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A Novel Label-Free Biosensor Using an Aptazyme–Suppressor-tRNA Conjugate and an Amber Mutated Reporter Gene

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DNA or RNA aptamers have great potential as biosensors for detecting target molecules owing to their high and specific target-binding ability.[1] Aptamer–target complex formation can be analyzed by mass-sensitive detection techniques, such as surface plasmon resonance (SPR) or quartz crystal microbalance (QCM), or by fluorescent, electrochemical, colorimetric, or turbidimetric methods by using appropriately labeled aptamers or probes.[2] Aptazymes, which are composed of an aptamer and a ribozyme, can convert the signal of the aptamer–target complex formation to that of ribozyme activity, so that they have also garnered attention as aptamer-based sensors.[3] Nevertheless, detection of these ribozyme signals also requires special detectors and/or labeling of ribozymes, much as in the case of the aptamer sensors described above. On the other hand, we have recently developed an aptazyme-based riboswitch as a label-free and detector-free biosensor.[4] In this riboswitch sensor, the aptamer–target complex formation signal is converted to an easily detectable signal—that is, expression of a downstream reporter protein—with the help of a prokaryotic cell-free translation system. As the expression signal of the reporter protein can be detected easily, in some cases visibly, labeling of aptazymes or special detectors is not required. Moreover, the benefits of using the translation system lie not only in the label- and detector-free sensing, but also to its applicability to regulating gene expression in vivo.[5] We report herein a new type of label-free and aptazyme-based biosensor that uses a translation system in combination with a nonsense codon suppression method, and is distinct from the riboswitch biosensor developed previously.[4]

The nonsense codon suppression method is a method by which a natural or unnatural amino acid is assigned to the nonsense codon (amber, opal, or ochre codon) on the mRNA.[6] The ribosome usually stops at the nonsense codon, and the synthesized protein is removed from the mRNA by the action of release factors (RFs). In a prokaryotic translation system, there are three RFs and two of these directly recognize the nonsense codons; release factor 1 (RF1) recognizes the amber and ochre codons and release factor 2 (RF2) recognizes the opal and ochre codons. However, in the presence of an anticodon-adjusted suppressor tRNA, which is aminoacylated, the suppressor tRNA competes with the RFs for being incorporated into the ribosome on the nonsense codon of the mRNA. If the suppressor tRNA wins this competition, translation continues (i.e., the nonsense codon is suppressed) but if the RFs win, translation stops. In the absence of RFs or in the presence of inhibitors for RFs, the suppression efficiency becomes much higher.[7] Recently, Ogawa and co-workers used this nonsense suppression method with the three kinds of suppressor tRNA that correspond to each of the three nonsense codons in a reconstituted prokaryotic cell-free translation system in the absence of RFs;[7a] this enabled the ribosome to read through all nonsense codons.[8] These suppressor tRNAs were derivatives of *E. coli* $tRNA^{SerU}$, which were recognized by Ser-tRNA synthetase (SerRS) and rapidly charged with Ser, even if their anticodon was changed into another anticodon and no modified base was used.[9] In this study, we tethered a theophylline-dependent aptazyme[10] to the 5′ terminus of the anticodon-adjusted suppressor $tRNA^{SerU}_{CUA}$ for an amber codon,[11] and combined the aptazyme–suppressor-tRNA conjugate (AST) with an amber-mutated reporter gene (luciferase) in the translation system to construct a novel aptazyme-based biosensor.

The basic concept of the aptazyme-based biosensor in this study is illustrated in Figure 1. This biosensor system consists of an RF1-free reconstituted prokaryotic cell-free translation system[7a] and two RNA molecules: an AST (Figure 1, upper left) and an amber-mutated reporter gene (Figure 1, lower left). In the absence of the target of the aptazyme (Figure 1, left; OFF state) it is expected that AST will not be aminoacylated because aminoacyl tRNA synthetase (ARS) is sensitive to the structure of an accepter stem of tRNA.[12,13] Under this condition, that is, in the absence of an "activated" suppressor tRNA, the ribosome stalls at the amber codon on the reporter gene because of the absence of RF1.[14] On the other hand, when the target binds to the aptazyme of AST (Figure 1, right; ON state) it induces self-cleavage of the aptazyme; this produces the "activated" suppressor tRNA, which can be recognized by the corresponding ARS and aminoacylated with the corresponding amino acid. This aminoacylated suppressor tRNA is incorporated into the stalling ribosome at the amber codon; this causes the ribosome to continue with the synthesis of the reporter protein until it reaches the "true" terminal codon (ochre codon: UAA), which is recognized by RF2. Therefore, the expression of a full-length active reporter protein is an indicator of the existence of the target molecule.

