ACS NANOSCIENCE Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Letter

Turning on Protein Function Inhibited by DNA Aptamers Employing a Covalent DNA-Binding Protein

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Cite This: ACS Nanosci. Au 2026, 6, 201–207
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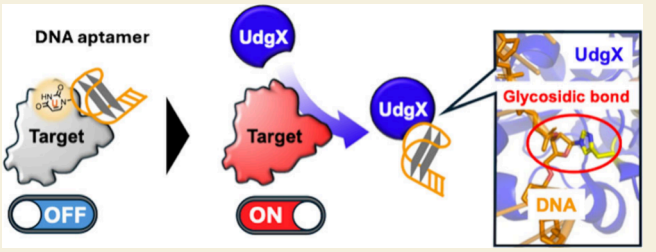
ABSTRACT: Certain DNA aptamers can serve as effective inhibitors, although their inhibitory action is typically unidirectional. Simple removal of these aptamers from their target complexes could improve their application in areas such as oligonucleotide therapeutics. In the current study, we used uracil-DNA glycosylase from *Mycobacterium smegmatis* (UdgX), which binds covalently to DNA after uracil removal. A suitable site for incorporating uracil-DNA into a G-quadruplex-structured DNA aptamer was identified, and subsequent restoration of thrombin activity previously inhibited by the DNA aptamers was examined. The addition of UdgX restored thrombin activity to nearly 100% within 1 min, demonstrating greater speed and efficacy than complementary strand addition targeting the thrombin aptamer. Furthermore, UdgX restored the binding capacity of the anti-VEGF antibody bevacizumab that had been inhibited by a hairpin-structured aptamer. These findings highlight the versatile potential of UdgX to turn on protein functions inhibited by DNA aptamers.



KEYWORDS: *Anticoagulant, DNA aptamer, DNA aptamer remover, UdgX, uracil-DNA glycosylase*

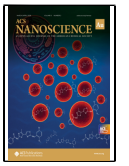
DNA aptamers have garnered considerable attention due to their ability to bind and inhibit various substances, including ions, chemical compounds, and proteins.[1−3] For instance, the anticoagulant thrombin aptamer binds to the blood coagulation factor thrombin, thereby inhibiting blood coagulation. Meanwhile, DNA aptamers can also function as drugs for cancer[4] and neurodegenerative diseases[5] by binding to and inhibiting their targets. Furthermore, unlike antibodies, DNA aptamers can be easily prepared by chemical synthesis based on known sequences.

Thus, if the DNA aptamers can be removed from their binding complex using a simple operation, we can turn on the target molecule's function. This enhances the utility of DNA aptamers for various applications. For instance, in the case of antithrombin aptamers that are utilized as anticoagulants, the function of thrombin must be rapidly restored when bleeding occurs.[6] Therefore, the development of a rapid, effective, and universal method for DNA aptamer removal is important to accelerate the application of aptamer-based drugs. To this end, researchers have employed complementary strands—allowing the aptamer to release the target by forming double-stranded DNA—and G-quadruplex (G4)-specific ligands, which recognize typical guanine-rich nucleic acid sequences, as inhibitors of antithrombin aptamers.[7,8] However, these operations are time-consuming, necessitating the development of a rapid turn-on operation.

Uracil-DNA glycosylase—commonly referred to as UdgX—is derived from *Mycobacterium smegmatis*. Distinct from other uracil-DNA glycosylases, UdgX establishes an irreversible covalent glycosidic bond at the abasic site through a histidine residue, following the recognition and excision of aberrant uracil in either single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA). Since its initial characterization in 2015, UdgX has predominantly been utilized for uracil detection.[9,10] More recently, we have leveraged UdgX's unique covalent DNA-binding capability to construct a versatile, reversible DNA−protein coupling module by designing an aptamer−enzyme complex that efficiently forms covalent bonds with uracil-DNA-containing aptamers.[11]

In this study, we focus on the remarkable properties of UdgX to develop a rapid, turn-on technology for protein functions inhibited by DNA aptamers. First, we examined where uracil-DNA could be incorporated into the antithrombin aptamer RE31[12] and evaluated the aptamer's functionality. Interestingly, certain RE31 mutants exhibited higher anticoagulant activity than the original RE31 sequence. Thrombin

