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Sensitive and specific detection of proteins based on target-responsive DNA polymerase activity

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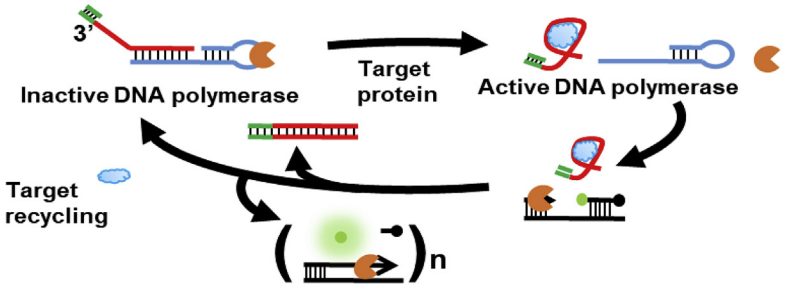
HIGHLIGHTS

- Two different types of DNA aptamers enable the sensitive analysis of a target protein.
- The target proteins are recycled by the active DNA polymerase, leading to the sensitive detection of target proteins.
- This method is designed to have a separate target recognition and signal transduction steps.
- It would be expanded to determine other target proteins or biological molecules with low-cost.

GRAPHICAL ABSTRACT



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ABSTRACT

We herein describe a novel method for the detection of target protein based on the target-responsive DNA polymerase activity. In the sensor, two different types of DNA aptamers with the respective functions: one binds to the target protein and the other binds to DNA polymerase, are rationally engineered and combined to form the detection probe that regulates DNA polymerase activity in response to the target protein. In the presence of target protein, the detection probe becomes destabilized and stops the inhibition of DNA polymerase activity. Consequently, the active DNA polymerase initiates the primer extension reaction on the target-specific DNA aptamer, which recycles the target protein to promote another activation cycle of DNA polymerase. In addition, DNA polymerase also catalyzes the primer extension reaction on the primer/template complex in conjugation with TaqMan probe, leading to the significantly enhanced fluorescence intensities. With this novel strategy, we detected a model target protein, lysozyme with a limit of detection as low as 0.80 nM. In addition, the practical applicability of this system was successfully demonstrated by determining lysozyme in human serum.

1. Introduction

Proteins, one of the most important macromolecules in living organisms, are involved in various biological processes including hormone regulation, cell signaling, immune response, biomolecule transportation and storage [1]. In addition, their abnormal levels are closely associated with a risk of diseases such as Alzheimer's

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disease, chronic kidney disease, and human malignant tumors and thus have been used as biomarkers for the early detection of diseases [2–4]. Owing to the biological and clinical significance of proteins, several analytical systems including polyacrylamide gel electrophoresis (PAGE) [5], high-performance liquid chromatography (HPLC) [6], and immunoassays such as immunoelectrophoresis (IEP) [7], and enzyme-linked immunosorbent assay (ELISA) [8,9] have been developed. Of these, ELISA has been regarded as a 'gold standard' method in analyzing target proteins and utilized in most central laboratories. However, it entails time/labor-intensive steps including probe immobilization and multiple washing. Above all, antibodies that function as the receptor for the specific recognition of target proteins are costly and unstable, hampering its widespread use [10,11].

Aptamers, nucleic acids that have strong affinities toward specific target molecules [12–18], have been emerged as a compelling alternative to antibodies, because they are advantageous in cost, robustness, and synthesis/modification [19]. With the unique features, they have been utilized in various detection platforms including surface plasmon resonance [20,21], electrochemiluminescence [22], electrochemistry [23,24], colorimetry [25,26], and fluorometry [27,28]. Notably, fluorescence-based methods, which work with the great simplicity, reliability, and sensitivity, have garnered special attention. The representative example in this type of sensing strategy relies on DNA aptamer modified with fluorophore and quencher, which has been utilized for the determination of target proteins such as platelet-derived growth factor (PDGF), thrombin, lysozyme, and Tat protein of HIV-1 [29–33]. However, the existing systems require the direct modification of DNA aptamer with fluorophore or quencher, which increases the total analysis cost and affects the binding affinity of DNA aptamer, consequently interfering with the effective analysis of target proteins. Therefore, it is in high demand to devise a novel and efficient fluorescent strategy with separated target recognition and signal transduction elements to overcome current drawbacks.