To first investigate whether or not a suppressor tRNA with an extra sequence at its 5′ terminus is recognized and aminoacylated by ARS, we prepared four DNA templates for two different kinds of suppressor tRNAs and two different kinds of mRNAs that coded luciferase genes (Figure 2A and 2B). One of the suppressor tRNAs is a normal suppressor $tRNA^{SerU}_{CUA}$ (supT) and the other (5SL-supT) is identical to supT except that it has an extra stem–loop sequence at its 5′ terminus. The two luci-

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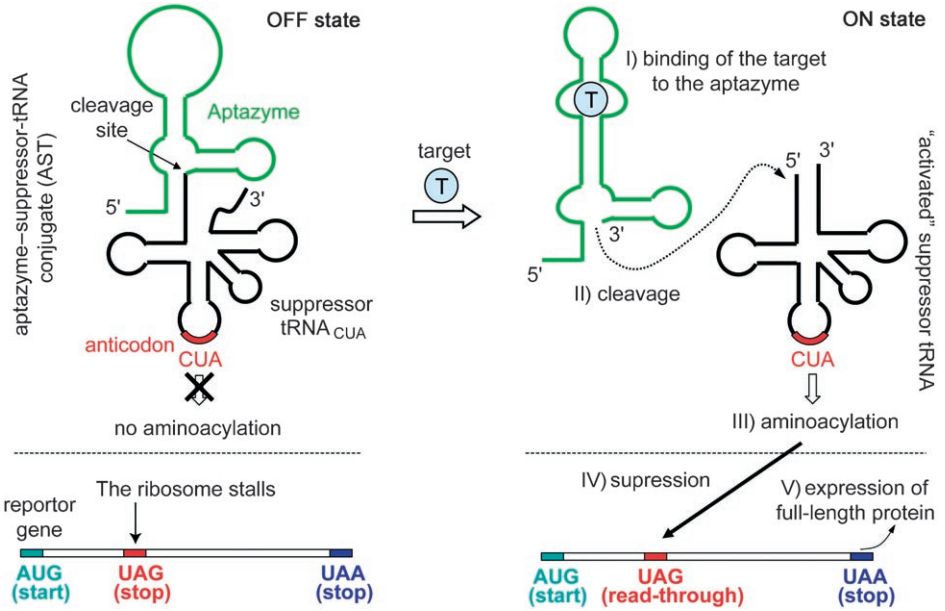

Figure 1. Illustration of the mechanism of the aptazyme-based biosensor. This biosensor is composed of two RNA molecules: an aptazyme–suppressor-tRNA conjugate (AST; upper left) and an amber-mutated reporter gene (lower left). The translation system used is a reconstituted prokaryotic cell-free translation system without RF1.[7a] In the absence of the target molecule (the cofactor of the aptazyme), the suppressor tRNA is not aminoacylated with any amino acids because of the aptazyme sequence at the 5′ terminus; so, the ribosome stalls at the amber codon (OFF state; left). When the target (T) binds to the aptazyme it induces self-cleavage of the aptazyme to release the aptazyme from the suppressor tRNA. The resulting "activated" suppressor tRNA is aminoacylated with an appropriate amino acid and is incorporated into the stalled ribosome at the amber codon (UAG; red) on the reporter gene. The ribosome then continues with translation and stops at the terminal ochre codon (UAA; blue), which is recognized by another release factor (RF2) to produce the full-length reporter protein (ON state; right).

