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# Expanding Targets of DNAzyme-Based Sensors through Deactivation and Activation of DNAzymes by Single Uracil Removal: Sensitive Fluorescent Assay of Uracil-DNA Glycosylase

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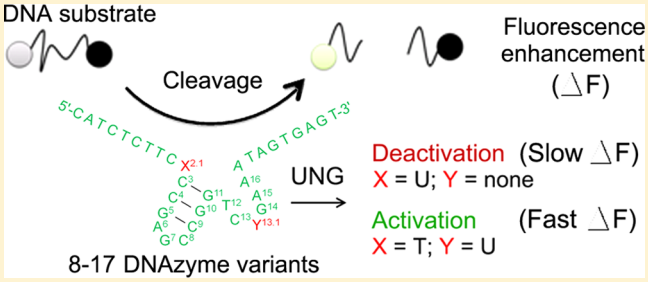
**ABSTRACT:** Although deoxyribozymes (DNAzymes) have been widely used as biosensors for the detection of their cofactors and the targets of related aptazymes, it is desirable to expand their range of analytes to take advantage of the DNAzyme-based signal amplification for more sensitive detections. In this study, the activity of uracil-DNA glycosylase (UNG) was successfully detected and quantified by deoxyuridine-modified DNAzymes that underwent UNG-dependent deactivation or activation. In one design, the indispensable thymidine T2.1 in the 8−17 DNAzyme was replaced with a deoxyuridine, resulting in minimal change of the DNAzyme's activity. Since UNG is capable of removing uracils from single- or double-stranded DNAs, the modified DNAzyme was deactivated when the uracil at the indispensable thymidine site was eliminated by UNG. In another design, introducing a deoxyuridine to the 3′ position of the deoxycytidine C13 in the catalytic core of the same DNAzyme caused significant decrease of the activity. The removal of the interfering deoxyuridine by UNG, however, activated the DNAzyme. By monitoring the activity change of the DNAzymes through the fluorescence enhancement from the DNAzyme-catalyzed cleavage of DNA substrates labeled by a fluorophore and quencher pair, the UNG activity was measured based on UNG-dependent deactivation and activation of the DNAzymes. This method was found to be able to detect UNG activity as low as 0.0034 U/mL. Such a method can be applied to the detection of other nucleotide-modifying enzymes and expand the analyte range of DNAzyme-based biosensors.




Since the first discovery of a DNAzyme (also called deoxyribozyme) through in vitro selection in the 1990s, DNA is no longer considered as only a genetic material; it is now recognized as also being capable of catalyzing many reactions.[1−6] Due to their ease of synthesis, versatility in modification, and high stability, DNAzymes have been widely applied in biological chemistry,[7,8] nanotechnology,[9−13] and analytical chemistry.[14−19] For example, numerous DNAzyme-based biosensors have been developed to detect their cofactors, including Pb(II),[10,19−23] $UO_2$(II),[23−26] Cu(II),[27,28] Hg(II),[29,30] and histidine.[31,32] In addition to these cofactors, other targets have also been successfully quantified by integrating their recognition modules such as DNA[33,34] and aptamers[35−37] with DNAzymes. Despite these successes, few works have been reported to take full advantage of the DNAzyme-based signal amplification to monitor a broader range of analytes, such as the activities of protein enzymes,[38] as a basis for developing sensitive sensors for more analytes; this situation is mainly due to the lack of a link between the target enzymes and the DNAzyme-catalyzed reactions.

In this work, we report a new method for the sensitive detection of uracil-DNA glycosylase (UNG) activities based on enzyme-induced deactivation and activation of DNAzymes, to achieve signal amplification by a single uracil removal from the catalytic core of the DNAzymes. UNG is a class of enzymes responsible for removing undesired uracil bases from DNA and yields an apyrimidinic site (abasic site) in the DNA for the subsequent DNA repair mechanisms involved in the base excision repair (BER).[39,40] Because of the importance of UNG in gene regulation (BER) and related diseases such as Bloom syndrome and chemotherapy resistance,[41,42] sensitive assays for UNG are required for the study of the mechanism and function of the enzyme. These assays will also facilitate the screening of UNG inhibitors as potential drugs and biochemical tools.[43,44] Classic methods for UNG assays require complicated nucleic acid labeling and gel electrophoresis procedures.[45−47] Alternatively, fluorescent sensors based on fluorophore-labeled oligonucleotides with uracil modifications are more simple and suitable for fast assays of UNG activities.[43,48−51] For example, Stivers[43] and the Baldwin group[48] utilized DNA duplexes containing a fluorescent 2-aminopurine deoxyriboside opposite to a deoxyuridine for rapid reaction analysis of UNG activity on the basis of the fluorescence enhancement of 2-aminopurine when the deoxyuridine was removed by UNG. Wang et al.[49] and Liu et al.[50] introduced multiple deoxyuridines in DNA molecular beacons for real-time monitoring of uracil

