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Hijacking the self-replicating machine of bacteriophage for PCR-based cascade signal amplification in detecting SARS-CoV-2 viral marker protein in serum

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1. Introduction

Since the outbreak of COVID-19 [1], many effort has been devoted to developing effective signal amplification methods to improve the sensitivity in detecting SARS-CoV-2 [2–5]. And many encouraging results have been reported [6–10]. One main theme in this field is to use enzymes to guide nucleic acids into cycles and cascades of reactions [11–14]. These inspiring works seem to offer infinite possibilities of new designs, and they are also reminiscent of similar naturally-occurring processes. In fact, viral infection also involves amplification and uses the host cellular machinery to rapidly produce hundreds of thousands of copies of a single virus particle in a short time [15]. Bacteriophages have also been used to develop biotechnological tools, such as phage display [16], which is as well-established and widely adopted as the nucleic acid-based polymer chain reaction (PCR). Based on these two well-known and proven methods, and inspired by the artificial DNA replication machine [19], we have developed a method, using a viral marker protein of SARS-CoV-2 to control the replication of a bacteriophage, m13 [18], thus converting the amount of serum viral marker protein into amplified Q-PCR signal readout.

As illustrated in Scheme 1, the m13 phage serves two purposes. First, it acts as a probe for the highly conserved viral target protein, the main protease of SARS-CoV-2. This protein is responsible for building the viral replication machine [19]. Using phage display technique, the tail filament protein (known as the “P3” protein of m13 [18]) of the m13 phage particle is capped by a short display peptide sequence, which is specific for the substrate peptide of the virus protease [19]. This sequence can only be recognized by the viral protease, so the sequence and the m13 phage particle can serve as the probe. Meanwhile, the phage particle also serves as the signal converter: before the SARS-CoV-2 virus protease cleaves the displayed substrate sequence, the tail of m13 is “capped”. But the phage needs its tail to anchor it to the surface of E. coli (Escherichia coli) bacteria to initiate m13 replication. So, in the absence of target viral protease, the m13 phage particle is “blocked” by the displayed substrate peptide.

In the presence of the SARS-CoV-2 protease, the substrate peptide is removed from the tail of m13 particle. It is then “activated”, and is allowed to attack E. coli bacteria freely, and then hundreds of new m13 particles can be generated in cycles of phage self-replication. The target protease of SARS-CoV-2 also participates in this signal amplification process: new batches of m13 also contains the substrate peptide “cap”, and the target protease can enzymatically “activate” them to start
The presence of the SARS-Cov-2 protease has resulted more than a hundred of new m13 particles [20], and these particles can be subsequently brought to Q-PCR application to start a second round of signal amplification. This method relies largely on fully developed biotechniques such as phage display and Q-PCR.

2. Experimental

2.1. Chemicals, reagents and biological materials

Phage displayed peptides were constructed by Science peptide, (Shanghai, China) using the method as detailed below in Section 2.2. Human recombinant main protease of SARS-Cov-2 was from R&D system (Minneapolis, USA), received as a solution (1.7 mg/mL (50 μM) main protease in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA). It was then diluted with 100 mM phosphate buffered saline (PBS) to various concentrations for the experimental applications, stored at – 80 °C. Freeze-thaw cycles were avoided. The source of m13 phage, the E. coli, as well as the PCR primer for m13 and E. coli were detailed in their relevant sections, respectively. Indium Tin oxide (ITO) glass was from Ossila, and cut to small pieces of roughly 2 cm × 1 cm for use. Au coated magnetic beads (average diameter – 15 nm) was from CD Bioparticles. Serum sample. Experiments on serum samples were conducted in the Clinical Laboratory of the Second Hospital of Nanjing, after the elected consent by Medical Ethics Committee of Nanjing Second Hospital. Before testing, the samples were centrifugated for 15 min at 2500 RPM, and the supernatant was collected. All experiments were performed in compliance with the Guidelines for Health-related Research Involving Humans of CIOMS(Council for International Organizations of Medical Science). All the chemical reagents were of analytical grade, and the water used for preparing all solutions was prepared with a Milli-Q water purification system to the standard of double-stilled water (dd water hereafter).