ferase genes are the wild type (WT) and a Ser60-to-amber mutated gene (S60UAG). Each linear DNA template has a T7 promoter sequence so that it can be transcribed to the desired tRNA or mRNA with T7 RNA polymerase (T7RNAP). By using these DNA templates, in vitro coupled transcription/translation was performed in an RF1-free reconstituted prokaryotic cell-free translation system that contained T7RNAP (Figure 2C). When the S60UAG gene was used, almost no active luciferase was expressed without suppressor tRNA (lane 1) or in the presence of 5SL-supT (lane 2),[14,15] whereas active luciferase was highly expressed in the presence of supT (lane 3). The efficiency of suppression by supT was almost 100% in comparison with when the WT gene was used in the presence of supT (lane 5).[16] These results strongly indicate that the suppressor $tRNA^{SerU}_{CUA}$ with an extra sequence at its 5′ terminus is not aminoacylated by ARS, or if aminoacylated, is not incorporated into the stalling ribosome.

We next constructed DNA templates for ASTs (Figure 3A). A theophylline-dependent aptazyme[10] was tethered to the 5′ terminus of supT such that one side of helix A of the aptazyme was the 5′ terminus of supT. Although both the sequence and length of helix A are expected to have a pronounced effect on the switching efficiency of the aptazyme, the length of helix A was first optimized because the sequence of the 5′ terminus of supT is accurately recognized by SerRS,[9] and thus it cannot be altered. We prepared five DNA templates for ASTs with helices A of various lengths (Figure 3B, AST1–AST5). Each AST template was mixed with the amber-mutated Ser60UAG template to prepare five biosensors (AST sensors). In vitro coupled transcription/translation was performed by using these biosensors in the absence or presence of theophylline (1 mM; Figure 3C). The most efficient AST sensor was the AST3-containing one, which has a 10 bp-long helix A, and gave in a high ON/OFF efficiency[17] of 6.8 ± 1.1, which is comparable to the aptazyme-based riboswitch sensor that used luciferase as reporter gene in a previous study.[4] In the case of ASTs with a shorter helix A (AST1 and AST2), luciferase was relatively highly expressed in the OFF state; this indicates that these ASTs were probably cleaved in the absence of theophylline, presumably because they were partly active as ribozymes even in the OFF state. In the case of ASTs with a helix A longer than AST3 (AST4 and AST5) the expression of active luciferase was very low in the ON state; this suggests that the aptazyme cannot separate from supT after self-cleavage because of the robust hybridization of helix A at the translation temperature (37 °C).

Finally, a mismatched base pair was inserted into helix A of AST4 and AST5 (Figure 4A; AST4m and AST5m), which showed low activity in the ON state, to promote the separation of the aptazyme from supT after self-cleavage in the ON state. As a result, the ON/OFF efficiency of AST4m and AST5m increased, as observed by higher luciferase activity in the ON state compared to that of the corresponding wild-type ASTs (i.e., AST4 and AST5).[18] This indicates that the aptazyme separated form supT more efficiently in the ON state (Figure 4B). On the other hand, the ON/OFF efficiency of the AST3 mutant decreased (Figure 4A, AST3m) as indicated by a reduction in luciferase activity in the ON state compared to the wild type; this is likely due to the instability of helix A of the active form of the aptazyme in the ON state (Figure 4B). The sensor from AST4m was approximately twofold more efficient than AST3, and showed a high ON/OFF efficiency of 11.6 ± 1.2 with or without theophylline (1 mM), as well as high theophylline dependency (Figure 4C and D). This ON/OFF efficiency is slightly higher than, or comparable to, that of other theophylline-dependent aptazymes[3b,e,4,19] although it is lower than that of the original aptazyme[10] because of the decrease of aptazyme activity caused by the addition of extra sequences, the use of a temperature that was slightly higher, and a magnesium ion con-