Aimed at this goal, we developed a novel method for the sensitive detection of target protein based on a combination of two independent target recognition and signal transduction events by the regulation of DNA polymerase activity in response to the target protein [34–36]. In this study, the previous finding was innovated for the detection of target proteins through combining two different types of DNA aptamers with the respective functions and introducing helper DNA for the target recycling. In the presence of a target protein, the detection probe composed of target-specific and DNA polymerase-specific aptamers becomes destabilized, which no longer binds to and inactivates DNA polymerase. As a result, the active DNA polymerase initiates the primer extension reaction on the target-specific aptamer displacing the target protein for another activation cycle of DNA polymerase. In addition, DNA polymerase executes the primer extension reaction on the primer/template complex in conjunction with TaqMan probes, resulting in the significant fluorescence enhancement. With this novel strategy, we successfully analyzed a model target protein, lysozyme whose elevated level is associated with an increased risk of diseases such as tuberculosis, leukemia, renal disease, and meningitis [37–39], with high sensitivity and selectivity even in human serum.

2. Experimental

2.1. Materials

All oligonucleotides employed in this study were synthesized from Genotech Co. (Daejeon, South Korea) and purified by desalting, except for Apt-$_{lysozyme}$ and template purified by PAGE and TaqMan probe purified by HPLC. The sequences of oligonucleotides are listed in Table S1. *Thermus aquaticus* DNA polymerase (Taq DNA polymerase) and bovine serum albumin (BSA) were purchased from New England Biolabs Inc. (Beverly, MA, USA). Lysozyme, cytochrome C (Cyt C), and thrombin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure DNase/RNase-free distilled water (DW) used in all the experiments was purchased from Bioneer® (Daejeon, Korea). All other chemicals were of analytical grade and used without further purification.

2.2. Lysozyme detection procedure

The reaction mixtures were separately prepared as part A and part B. Part A (total volume of 20 μL) consisting of 400 nM Apt-$_{pol}$, 200 nM Apt-$_{lysozyme}$, 400 nM HP and 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$) was heated at 90 °C for 5 min, cooled slowly to 25 °C (0.1 °C/s), and incubated for 30 min. Taq DNA polymerase (11 nM) and lysozyme at varying concentrations or other proteins were then added to the part A and incubated at 25 °C for 30 min. Part B (total volume of 20 μL) composed of 600 nM template, 600 nM primer, 500 nM TaqMan probe, 500 μM dNTPs, and 1X reaction buffer was heated at 90 °C for 5 min, cooled slowly to 25 °C (0.1 °C/s), and incubated for 60 min. Part A and B were mixed, and the fluorescence signal from TaqMan probe was immediately monitored at every 2 min at 25 °C during the primer extension reaction on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA).

2.3. Polyacrylamide gel electrophoresis (PAGE) to confirm the interaction between the detection probe and DNA polymerase

The reaction mixtures (total volume of 40 μL) composed of 500 nM Apt-$_{pol,}$ 500 nM Apt-$_{lysozyme}$, and 1X reaction buffer were heated at 90 °C for 5 min, cooled slowly to 25 °C (0.1 °C/s), and incubated for 30 min. Taq DNA polymerase (110 nM) and lysozyme (1 μM) were then added to the mixtures, and incubated at 25 °C for 30 min. After that, the product solutions were mixed with 6X loading buffer (Bioneer®, Daejeon, Korea) and resolved on 15% polyacrylamide gel. The electrophoresis was carried out in 1X TBE for 90 min at 100 V with Mini-PROTEAN® Tetra Cell Systems (Bio-rad, Hercules, USA). After staining with ethidium bromide (EtBr) (Bioneer®, Daejeon, Korea), the gel image was obtained with Gel Doc EZ Imager (Bio-rad, Hercules, USA).

2.4. PAGE to confirm the primer extension reactions promoted by active DNA polymerase

The reaction mixtures (total volume of 40 μL) were prepared according to 'Lysozyme detection procedure' except that 400 nM Apt-$_{lysozyme}$ was used instead of 200 nM of Apt-$_{lysozyme}$ in Part A and TaqMan probe was excluded in Part B, and it was incubated at 25 °C for 180 min. The product solutions were mixed with 6X loading buffer and resolved on 15% polyacrylamide gel. The electrophoresis was run in 1X TBE for 180 min at 120 V with Mini-PROTEAN® Tetra Cell Systems (Bio-rad, Hercules, USA). After staining with EtBr, the gel image was taken with Gel Doc EZ Imager (Bio-rad, Hercules, USA).

