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CRISPR/Cas12a collateral cleavage activity for simple and rapid detection of protein/small molecule interaction

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ABSTRACT

To realize the full potential of the CRISPR/Cas system and expand its applicability up to the detection of molecular interactions, we herein describe a novel method to identify protein/small molecule interactions by utilizing the CRISPR/Cas12a collateral cleavage activity. This technique employs a single-stranded activator DNA modified with a specific small molecule, which would switch on the CRISPR/Cas12a collateral cleavage activity upon binding to crRNA within the CRISPR/Cas12a system. When the target protein binds to the small molecule on the activator DNA, the bound protein sterically hinders the access of the activator DNA to crRNA, thereby promoting less collateral cleavage activity of CRISPR/Cas12a. As a consequence, fewer reporter probes nearby are cleaved to produce accordingly reduced fluorescence signals in response to target protein. Based on this unique design principle, the two model protein/small molecule interactions, streptavidin/biotin and anti-digoxigenin/digoxigenin, were successfully determined down to 0.03 nM and 0.09 nM, respectively, with a fast and simple detection workflow (11 min). The practical applicability of this method was also verified by reliably detecting target streptavidin spiked in heterogeneous human serum. This work would provide great insight to construct novel strategies to identify protein/small molecule interaction by making the most of the CRISPR/Cas12a system beyond its superior capabilities in genome editing and molecular diagnostics.

1. Introduction

Numerous types of molecular interactions, such as the interactions between proteins, protein/nucleic acid, and protein/small molecule, serve as the fundamental principles of functioning of living systems by being involved in almost all the important biological processes, which include hormone/receptor and antigen/antibody interactions, various enzymatic reactions, and ligand-specific transport phenomena. Interpreting these interactions and understanding how they participate in the biological processes are critically demanded for the advancement of biological science as the most representative scientific goals (Ideker and Sharan, 2008; Rual et al., 2005; Stelzl et al., 2005). In particular, protein-small molecule interaction (PSMI) plays key biological roles by regulating protein functions and is deeply involved in many major human diseases (Marazuela, 1996; Smith and Hall, 1974). Therefore, the elucidation of PSMI would offer great potentials for drug development and biomedical research (Li et al., 2013b; McFedries et al., 2013).

Conventional techniques to identify PSMI mainly include affinity chromatography (Ong et al., 2002), kinetic capillary electrophoresis (Drabovich et al., 2009), protein-fragment complementation assay (Michnick et al., 2007), and surface plasmon resonance (SPR) (Cooper, 2002; Wear et al., 2005). These techniques possess their own intrinsic merits to identify PSMI but require sophisticated instruments and/or cumbersome assay procedures including specific immobilization step along with limited sensitivities. By overcoming these limitations, fluorescence-based approaches have been also extensively established, which are advantageous in terms of high sensitivity, simplicity, and operation versatility (He and Jiao, 2016; Shi et al., 2017; Wang et al., 2015). Notably, the functional DNA components have been recently exploited to identify the interactions between small molecules and their protein receptors by taking advantage of their functional properties, which have resulted in several satisfactory performances for the detection of PSMI (Harris et al., 2008; Kim et al., 2018; Lee et al., 2018; Song et al., 2021a). Nevertheless, there still exists a large room for further improvement with respect to the analytical performance such as sensitivity, specificity, rapidity, and convenience.

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas), together referred to as

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the CRISPR/Cas system, were first discovered as a defense mechanism of bacteria against foreign nucleic acids (Makarova et al., 2011; Philippe and Rodolphe, 2010). By precisely creating intended gene deletions and mutations down to the single nucleotide level, the CRISPR/Cas system has revolutionized genome editing, far surpassing the existing technologies such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Gaj et al., 2013; Hsu et al., 2014). In recent years, the CRISPR/Cas system's ability to identify and cut nucleic acid sequence with single nucleotide precision has been exploited to create new approach termed CRISPR-based molecular diagnostics, which might enable highly programmable molecular diagnostics allowing easy adaptation to different targets while keeping high sensitivity and specificity (Aman et al., 2020).

