discriminate the PM and MM via FWHM.

Fig. 5. Feasibility of the dissipative self-resetting probe for pathogen nucleic acid detection. (A) Schematic illustration of the TWJ probe for the economical use of fluoro-phore- and quencher-labeled strands for multiplexed detection. (B) Procedure for the combination of isothermal amplification (RT-asyRP) and detection by self-resetting probes. (C) In-sample multiplexed detection of pathogen RNAs with dissipative probes labeled with different dyes. The data are presented cycle by cycle. (D) Genome map showing the amplified fragments for SARS-CoV-2 detection. (E) Kinetic traces of five cycles of probe use for detecting SARS-CoV-2 genes. (F) Quantification of the SARS-CoV-2 orf1ab gene fragment with different cycles of self-resetting probe use (* * * P < 0.001; **** P < 0.0001; two-sided t-test, n = 15, each cycle, n = 3). (G) Each probe use cycle yielded R² values > 0.9. (H) Sequences for the detection of the D614G gene mutation of SARS-CoV-2. The SNV is marked with a gray circle. (I) Dissipative kinetic traces of D614G and D614A targets. All dissipative reactions were performed at 25 °C.
the probe. The two targets have the same complementary region as the probe (Fig. S22A). They yielded fast TMSD (Fig. S22B) and underwent a similar dissipative process (Fig. S22C), indicating that the probe design is feasible. To enhance the sensitivity, reverse transcription asymmetric RTA (RT-asypRA), which involves different ratios of forward and reverse primers, was utilized to rapidly increase the target concentration and generate ssDNA for the self-resetting probe (Fig. 5B). The RT-asypRA products were characterized by gel electrophoresis, indicating successful generation of ssDNA targets as expected (Fig. S23).

Multiplexed detection of the four targets was performed to demonstrate superior orthogonality by leveraging orthogonal probes. The presence of DNA from different combinations of pathogens could be inferred from the presence or absence of the corresponding fluorescence signals. To our satisfaction, little or no signal was observed in the absence of the corresponding pathogens, and highly orthogonal detection was achieved for all probe use cycles (Fig. 5C). By testing various concentrations of each target RNA, the self-resetting probes specifically produced fluorescence that responded only to the respective targets throughout all cycles, thereby enabling quantitative detection of the target of interest in a complex sample (Fig. S24).

Next, we designed probes to detect the gene domains of SARS-CoV-2: the open reading frame 1ab (orf1ab), nucleocapsid protein (N), and envelope (E) genes (Fig. 5D). As expected, the probes could be used for five times to detect the above gene fragments (Fig. 5E). All target gene fragments yielded almost identical peak heights for each cycle (Fig. S25A). Although the FWHM slightly increased as the number of cycles increased due to enzyme activity decay, it does not affect the detection (Fig. S25B). Moreover, high orthogonality was realized in each cycle for all targets (Fig. S26). To realize quantitative detections, we optimized the RTA primer concentration (see Supporting note 4 for details), which, according to the linear coefficients, was 100 nM (Fig. S27). Target quantification was also achieved for each cycle with an R² value > 0.9 as well as quantification limit of 0.01 fm utilizing the amplification step (Fig. 5F and G, Fig. S28A and B). Moreover, the dissipative probe was used to detect SARS-CoV-2 mutation D614G (Fig. 5H). As expected, the two targets yielded distinct dissipative kinetics (Fig. S1) with the wild type yielding a significantly broader FWHM (Fig. S29).

Overall, we demonstrated that the dissipative self-resetting probe could be easily configured to detect various pathogen RNA markers by simply redesigning the probes. High orthogonality was achieved not only for SARS-CoV-2 gene fragments but also for different pathogen targets.