2.5. PAGE to confirm the stability of complex of the detection probe and DNA polymerase in human serum (1%)

The complex of the detection probe and DNA polymerase (Apt-$_{pol}$/Apt-$_{lysozyme}$/DNA polymerase) was prepared by mixing 500 nM Apt-$_{pol,}$ 500 nM Apt-$_{lysozyme}$, and 1X reaction buffer. The mixtures were subsequently heated at 90 °C for 5 min, cooled slowly to 25 °C (0.1 °C/s), and incubated for 30 min. Taq DNA

polymerase (110 nM) was then added to the mixtures, and incubated at 25 °C for 30 min. After being incubated in diluted human serum at 25 °C for different times, the complexes were resolved on 15% polyacrylamide gel. The electrophoresis was run in 1X TBE for 90 min at 100 V with Mini-PROTEAN® Tetra Cell Systems (Bio-rad, Hercules, USA). After staining with EtBr and taking the gel image with Gel Doc EZ Imager (Bio-rad, Hercules, USA), the band intensities for the complexes were evaluated with Image J (Fig. S4).

2.6. Detection of lysozyme in human serum (1%)

Without any pretreatment steps for human serum, lysozyme-spiked human serum (25%) was prepared by mixing varying concentrations of lysozyme and human serum. It was then added to part A and analyzed according to the same procedures described in 'Lysozyme detection procedure'. The final concentration of diluted human serum was 1%.