Received: September 25, 2025
Revised: December 13, 2025
Accepted: December 15, 2025
Published: December 20, 2025



https://doi.org/10.1021/acsnanoscienceau.5c00143
ACS Nanosci. Au 2026, 6, 201−207

Table 1. Oligonucleotide Sequences for RE31, RE31 Mutants, and Complementary Strand

Sample	Sequence (5′ to 3′)
RE31	GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC
RE31_T11U	GTGACGTAGGUTGGTGTGGTTGGGGCGTCAC
RE31_T12U	GTGACGTAGGTUGGTGTGGTTGGGGCGTCAC
RE31_T15U	GTGACGTAGGTTGGUGTGGTTGGGGCGTCAC
RE31_T17U	GTGACGTAGGTTGGTGUGGTTGGGGCGTCAC
RE31_T20U	GTGACGTAGGTTGGTGTGGUTGGGGCGTCAC
RE31_T21U	GTGACGTAGGTTGGTGTGGTUGGGGCGTCAC
RE31_5′(AAGTGUAGGCA)	AAGTGUAGGCA GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC
RE31_5′(TGUAG)	TGUAG GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC
RE31_5′(GUAG)	GUAG GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC
RE31_5′(TGU)	TGU GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC
RE31_5′(GU)	GU GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC
RE31_5′(U)	U GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC
RE31_3′(AAGTGUAGGCA)	GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC AAGTGUAGGCA
RE31_3′(GUAG)	GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC GUAG
RE31_3′(UAG)	GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC UAG
RE31_3′(U)	GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC U
Complementary strand	GTGACGCCCCAACCACACCAACCTACGTCAC

function, initially inhibited by RE31, was restored upon the introduction of UdgX, which formed a covalent bond with uracil-DNA. The turn-on property induced by UdgX was faster and more effective than that induced by adding a complementary strand to RE31. Furthermore, we validated the restored binding capacity of the antivascular endothelial growth factor (VEGF) antibody, bevacizumab, previously inhibited by an anti-ideotype aptamer, A14#1,[13] which adopts a stem−loop structure. These findings establish that the UdgX-based turn-on technology is universally applicable, irrespective of aptamer structure. Moreover, this work represents the first report of a rapid turn-on technology for protein function inhibited by DNA aptamers.

INCORPORATION OF URACIL-DNA INTO A THROMBIN-BINDING APTAMER, RE31

RE31 is a thrombin-binding aptamer that exhibits anticoagulant activity by inhibiting the enzymatic activity of thrombin.[12] Considering that UdgX recognizes uracil-DNA, we first investigated the introduction of uracil-DNA into RE31. We substituted thymine in RE31 with uracil due to their similar structures. Specifically, we replaced thymine on the loop of RE31 with a uracil base, or added a uracil-DNA-containing sequence to the 3′ or 5′ end of RE31 (Table 1). All uracil-DNA-replaced or introduced mutants are referred to as RE31 mutants in this study.

Circular dichroism (CD) spectroscopy was used to confirm the secondary structure of the RE31 mutants. All RE31 mutants exhibited positive peaks at 245 and 295 nm and a negative peak at 265 nm, indicating that they formed an antiparallel G4 structure similar to that of the original RE31[14,15] (see Supporting Information Figure S1 for CD spectra). In contrast, some mutants with a long sequence added at either the 5′ or 3′ terminus—i.e., RE31_5′(AAGTGUAGGCA), RE31_5′(GUAG), RE31_5′(TGUAG), RE31_3′(AAGTGUAGGCA), and RE31_3′(GUAG)—demonstrated smaller peaks at 265 nm than that of RE31. These sequences were predicted to form an antiparallel G4 structure; however, the introduction of longer sequences might lead to loosening of the structure.[16]

Covalent binding of UdgX to the RE31 mutants was confirmed by SDS-PAGE (see Supporting Information Figure S2 for SDS-PAGE of RE31 and RE31 mutant). UdgX did not form covalemt binding to the original RE31 aptamer, which did not contain uracil-DNA (cFigure S2C1 and S2C2). In contrast, the formation of the complex with UdgX was confirmed for the RE31 mutants, excluding RE31_5′(U) and RE31_3′(U). These results indicate that UdgX is capable of binding to uracil-DNA located in all loop regions, as well as at