**SARS-CoV-2 detection in clinical samples**

Eventually, we proved the feasibility of the self-resetting probe for the detection of SARS-CoV-2 in clinical samples (nasopharyngeal swabs) obtained from patients with COVID-19 (see Table S4 for patient information) and healthy control individuals. COVID-19 status was also independently confirmed by the clinical laboratory at hospitals for comparison and to show objectivity. The extracted viral RNAs went through a reverse transcription and amplification by RT-asypRA for 30 min, and the products were detected by using the self-resetting probes on a fluorescence multiplate reader (Fig. 6A). A total of 110 samples, 55 COVID-19 patients and 55 healthy controls, were used as a discovery cohort. We used 22 sets of probes to detect these samples. Each set was composed of orthogonal probes for orf1ab (FAM), E (HEX), N (ROX), and RNase P (Cy5) which served as an internal control. The fluorescence for orf1ab (FAM), E (HEX), and N (ROX) was significantly higher (P < 0.0001 for all; two-sided t-test) in samples from the patients with COVID-19 than those from the controls, but there was no significant difference for RNase P (Cy5) between patients and controls (Fig. 6B). The results analyzed by cycles showed no difference among different cycles, indicating that each cycle could effectively distinguish patient and control samples (Fig. 6C, Fig. S30). Overall, the 22 sets of self-resetting probes accurately diagnose COVID-19 with greatly reduced cost (Fig. S31). Additionally, independent replicate assays confirmed the high reproducibility (Table S5). Moreover, this probe is also adopted to...
asymmetric PCR, each cycle of probe use can correctly discriminate positive and negative samples (Fig. S32), we further determined receiver operating characteristic (ROC) curves for the orf1ab, E, and N genes. The diagnostic accuracy was excellent, with an area under the curve (AUC) of 1 for the three genes (Fig. 6D). We used the bright dye Alexa Fluor 488 to label the DNA probe to make this method compatible with inexpensive transilluminators. Based on images acquired by a transilluminator (< $200), each cycle could correctly classify the 110 clinical samples tested (Fig. 6E).

Discussion

Nucleic acid tests play a critical role in the containment of COVID-19 by permitting the rapid implementation of disease control such as case identification, isolation, and contact tracing. However, the use of disposable reagents in current methods results in insufficient test availability in some developing countries, costs heavy resources and labor, and generates a large scale of BMW. While probe regeneration could solve those problems, conventional approaches are time-consuming as well as generate hazardous waste, and thus cannot satisfy the requirement of rapid and onsite SARS-CoV-2 diagnostics.

The fuel dissipation-based probe developed in this work is the first molecular probe for repeated SARS-CoV-2 detection, thus representing a new class of self-resetting probes. It has promising advantages for molecular diagnostics: autonomous probe resetting in homogeneous solution without external reagents, at least 5 SARS-CoV-2 RNA detections, a short turnaround time for each cycle (about 10 min), a low limit of quantification (0.01 fm) in combination with isothermal amplification, and room temperature operation. Interestingly, this probe provides concentration-independent FWHM fingerprints to discriminate single-base mismatches, allowing for identification of SARS-CoV-2 SNVs whose credibility is superior than conventional molecular probes. In this work, we demonstrated the successful application of the self-resetting probe to four other pathogens using minimal redesign based on the highly modular structure of the probes. Moreover, the design of TWJ probes enables the economical use of modified strands since the strands do not need to be altered even if detection targets change. The simple enzymatic composition is another reason for the high robustness of probe reuse. We have shown the feasibility of this method for inexpensive and portable instruments such as transilluminators that can meet the demands of point-of-care applications. This probe can be further developed into a portable, easy-to-use, paper-based or lateral-flow test as exemplified by recent developments in nucleic acid diagnostics.[6,33] The reproducibility is of great significance for robust diagnostic methods. To evaluate the reproducibility, parallel tests were performed 3 times not only for synthetic targets but also for clinical samples. The average CV values for the quantification of synthetic target (Fig. 3I), the quantification of amplification products (Fig. 5F), and the detection clinical samples (Fig. 6B) were 4.52%, 7.67%, and 7.06%, respectively. Thus, the assay showed good reproducibility for the detection of nucleic acid targets.