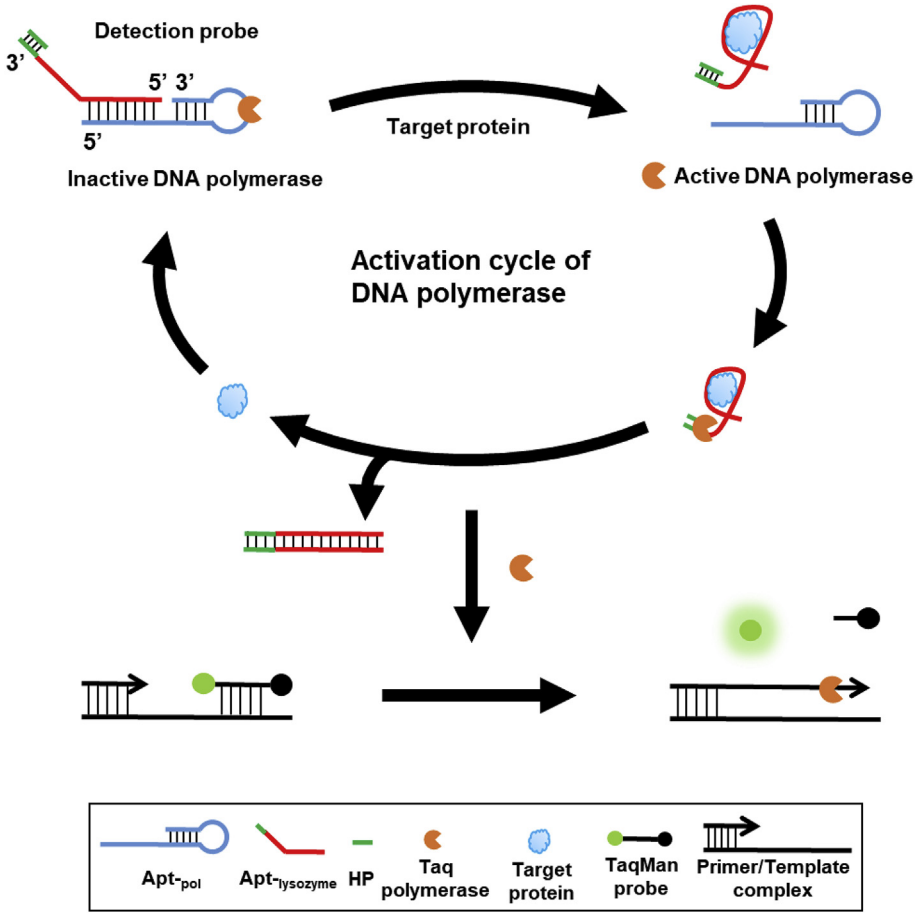
3. Results and discussion

3.1. Overall detection principle

The basic principle of a new strategy to detect a target protein is illustrated in Scheme 1. In the sensor, the detection probe is formed by the hybridization of the two-engineered DNA aptamers specific to the target proteins (Apt-$_{lysozyme}$) and DNA polymerase (Apt-$_{pol}$) [34–36]. In the absence of a target protein, the rationally designed detection probe binds to and inhibits DNA polymerase activity. On the other hand, the presence of lysozyme, which interacts with Apt-$_{lysozyme}$, destabilizes the detection probe by detaching Apt-$_{lysozyme}$ from Apt-$_{pol}$. This process activates DNA polymerase, leading to the primer extension reaction on the 3'-end of Apt-$_{lysozyme}$ with the helper primer (HP) while displacing lysozyme to promote another activation cycle of DNA polymerase. In addition, the activated DNA polymerase with 5'→3' exonuclease activity also catalyzes the primer extension reaction on the primer/template complex in conjugation with TaqMan probe labeled with a fluorophore (FAM) and a quencher (BHQ1) at each end, resulting in the significantly enhanced fluorescence intensities.

3.2. Feasibility of the strategy

We first validated the feasibility of the proposed strategy by measuring the time-dependent fluorescence signals under different conditions. As illustrated in Fig. 1, the detection probe composed of target-specific and DNA polymerase-specific aptamers inhibited DNA polymerase activity, thereby resulting in the negligible fluorescence enhancement (curve 1). However, upon the addition of lysozyme, the fluorescence signal was significantly enhanced, indicating that lysozyme dissociates the detection probe and recovers the DNA polymerase activity (curve 2). In addition, to demonstrate that the fluorescence increase is attributed to the specific interaction of Apt-$_{lysozyme}$ with lysozyme, we designed the control detection probe in which lysozyme-specific and DNA polymerase-specific aptamers were replaced with random sequences (Table S1), respectively. As envisioned, the control



Scheme 1. Schematic illustration of the protein detection method based on the target-responsive DNA polymerase activity.