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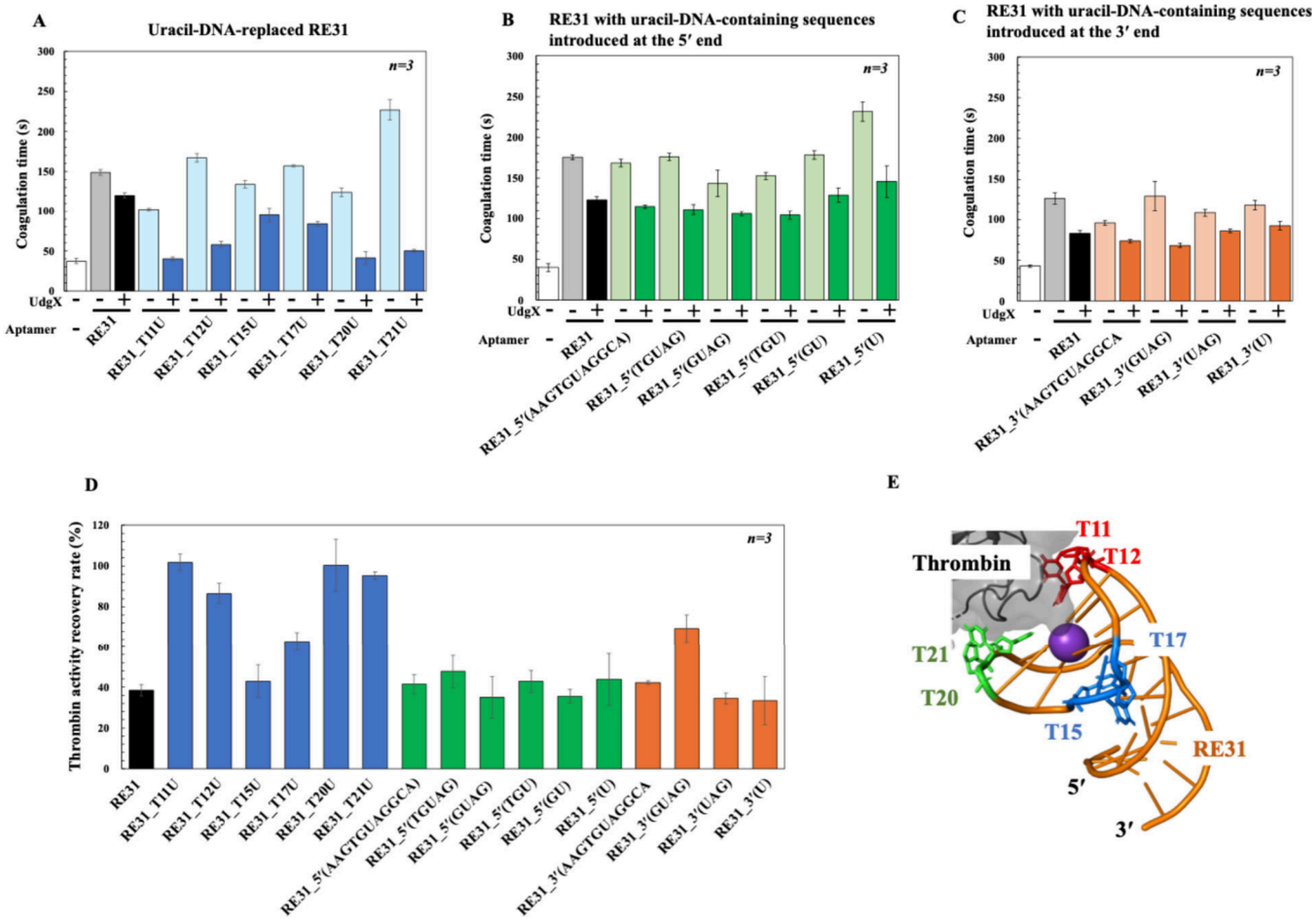