2.2. Construction of m13 P3 protein carboxyl-end fused peptide display

The m13 phage display was constructed following established methods. The substrate peptide of the viral main protease was displayed on the C-terminal of the P3 shell protein of m13 phage [21]. Each phage particle only contained 5 copies of this protein at one end of the cylindrical phage particle [18]. This protein was responsible for recognition of the E. coli host [18]. Traditional phage display method directly using the genetically modified m13 phage particle to directly infect the host would not produce functional copies, as the peptide sequence was fused to the N-terminal of the P3 protein, resulting in loss of P3 function [18]. So the hybrid display method was adopted. Briefly, VCS m13 Coliphage and E. coli XL-1 blue from Stratagene was employed as the helper phage and the host cell, respectively. The host cells were infected by the helper phage which could provide wild-type functional variant of all 5 types of the m13 phage protein. Meanwhile, a special plasmid called the “phagemid”, as designed and tested by a previous work, was co-infected with the helper phage. The phagemid contained an open reading frame at the C-end of P3 protein, and the desired peptide sequence was inserted: first, the effective linker from the previous report, LPITQRDH was inserted. This would guarantee that the displayed sequence was not buried inside the p3 protein, as determined by the previous report. Then the specific substrate peptide for the SARS-Cov-2 main protease, SAG was inserted, followed by a H4 linker, ended with a Cysteine moiety. Then the phage was allowed to propagate within the host cells, still with the help of the helper phage. After each step of phage replication, the resulted copies were selected using slide-immobilized anti-H4 antibodies. For a last round of panning, the helper phage was withdrawn, and the off-spring copies were tested. Their ability to infect E. coli was effectively blocked. The constructed phage particles were kept in 15% glycerol solution at 4 °C. The nominal concentration of the “capped” phage was around 1.35 × 10¹⁰ plaque forming unit per milliliter. The enumerating was conducted using the helper phage as the substitute of the blocked phage, following the United States Environmental Protection Agency’s method 1601. Briefly, the helper phage was diluted 10x, using dd water. Then 100 μL of this phage suspensions was seeded into 500 µL of an early-log phase culture of E. coli. Then the whole mixture was in return seed into 5 mL of semi-solid tryptic soy agar (TSA), mixed again, followed by be poured onto a bottom layer of TSA. The agar media then gradually solidified. And the dash could then be inverted for overnight incubation at 37 °C. Then the number of plaques were calculated as pfu/mL.

2.3. Immobilization of phage

To validate the sensing mechanism, phages were immobilized to

![Scheme 1](image-url). The proposed phage amplification method to detect the viral protease of SARS-Cov-2. Not drawn to scale.
sensing surfaces using the terminal Cysteine moiety of the displayed peptide. The ITO slide was first ion-sprayed with a transparent layer of gold, with a thickness of roughly 10 Å, while the Au-coated magnetic beads were directly brought to the experimental application. The stock solution of the P3 displayed phage was diluted 10x using 100 mM PBS and incubated with the slide or the beads overnight at 37 °C. Then the slide was thoroughly rinsed with dd water and ready for test, while the beads were separated from the buffer system using externally applied magnetic field, followed by dd water rinsing and re-suspension with 100 mM PBS.

2.4. Detection of viral protease

Incubation with either PBS buffered or serum spiked recombinant target protease, or directly with the serum of SARS-Cov-2 infected patients, was at room temperature, and was allowed to proceed for proper time as investigated in Fig. S3. Then the phage could either be brought directly to PCR detection or first being sent for self-replication and then PCR detection. The type of sample as well as the method of detection was specified in the caption for each figures. The method of phage self-replication was the same as the above enumeration process. While the method of PCR detection of m13 genome, as well as the genome of E. coli, was as specified below:

The forward and reverse primers were designed using Primer Designer 2.01. The sequences of these primers were: 5′-AGC CTT TGT AGA CCT CTC A-3′ (Forward) and 5′-AGC CTT TAT TTC AAC GCA AG-3′ (Reverse). The PCR reaction was realized in 25 µL small sterile vial. The reaction mixture employed the following recipe: 12.5 µL of 2x QuantiTect SYBR Green PCR master mix, which contains 2.5 mM MgCl₂, 0.28 µM of the above primers each; mixed with 5 µL of nucleic acid extract, and finally, nuclease-free water was added to bring the volume up to 25 µL. Real time PCR was conducted using Thermal Fisher’s Applied Biosystems Thermal Cycler 2720. The temperature cycling scheme was set as follows: hot start at 95 °C for 15 min, followed by 38 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. Real-time monitoring of the PCR product was through measuring the fluorescent signal at the end of the extension phase for each cycle. After the amplification step, a melting curve was produced at 60 °C for 1 s, followed by heating slowly at 0.2 °C/s to 98 °C, with continuous streaming of fluorescent signal.