Early CRISPR-based molecular diagnostics largely relied on the CRISPR/Cas9 system, in which Cas9 was used to cleave target nucleic acid, producing double strand break. Some methods employed Cas9 variants such as Cas9 nickase to produce single strand break or Cas9 without endonuclease activity (dCas9) (Guk et al., 2017; Huang et al., 2018; Moon et al., 2020; Song et al., 2021b; Wang et al., 2020; W. Zhou et al., 2018). In these methods, the cleavage or dCas9 binding of target DNA was directed to initiate downstream reactions to produce the signals used to detect target nucleic acids. Newer methods made use of the collateral cleavage activity found in Cas12 or Cas13 which could indiscriminately cleave any nearby non-target single-stranded DNA (ssDNA) or RNA, respectively, upon target recognition (Chen et al., 2018). By employing ssDNA or RNA labeled with fluorophore and quencher, the collateral cleavage activity could produce enormously amplified fluorescent signals in response to target nucleic acid, which has led to the active development of a variety of ultrasensitive nucleic acid detection strategies such as SHERLOCK, HOLMES, and DETECTR (Chen et al., 2018; Gootenberg et al., 2018; Kellner et al., 2019; Li et al. 2018, 2019).

Particularly, the collateral cleavage activity of CRISPR/Cas12a has been further exploited to construct novel strategies for the detection of various biological analytes beyond nucleic acid detection. Representatively, CRISPR/Cas12a system was utilized to detect small molecules (ATP) by using cognate aptamers (Peng et al., 2020) and metal ions by coupling with DNAzyme activity (Xiong et al., 2020). More recently, the activities of various enzymes, including T4 PNK, telomerase, and APE1, were successfully detected by incorporating the activity of terminal deoxyribonucleotidyl transferase (TdT) into the CRISPR/Cas12a system (Chen et al., 2021). These active applications of the CRISPR/Cas12a system verify its tremendous potentials to be adapted for a much wider range of targets.

To unlock the potentials and expand the applications of CRISPR/Cas12a, we herein describe a novel CRISPR/Cas12a-based strategy to identify protein/small molecule interaction. By employing a ssDNA modified with a specific small molecule to serve as an activator DNA of Cas12a, we successfully translated the protein/small molecule interaction into CRISPR/Cas12a collateral cleavage activity. As a consequence, the two model target interactions, streptavidin/biotin and anti-digoxigenin/digoxigenin, were very reliably identified with outstanding sensitivity and specificity, by producing fluorescence signal to inversely reflect the amount of the corresponding target protein.

2. Materials and methods

2.1. Materials

All the oligonucleotides employed in this study were synthesized and purified with polyacrylamide gel electrophoresis (PAGE) by Bioneer® (Daejeon, Korea). The sequences of the oligonucleotides used in this study are listed in Table S1. Alt-R® A.s. Cas12a (Cpf1) Ultra and Alt-R® A.s. Cas12a crRNA were purchased from IDT (Coralville, IA, USA). Human serum, lysozyme, papain, and anti-digoxigenin were purchased from Sigma-Aldrich (St Louis, MO, USA). Streptavidin, BSA, and thrombin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ultrapure DNase/RNase-free distilled water was purchased from Bioneer® and used throughout all the experiments. All other chemicals were of analytical grade and used without further purification.

2.2. Experimental procedures for the proposed assay

In a typical protein/small molecule interaction assay, the reaction mixtures were separately prepared as PSMI solution and detection solution. The PSMI solution consisted of 1 μL of activator DNA (1 μM) and 1 μL of target protein at varying concentrations. For the experiments to detect streptavidin present in human serum, 0.5 μL of streptavidin at varying concentrations was spiked to 0.5 μL of 40% human serum solution, which was then used as target protein solution. The detection solution consisted of 10 μL of DW, 2 μL of 10X NEBuffer 2.1 (500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, 1 mg/mL BSA, pH 7.9), 4 μL of FAM- and BHQ1-labeled reporter probe (F-Q reporter) (1 μM), 1 μL of crRNA (1 μM), and 1 μL of Cas12a (1 μM). After the PSMI solution was incubated at room temperature for 1 min, the detection solution was added to the PSMI solution, which was then incubated at 32 °C for 10 min. The fluorescence signal from the solution was measured in the range of 500–600 nm at an excitation wavelength of 460 nm by using a Tecan Infinite M200 pro microplate reader (Männedorf, Switzerland) and black 384-well Greiner Bio-One microplates (Ref. 781077, Courtaboeuf, France). For the real-time fluorescence analysis, the reaction solution was incubated at 28 °C, from which the fluorescence intensity was monitored at intervals of 30 s by a CFX Connect™ Real-Time System (Bio-Rad, CA, USA).

2.3. Gel electrophoresis analysis

For PAGE analysis, 10 μL aliquot of the reaction solution was mixed with 2 μL loading buffer (6X) purchased from Bioneer®, which was resolved on 15% polyacrylamide gel at a constant voltage of 120 V for 120 min using 1X TBE as the running buffer. After GelRed staining, the gel image was taken with a ChemiDoc™ Imaging System (Bio-Rad, Hercules, USA). In the experiments employing FAM-labeled reporter probe (FAM reporter), the staining step was omitted.