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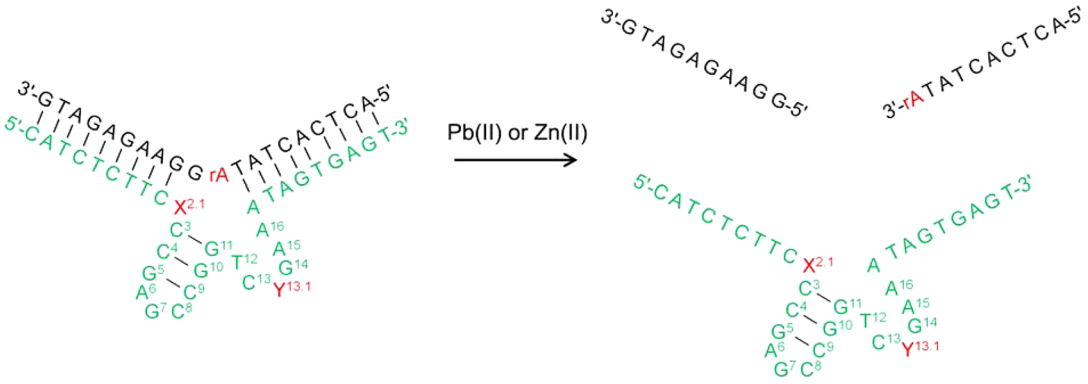


**Figure 1.** Cleavage reactions of the substrate DNA (black) containing an internal ribonucleotide (rA) catalyzed by the 8−17 (*X* = T, *Y* = none), E-U2.1 (*X* = U, *Y* = none), and E-C13 (*X* = T, *Y* = U) DNAzymes (green). Here "U" indicates a deoxyuridine.

removal by UNG through the UNG-induced unfolding of the molecular beacons. Kool's group[51] developed a new UNG fluorescent assay based on novel small-sized oligonucleotides containing pyrene deoxyribosides, whose fluorescence was quenched by neighboring deoxyuridines. Such a system was used for UNG detection in vitro with fluorescence enhancement up to 90-fold and for efficient cellular delivery of the oligonucleotides to realize in vivo imaging of UNG activities.[51] Built upon these successes, it is desirable to introduce signal amplification mechanisms into fluorescent assays of UNG for more sensitive detections.[38] However, due to the lack of a link between the enzyme activity and amplification reactions, it has been difficult to achieve this goal. To overcome the challenge, we herein demonstrate the construction of an enzyme cascade that could be initiated by UNG-dependent deactivation or activation of the DNAzymes through the modification of their catalytic cores with deoxyuridines. By signal amplification of the DNAzyme-catalyzed reaction, sensitive assays of UNG activity based on fluorescence enhancement have been successfully achieved.

## RESULTS AND DISCUSSION

**General Design of the UNG-Induced Deactivation and Activation of DNAzymes for UNG Activity Assays.** In this study, the 8−17 DNAzyme (Figure 1)[19,52−55] was chosen for uracil modification because the DNAzyme was well characterized in previous studies, including comprehensive mutation studies to identify the conserved sequence within its catalytic core.[52,53,56] To design UNG-dependent DNAzymes, one deoxyuridine was introduced into each variant of the 8−17 DNAzyme, either by replacing the indispensable thymidine T2.1 with a deoxyuridine (the corresponding DNAzyme is named as E-U2.1, Figure 1)[53] or by inserting a deoxyuridine to the 3′ position of a deoxyribonucleotide in the catalytic core (such as $C^{13}$, the corresponding DNAzyme is called E-C13, Figure 1). Since UNG is capable of removing uracils from single- or double-stranded DNAs, we hypothesized that the activity of the modified DNAzymes could be deactivated when the uracils at the indispensable thymidine sites were removed by UNG (such as E-U2.1 in Figure 2a). In contrast, the DNAzymes could also be activated when the disrupting uracils inserted in the catalytic core of the DNAzymes were eliminated by UNG (such as E-C13 in Figure 2b).