3. Results and discussion

3.1. Study of the probe-protease interaction

The molecular recognition between the designed m13 phage displayed probe and the target protease is first studied. To show their interaction with Q-PCR signal readout, the m13 phage’s tail filament protein is genetically modified to display the substrate peptide sequence of the protease. Specifically, to directly study the interaction between the m13 particle and the protease, this substrate peptide sequence is further elongated by several glycine, terminated by a cysteine. (Scheme S1) Then using the free thiol group of this terminal cysteine, the whole m13 phage particle can be immobilized on a gold sprayed mica surface, as can be observed by atomic force microscopy (Fig. S1), which shows the characteristic filament-like form of the m13 phage on the surface. Then, the surface immobilized phage array is incubated with recombinant SARS-Cov-2 viral protease of several different concentrations. Subsequently, both the reaction solution and the slide are sent for Q-PCR amplification, or the PCR detection of m13 genome. As shown in Fig. 1a, in the solution phase, the Q-PCR signal increases in proportion to protease concentration, indicating that more m13 phage particles are released into the solution by higher concentrations of protease in the solution sample. While Fig. 1b shows a reciprocating decrease of surface m13 phage signal as the protease concentration in the solution sample grows higher. It is worth noting that PCR from surface immobilized template is also a well-known technique for over two decades. As a control, the same experiments in Fig. 1 are repeated in serum spiked recombinant SARS-Cov-2 protease sample (Fig. S2a, b). This is necessary because the intended working scenario for the proposed sensing method inevitably involves serum samples that contain many interfering proteins. As can be observed from Fig. S2, the protease can still reliably recognize and cleave the m13 phage displayed substrate peptide, since Fig. S2 is all most the same as Fig. 1.

Then the kinetic aspects of the target viral protease are studied. First, the amount of m13 particles immobilized on a surface, as in the above experiment, is very limited. Because the surface area of the slide is limited. This poses a problem: higher concentrations of protease may become surplus, then the Michaelis-Menten constant of the protease cannot be reliably assayed using the surface immobilized phages. To overcome this problem, this phage with cysteine terminated tail protein is immobilized on commercially available magnetic beads, so that the cleaved and un-cleaved phages can still be separated as in the case of the surface immobilized phage, while the amount of immobilized phage is greatly increased, by virtue of the large surface-to-volume ratio of the beads. Now, using surplus amount of bead-presented m13 phage-displayed substrate peptides, the reaction time of protease cleavage is studied (Fig. S3). Using magnetic field, the unreacted m13 phage can be separated, and then the phages released into the solution is brought to PCR detection of their genome. It is evident that the protease cleavage process is relatively slow, over 30 min is needed for the protease to cleave all the available substrates. Then, under the optimized cleavage time, target viral protease samples of different concentrations are incubated with the bead-presented m13 phage particles (Fig. 2a).
Michaelis-Menten constant can be calculated to be around 3 nM (Fig. 2b). This indicates the target protease can bind with the target with high affinity while also being able to turn the substrate into the product relatively quickly. With these kinetic parameters of the target proteins determined, the quantification of the target protein is carried out, using only the PCR detection and amplification, but without the E. coli-assisted m13 phage replication step. This shows PCR signal in proportion to viral protease concentration, and a working curve between the PCR signal peaks and the logarithm of protease concentration can be established (Figs. 3a and b). The limit of detection is as low as 0.8 nM (The lowest point in Fig. 3b). This quantitative result can serve as a baseline for evaluating the effectiveness of the proposed m13-replication and PCR two-fold amplification in the following experiments.

3.2. Effectiveness of m13-replication as signal amplification method

The m13 phage needs E. coli as the host for replication, so the concentration of E. coli should be optimized. Higher concentration of E. coli can enhance the signal amplification, as the same amount of m13 phage can generate larger amounts of copies. However, to take advantage of higher E. coli concentration, longer incubation for the m13 replication is needed. Besides, the genome of E. coli does constitute some interference during the PCR detection of m13 genome, since the phage genome is also integrated inside the genome of the bacteria. To maximize the effect of signal amplification, at the same time minimizing the interference from the E. coli genome, and also lowering the reaction time of m13 replication as far as possible, E. coli of different concentrations are employed for m13 replication, and the time course is recorded as PCR signal readout (Fig. S4). The upper row (Fig. S4a, a’, a’’) are three representative time points using relatively high concentration of E. coli. It can be observed that longer replication time can indeed give rise to higher PCR signal representing the genome of m13, but this PCR signal peak may contain possible false positive. So the intermediate 30 min is chosen for the following applications. As explained above, the m13 genome is also integrated in the genome of E. coli. As a control, using another primer for amplifying the E. coli genome, the signal of remaining E. coli genome is also evident when the concentration of E. coli is high, after the m13 replication process (Fig. S4a’’). Then the replication is traced a second time using the relatively low concentration of E. coli (Fig. 3b, b’ and b’’). It can be observed that all the E. coli host cells seem to be destroyed in a shorter interval, if compared with the above case of higher concentration of E. coli. Consequently, the PCR signal is correspondingly smaller, since fewer copies of the m13 is produced. On the other hand, as shown in Fig. S4b’’’, the residual E. coli genome signal is relatively small. So, using an intermediate E. coli concentration, the replication process is followed a 3rd time. As shown from Fig. S4c to S4c’’’, the PCR signal of m13 genome is relatively large, while the residual PCR signal of E. coli genome is suppressed. So, this concentration of E. coli is chosen for the following quantitative detection.