Figure 1. (A) Coagulation time without aptamer (white), or with RE31 (gray), RE31 after the addition of UdgX (black), uracil-DNA-replaced RE31 mutant (light blue), and uracil-DNA-replaced RE31 mutant (with UdgX) (deep blue). (B) Coagulation time without aptamer (white), or with RE31 (gray), RE31 after the addition of UdgX (black), RE31 with uracil-DNA-containing sequences introduced at the 5′ end (light green), and RE31 with uracil-DNA-containing sequences introduced at the 5′ end (with UdgX) (deep green). (C) Coagulation time without aptamer (white), or with RE31 (gray), RE31 after the addition of UdgX (black), RE31 with uracil-DNA-containing sequences introduced at the 3′ end (light orange), and RE31 with uracil-DNA-containing sequences introduced at the 3′ end (with UdgX) (deep orange). All the results are represented as mean ± SD (n = 3). (D) Thrombin activity recovery rate of RE31 (black), uracil-DNA-replaced RE31 (deep blue), uracil-DNA-containing sequences introduced at the 5′ end (deep green), and RE31 with uracil-DNA-containing sequences introduced at the 3′ end (deep orange). (E) A cocrystal structure of thrombin and RE31 (Protein Data Bank (PDB) ID: 5CMX).

the 5′ and 3′ termini of the G4-forming thrombin aptamer. Furthermore, as previously reported,[10,15] our results suggest that the presence of at least two additional nucleotides beside the uracil base was required at the 5′ or 3′ terminal for the efficient DNA binding of UdgX.

MEASUREMENT OF COAGULATION TIME USING THE RE31 MUTANTS

We investigated the effect of the RE31 mutants on coagulation activity by measuring fibrinogen coagulation time. Figures 1A–C show the results for the uracil-DNA-replaced RE31 mutant, RE31 with uracil-DNA containing sequences introduced at the 5′ end, and RE31 with uracil-DNA containing sequences introduced at the 3′ end, respectively. All RE31 mutants exhibited extended coagulation times, indicating that RE31 mutants with thymine replaced by uracil-DNA also possess thrombin inhibitory activity (Figure 1A–C, and Supporting Information Figure S3A–C for Time-course measurement of absorbance at 380 nm monitoring fibrinogen coagulation). Interestingly, certain RE31 mutants demonstrated higher inhibitory activity; in particular, RE31_T21U showed a 1.5-fold higher activity than RE31. The replacement of T13 of thrombin binding aptamer (TBA) (the original sequence of RE31, Supporting Information Table S1 for Oligonucleotide sequences for TBA) with a thymine analog, 5-(indolyl-3-acetyl-3-amino-1-propenyl)-2-deoxyuridine, reportedly decreases the binding affinity for thrombin,[17] suggesting the importance of T21 for RE31 binding. In addition, the replacement of uracil-DNA in antisevere acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleocapsid protein DNA aptamers to fit the binding pocket and enhance stereocomplementarity increased binding affinity.[18] Considering these factors, the increased steric complementarity between RE31_T21U and thrombin may also have contributed to the enhanced binding affinity, which, in turn, contributed to the enhanced thrombin inhibitory activity of RE31_T21U.

In contrast, RE31_T11U exhibited a 0.6-fold decrease in thrombin inhibitory activity. We hypothesized that substituting thymine with uracil, which lacks a methyl group, would alter its molecular structure. This structural modification may contribute to diminished binding affinity for thrombin. Furthermore, prior studies have demonstrated that replacing T11 with

https://doi.org/10.1021/acsnanoscienceau.5c00143
ACS Nanosci. Au 2026, 6, 201–207

unlocked nucleic acid−uracil (UNA−uracil) leads to a complete loss of thrombin-binding ability.[19] Collectively, these findings suggest that T11 plays an important role in thrombin binding. This highlights the importance of carefully selecting the position at which the uracil-DNA substitutions are introduced.

Most RE31 mutants with uracil-DNA-containing sequences introduced at the 5′ or 3′ end exhibited structural changes (see Supporting Information Figure S1 for CD spectra). Interestingly, among them, RE31_5′(U) alone exhibited high inhibitory activity, and the addition of a single uracil-DNA at the 5′ end induced favorable structural changes that enhanced thrombin binding.