To transduce the above UNG-dependent DNAzyme activities into detectable signals, we employed the catalytic beacon approach by attaching a fluorophore at the 5′ end and a

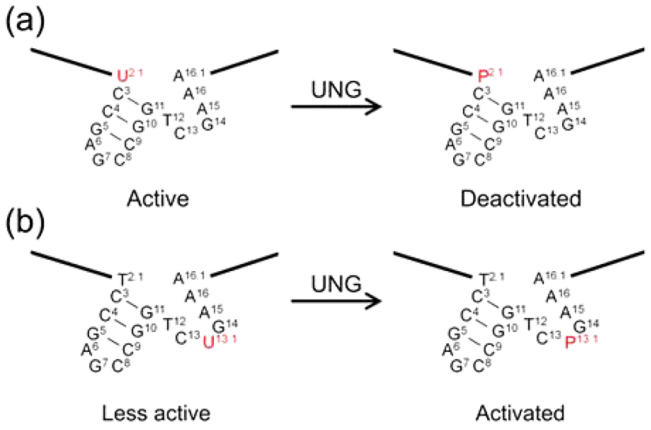


**Figure 2.** UNG-induced (a) deactivation of E-U2.1 and (b) activation of E-C13 through a single uracil removal by UNG. Here "U" and "P" indicate a deoxyuridine and an apyrimidinic site, respectively.

quencher at the 3′ end of the DNA substrate (Figure 1).[32,57] As shown in Figure 3, in the absence the DNAzyme, the fluorescence signal of the fluorophore is quenched by the quencher due to the close proximity between the two in the unstructured single-stranded substrate. Upon binding to the

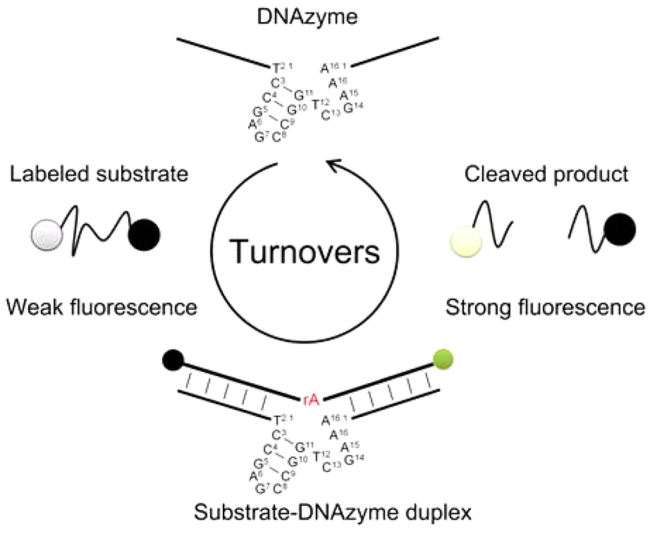


**Figure 3.** DNAzyme-catalzyed cleavage of dual-labeled substrates with multiple turnovers for signal amplification.


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**Table 1. MALD-TOF MS Peaks ($m/z$) of 8−17, E-U12, and E-C13 DNAzymes before and after Treatment by 1 U/mL UNG for 1 h**

| sample | 8−17 | 8−17+UNG | E-U2.1 | E-U2.1+UNG | E-C13 | E-C13+UNG |
|---|---|---|---|---|---|---|
| measured | 10097.7 | 10103.2 | 10085.2 | 9993.3 | 10389.8 | 10301.6 |
| calculated | 10104.6 | 10104.6 | 10090.6 | 9996.5 | 10394.8 | 10300.7 |