3. Results and discussion

3.1. Design strategy

As illustrated in Fig. 1, the new strategy to detect protein/small molecule interaction employs a single-stranded activator DNA (AD) modified with a small molecule involving in the interaction as a key component to recognize target protein and initiate the downstream signaling process. Since the small molecule-modified single-stranded activator DNA was designed to be complementary with crRNA, it freely binds to crRNA within crRNA/Cas12a (RNP), in the absence of target protein, activating the collateral cleavage activity of Cas12a. As a consequence, Cas12a indiscriminately cleaves multiple F-Q reporter probes nearby, producing intense fluorescence signal. In the presence of target protein, on the other hand, target protein binds to the small molecule on AD and the bound protein sterically hinders the hybridization of AD with crRNA, consequently activating fewer Cas12a molecules and producing accordingly reduced fluorescence signal. The reduced signal could be used to identify and quantify target protein.

3.2. Detection feasibility of the proposed assay

To demonstrate the feasibility of this approach, we first investigated whether the AD with biotin modification binds to crRNA within RNP and switches on the collateral cleavage activity of Cas12a, by employing streptavidin and biotin as a model target interaction. First, the RNP alone was not able to exhibit the collateral cleavage activity to cleave

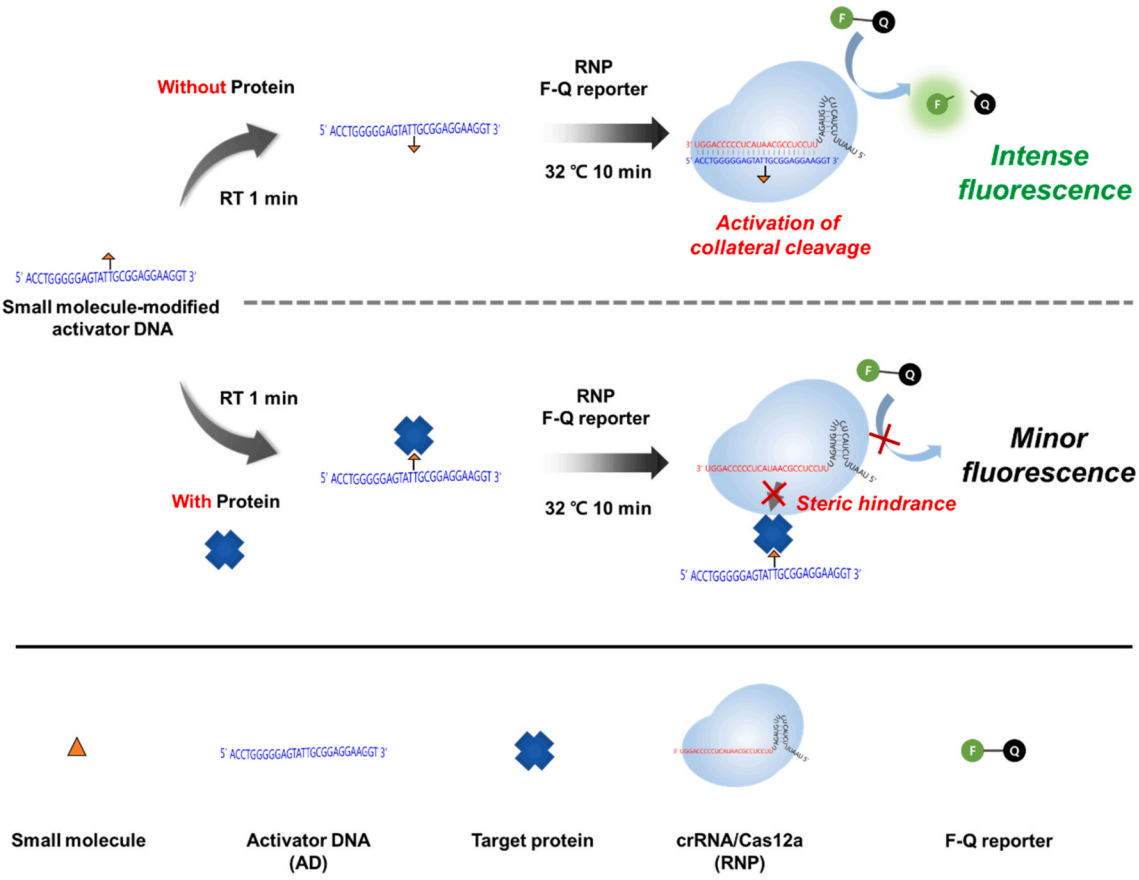


Fig. 1. Schematic illustration of the strategy to identify protein/small molecule interaction based on the CRISPR/Cas12a collateral cleavage activity.