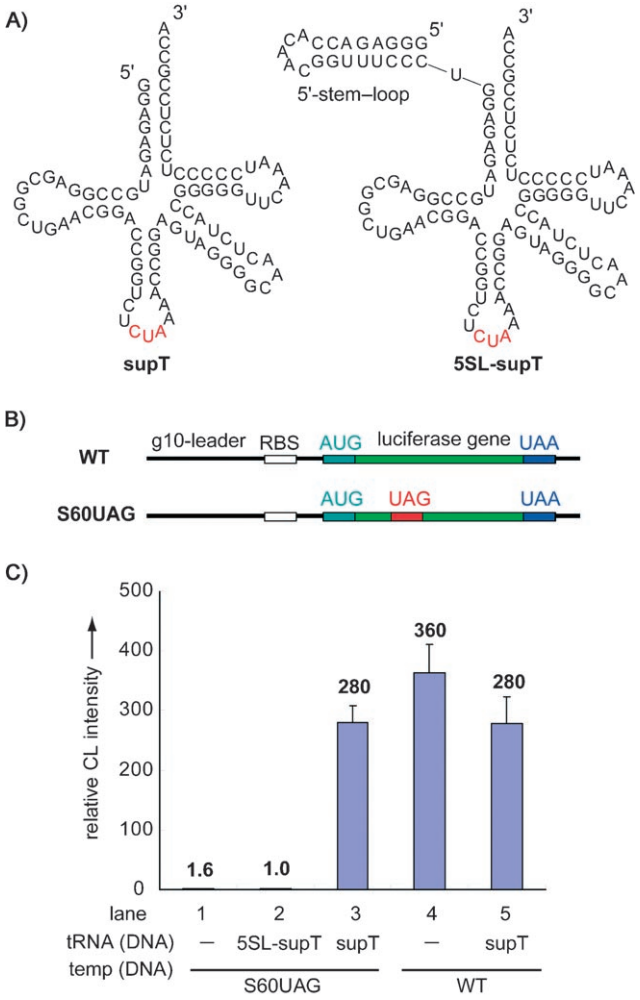

Figure 2. Control experiments with suppressor $tRNA^{SerU}_{CUA}$ to which no aptazyme was fused. A) RNA sequences and proposed secondary structures of a normal suppressor $tRNA^{SerU}_{CUA}$ (supT) and a negative control tRNA (5SL-supT), which has an extra stem–loop sequence at its 5′ terminus. B) Two different luciferase-coding mRNA molecules: the wild-type (WT) and Ser60-to-amber mutant (S60UAG). C) The relative activity (chemiluminescence; CL) of the translated luciferase by using either the S60UAG or WT luciferase gene, and 5SL-supT or supT as suppressor tRNA.

centration that was slightly lower than optimal for the original aptazyme.[20]

In summary, we have constructed a novel, label-free, and aptazyme-based biosensor system using a prokaryotic cell-free translation system in combination with a nonsense codon suppression method. We tethered a theophylline-dependent aptazyme to the 5′ terminus of the anticodon-adjusted suppressor $tRNA^{SerU}_{CUA}$ (aptazyme–suppressor-tRNA conjugates; AST) and combined the AST with an amber-mutated luciferase gene in the translation system to construct AST sensors. AST was self-cleaved and activated to suppress the amber codon on the luciferase gene only in the presence of the aptazyme cofactor; these actions did not take place in the absence of cofactor. As a result of the optimization of helix A of the AST, we obtained the most efficient AST, AST4m, the sensor of which (AST4m sensor) showed high theophylline dependency and was slightly more efficient than the riboswitch sensor in the previous study.[4] Although the ON/OFF efficiency of the AST4m sensor was lower than that of the original aptazyme, AST and riboswitch sensors have an advantage over the original aptazyme as they are "label-free" and, in some cases, "detector-free" because of the translation system. In addition, in vitro reselection of the aptazyme with a suppressor tRNA sequence at the optimal translation temperature and magnesium ion concentrations could significantly improve the ON/OFF efficiency. Moreover, these AST sensor systems might be applicable for gene regulation in living cells although we need to inhibit 5′ processing by RNase P.[21]

Experimental Section

General: Reagents, solvents, and enzymes were purchased from standard suppliers and used without further purification. Oligonucleotides were synthesized and OPC-purified by Operon Biotechnologies (Tokyo, Japan) or Hokkaido System Science (Sapporo, Japan). All PCR reactions were performed with PrimeSTAR Max or PrimeSTAR HS DNA polymerase from Takara Bio (Ohtsu, Japan). PCR products were purified with a GFX column from GE Healthcare (Buckinghamshire, UK) and quantitated by measuring absorbance at 260 nm. All plasmid constructions were verified by sequencing.

