Letter

Nucleic Acid Detection by a Target-Assisted Proximity Proteolysis Reaction

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Nucleic Acid Detection by a Target-Assisted Proximity Proteolysis Reaction

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Supporting Information Placeholder

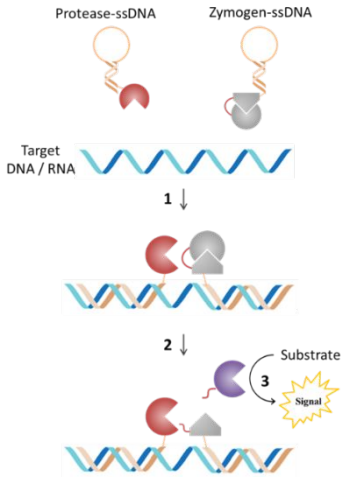
ABSTRACT: Nucleic acid analysis plays an important role in diagnosing diseases as well as understanding biology. Despite advances in technology, there is still a need to develop a rapid and simple method to detect specific nucleic acids, especially in remote locations and low-resource cases. Here, we proposed a proximity proteolysis reaction in which the reaction between protease and zymogen is enhanced in the presence of a target molecule. The pair of proteins was site-specifically modified with oligonucleotides, and the conjugates were used to develop a method of detecting nucleic acids. Target DNA and RNA could be detected in less than one hour at sub-nanomolar concentrations based on an absorbance signal. The assay method was resistant to interference by biological matrixes, and its sensitivity could be improved when combined with an isothermal nucleic acid amplification method. The results demonstrated the feasibility of this proximity proteolysis reaction as a new platform technology for detecting specific nucleic acid sequences.

KEYWORDS: biosensor, nucleic acid, proximity proteolysis reaction, zymogen, protease, site-specific conjugation

Nucleic acids provide a significant amount of biological information, and various methods have been utilized to access them.[1-3] In particular, the concentration of specific nucleic acid molecules can be an important indicator of certain disease states, and developing an efficient and simple method to determine the concentration of target DNA or RNA has been researched intensively for several decades.[4-6] Leveraging the simple principle of designing probes specific for target nucleic acids based on Watson-Crick base pairing, diverse technologies have been devised to convert hybridization into detectable signals such as absorbance, fluorescence, luminescence, and electrochemistry.[7-10] Because of their high sensitivity, methods based on the latter three signals have been the focus of most research. However, absorbance signals have advantages over other signals, such as their simple detection instrumentation[11-14], which is an important factor for developing on-site diagnostic methods. Several methods have been reported for signal amplification to overcome the low sensitivity limitation of absorbance signals[12, 15-16], but the addition of multi-step or time-consuming processes has inevitably resulted in increased complexity.

Reaction rates can be enhanced by positioning reactants close each other, and this principle has been utilized for detecting target molecules. The most outstanding example is the proximity ligation assay: two DNA molecules conjugated to different antibodies are in close proximity in the presence of an antigen or protein-protein interaction and can participate in the amplification process of rolling circle DNA synthesis.[17-19] In addition, the same principle has been applied for detecting various molecules such as proteins[20-21], antibodies[22-24] and nucleic acids[25-27]. In this letter, we report a target-assisted proximity proteolysis reaction and its application for developing a simple and sensitive nucleic acid detection method (Scheme 1). The zymogen is activated by the proximity proteolysis reaction in the presence of a target nucleic acid, and the active enzyme generates a detectable signal. Notably, the catalytic turnover results in signal amplification, which enables detection at sub-nanomolar sensitivity with a chromogenic substrate.

Scheme 1. Proposed nucleic acid detection method consisting of three steps: 1) hybridization of protease-ssDNA and zymogen-ssDNA with target nucleic acid, 2) proximity proteolysis reaction between protease and zymogen, and 3) signal production by activated zymogen enzyme.