the employed F-Q reporter and there was no significant fluorescent signal produced from the F-Q reporter in the absence of the AD (Fig. 2 (A), curve a). When the AD was applied to the sample solution, the AD properly activated the collateral cleavage activity of Cas12a within RNP upon binding to crRNA and produced intense fluorescent signal by cleaving the F-Q reporter (curve b). The biotin-modified AD also activated the collateral cleavage activity of Cas12a and produced the almost same level of fluorescent signal with the free AD, indicating that the

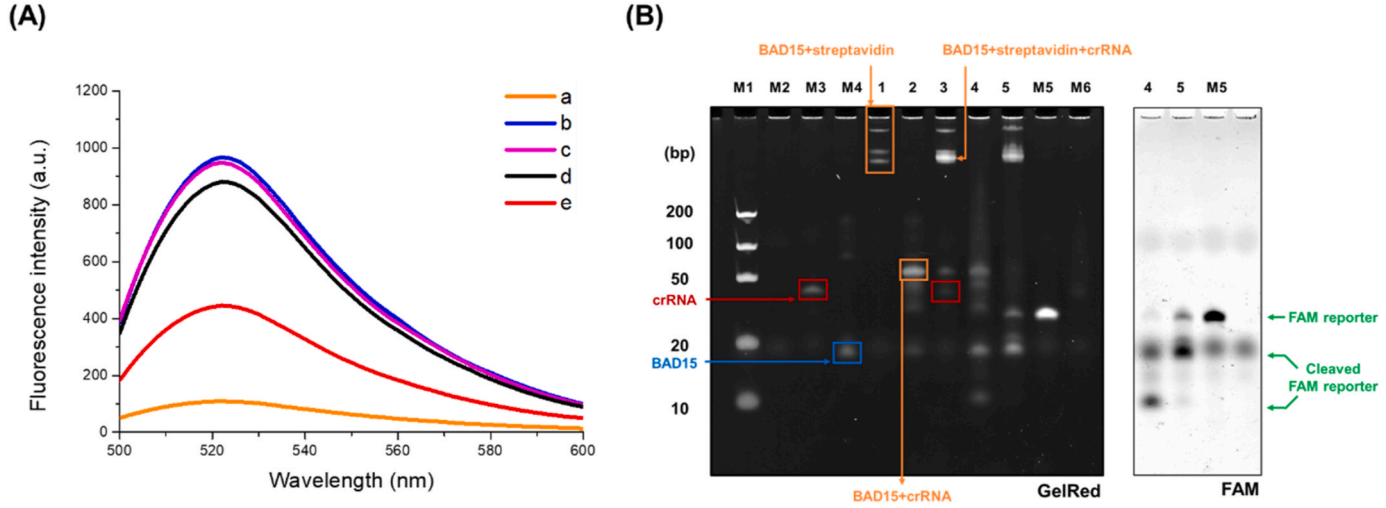


Fig. 2. Feasibility of the proposed assay. (A) Fluorescence signals produced form F-Q reporter under various combinations of reaction components to identify streptavidin/biotin interaction. a: RNP + F-Q reporter, b: RNP + F-Q reporter + AD, c: RNP + F-Q reporter + AD + streptavidin, d: RNP + F-Q reporter + BAD15, e: RNP + F-Q reporter + BAD15 + streptavidin. The final concentrations of AD, BAD15, F-Q reporter, RNP, and streptavidin were 5 nM, 5 nM, 100 nM, 50 nM, and 5 nM, respectively. The reaction temperature, reaction time, streptavidin-BAD15 incubation time were 28 °C, 10 min, and 1 min, respectively. (B) Non-denaturing polyacrylamide gel electrophoresis (PAGE) image of the reaction products. M1: Ultra-low DNA ladder, M2: streptavidin, M3: crRNA, M4: BAD15, Lane 1: BAD15 + streptavidin, Lane 2: BAD15 + crRNA, Lane 3: BAD15 + streptavidin + crRNA, Lane 4: BAD15 + RNP + FAM reporter, Lane 5: BAD15 + streptavidin + RNP + FAM reporter, M5: FAM reporter (FAM-labeled ssDNA), M6: RNP. The final concentrations of BAD15, streptavidin, crRNA, FAM reporter, and RNP were 100 nM. The reaction temperature, reaction time, streptavidin-BAD15 incubation time were 28 °C, 10 min, and 1 min, respectively.

biotin modification does not interfere with the interaction of the AD with crRNA (curve d).