Preparation of DNA templates for tRNA or aptazyme–suppressor-tRNA conjugates: DNA templates for SupT, 5SL-supT, and aptazyme-suppressor–tRNA conjugates AST1–5, and AST3m–5m were prepared with PCR by using 5′-primers (5′-GAA *ATA ATA CGA CTC ACT ATA* GGA GAG ATG CCG GAG CGG CTG AAC-3′ for SupT; 5′-GAA AT*T AAT ACG ACT CAC TAT A*GG GAG ACC ACA ACG GTT TCC CTG GAG AGA TGC CGG AG-3′ for 5SL-supT; 5′-GAA AT*T AAT ACG ACT CAC TAT A*GG GAG ACC ACA ACG GTT TCC CT-$(X)_n$-CTT TCC CTG ATG AGC CTG GAT GAA AAT CCA GGC GAA ACG GTG AAA G-3′ for AST1–5 and AST3m–5m, where $(X)_n$ is AT for AST1, CAT for AST2, GCAT for AST3, GGCAT for AST4, CGGCAT for AST5, GCAA for AST3m, GGCAA for AST4m, and CGGCAA for AST5m; the T7 promoter sequence is italicized), a 3′-primer (5′-TGG CGG AGA GAG GGG GAT TTG AAC CCC CGG TAG AGT TGC CCC TAC TCC GGT TTT AGA GAC CGG TCC GTT C-3′), and template (5′-TGA TGA GCC TGG ATA CCA GCC GAA AGG CCC TTG GCA GTT AGA CGA AAC GGT GAA AGC CGT AGG AGA GAT GCC GGA GCG GCT GAA CGG ACC GGT CTC TAA-3′).

Preparation of DNA templates for the WT or S60UAG luciferase gene: The wild-type luciferase gene with a T7 promoter was prepared by three-step PCR. The first step was performed by using a 5′-primer (5′-AGG AGA TAT ACC AAT GGA AGA CGC CAA AAA CAT AAA GAA AG-3′), a 3′-primer (5′-CAA TTT GGA CTT TCC GCC CTT CTT GG-3′), and pBESTLuc from Promega (Tokyo, Japan) as template. The second step was carried out by using a 5′-primer (5′-GAA AT*T AAT ACG ACT CAC TAT A*GG GAG ACC ACA ACG GTT TCC CTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA TAT ACC A-3′; the T7 promoter sequence is italicized), a 3′-primer (5′-TAT TCA TTA CAA TTT GGA CTT TCC GCC CTT CTT GG-3′), and the agarose gel-purified PCR product from the first step as template. The third step was implemented by using a 5′-primer (5′-GAT CGA GAT CTC GAT CCC GCG AAA T*TA ATA CGA CTC ACT ATA* GG-3′; the BglII site is underlined), a 3′-primer (5′-TTA TTG CTC AGC TAT TCA TTA CAA TTT GGA CTT TCC GCC CTT CTT GG-3′; the Bpu1102I site is underlined), and the column-purified PCR product from the second step as template. The column-purified PCR product from the third step was then cloned into the BglII–Bpu1102I site of pET-15b from Merck Biosciences (Tokyo, Japan) to construct a plasmid, pT7-Luc.