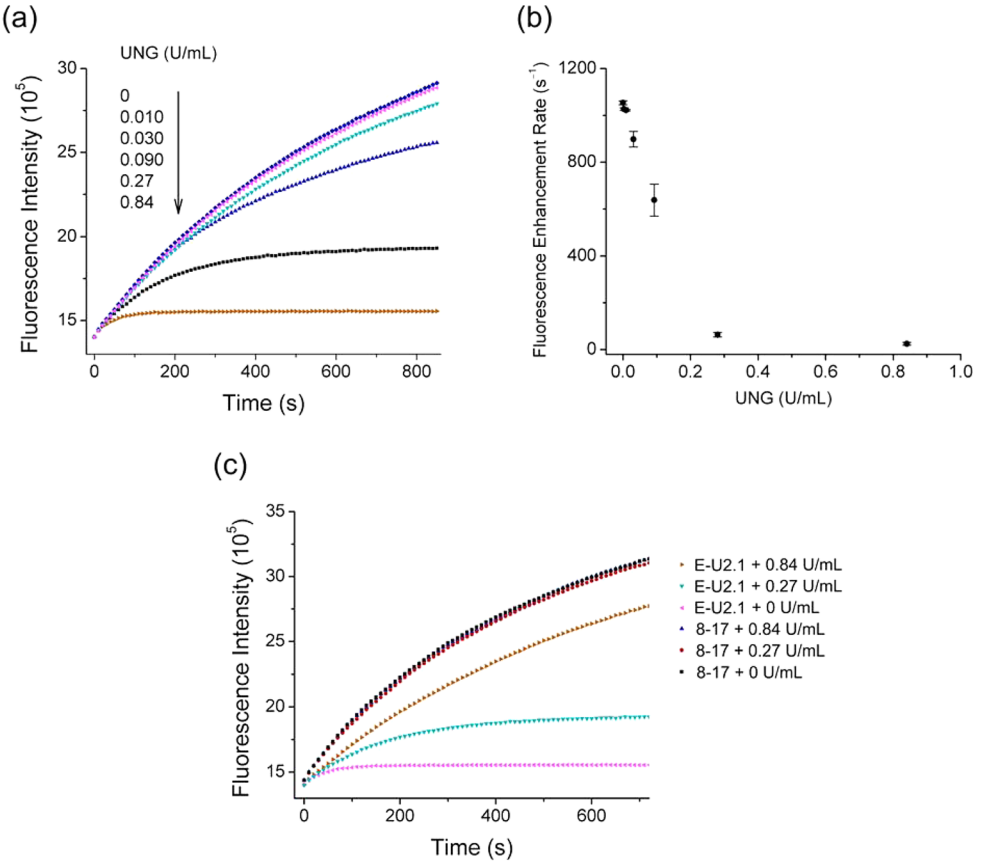


**Figure 4.** (a) Kinetics of fluorescence enhancement caused by DNAzyme E-U2.1-catalyzed cleavage of the dual-labeled substrate A in the presence of different amounts of UNG. (b) Dependence of UNG activity and fluorescence enhancement rate at 800 s for the E-U2.1-catalyzed cleavage of the dual-labeled substrate A. (c) Kinetics of fluorescence enhancement caused by the 8−17 DNAzyme- or E-U2.1-catalyzed cleavage of the dual-labeled substrate A in the absence and presence of UNG.

DNAzyme, the substrate is cleaved into two pieces of products by the DNAzyme-catalyzed multiple-turnover reaction, significantly increasing the distance between the fluorophore and the quencher. As a result, the quenched fluorescence is recovered so that the fluorescence signal is increased. By continuously measuring the rate of fluorescence enhancement, the activity of the DNAzyme is monitored in real time, and its change over time can be further used to quantify the activity of UNG. In this approach, we have introduced only one uracil modification to each DNAzyme (Figure 1 and Table 2). Introducing multiple uracils to each DNAzyme is possible, but it can complicate the kinetics of the DNAzyme's activity due to the fact that UNG needs to remove multiple uracils before the DNAzyme can be fully activated or deactivated.