Since our strategy basically relies on the steric hindrance caused by the target protein bound to the small ligand molecule, we reasoned that the spatial position of the protein should greatly influence the final performance and conducted the experiments to identify the streptavidin/biotin interaction by employing the ADs modified with biotin at several different positions. Very importantly, when streptavidin was applied to the biotin-modified ADs, the fluorescent signals were generally reduced for all cases (Figure S1(B)–(F)), compared with the samples without streptavidin. Among the tested ones, the AD modified with biotin at the 15th base, referred to as BAD15, led to the highest reduction of the fluorescent signal by the interaction with the target streptavidin protein (Figure S1(D) and Fig. 2(A), curve d and e), which was then employed for the experiments in this work. When the AD without biotin modification was employed as negative control experiment, the addition of streptavidin did not influence the fluorescence signal from the F-Q reporter and the intense fluorescence signal was just kept unreduced (Figure S1(A) and Fig. 2(A), curve c). All these results confirm that the specific binding of streptavidin to biotin on the AD hinders the interaction of BAD15 with RNP and prevents the activation of the collateral cleavage activity of Cas12a, consequently diminishing the fluorescence signal from the F-Q reporter, which could be used to quantify the target streptavidin according to the mechanism envisioned in Fig. 1.

To further support the fluorescence results, we conducted PAGE analysis of the reaction components and products for the assay of streptavidin/biotin interactions. First, we confirmed that BAD15 properly binds to streptavidin and crRNA based on the bands corresponding to the respective complexes on the upper positions (Fig. 2(A), Lane 1 and 2). The multiple bands in Lane 1 is due to the binding of multiple BAD15 probes to single streptavidin having four biotin-binding sites. Most importantly, when the mixture of BAD15 and streptavidin was applied to crRNA (Lane 3), the crRNA band remained unchanged to some extent and the band corresponding to the mixture of BAD and crRNA was marginal, verifying that streptavidin bound to BAD15 significantly inhibit the binding of crRNA to BAD15. We also found that one band for the BAD15-streptavidin complex was thickened on a slightly higher position, which could be assumed to correspond to the BAD15-streptavidin-crRNA complex.

To further verify the collateral cleavage of a reporter probe, FAM-labeled single-stranded DNA (FAM reporter) was particularly employed and applied to the solution of BAD15 and RNP. As a result (Lane 4), the FAM reporter band almost disappeared and the new bands corresponding to the digested short fragments appeared, confirming that BAD15 successfully activated the collateral cleavage activity of Cas12a. On the other hand, significant amount of the FAM reporter remained undigested in the solution where BAD15 was allowed to interact with streptavidin prior to RNP, indicating that the binding of streptavidin successfully repressed the activation of Cas12a (Lane 5). Overall, these results are quite consistent with the above fluorescence results, ensuring that the binding of streptavidin sterically hinders the binding of activator DNA to RNP and significantly prevent the activator DNA from activating the collateral cleavage activity of Cas12a.

3.3. Sensitivity and specificity of the proposed assay

To find the optimal reaction conditions maximizing the efficiency of the assay, various reaction conditions were optimized by comparing the fluorescence intensities from the reaction samples containing streptavidin to those from the reaction samples without streptavidin. As presented in Figures S2~S7, 2.5 nM of BAD15, 50 nM of RNP, 200 nM of F-Q reporter, 10 min of reaction time, 1 min of streptavidin-BAD15 incubation time, and 32 °C of reaction temperature were selected as the optimal conditions for the proposed strategy and used for further experiments.

To determine the sensitivity of the proposed system, streptavidin at various concentrations in the range from 0 to 50 nM was subjected to the assay procedure and the produced fluorescence signals were measured (Fig. 3). The results showed that the fluorescence signal at 522 nm (F_{522}) decreased in proportion to the increasing streptavidin concentration (C_{STV}). When F_{522} was plotted against C_{STV}, an excellent linear relationship was given over the range of 0.1–2.5 nM ($F_{522} = -381.250\ C_{STV}$ (nM) + 1260.794, $R^2 = 0.9987$), confirming that the developed method is capable of reliably quantifying streptavidin. The limit of detection (LOD) was determined to be 0.03 nM according to the equation: LOD = 3σ/S, where σ is the standard deviation of the blank, and S is the slope of the calibration line. This value is comparable or even better than those from previously reported techniques to detect streptavidin (Table 1).

The specificity of this method was next assessed by examining the extents of fluorescence reduction induced by other proteins such as BSA, papain, thrombin, and lysozyme. The results in Fig. 4 show that only target streptavidin induces the significant fluorescence reduction, while other proteins do not influence the fluorescence signals at all although 20 times higher concentration (50 nM) was used than that of streptavidin (2.5 nM). These results confirm the excellent specificity of our strategy toward the target streptavidin.