TURNING ON THROMBIN FUNCTION BY REMOVING RE31 MUTANTS WITH UDGX

The turning on of thrombin activity by removal of the RE31 mutants from thrombin induced by UdgX was evaluated as a decrease in fibrinogen coagulation time. The addition of UdgX resulted in a shorter coagulation time in all RE31 mutants, indicating that thrombin activity was turned on by the covalent binding of UdgX to RE31 mutants (Figure 1A−C, S3A−C). T11U, T12U, T20U, and T21U mutants exhibited nearly 100% thrombin activity recovery (Figure 1D). This was likely attributed to the uracil-DNA substitutions being at the RE31−thrombin interaction interface, allowing UdgX to restore activity (Figure 1E).[15] In contrast, a variant with uracil in the second RE31 loop—a region not directly involved in thrombin interactions—demonstrated low functional recovery. These findings suggest that introducing uracil-DNA at the aptamer−target interface enables UdgX to effectively inhibit the aptamer.

A turning-on effect was not observed upon the addition of bovine serum albumin (BSA) to RE31 lacking uracil-DNA substitution. In contrast, the addition of UdgX to RE31 induced a turning-on effect (see Supporting Information Figure S4 for coagulation time of adding BSA and UdgX). One possible reason is that UdgX is a DNA glycosylase that can interact with DNA and result in the removal of RE31, although not to the same extent with the RE31 mutants, such as T11U, T12U, T20U, and T21U.

To investigate whether UdgX removes the aptamer from its target, we used AlphaAssay to assess the binding of RE31 and its mutant, RE31_T21U. In the absence of UdgX, both RE31 and RE31_T21U were able to bind thrombin (Figure 2; *KD* value: RE31, 0.61 nM; RE31_T21U, 0.50 nM). However, when UdgX was present, RE31 retained its ability to bind thrombin (*KD* value: 0.53 nM), while RE31_T21U did not produce any detectable binding signal (*KD* value: below detection limit). These results imply that adding UdgX activates thrombin by binding with the aptamer and removing it from the target. Notably, RE31_T21U showed a lower *KD* for thrombin than RE31, suggesting it has a greater affinity for thrombin. Additionally, RE31_T21U exhibited the strongest inhibitory effect on thrombin (Figure 1A). These findings suggest that the substitution of T21 with uracil altered its structural complementarity with thrombin, thereby affecting its activity.

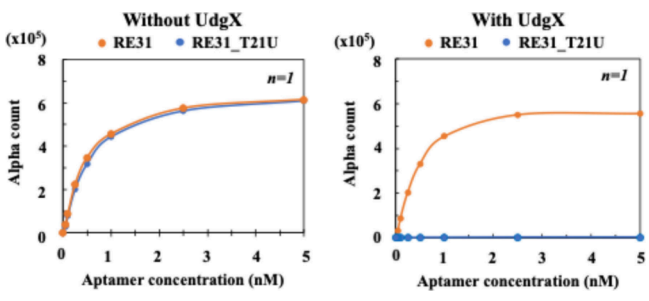

Figure 2. Alpha assay of RE31 (orange) and RE31_T21U (blue). Their binding was not inhibited in the absence of UdgX. Contrastingly, the addition of UdgX inhibited the binding of RE31_T21U.

Previously, we applied SpyCatcher-fused UdgX as a simple nucleic acid−protein linkage tool,[11] revealing that the fusion with SpyCatcher improved the expression of UdgX in *Escherichia coli* and its final yield after purification. Considering that truncated SpyCatcher (TrSC) is more structurally stable than SpyCatcher,[20] we fused it with UdgX in this study. Specifically, we used TrSC-fused UdgX as a turn-on inducer in thrombin protein complexes with DNA aptamers. This allowed us to produce a homogeneous protein with a high yield (29.2 mg/L culture). We also confirmed that untagged or unfused UdgX exhibited a similar ability to turn on thrombin (see Supporting Information Figure S5 for coagulation times with unfused UdgX, and SpyTag-fused UdgX). Furthermore, comparison of the turn-on activity of a construct in which UdgX was fused with a 13-amino-acid SpyTag (AHIVMVDAYKPTK) revealed no difference relative to unfused UdgX or UdgX. This suggests that UdgX's turn-on activity remains unchanged even after fusion, expanding its potential applications.