Although DNA-based dissipation systems have been reported, none have focused on target detection. Moreover, in some systems, hours and even days are needed to complete dissipation which is not suitable for detection applications.[34,35] Simulation played an important role in leveraging fuel dissipation for target detection. The forward and backward reactions in the fuel-driven dissipation system need to be programmed to satisfy the two requirements for sensitive and highly efficient target detection: a large signal difference between the equilibrium state and high-energy state as well as a short dissipation time. However, these two requirements are contradictory as reduced lifetime of the dynamic transient high-energy state will decrease the yield. Accordingly, we performed kinetics simulations to obtain the optimized experimental conditions that balance the sensitivity and the detection time. Furthermore, MD simulation assists in optimizing the principal probe structure to enhance fluorescence signals. This set of simulation toolkits shows potential to develop more complicated DNA circuits and dynamic networks.

The increased usage time for resetting probes with multiple use cycles indicates the need to maintain enzyme activity. Exo III activity decay delays dissipation, resulting in not only a long time for probe resetting (Fig. S12) but also a small difference in signal between SNVs (Fig. S19). In the future, enzyme immobilization may be used to stabilize the nucleases in this system. Furthermore, the cumulative waste deoxyribonucleoside monophosphate (dNMP) generated by Exo III may also inhibit the long-term operation of the dissipative system. Effective approach to remove dNMP can be helpful to extend the usage count.

Conclusion

Overall, responding to the ongoing COVID-19 pandemic and increasing number of daily tests performed worldwide, we for the first time reported a self-resetting probe via dynamic DNA nano-technology to detect SARS-CoV-2 RNA. The concept of fuel dissipation in biological systems was utilized, and effective target detection and autonomous probe resetting were realized in homogeneous solution. This approach would greatly enhance the testing capability of emerging infectious diseases and circumvent the lack of sustainability in clinical diagnostics.

Experimental section

Reagents

The modified and unmodified oligonucleotides were purified by HPLC and ULTRAPAGE, respectively; and synthetic RNAs were obtained from ExonanorNA. Their sequences are listed in Table S1. The enzymes and their corresponding buffers were obtained from New England Biolabs (NEB). A reverse transcriptase-RPA kit was purchased from GenDx Biotech (KS104). DNase/RNase-free deionized water was used in all experiments.

oxDNA simulation

Structural analysis of the hairpin probes and the TWJ probes was achieved by using oxDNA, which is a coarse-grained MD simulation software program. The analysis contains two steps: (1) a DNA probe structure construction step and (2) a structural movement analysis step. oXView (https://sulggroup.github.io/oxdna-viewer/) provides some simple functions to build DNA structures. In our case, the initial DNA structures were generated from oXView, and then, two simulation files, “example.top” and “example.dat”, could be downloaded for oxDNA simulation. In this way, preparation of the basic structure of the DNA nanostructures from ssDNA in a box through the function “mutual trap” in oxDNA could be omitted. Graphical representations of generated DNA probes are shown in Fig. S5 and Fig. S20. Usually, the abovementioned simulation files need to be “relaxed” in oxDNA, as shown in Table S2, to produce initial structure files for virtual move Monte Carlo (VMMC) simulation. After each initial structure was generated as expected, the molecular behavior of the DNA structures was simulated by sequence-dependent VMMC conditions without mutual trapping. The detailed simulation target parameters and conditions are noted in Table S2. Default values were used for additional settings. During simulation, DNA conformations over time were recorded in “trajectory.dat”. In these cases, the conformations were recorded every 10 steps, and the total simulation step was 100,000. Therefore, 10,000 data points could be extracted from the trajectory through a Python script named
“distance.py” in the oxDNA analysis tool. The distance from the fluorophore-modified base to the quencher-modified base was measured in each recorded conformation. The obtained data were used to construct distance histograms.

Kinetics simulation of the fuel dissipation reactions

We computationally simulated the time-dependent concentration changes in the dissipation reactions by solving the differential equation (see Supplemental note 1) via Runge–Kutta methods (see Supplemental note 2 for additional details regarding the parameter setup).