**UNG Assay Based on UNG-Induced Deactivation of DNAzymes.** Previous mutation studies have revealed the essential roles of T2.1 for the activity of the 8−17 DNAzyme in catalyzing the cleavage of its substrates at the rA linkage.[53,56] For example, Brown et al. found that the DNAzyme completely lost its activity when the T2.1 was mutated to C2.1,[53] while Wang et al. also observed a loss of activity when the T2.1 was replaced by either a dSpacer or a C3 Spacer.[56] On the basis of these findings and the fact that uracils exhibit similar functions as thymines in DNA, we substituted T2.1 in the catalytic core of the 8−17 DNAzyme by a deoxyuridine and predicted that the variant (named as E-U2.1) could be deactivated by UNG after the removal of the essential U2.1 (Figure 2a). In this case, UNG activity would be proportional to the amount of U2.1 eliminated from the E-U2.1 DNAzyme, and this relationship could be quantitatively measured by monitoring the decrease of fluorescence enhancement rate caused by the activity loss of E-U2.1.

To investigate whether the uracil in E-U2.1 could be removed by UNG, a solution of E-U2.1 (42 nM) was treated with UNG (1 U/mL) in the buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5 mM EDTA, 1 mM $ZnCl_2$) for 1 h, after which salts and nucleotides were separated from the DNA using Amicon centrifugal filters and the DNA was characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). As shown in Table 1, the mass peaks ($m/z$) of E-U2.1 before and after UNG treatment are 10085.2 and 9993.3, corresponding well with the


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calculated value of 10090.6 (E-U2.1) and 9996.5 (E-U2.1 with loss of one uracil (−94.1)), respectively, indicating the successful elimination of U2.1 in E-U2.1 by UNG. In contrast, the mass peaks of the unmodified 8−17 DNAzyme before and after UNG treatment (10097.7 and 10103.2, respectively) were close to each other, within the experimental error, and similar to the calculated value of 10104.6, suggesting that the T2.1 in the unmodified 8−17 DNAzyme did not undergo any change in the presence of UNG under the same condition. These results confirmed the fact that UNG could remove uracils but not thymines from the DNA.[39,40]

To quantify the activity of UNG in solution, UNG (0−0.84 U/mL), the uracil-modified E-U2.1 DNAzyme (42 nM), the dual-labeled substrate A (500 nM), and the DNAzyme cofactor $Zn^{2+}$ (1 mM) were mixed in the reaction buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5 mM EDTA) and the fluorescence was monitored continuously. As illustrated in Figure 4a, the initial rate of fluorescence enhancement was high because E-U2.1 was fully active before being processed by UNG. In the presence of UNG, however, the rate of fluorescence enhancement underwent gradual decrease over time until almost reaching a constant intensity, suggesting that E-U2.1 was deactivated due to the loss of the uracil.

By measuring the rate of fluorescence enhancement at 800 s (the average rate between 750−850 s was used to minimize deviations), the activity of UNG in the samples could be successfully quantified based on the calibration curve shown in Figure 4b. The detection limit, according to the definition of $3\delta_b$/slope, was found to be 0.0034 U/mL, which is lower than other published fluorescent assays of UNG activity.[43,48−51] The high sensitivity of our method is most probably ascribed to (1) the signal amplification via DNAzyme-catalyzed multiple-turnover reactions and (2) the measurement of the change in fluorescence enhancement rate, which is more resistant to background fluctuations than intensity measurement.

To confirm that UNG activity was responsible for the decrease of fluorescence enhancement rate caused by the loss of the DNAzyme activity, the unmodified 8−17 DNAzyme was used instead of E-U2.1 to carry out the fluorescence assays under the same conditions. As depicted in Figure 4c, different from the results for E-U2.1, the rate of fluorescence enhancement was independent of UNG activity since the DNAzyme without any uracil modification underwent no change in the presence of UNG. The results suggested that UNG-induced removal of uracils and deactivation of the E-U2.1 DNAzyme were essential for the observed decrease of fluorescence enhancement rate.

**UNG Assay Based On UNG-Induced Activation of DNAzymes.** In the above approach, the quantification of UNG activity was based on the deactivation of E-U2.1 by UNG. Compared to deactivation, UNG-induced activation of DNAzymes is more interesting because the latter is less vulnerable to deactivation due to the presence of interference and because it can be more efficiently monitored for higher sensitivity. Unlike the UNG-induced deactivation of the E-U2.1 DNAzyme by eliminating the uracil from the essential U2.1, however, no previous study has provided any information or example of enhancing the 8−17 DNAzyme's activity through the removal of a thymine from its catalytic core, making the rational design challenging. Therefore, we carried out a screening experiment by inserting a deoxyuridine to the 3′ side of each nucleotide in the catalytic core of the 8−17 DNAzyme to search for the variant that could display activity enhancement after the uracil was removed by UNG (see Table 2 for the sequences of the variants of 8−17 DNAzyme).