COMPARISON OF THE THROMBIN ACTIVITY RECOVERY RATES BETWEEN THE COMPLEMENTARY STRAND AND UDGX

Considering the therapeutic application of aptamers as anticoagulants, a key factor for an optimal drug candidate is the ability to rapidly restore thrombin activity by efficient aptamer removal. To date, complementary strands have been used to remove aptamers. Therefore, we compared the recovery rates of thrombin activity following aptamer removal via UdgX with those following removal of the complementary strand, using RE31_T21U as the aptamer model. The complementary strand (final concentration 100 nM) resulted in approximately 13% recovery after 1 min, increasing to 73% at 10 min postaddition. Notably, only approximately 56% removal was observed at 1 min, even at 1000 nM of the complementary strand. Conversely, UdgX (final concentration 100 nM) achieved nearly complete recovery—approximately 100%—within 1 min (Figure 3A). Wakui et al. sought to enhance the DNA aptamer removal rate by extending the DNA aptamer and complementary strand by 10 bases;[6] however, this modification resulted in an increase to 83%, which was lower than the rate achieved by UdgX in the current study. UdgX carries a positive charge at its DNA-binding site (see Supporting Information Figure S6 for Surface electrification of UdgX) and forms covalent bonds with DNA, which is believed to contribute to effective aptamer removal. Unlike the complementary strand, the positive charge facilitates interactions with DNA via electrostatic and covalent binding, thereby preventing dissociation. Overall, UdgX can turn on the activity inhibited by DNA aptamers more efficiently than complementary strands. Thus, UdgX is expected to rapidly and effectively inhibit such as anticoagulant aptamers.

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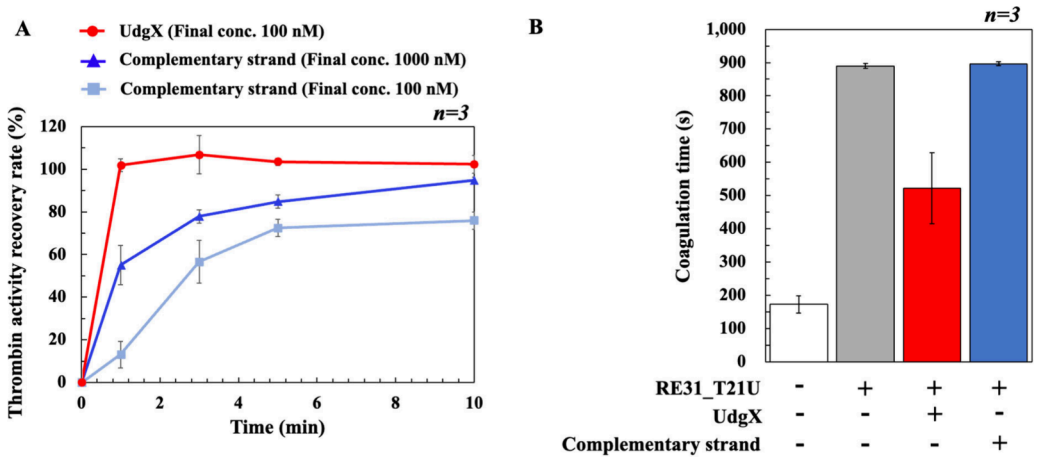

Figure 3. (A) Thrombin activity recovery rate of UdgX (Final concentration 100 nM: red), complementary strand (Final concentration 1000 nM: deep blue), and complementary strand (Final concentration 100 nM: light blue). All the results are represented as mean ± SD (n = 3). (B) Coagulation time in serum without aptamer (white), or with RE31_T21U (gray), RE31_T21U plus UdgX (red), and RE31_T21U plus complementary strand (blue). The reactions were performed in buffer supplemented with serum at a final concentration of 40%.