Besides the analytical performances, our strategy neither require laborious synthesis of nanomaterials nor involve time-consuming multiple enzymatic reactions and these superior features enable accurate quantitation of the target molecules in 11 min, which is far faster than most of the previous methods.

3.4. Practical utility test

To verify the general applicability of this method for heterogeneous biological sample, streptavidin at varying concentrations was spiked into human serum and subjected to the proposed assay. As shown in Figure S8, an excellent linear relationship ($R^2 = 0.9973$) was also obtained between F_{522} and C_{STV} in human serum. Based on this calibration curve, the concentrations of streptavidin in human serum were successfully determined with high reproducibility and precision, as evidenced by the coefficients of variation (CV) (0.614–4.607%) and the recovery rates (99.6–105.0%) (Table 2). These results indicated that this method could identify streptavidin/biotin interaction in complex heterogeneous specimens, verifying its robust applicability for real biological sample analysis.

3.5. Universality of the proposed assay

Finally, the universal applicability of the proposed assay was evaluated by detecting another target interaction: anti-digoxigenin/digoxigenin (ADIG/DIG). For this purpose, we conducted the assay procedure to detect the target ADIG protein by employing a new activator DNA with DIG modification (DAD15). As a result shown in Fig. 5 (A), the fluorescence signal decreased proportionally to increasing ADIG concentration and an excellent linear relationship between F_{522} and the ADIG concentration (C_{ADIG}) was obtained over the range of 0.2–5 nM ($F_{522} = -139.46\ C_{ADIG}$ (nM) + 1063, $R^2 = 0.9934$). The limit of detection (LOD) was determined to be 0.09 nM (Fig. 5(B)) and target ADIG protein was also very specifically discriminated from other proteins (Fig. 5(C)), confirming the universal utility of the proposed strategy.

Besides streptavidin/biotin and ADIG/DIG, we believe that this strategy can be employed to analyze plenty of small molecules and their target proteins such as folate receptor/folate and thyroid hormone/thyroid receptor (Cao et al., 2016; Song et al., 2021a). For this method to be applicable to wider target pairs, modification of small molecules onto DNA is the most important issue. Fortunately, numerous research have been reported to label small molecules onto DNA utilizing their functional groups such as hydroxyl group and carboxyl group (Gothelf, 2017; Li et al., 2013a). In addition, click chemistry can facilitate the modification by utilizing azide-modified small molecule and alkyne-modified

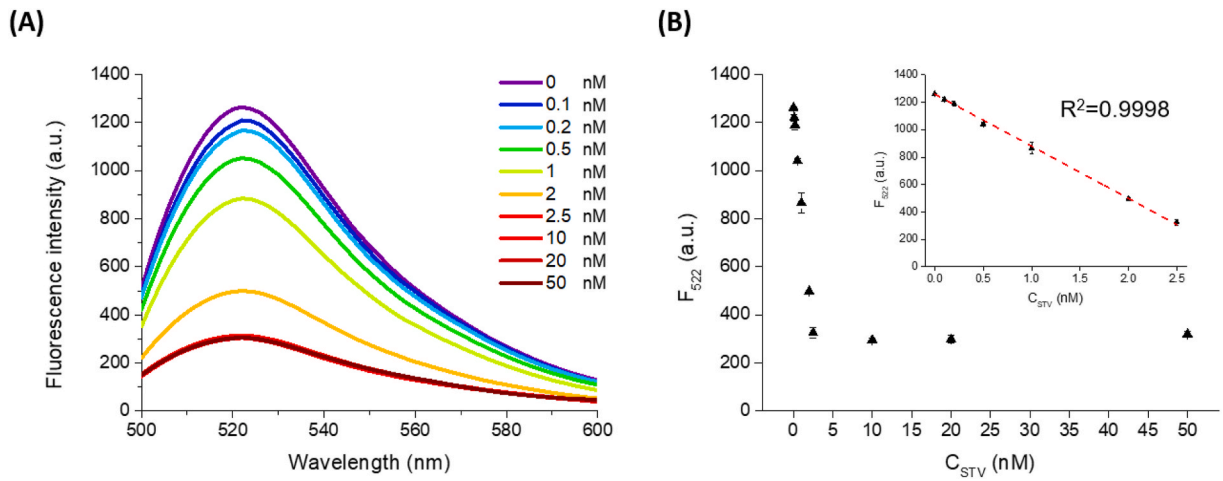


Fig. 3. Sensitivity of the proposed assay. (A) Fluorescence emission spectra of the sensing system in the presence of different concentrations of streptavidin from 0 to 50 nM. (B) Relationship between the fluorescence intensity at 522 nm (F_{522}) and the concentration of streptavidin (C_{STV}). Inset: Linear relationship between F_{522} and C_{STV}. The error bar was determined from triplicate measurements.