We chose Tobacco Etch Virus (TEV) protease and β-lactamase zymogen for the proximity proteolysis reaction. Because of its origin and substrate specificity, the TEV protease has been widely used for biology research.[28] We previously reported β-lactamase zymogen that was constructed by connecting a circularly permutated β-lactamase enzyme and its inhibitor protein, β-lactamase inhibitory protein (BLIP), via a linker cleavable by a protease of interest.[29] In this study, a peptide linker containing a TEV protease cleavage site (ENLYFQ/G, /: peptide bond cleaved by TEV protease) was inserted between β-lactamase and BLIP, and the zymogen could be activated via proteolysis by TEV protease (Figure S2c). β-lactamase has been used as a reporter enzyme for molecular sensing in a variety of ways.[30-33]

To apply the protease and zymogen pair for detecting nucleic acids, the two proteins were covalently linked to single-stranded DNA (ssDNA) respectively. The sequences of ssDNAs were adopted from the previously reported dual molecular beacons designed for targeting the *KRAS* transcript.[34] The site-specific conjugation between β-lactamase zymogen and ssDNA was achieved via the strain-promoted click reaction between azide and cyclooctyne[35] (Figure 1b). 4-Azido-L-phenylalanine (AzF) (Figure 1a) was introduced into the β-lactamase zymogen using an orthogonal pair of tRNA and aminoacyl-tRNA synthetase engineered for incorporating the amino acid analogue into the amber codon[36-38]; the N-terminus of the zymogen was chosen as the conjugation site for the release of β-lactamase into solution after cleavage by the TEV protease. An amino-functionalized ssDNA was reacted with N-hydroxysuccimide ester-(polyethyleneglycol)$_4$-dibenzocyclooctyne (NHS-PEG$_4$-DBCO) (Figure S1), and then the DBCO-modified ssDNA was conjugated to the β-lactamase zymogen with the azide group. The protein-ssDNA conjugate was purified by a series of anion exchange and size-exclusion chromatography steps (Figure 1d). The β-lactamase zymogen modified with ssDNA was processed specifically by the TEV protease (Figure 1e), and the chemical conjugation minimally affected the catalytic activities of uncleaved and cleaved zymogen enzyme (Figure S3).

TEV-ssDNA conjugates prepared using a similar method used for β-lactamase zymogen showed notably lower activities than that for the unconjugated TEV protease (Figure S4). We suspected the loss of activity was caused by the covalent linkage of ssDNA or the purification procedure, and we therefore used a different strategy to conjugate the TEV protease and ssDNA (Figure 1c). The SpyTag/Catcher system, originally reported by Howarth et al., is based on an efficient isopeptide bond formation reaction between the two proteins of SpyTag and SpyCatcher.[39] The SpyCatcher with one AzF residue (Figure S2a) was conjugated to the DBCO-modified ssDNA, and after removing the unconjugated SpyCatcher (Figure 1f), the protein-ssDNA conjugate was incubated with the TEV-SpyTag fusion protein (Figure S2b). The product of isopeptide bond formation was purified using the Strep-tag purification system; the TEV-SpyTag protein has Strep-tag at its N-terminus. Because of the high yield of SpyTag/Catcher system and using the excess amount of SpyCatcher-ssDNA conjugate, unconjugated TEV-SpyTag was minimally detected in the SDS-PAGE analysis (Figure 1g). The conjugate of TEV and ssDNA using the SpyTag/Catcher system showed about 80% activity relative to that of the unconjugated TEV protease (Figure S4b), which was much higher than that prepared by direct conjugation of the TEV protease and ssDNA. The results indicate that a protein can lose its activity upon chemical modification even when the position for modification is carefully selected and a specific chemical reaction is employed. The approach of preparing TEV-ssDNA provides an alternative method to conjugate a protein with molecules, especially when the protein is sensitive to the intended modification.