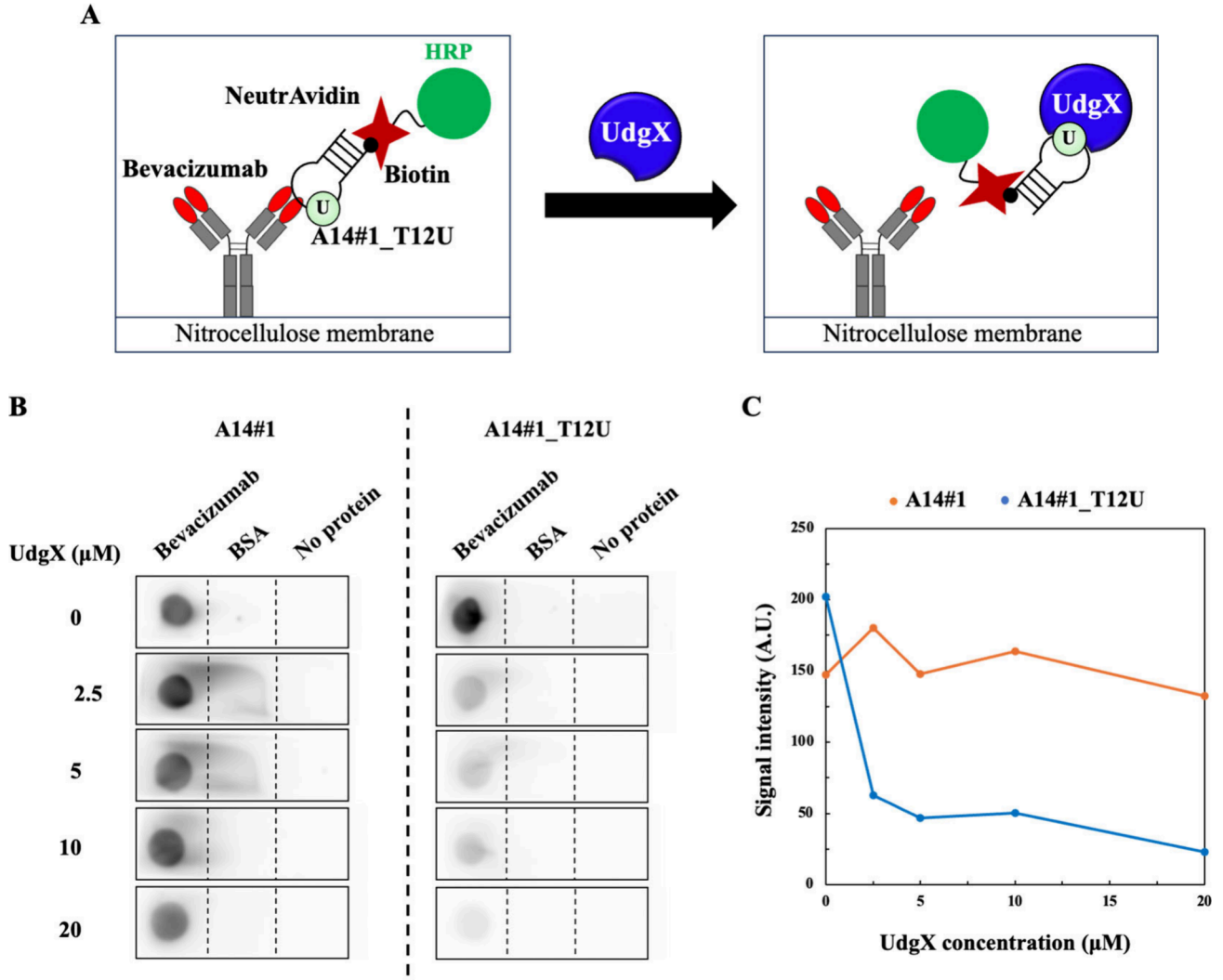

Figure 4. Determination of the aptamer removal effect of UdgX via aptamer blotting. BSA was used as the negative control and NeutrAvidin-HRP for detection. (A) Schematic diagram turn-on technology for Bevacizumab employing UdgX. (B) Determination of the aptamer removal effect of UdgX via aptamer blotting. BSA was used as the negative control, and NeutrAvidin-HRP for detection. (C) Signal intensities quantified at each UdgX concentration using ImageJ.

https://doi.org/10.1021/acsnanoscienceau.5c00143
ACS Nanosci. Au 2026, 6, 201−207

Next, to demonstrate the potential of UdgX in practical utility, fibrinogen coagulation reactions were performed in serum. In serum at a final concentration of 40%, adding RE31 T21U prolonged fibrinogen coagulation times (Figure 3B and Supporting Information Figure S7 for Effect of serum on fibrinogen coagulation reaction). Introducing UdgX reduced coagulation time, whereas the complementary strand had no effect—potentially due to DNase degradation. These results indicate that the turning on effects of UdgX are retained in serum.