It is worth noting that in these m13 replication experiments, the displayed substrate peptide sequence contains no terminal cysteine, and magnetic beads are dropped out of use. This is because the number of copies after m13 replication is over two orders of magnitude higher than the number of the original m13 probe, and this is evident in Fig. S5,

![Fig. 2. Evaluating the Michaelis-Menten constant. (a) PCR product melting curves representing the m13 genome detected in solution after incubating surplus amount of beads-immobilized m13 particles with different concentrations of target protease (PBS buffered). Protease concentrations are marked on the graph. (b) Plotting of peak signals in (a) as a function of protease concentration (n = 3, error bars represent standard deviation).](image)

![Fig. 3. Quantification of target protease using PCR alone, without m13 phage replication. (a) PCR product melting curves representing the m13 genome detected in solution after incubating bead-immobilized m13 with different concentrations (as marked on the graph) of target protease (serum spiked) (b) Peak signals in (a) plotted as a function of protease concentration, inset is the linear range between peak signal and the logarithm of protease concentration (n = 3, error bars represent standard deviation).](image)
which directly compares the magnitude of the PCR signal before and after m13 replication. Both interface and solution suspended beads have been employed to study the proposed method, because that both are useful for clinical applications. The proposed method has also been examined using dynamic light scattering technique (Fig. S6), which can observe the size of the particles such as the bacteria and the phage, suspended in the solution. Fig. S6a represents the observed particle size for the interaction between the bacteria-phage mixture and the blank buffer, while Fig. S6b represents the interaction with the target protease. The observed size of suspended particle in Fig. S6a is evidently much larger than that recorded in Fig. S6b, indicating that the blank buffer cannot initiate the lysis of *E. coli*, excluding the possibility of false positive.

### 3.3. Analytical performance

Under the above optimized conditions, serum spiked SARS-Cov-2 protease samples are detected using the in-solution two-fold signal amplification method. As shown in Figs. 4a and 4b, the PCR signal increases in proportion to viral protease concentration, and a working curve between the PCR signal peaks and the logarithm of protease concentration can be established (Figs. 4a and 4b). The limit of detection is as low as 1 pM, or two orders of magnitude higher than that of the above single step amplification using PCR alone. The standard deviation is lower than 5%, shown as the error bars, representing good repeatability. As shown in Fig. 4c, the specificity with respect to other serum interfering proteins is also satisfactory. The specificity has also been examined using other members of the papain-like protease (Fig. S7). The comparison with commercially available methods has been summarized in Table S1. And compared with enzymatic assays using fluorescent substrate peptides, the proposed method can not only achieve higher sensitivity, but the chance of false positive can also be lowered. Since the capped tail peptide cannot infect the bacteria, while residual fluorescent resonance can still occur between the fluorophore and the quencher. The proposed method can also be readily generalized to the detection of other proteases, while the detection of other types of enzymes is also potentially compatible with the design.

Then, the proposed method is brought to clinical application, using the serum samples of SARS-Cov-2 infected patients, the serum samples of healthy volunteers are employed as a control. The serum samples of the patients are grouped according to their symptom, and it can be observed that the group showing severe acute respiratory syndrome is detected to have elevated serum level of the viral marker protein (Fig. 4d). This difference is of statistically significant. These results may validate the feasibility of the proposed method in evaluating the viral load of SARS-Cov-2 for infected patients.

### 4. Conclusion

In this work, a method to sensitively detect a viral marker protein, namely, the main protease of SARS-Cov-2, has been developed. This method first utilize the well-established phage display technique to display the substrate peptide on the tail protein of m13 phage, so that the phage become “inactivated”, thus cannot infect its host cell the *E. coli*. In the presence of the target protease, through enzymatic cleavage of the displayed substrate peptide, the m13 phage is “activated” and can infect *E. coli* to initiate a self-replication process. After this process, the genome of amplified m13 phage population can be detected using PCR, which is a second round of signal amplification. Using this method, the target viral protein can be detected directly from serum samples of patients, though the abundance of such protein is much higher than the limit of detection of the proposed method. This method utilizes two proven techniques of biological analysis, showing promising analytical performance for clinical application in the near future.
CRediT authorship contribution statement

Jiaxu Du: Conceptualization, Methodology, Investigation, Writing – original draft. Daili Xiang: Methodology, Investigation, Validation. Fushan Liu: Investigation, Validation. Leichen Wang: Investigation, Data curation. Hao Li: Supervision, Writing – review & editing. Liu Gong: Formal analysis, Data curation. Xiangyu Fan: Validation, Project administration.

Declaration of Competing Interest

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

Data Availability

The authors do not have permission to share data.

Acknowledgement

We gratefully acknowledge the National Natural Science Foundation of China (52002146), the Key Research and Development Project of Shandong Province (2019GSF109013).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.132780.

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