The proximity proteolysis reaction of the TEV-ssDNA and β-lactamase zymogen-ssDNA conjugates was established using a 46-nt DNA oligonucleotide (Target DNA-4 in Table S3) sequence that corresponds to a part of the *KRAS* transcript.[40] We aimed to develop a simple assay method and thus set up the reaction procedure as the two protein-ssDNA conjugates and the chromogenic substrate for β-lactamase (CENTA) were added simultaneously into a sample including the target nucleotide (Figure 2a). At first, we optimized factors that can influence hybridization and proteolysis, such as $MgCl_2$ concentration and temperature (Figure 2b); the concentration of each conjugate was decided based on the background signal without a target nucleic acid (data not shown). Then, the distance between the binding sites in the template DNA for the two ssDNA species was varied, and a three-nucleotide space showed a higher signal than that for other cases tested (Figure 2c); the DNA oligonucleotide with a three-nucleotide spacer was therefore used for further evaluation.

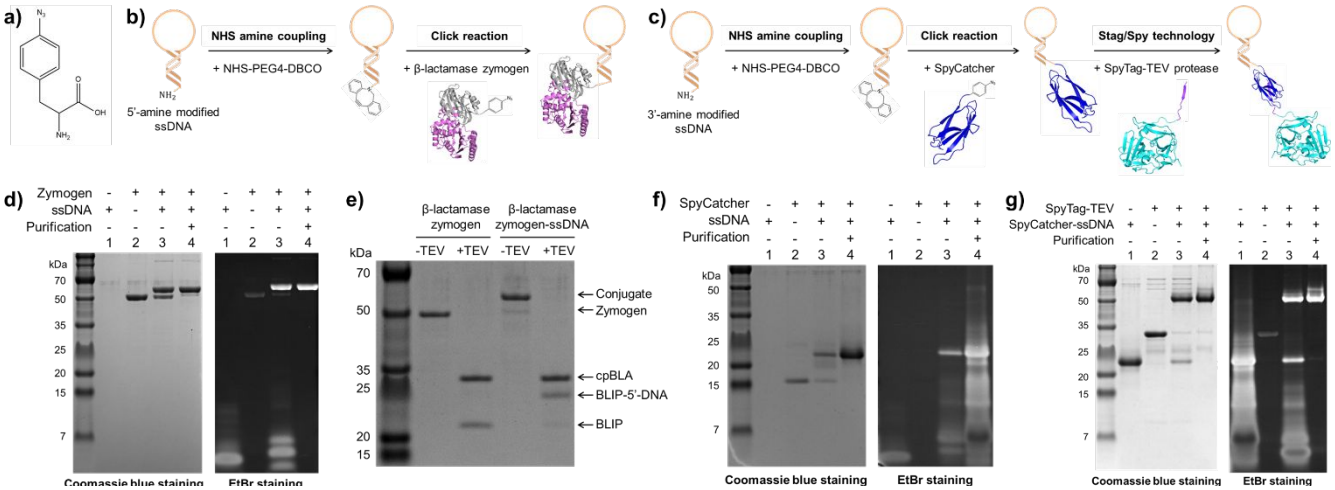


Figure 1. Conjugation of protein and ssDNA a) Structure of 4-azido-L-phenylalanine b) Scheme for conjugating β–lactamase zymogen and ssDNA. c) Scheme for conjugating TEV protease and ssDNA. d) SDS-PAGE results for the reaction between the DBCO-modified ssDNA and the β-lactamase zymogen containing AzF and the purified β-lactamase zymogen-ssDNA. e) Comparison of β-lactamase zymogen and β-lactamase zymogen-ssDNA for the hydrolysis by TEV protease. f) SDS-PAGE results for the conjugation of SpyCatcher including AzF and DBCO-modified ssDNA and the purified SpyCatcher-ssDNA conjugate. g) SDS-PAGE results for the reaction between SpyCatcher-ssDNA and SpyTag-TEV and the purified TEV-ssDNA.