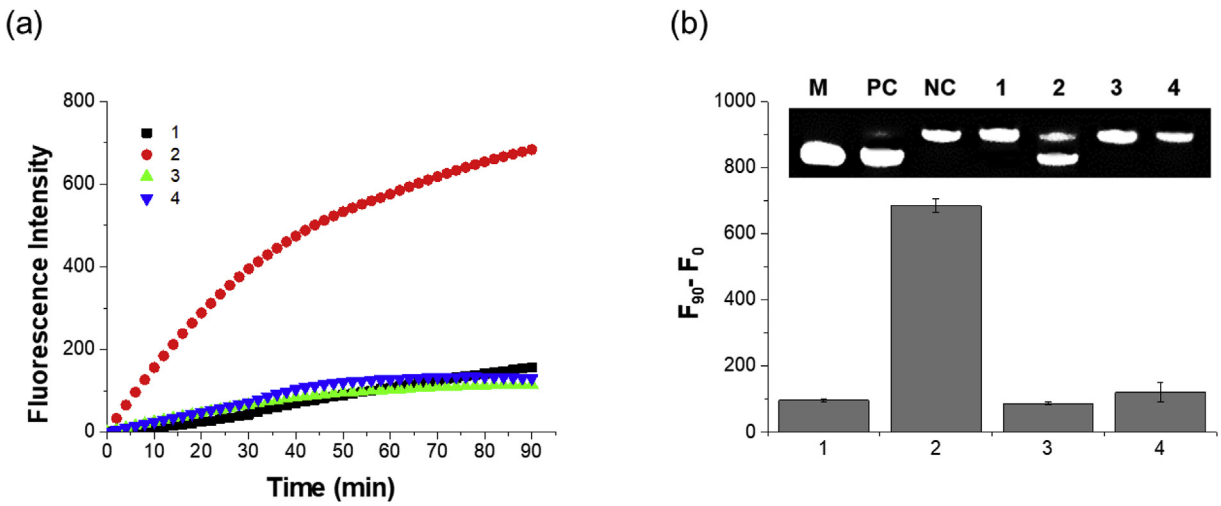


Fig. 1. Detection feasibility of the protein detection method. (a) Time-dependent fluorescence intensities from TaqMan probe during the primer extension reaction. (b) Fluorescence signal changes (F_{90}-F_0), where F_{90} and F_0 are the fluorescence intensities measured at 90 min and 0 min in the primer extension reaction, respectively. Inset in (b) shows the polyacrylamide gel electrophoresis image of the primer extension products. (M) Template/complement, (PC) Template + primer + DNA polymerase, (NC) Template + primer, (1) Apt-$_{pol}$ + Apt-$_{lysozyme}$, (2) Apt-$_{pol}$ + Apt-$_{lysozyme}$ + lysozyme, (3) Control Apt-$_{pol}$ + Control Apt-$_{lysozyme}$, and (4) Control Apt-$_{pol}$ + Control Apt-$_{lysozyme}$ + lysozyme. The final concentrations of Apt-$_{pol}$, Apt-$_{lysozyme}$, HP, DNA polymerase, and lysozyme are 200 nM, 200 nM, 200 nM, 5.5 nM, and 1 μM, respectively.

detection probe led to quite a low fluorescence enhancement regardless of the presence of lysozyme (curve 3 and 4). To further support the fluorescence results, polyacrylamide gel electrophoresis (PAGE) analysis, which confirms the generation of primer extension products, was performed. As presented in the inset of Fig. 1(b), an intense band corresponding to the extension product was observed under the condition where the significant fluorescence enhancement was resulted (lane M, PC and 2). In contrast, a band of primer/template complex was only displayed in the other conditions where quite a low fluorescence enhancement was produced (lane NC, 1, 3, and 4). Overall, these results are consistent with the fluorescence results, ensuring the feasibility of this strategy.

We also carried out the PAGE analysis to confirm that the DNA polymerase binds to the rationally designed detection probe. The results in Fig. S1 show that the band intensity of the detection probe is weakened, while that of Apt-$_{pol}$ is enhanced when lysozyme is added to the detection probe (lane 3 and 4), proving that lysozyme interacts with Apt-$_{lysozyme}$ and releases Apt-$_{pol}$. Importantly, upon the addition of DNA polymerase to the detection probe, a new intense band near the loading well was observed (lane 5), which indicates the binding of DNA polymerase to the detection probe. However, when lysozyme was applied to the sample in lane 5, the intense band near the loading well becomes weakened with the appearance of a band corresponding to Apt-$_{pol}$ (lane 6). These results clearly confirm that the detection probe binds to the DNA polymerase in response to lysozyme.

3.3. Optimization and performances of the strategy

Next, we optimized the reaction conditions required for the efficient analysis of lysozyme by examining the fluorescence signal change (F_{90}-F_0) at different conditions. Importantly, F_{90} in which the fluorescence signal measured at 90 min was selected for the optimal assay time because the fluorescence signal difference between in the presence and absence of lysozyme was the maximum (Fig. 1(a)). The results of experiments in which the hybridization length between Apt-$_{pol}$ and Apt-$_{lysozyme}$ (Fig. 2(a)), the concentrations of Apt-$_{lysozyme}$ (Fig. S2), and the concentration of HP (Fig. 2(b)) were varied, show that 10-mer hybridization, 100 nM of Apt-$_{lysozyme}$, and 200 nM of HP are ideal for further experiments. In particular, it should be noted that the presence of HP results in the higher S/N value than that in the absence of HP, confirming that HP, which displaces lysozyme during the primer extension reaction on Apt-$_{lysozyme}$, promotes multiple activation cycles of DNA polymerase and enables the sensitive detection of lysozyme.

With the optimized conditions, the detection sensitivity of the present method was determined by measuring the fluorescence signal change (F_{90}-F_0) as a function of lysozyme concentration. As shown in Fig. 3(a), the fluorescence signal changes were elevated with increasing concentrations of lysozyme up to 1 μM, but reached a plateau at concentrations above 1 μM. An excellent linear relationship ($R^2 = 0.9946$) of fluorescence signal change (F_{90}-F_0) versus lysozyme concentration was observed in the range from 0 to 20 nM, and the limit of detection (LOD) (3σ/slope) was calculated as 0.80 nM, a value that is lower or comparable to those from previous fluorescent methods for the detection of lysozyme (Table S2).

The specificity of this strategy was also demonstrated by employing other proteins such as thrombin, Cyt C, and BSA. The results in Fig. 3(b) show that lysozyme only leads to the significant fluorescence signal change whereas the other proteins generate the negligible fluorescence signal changes comparable to that from blank sample even though they are applied at ten times higher concentration than that of lysozyme. These results clearly prove that this sensing system is highly selective to lysozyme, which is attributed to the specific interaction of Apt-$_{lysozyme}$ with lysozyme.