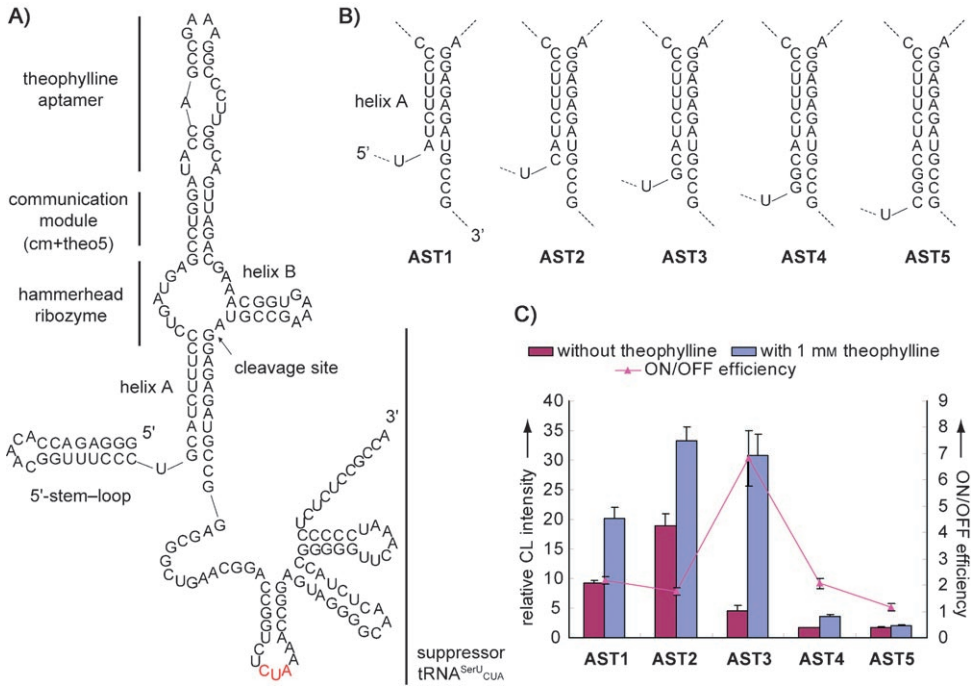

Figure 3. The theophylline-dependent aptazyme–suppressor-tRNA conjugate (AST). A) The RNA sequence and proposed secondary structure of AST in the presence of theophylline.[10] B) Five variants of AST with various lengths of helix A. C) The activity (chemical luminescence; CL) of the translated luciferase with each AST sensor (combination of S60UAG with each AST template) with or without theophylline (1 mM) relative to the activity when 5SL-supT was used without theophylline (Figure 2C, lane 2).[17]

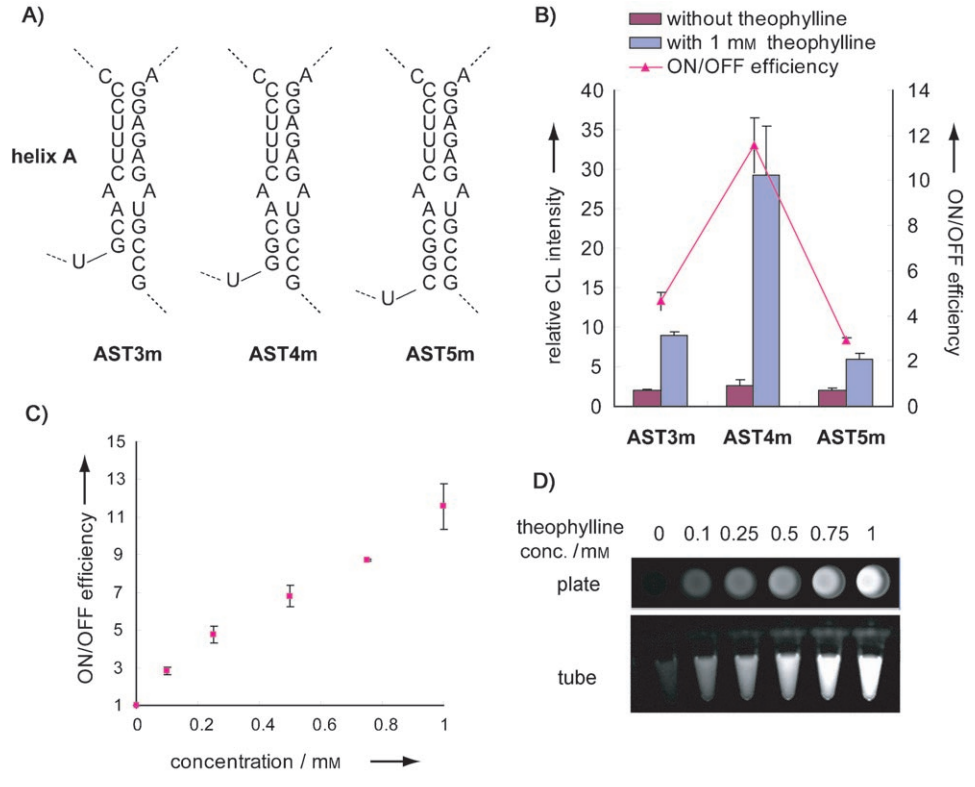

Figure 4. A) Three variants of AST with an A–A mismatch base pair in helix A. B) The activity (chemical luminescence; CL) of the translated luciferase by using each AST sensor with or without theophylline (1 mM), relative to the activity when 5SL-supT was used without theophylline (Figure 2C, lane 2). C) Theophylline dependency of the AST4m sensor. D) Chemical luminescence images of the luciferase assay solutions of the AST4m sensor at various theophylline concentrations.