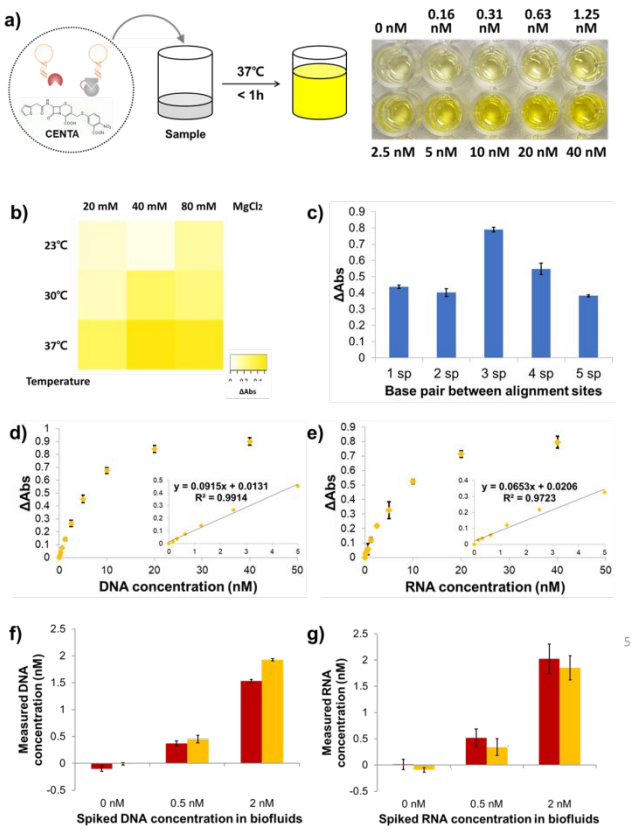


Figure 2. Nucleic acid detection by proximity protease assay. a) One-step method and an assay result for DNA detection. b) Optimization of the reaction condition for temperature and $MgCl_2$ concentration. c) Optimization of the nucleotide spacer between the binding sites for β-lactamase zymogen-ssDNA and TEV-ssDNA. d, e) Absorbance at 405 nm for a range of concentrations (0-40 nM) of target DNA-3 (d) and target RNA (e) relative to that for the blank. The insets display the linear relationships at the low target concentrations (0-5 nM). f, g) Effects of biological matrixes of mouse serum (red) and HEK 293F lysate (yellow) on nucleic acid detection by the proximity proteolysis reaction for DNA (f) and RNA (g).

Using the optimized conditions, the proximity proteolysis reaction was applied for different concentrations of the target DNA oligonucleotide (Target DNA-3 in Table S3). As shown in Figure S5a, the signal difference was observed according to the DNA concentrations immediately after adding the protein-ssDNA conjugates and CENTA. The highest signal difference was observed at 45 minutes, and the absorbance difference at 405 nm at this time was plotted against the target concentrations (Figure 2d). A hyperbolic curve was obtained for the concentrations in the range tested, and a linear relationship was observed up to 5 nM with 94 pM as the limit of detection (LOD). To test the possibility of using a linear ssDNA for sensing target nucleotides, a short ssDNA was conjugated to β-lactamase zymogen instead of using an ssDNA with a hairpin structure. A faster signal increase was observed with the linear ssDNA than with the hairpin structure (Figure S6b), which was not surprising since the former can associate faster with the target DNA than the latter. The two systems exhibited similar responses for the target DNA nucleotides (Figure S6a). Since the ssDNA conjugated to the TEV protease and β-lactamase zymogen were originally designed for detecting *KRAS* mRNA, the proximity proteolysis assay was applied for a synthesized RNA nucleotide (Target RNA in Table S3). The color development took longer (60 minutes) than that for the DNA target (45 minutes) (Figure S5a and b), possibly because of increasingly formation of secondary structure of RNA target.[41] A hyperbolic curve was observed for the whole range of target concentrations, and a linear relationship was observed up to 5 nM with a LOD of 93 pM (Figure 2e). The interference by biological matrixes on the assay method was evaluated using HEK293F cell lysate and mouse serum (Figure 2f and 2g), and the results suggested the developed method can be applied for detecting nucleotides of DNA and RNA present in biological samples. In addition, the sequence specificity of the assay was assessed using modified Target DNA-3 oligonucleotides in which a subsitution, a deletion, or an insertion was introduced into Target DNA-3; the sequence of each oligonucleotide, named Target DNA-subsitution, Target DNA-deletion, and Target DNA-insertion respectively, was shown in Table S3. The modifications resulted in more than 70 % reduction of signal compared to the Target DNA-3 (Figure S7).