**Table 2. Oligonucleotides Used in This Study**

| name | DNA sequence[a] |
|---|---|
| substrate A | 5′-Alex488-ACTCACTATrAGGAAGAGATG-Dabcyl-3′ |
| substrate B | 5′-Iowa Black-ACTCACTATrAGGAAGAGATG-Fluorescein-3′ |
| 8−17 DNAzyme | 5′-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3′ |
| E-U2.1 | 5′-CAT CTC TTC UCC GAG CCG GTC GAA ATA GTG AGT-3′ |
| E-U12 | 5′-CAT CTC TTC TCC GAG CCG GUC GAA ATA GTG AGT-3′ |
| E-T2.1 | 5′-CAT CTC TTC TUCC GAG CCG GTC GAA ATA GTG AGT-3′ |
| E-C3: | 5′-CAT CTC TTC TCUC GAG CCG GTC GAA ATA GTG AGT-3′ |
| E-C4: | 5′-CAT CTC TTC TCCU GAG CCG GTC GAA ATA GTG AGT-3′ |
| E-G5 | 5′-CAT CTC TTC TCC GUAG CCGT GTC GAA ATA GTG AGT-3′ |
| E-A6 | 5′-CAT CTC TTC TCC GAUG CCG GTC GAA ATA GTG AGT-3′ |
| E-G7 | 5′-CAT CTC TTC TCC GAGU CCG GTC GAA ATA GTG AGT-3′ |
| E-C8 | 5′-CAT CTC TTC TCC GAG CUCG GTC GAA ATA GTG AGT-3′ |
| E-C9 | 5′-CAT CTC TTC TCC GAG CCUG GTC GAA ATA GTG AGT-3′ |
| E-G10 | 5′-CAT CTC TTC TCC GAG CCGU GTC GAA ATA GTG AGT-3′ |
| E-G11 | 5′-CAT CTC TTC TCC GAG CCG GUTC GAA ATA GTG AGT-3′ |
| E-T12 | 5′-CAT CTC TTC TCC GAG CCG GTUC GAA ATA GTG AGT-3′ |
| E-C13 | 5′-CAT CTC TTC TCC GAG CCG GTCU GAA ATA GTG AGT-3′ |
| E-G14 | 5′-CAT CTC TTC TCC GAG CCG GTC GUAA ATA GTG AGT-3′ |
| E-A15 | 5′-CAT CTC TTC TCC GAG CCG GTC GAUA ATA GTG AGT-3′ |
| E-A16 | 5′-CAT CTC TTC TCC GAG CCG GTC GAAU ATA GTG AGT-3′ |

[a]The underlined "rA" and "U" indicate an adenosine and a deoxyuridine, respectively.

As demonstrated in Figure 5a, the original 8−17 DNAzyme showed little activity change upon the addition of UNG because there was no uracil in the DNAzyme. In contrast, E-U2.1 and E-U12, with the essential thymines replaced by uracils, underwent significant activity loss after UNG-catalyzed removal of the uracils. For other variants of the DNAzyme with a deoxyuridine inserted at the 3′ position of each nucleotide in the catalytic core, it is very interesting to find that the cases of inhibition (E-T2.1, E-G7, E-G14), enhancement (E-C3, E-C9, E-G10, E-T12, E-C13, E-A15, E-A16), and little change (E-C4, E-G5, E-A6, E-C8, E-G11) of activity were all observed after UNG treatment. In addition to the use of dual-labeled substrate B (5′-Iowa Black and 3′-Fluorescein, Table 2) in Figure 5a, another dual-labeled substrate A (5′-Alex488 and 3′-Dabcyl, Table 2) was also used to test the selected DNAzymes with activity enhancement after UNG treatment (Figure 5b) to demonstrate the effect of the fluorophore/quencher pair's position. Among the candidates, E-C13 and E-A16 showed the largest fold of activity enhancement when used with substrate A. Compared with E-C13, the insert of deoxyuridine in E-A16