Table 1
Comparison of the proposed assay with alternative methods.

Key components/Methods	Limit of detection	Analysis time	Limitations	Reference
Protein-induced fluorescence enhancement	2.93 nM	10 min	• Low sensitivity	Kim et al. (2018)
Silver nanoclusters	2.6 nM	140 min	• Low sensitivity • Long assay time • Synthesis of nanomaterials	He and Jiao (2016)
Branch migration	2 nM	N.A.	• Low sensitivity	Zhou et al. (2018)
Rolling circle amplification	0.27 nM	110 min	• Preparation of circular template • Long assay time	Shi et al. (2017)
Copper nanoclusters	0.1 nM	25 min	• Low sensitivity • Synthesis of nanomaterials	Wang et al. (2015)
Exponential strand displacement amplification	16 pM	95 min	• Long assay time • Use of three different enzymes	Lee et al. (2018)
G-triplex molecular beacon	86.1 fM	150 min	• Long assay time	Liu et al. (2020)
Collateral cleavage of Cas12a	0.03 nM	11 min	–	This work

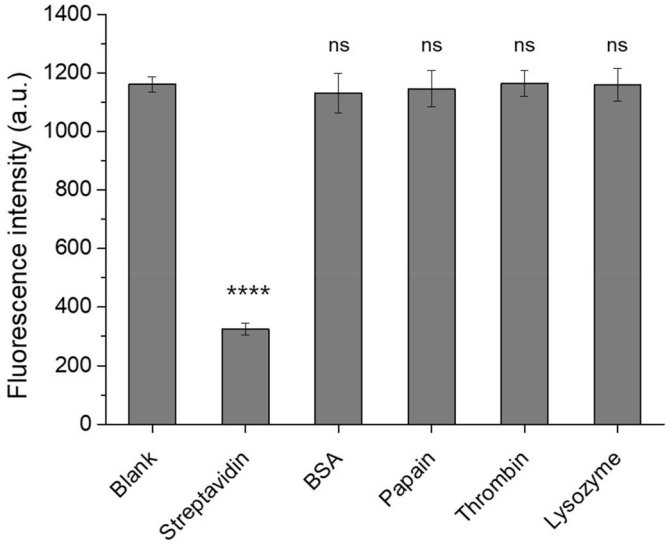


Fig. 4. Selectivity of the proposed assay. Fluorescence intensities at 522 nm (F_{522}) were measured in the samples containing streptavidin (2.5 nM) or other proteins: BSA, papain, thrombin, and lysozyme (50 nM). The error bars indicate the standard deviations obtained from triplicate measurements. Asterisks indicate different p-values of the sample containing streptavidin compared to the negative control without streptavidin. (****$p < 0.00005$; ns, not significant; unpaired two-tailed *t*-test).

Table 2
Determination of streptavidin in 1% human serum samples.

Added streptavidin (nM)	Measured streptavidin[a] (nM)	CV[b] (%)	Recovery[c] (%)
0.1	0.105	2.311	105.0
0.5	0.510	0.614	102.0
1	0.996	4.607	99.6

[a] Mean of three measurements.
[b] Coefficient of variation = (standard deviation)/mean × 100.
[c] Recovery = (measured streptavidin)/(added streptavidin) × 100.

DNA, or vice versa (Gierlich et al., 2006). Moreover, many modifications are also commercially available. Therefore, we believe that the developed method can be easily expanded to other protein and small molecule pairs and has the tremendous potential for clinical application by reliably detecting various disease biomarkers in human serum.

4. Conclusions

In this work, we developed the new fluorometric strategy to identify protein/small molecule interaction by utilizing collateral cleavage activity from CRISPR/Cas12a system. A small molecule-modified ssDNA was employed to activate the CRISPR/Cas12a collateral cleavage activity that would produce fluorescence signals by cleaving reporter probes nearby. The binding of a pair protein to the small molecule on the activator DNA inhibited the binding of the activator DNA with Cas12a,