3.4. Practical applicability of the strategy

Finally, the practical applicability of the developed strategy was demonstrated by determining lysozyme spiked in human serum. As shown in Fig. S3, a good linear correlation ($R^2 = 0.9975$) between fluorescence signal change and lysozyme concentration in diluted human serum (1%) was established. Based on this standard curve, the concentrations of lysozyme were successfully determined with high reproducibility and precision, as evidenced by the coefficients of variation (CV) (0.33–7.88%) and the recovery rates (96.5–100.0%) (Table 1). These results verify that the proposed strategy has a potential to reliably determine the lysozyme in complex biological samples.

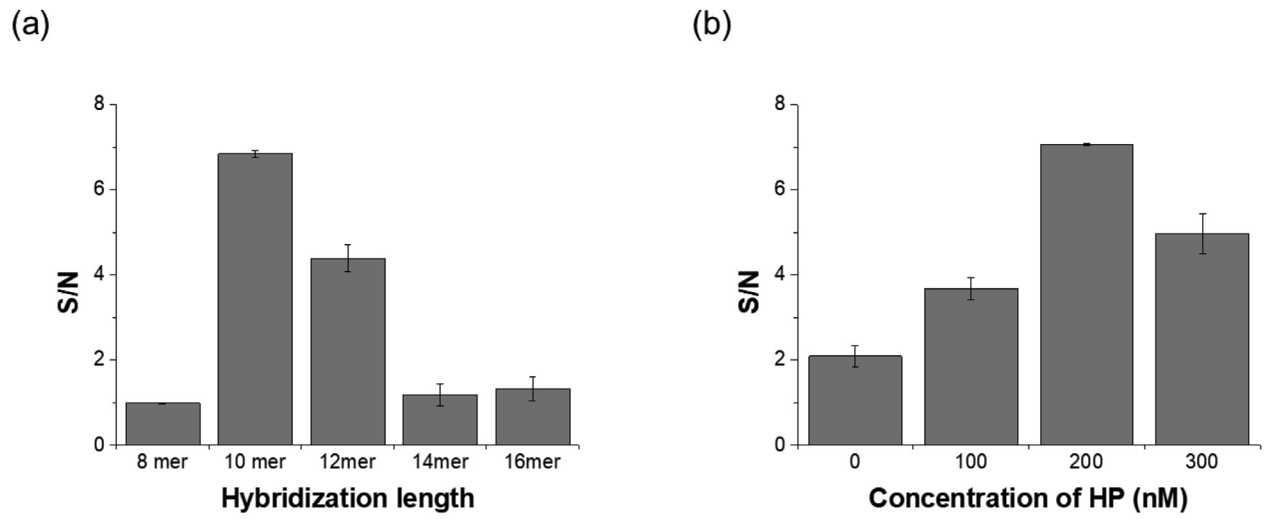


Fig. 2. Optimization of the reaction conditions. (a) S/N at different hybridization length between Apt-$_{pol}$ and Apt-$_{lysozyme}$, where S and N are the fluorescence signal changes (F_{90}-F_0) obtained in the presence and absence of lysozyme, respectively. F_{90} and F_0 are the fluorescence intensities measured at 90 min and 0 min in the primer extension reaction, respectively. The concentrations of Apt-$_{pol}$, Apt-$_{lysozyme}$, and HP are 200 nM, 200 nM, and 200 nM, respectively. (b) S/N at different concentrations of HP. The concentrations of Apt-$_{pol}$, Apt-$_{lysozyme}$, and the hybridization length between Apt-$_{pol}$ and Apt-$_{lysozyme}$ are 200 nM, 100 nM, and 10 mer, respectively.