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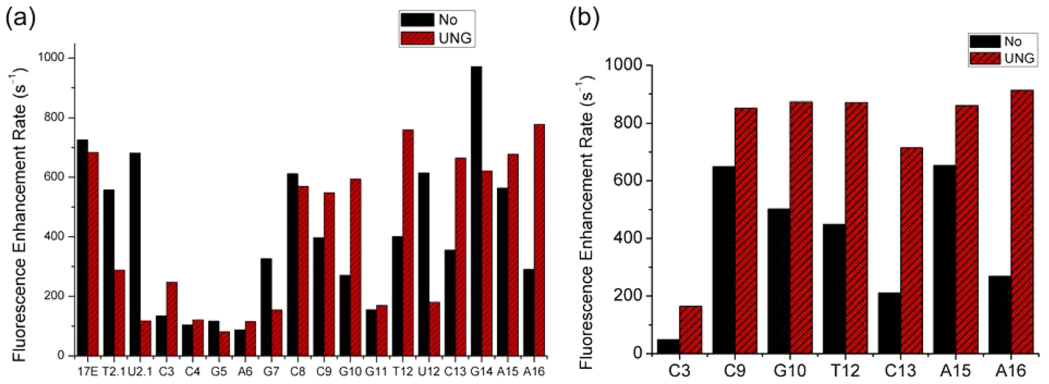


**Figure 5.** Fluorescence enhancement rate at 450 s for the DNAzyme-catalyzed cleavage of (a) dual-labeled substrate B (5′-Iowa Black and 3′-Fluorescein) and (b) dual-labeled substrate A (5′-Alex488 and 3′-Dabcyl) in the absence and presence of 1 U/mL UNG for screening the variants of the 8−17 DNAzyme that are activated by UNG.

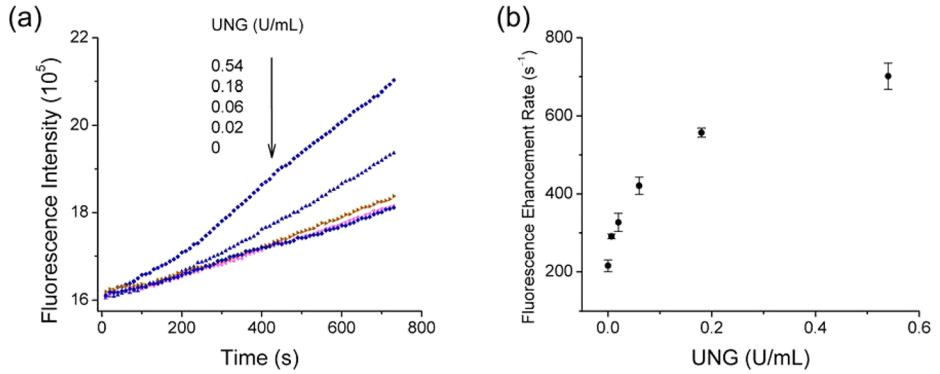


**Figure 6.** (a) Kinetics of fluorescence enhancement caused by the E-C13-catalyzed cleavage of the dual-labeled substrate A in the presence of different amounts of UNG. (b) Dependence of UNG activity and fluorescence enhancement rate at 650 s for the E-C13-catalzyed cleavage of the dual-labeled substrate A.

was at the edge of the catalytic core, and it was very possible that the inserted "U" deactivated the DNAzyme by forming a base pair with the rA in substrate A. This substrate dependence of deactivation may not be generally applicable for other substrates when the rA is replaced by other nucleotides to perturb the U-rA base pair. Therefore, we chose the E-C13 variant (Figure 2b) to carry out similar fluorescent assays for UNG under the same conditions as those for E-U2.1 in the previous sections (Figure 4). The MALDI-TOF MS also indicated that the uracil in E-C13 could be efficiently removed by UNG (Table 1).

According to the results of continuous fluorescence measurement in Figure 6a, E-C13 exhibited a lower activity compared to E-U2.1 or unmodified 8−17 DNAzyme, most probably due to the activity being perturbed by the inserted "disrupting" deoxyuridine. After removal of the uracil from E-C13 by UNG, the perturbation was reduced and the activity of the DNAzyme was enhanced, as shown in the increase of the slope of the curves for samples containing more UNG in Figure 6a.