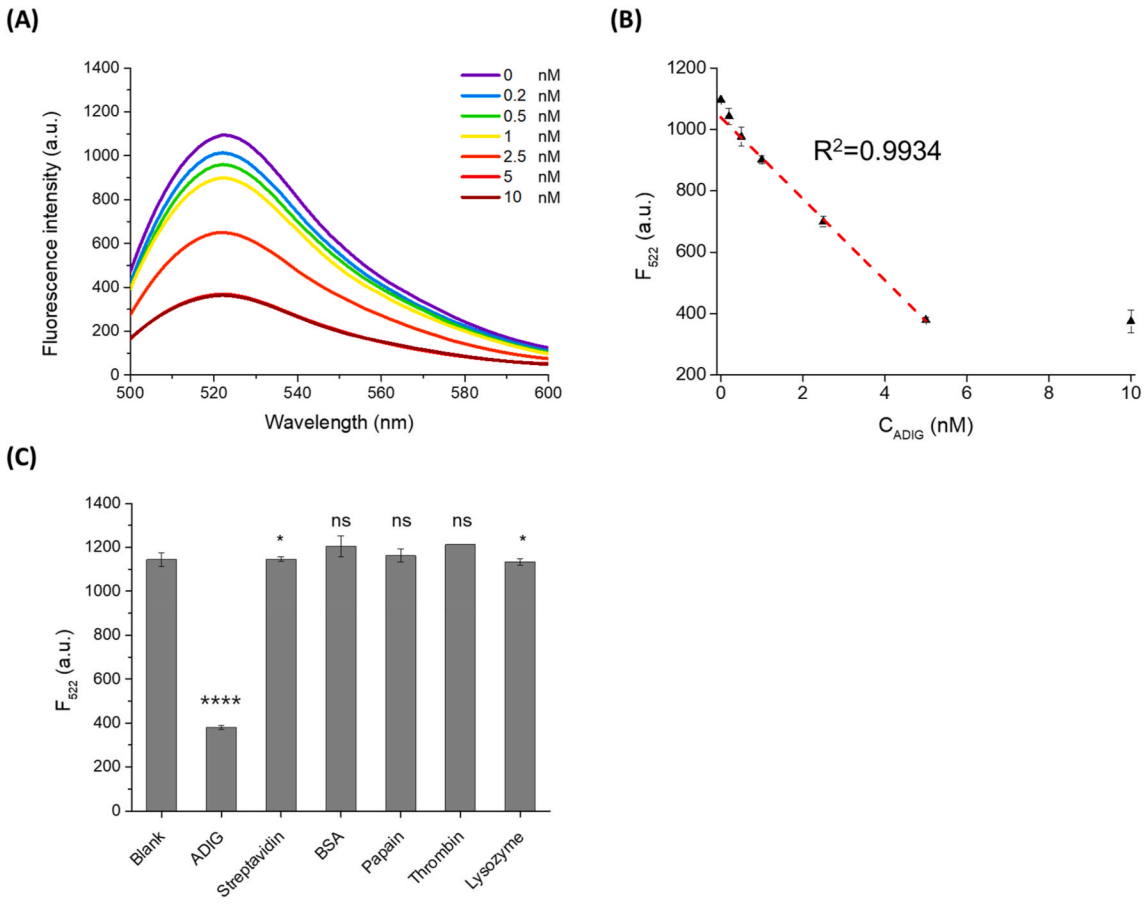


Fig. 5. Universality of the proposed assay. (A) Fluorescence emission spectra of the sensing system using DAD15 in the presence of different concentrations of ADIG from 0 to 10 nM. (B) Relationship between the fluorescence intensity at 522 nm (F_{522}) and the concentration of ADIG (C_{ADIG}). (C) Selectivity of the sensing system using DAD15. F_{522} were measured in the samples containing ADIG (5 nM) or other proteins: streptavidin, BSA, papain, thrombin, and lysozyme (50 nM). Asterisks indicate different p-values of the sample containing ADIG compared to the negative control without ADIG. (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ****$p < 0.00005$; ns, not significant; unpaired two-tailed *t*-test).

consequently resulting in decreased fluorescence signal. Based on this unique design principle, we successfully detected two model targets, streptavidin and ADIG, with a detection limit of 0.03 nM and 0.09 nM, respectively. Moreover, practical and robust applicability of this strategy was demonstrated by successfully detecting streptavidin in heterogeneous human serum.

To the best of our knowledge, this is the first attempt to utilize the collateral cleavage activity of Cas12a for the analysis of protein/small molecule interactions. This work offers a new opportunity that the collateral cleavage activity of Cas12a could be exploited to construct novel methods for protein/small molecule interaction assay as well as conventional molecular diagnostics. Based on the aforementioned excellent analytical performances, we believe that this work could serve as a platform technology to detect various biological entities such as small molecules, proteins, and cells based on the CRISPR/Cas system.

Author contributions

Hansol Kim: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. Seoyoung Lee: Investigation, Validation, Formal analysis, Investigation, Resources. Junhyeok Yoon: Validation, Resources. Jayeon Song: Validation, Resources. Hyun Gyu Park: Writing - review & editing, Supervision, Project administration, Funding acquisition.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2021.113587.

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