Even though the developed method showed high sensitivity, some target nucleotides are present in a much lower concentration in biological fluids; for example, virus RNA is present in patient serum at the femtomolar range.[42] To improve the detection limit further, an isothermal RNA amplification method, nucleic acid sequence-based amplification (NASBA)[43], was applied for *KRAS* mRNA that was prepared by *in vitro* transcription. Then, the samples were analyzed with the proximity proteolysis reaction (Figure 3a). A signal distinct from the baseline was observed in the sample, including at *KRAS* transcript concentrations as low as 10 fM, which is 10,000 times lower than the LOD without the amplification (Figure 3b). However, the results exhibited relatively large errors, which could be overcome by optimizing NASBA assay conditions such as primer sequences and buffer composition.[43-44]

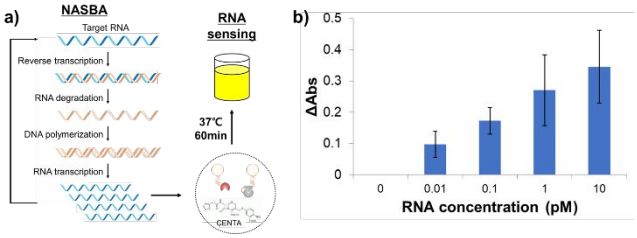


Figure 3. Combination the proximity protease reaction with nucleic acid sequence-based amplification (NASBA). a) Use of NASBA for amplifying RNA transcript and detecting the product with the proximity proteolysis reaction. b) Absorbance at 405 nm for the RNA transcript concentration range of (0.01 pM to 10 pM) relative to that for blank.

In this study, we proposed a proximity proteolysis reaction that enables the enhancement of a proteolysis reaction in the presence of target molecules, and applied this concept for developing a rapid and simple method for detecting nucleic acids. In the presence of the target nucleotides of the *KRAS* transcript, the proteolysis reaction between the two protein-DNA conjugates was enhanced, and the generated signals depended on the target concentrations down to 100 pM. The assay procedure was composed of one step of adding the two protein-DNA conjugates and the colorimetric substrate for β-lactamase into samples, and the color development took less than one hour. The assay also offered the selectivity toward single-base mismatches and the compatibility with biofluids. Using an isothermal amplification method (NASBA), its sensitivity was improved to a femto-molar range. Even though the NASBA step was done in a separate step in this study, we expect that one of isothermal amplification methods could be combined with our assay method, resulting in a

highly sensitive assay method that could be conducted at a constant temperature. Besides an amplification method of nucleic acids, the sensitivity of our assay method could be improved by using a fluorescent substrate of β-lactamase rather than chromogenic substrates.[31] The background signal of our assay method is dependent on the basal activity of the β-lactamase zymogen, and we are also engineering the zymogen to decrease its activity without an activation, which is believed to increase the sensitivity of the proximity proteolysis assay by allowing a higher concentration of the reporter enzyme in the reaction solution. The overexpression of *KRAS* is an important indicator of several cancers, where its mRNA can be present at more than 10,000 copies per cell.[45] The developed method was sensitive enough to detect the *KRAS* transcript in cell lysate. DNA and RNA molecules are released into biological fluids as cell-free nucleic acids and they have drawn much attention as biomarkers.[3, 46-47] Nucleic acid detection is also an important part for the diagnosis of pathogenic infections.[48] We expect that the proximity proteolysis reaction method can also be applied for detecting cell-free nucleic acids and pathogens.

ASSOCIATED CONTENT

Supporting Information

Method of protein purification, protein-ssDNA conjugation, nucleic acid detection by proximity proteolysis reaction and NASBA; plasmids and DNA sequences of primers used; structure of chemical compounds used; schematic structures and DNA sequence of proteins used in this study; property of protein-ssDNA conjugates; signal responses by the proximity proteolysis reaction; assay using β-lactamase zymogen conjugated with a linear form of DNA (ssDNA-2); assay with Target RNA; sequence specificity of the proximity proteolysis assay (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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