The rate of fluorescence enhancement at 650 s (the average rate between 600−700s) was calculated for each sample to estimate the UNG activity from 0−0.54 U/mL (Figure 6b). The detection limit ($3\delta_b$/slope) was found to be 0.0051 U/mL. The detection limit of this method is lower than that based on the UNG-induced deactivation of E-U2.1 (Figure 4), because the enhancement factor (<3 fold) is still moderate compared to the inhibition factor (>95%). The sensitivity can be significantly improved if DNAzymes with larger enhancement factors are found.

## ■ CONCLUSION

By introducing a single deoxyuridine modification to the 8−17 DNAzyme and taking advantage of the DNAzyme-catalyzed signal amplification, new highly sensitive fluorescent assays for UNG activities have been developed. After removal of uracils by UNG, the DNAzyme variants were either deactivated or activated depending on the location of the deoxyuridine modifications. Subsequently, the DNAzyme catalyzed the cleavage of a DNA substrate with a ribonucleotide (rA) in the middle and a fluorophore/quencher pair labeled at the 5′ and 3′ ends, respectively, resulting in fluorescence enhancement. The rate of fluorescence enhancement was dependent on the activity of the DNAzymes. This change over time, which was related to the UNG activies of the samples, could be used for UNG assays. A detection limit as low as 0.0034 U/mL UNG has been achieved based on the method. Such a method has expanded the capability of DNAzymes as biosensors for detecting a broader range of analytes.

## ■ EXPERIMENTAL SECTION

**Materials.** Amicon centrifugal filters (10K molecular weight cutoff) were purchased from Millipore Inc. (Billerica, MA). Uracil DNA glycosylase from *Escherichia coli* and other chemicals for buffers and solvents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). MALDI-TOF MS spectra


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were obtained from an Applied Biosystems Voyager DE PRO (Life Technologies Corp., Grand Island, NY) in the Mass Spectrometry Laboratory at UIUC. The oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA), and their sequences are shown in Table 2.

**Typical Procedures for the Fluorescent UNG Assay Using E-U2.1 and E-C13 DNAzymes.** To an 800 $\mu$L solution containing 0−0.84 U/mL UNG in the reaction buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl 0.5 mM EDTA), 4 $\mu$L 100 $\mu$M dual-labeled substrate A, 3.4 $\mu$L 10 $\mu$M DNAzyme, and 10 $\mu$L 80 mM $ZnCl_2$ stock solution were sequentially added. After being vortexed, 750 $\mu$L of the mixture solution was transferred to a cuvette and measured continuously in a fluorimeter at ex/em = 490/510 nm.

**Typical Procedures for the Screening "Deoxyuridine-Inserted" E-T2.1∼E-A16 DNAzymes.** To an 800 $\mu$L solution containing 0 or 1 U/mL UNG in the reaction buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl 0.5 mM EDTA), 4 $\mu$L 100 $\mu$M dual-labeled substrate B or A, 3.4 $\mu$L 10 $\mu$M DNAzyme, and 10 $\mu$L 80 mM $ZnCl_2$ stock solution were sequentially added. After being vortexed, 750 $\mu$L of the mixture solution was transferred to a cuvette and measured continuously in a fluorimeter at ex/em = 490/510 nm. The rate of fluorescence enhancement at 450 s (the average rate between 400−500s) was used for the calculation of UNG activity in each sample.

**MALD-TOF MS Analysis.** To an 800 $\mu$L solution containing 0 or 1 U/mL UNG in the reaction buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl 0.5 mM EDTA), 8 $\mu$L 100 $\mu$M DNAzyme (8−17, E-U2.1 or E-C13) and 10 $\mu$L 80 mM $ZnCl_2$ stock solution were sequentially added. After 1 h of reaction, the DNA was desalted by centrifuging 8 times, at 10 000 rpm for 10 min each time with water as the solvent, in an Amicon-10K. The resultant solution was concentrated to 20 $\mu$L and sent for MALD-TOF MS analysis.

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### Notes

The authors declare no competing financial interest.

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