Site-directed mutagenesis of Ser60 to an amber codon (UAG) was performed with QuikChange from Stratagene (La Jolla, CA, USA) by using double-stranded DNA (5′-GTA CGC GGA ATA CTT CGA AAT GTA GGT TCG GTT GGC AGA AG-3′ and the complementary strand; the amber codon is underlined), and pT7-Luc as template to construct pT7-S60UAG-Luc. The DNA templates for coupled transcription/translation were prepared with PCR by using a 5′-primer (5′-*CTT AAT ACG ACT CAC TAT A*GG GAG AC-3′), a 3′-primer (5′-AGC AAA AAA CCC CTC AAG ACC CGT TTA GAG-3′), and pT7-Luc or pT7-S60UAG-Luc as template.

In vitro coupled transcription/translation: To maximize the suppression efficiency, we used a reconstituted prokaryotic cell-free translation system (PURESYSTEM custom, Post Genome Institute, Tokyo, Japan), which included T7 RNA polymerase but did not include added release factor one (RF1). In vitro coupled transcription/translation was performed with this system according to the manufacturer's protocol with slight modifications. The reaction mixture (10 μL) in the absence or presence of theophylline (0–1 mM), the DNA template for tRNA or aptazyme–suppressor-tRNA conjugates (0 or 0.1 μg), and the DNA template for the luciferase gene (0 or 0.1 μg) were incubated at 37 °C for 1 h. The resulting translation solution was used for the next assay without purification.

Luciferase assay: The translation solution was diluted twofold with double-distilled water and then the diluted solution (5 μL) was mixed with luciferase assay reagent (100 μL; Promega). The chemiluminescence (CL) intensities were measured by using a Wallac 1420 ARVOsx instrument (Perkin–Elmer, Yokohama, Japan) with a black 96-well plate (Corning Costar 3915; Corning, Tokyo, Japan). Chemiluminescence images were acquired by using a Las4000 system (Fujifilm, Tokyo, Japan).

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Keywords: biosensors · cofactors · ribozymes · RNA recognition · tRNA

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[13] In the reconstituted cell-free translation system used in this study,[7a] there is very little RNase P, which cleaves the 5′ terminus of an immature tRNA to produce a mature tRNA (tRNA processing).[21] Therefore, tRNA processing does not occur.
[14] The ribosome is not removed from the mRNA and stalls because RF1 is absent in the system. Therefore, the amber codon can sometimes be misread by $tRNA^{Gln}_{GUC}$.[15] In fact, even in the absence of suppressor tRNA, a full-length luciferase (probably the S60Q mutant) from the S60UAG template can be detected by Western blotting (~20% compared to that from the WT template). However, because almost no luciferase activity was detected in the absence of suppressor tRNA (Figure 2C, lane 1) the S60Q luciferase mutant has a much lower activity than WT luciferase.
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[16] The reason the expression efficiency without suppressor tRNA is slightly higher than that with suppressor tRNA (lane 1 versus 2 or lane 4 versus 5 in Figure 2C) is that T7RNAP translates both the luciferase gene template and the tRNA template in lanes 2 and 5.
[17] The ON/OFF efficiency is the ratio of the synthesized luciferase activity in the presence of theophylline (1 mM) to that in its absence. Note there were no detectable changes in luciferase activity with or without theophylline when using either supT or 5SL-supT.
[18] AST4m maintained the low activity in the OFF state compared to its parent (AST4); this indicates that it took the same inactive form as the parent without theophylline.
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[20] AST4m (1.7 μM) was ~30% self-cleaved with theophylline (1 mM) under the translation conditions used (see the Supporting Information). This aptazyme activity was lower than that of the original aptazyme (the apparent dissociation constant K_D of the original aptazyme was 40 μM).[10] This is one of the reasons why CL intensity of the AST4m sensor at 1 mM theophylline was ~0.1-fold lower than that with supT (Figure 2C, lane 3). Another possible reason is that the aptazyme did not completely separate form supT after self-cleavage.
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