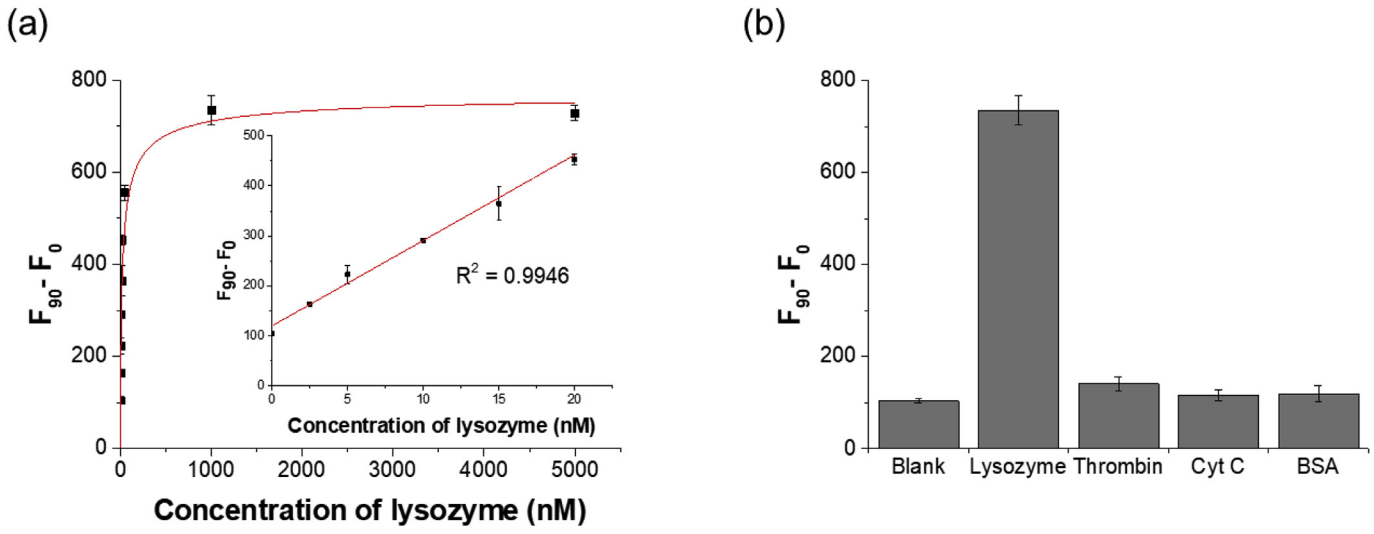


Fig. 3. Detection sensitivity and specificity of the protein detection method. (a) Fluorescence signal changes (F_{90}-F_0) in the presence of varying concentrations of lysozyme, where F_{90} and F_0 are the fluorescence intensities measured at 90 min and 0 min in the primer extension reaction, respectively. Inset in (a) shows the linear relationship between fluorescence signal change (F_{90}-F_0) and lysozyme concentration (0–20 nM). (b) Fluorescence signal change (F_{90}-F_0) in the presence of lysozyme (1 μM) and other proteins (10 μM). The final concentrations of Apt-$_{pol}$, Apt-$_{lysozyme}$, HP and DNA polymerase are 200 nM, 100 nM, 200 nM and 5.5 nM, respectively.

Table 1
Determination of lysozyme in diluted human serum (1%). The concentrations of lysozyme selected in this study are good enough to satisfy the clinical requirements, especially for the diagnosis of tuberculosis patients whose levels of lysozyme are ranging from 620 nM–1.27 μM.

Added lysozyme (nM)	Measured lysozyme[a](nM)	SD[b]	CV[c] (%)	Recovery[d] (%)
1	0.97	0.08	7.88	96.5
7.5	7.46	0.40	5.39	99.5
12.5	12.40	0.04	0.33	99.2
17.5	17.49	1.21	6.92	100.0

[a] Mean of three measurements.
[b] Standard deviation (SD) of three measurements.
[c] Coefficient of variation (CV) = SD/mean × 100.
[d] Measured value/added value × 100.

4. Conclusion

In summary, we have developed a novel and sensitive detection strategy for target protein by rationally designing the detection probe composed of two different types of DNA aptamers with the respective functions: one binds to the target protein and the other binds to DNA polymerase. The presence of target protein, which binds to the target-specific aptamer, destabilizes the detection

probe, and thus DNA polymerase becomes activated to execute the primer extension reactions on the primer/template complex in conjunction with TaqMan probe, consequently leading to the significant fluorescence enhancement. With this detection principle, we selectively detected the model target, lysozyme down to 0.80 nM, which exhibits better or comparable sensitivity to the previous fluorescent methods. The practical applicability of developed method was also verified by reliably determining lysozyme in human serum. Importantly, this approach is ingeniously designed to have a separate target recognition and signal transduction, which enables the non-labeled detection probe to effectively recognize the target protein (see the Supporting Information). Furthermore, it would be readily adapted to the detection of other target proteins with low-cost by simply changing the non-labeled detection probe while retaining the same primer/template complex with TaqMan probe.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.01.025.

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