Real-time monitoring of the reactions by fluorescence dequenching

DNA probes were prepared by mixing the corresponding single strands with equal concentrations (e.g., 200 nM) in 1 × NEB buffer 1 (1 mM Bis-Tris-Propane-HCl, 1 mM MgCl₂, 0.1 mM DTT, pH 7) in 50 µl PCR tubes. The strands were annealed in a PCR thermal cycler from 90 °C to 37 °C at a rate of 1 °C/min. Next, Exo III was added, and the solutions were mixed thoroughly. Upon the addition of target strands, fluorescence was recorded immediately in a real-time PCR machine. The obtained data were verified by native 8% polyacrylamide (19:1 acrylamide/bisacrylamide) gel electrophoresis (PAGE). The experiments were performed in Mg²⁺-containing TBE buffer (89 mM Tris-Borate, 2 mM EDTA, 12.5 mM MgCl₂, pH 8.0). Five microliters of each sample (500 nM) was mixed with 2 µl of loading buffer, and then, the mixture was added to the gel for electrophoresis. All samples were run at 120 V for 45 min at 4 °C. After 15 min of staining in SYBR gold (Invitrogen) dissolved in TBE buffer at pH 8.0, the gel was photographed with a gel imaging system.

Characterization of the products of dissipative reactions and amplification by polyacrylamide gel electrophoresis (PAGE)

The products of dissipative reactions and isothermal amplification were verified by native 8% polyacrylamide (19:1 acrylamide/bisacrylamide) gel electrophoresis (PAGE). The experiments were performed in Mg²⁺-containing TBE buffer (89 mM Tris-Borate, 2 mM EDTA, 12.5 mM MgCl₂, pH 8.0). Five microliters of each sample (500 nM) was mixed with 2 µl of loading buffer, and then, the mixture was added to the gel for electrophoresis. All samples were run at 120 V for 45 min at 4 °C. After 15 min of staining in SYBR gold (Invitrogen) dissolved in TBE buffer at pH 8.0, the gel was photographed with a gel imaging system.

Reverse transcription asymmetric RPA

To perform RT-assyRPA, the ratio of forward primers to reverse primers was fixed at 10:1. For the quantification assay, the forward primer concentration was optimized from 50 to 400 nM, and 100 nM exhibited the best quantification ability. RPA reactions were further run for 20 min. Considering that ‘quantification’ is more important than ‘quantification’ for SARS-CoV-2 detection in clinical samples, we adjusted the forward primer to a final concentration of 400 nM for highly efficient amplification.

Human clinical sample collection, RNA extraction, and SARS-CoV-2 detection

Negative nasopharyngeal swabs were acquired from healthy individuals with the approval of the Ethics Committee of Beijing University of Chemical Technology. Clinical nasopharyngeal and oropharyngeal swab samples from patients infected with SARS-CoV-2 (see Table S4 for patient information) were collected in universal transport medium (UTM) and transported to the clinical laboratory of hospitals. SARS-CoV-2 RNA was extracted following the instructions of the Qiagen DSP Viral RNA Mini Kit (Qiagen) and the MagNA Pure 24 instrument (Roche). The extracted RNA was amplified by RT-assyRPA for 30 min, where the forward primer and reverse primer concentrations were 400 and 40 nM, respectively, followed by enzyme deactivation by heating to 90 °C for 15 min 5 µl of the amplification products and 50 µl of the self-resetting probes (DNA and Exo III final concentrations: 200 nM and 8.6 nM, respectively) were mixed thoroughly, and the fluorescence of FAM, HEX, ROX, and Cy5 was immediately recorded at 25 °C by a multiplate reader. The DNA strand was labeled with Alexa Fluor 488 for fluorescence visualization on a transilluminator, orf1ab was chosen for demonstration, and the images (Fig. 6E) were taken 1 min, 2 min, 3.5 min, 5 min, and 7.5 min after the addition of the clinical sample for 1–5 cycles, respectively.

CRediT authorship contribution statement

X.S. and D.Z. conceived of the idea. N.L., Y.Z., Y.L., D.Z., L.M., and X.S. designed experiments. N.L., Y.Z., Y.L., and V.Z. performed experiments. Y.L., L.Z., Y.Z., and X.S. built up the simulation model. N.L., Y.Z., C.Y., Y.L., L.Z., Y.J., D.Z., and X.S. analyzed the results. L.M. provided the clinical sample and performed the detection of clinical samples. X.S., D.Z., N.L., Y.Z., R.L., X.D., C.Y., and L.Z. wrote the manuscript. All authors discussed the results, revised or commented on the manuscript.

Declaration of Competing Interest

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

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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.nantod.2021.101308.

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