EVALUATION OF THE EFFECT OF TURNING ON TARGET ACTIVITY USING A STEM−LOOP TYPE BEVACIZUMAB-BINDING APTAMER

To demonstrate how protein functions suppressed by DNA aptamers can be reactivated using a versatile process, another aptamer was analyzed. We previously developed a bevacizumab-binding aptamer, A14#1,[13] that possesses a hairpin structure. In contrast, the RE31 aptamer features a G-quadruplex (G4) structure—a typical conformation formed by guanine-rich nucleic acid sequences—and interacts with thrombin through this G4 configuration.[12] While A14#1 binds bevacizumab via its loop within the step−loop structure, both aptamers use distinct structures and modes for target binding.

To explore further, we substituted a thymine base near the binding site of A14#1 with a uracil, generating the variant A14#1_T12U (see Supporting Information Table S1 for Oligonucleotide sequences for A14#1, and A14#1_T12U), and compared its bevacizumab-binding ability using aptamer blotting. No differences were observed in binding strength between A14#1 and A14#1_T12U when UdgX was absent (Figure 4). However, in the presence of UdgX, chemiluminescence at the bevacizumab spot disappeared, indicating that UdgX inhibited A14#1_T12U binding. This inhibition was observed quantitatively at UdgX concentrations from 0 to 20 μM, but not with A14#1.

Typically, aptamer binding is disrupted by complementary strands designed to destabilize secondary structures, such as hairpins or G4 structures.[21,22] However, designing such complementary strands for each aptamer can be challenging. The findings of the current study indicate that UdgX can disrupt aptamer binding with hairpin or G4 structures by substituting thymine with uracil-DNA, without significantly affecting the aptamer's binding ability. In conclusion, UdgX effectively removes DNA aptamer binding across various structures, making it a valuable tool for reactivating proteins inhibited by DNA aptamers.

CONCLUSIONS

We have developed a rapid turn-on technology for restoring protein functions inhibited by DNA aptamers, using the DNA-binding protein, UdgX. When thrombin was inhibited by an antithrombin aptamer with a G-quadruplex structure, adding UdgX restored its function more quickly and effectively than simply introducing a complementary strand. Remarkably, UdgX retained efficacy in serum. The approach was also broadly applicable, as demonstrated with an anti-ideotype aptamer featuring a hairpin structure. While issues such as delivery, activation timing, and immunogenicity associated with UdgX require further investigation, this technology enables DNA aptamers to undergo reversible reactions, potentially expanding their utility in applications like oligonucleotide therapeutics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnanoscienceau.5c00143.

CD spectra of RE31 and RE31 mutants, SDS-PAGE of RE31 mutant and UdgX, Time-course measurement of absorbance at 380 nm monitoring fibrinogen coagulation, Coagulation time of only RE31 and RE31 with UdgX or BSA, Coagulation times with unfused UdgX or SpyTag-fused UdgX, Surface electrification of UdgX, Effect of serum on fibrinogen coagulation reaction, Table of oligonucleotide sequences for TBA, A14#1, and A14#1_T12U, Table of TrSC-fused UdgX protein sequence, and Material and method of this paper (PDF)

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https://doi.org/10.1021/acsnanoscienceau.5c00143
ACS Nanosci. Au 2026, 6, 201−207

https://pubs.acs.org/10.1021/acsnanoscienceau.5c00143

Author Contributions

Takeru Kanazu: Methodology, Investigation, Validation, Formal Analysis, Writing—original draft. Erika Komiya: Methodology, Investigation. Daimei Miura: Methodology, Validation, Supervision, Writing—review and editing. Kaori Tsukakoshi: Conceptualization, Methodology. Kazunori Ikebukuro: Supervision, Validation, Writing—review and editing. Tomohiko Yamazaki: Supervision, Conceptualization, Validation, Writing—review and editing, Funding Acquisition. Ryutaro Asano: Supervision, Validation, Writing—review and editing, Funding Acquisition. All the authors participated in the discussion and coordination of the manuscript submission. All the authors have read and approved the final version of this manuscript.

Funding

This study was supported by a Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science [JSPS; grant number 21K18321] and the National Institute for Materials Science Joint Research Hub Program.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Shinya Hattori, Ms. Tomoyo Umezawa, and Dr. Miwako Shobo [National Institute for Materials Science (NIMS)] for their excellent technical assistance. Part of this work was conducted at the NIMS Molecule and Material Synthesis Platform, supported by the Nanotechnology Platform Program of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan. We would like to thank Editage (www.editage.jp) for English language editing.

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