

Suppressor tRNA-based Biosensors for Detecting Analytes

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A nonsense suppressor tRNA (sup-tRNA) allows a natural or non-natural amino acid to be assigned to a nonsense codon in mRNA. Sup-tRNAs were utilized initially for studying tRNA functions but lately are used more for protein engineering and gene regulation. In the latter application, a sup-tRNA that is aminoacylated with a natural amino acid by the corresponding aminoacyl-tRNA synthetase is used to express a full-length natural protein from its mutated gene with a nonsense codon in the middle. This type of sup-tRNA has recently been artificially evolved to develop biosensors. In these biosensors, an analyte induces the processing of an engineered premature sup-tRNA into a mature sup-tRNA, which suppresses the corresponding nonsense codon incorporated into a gene, encoding an easily detectable reporter protein. This review introduces sup-tRNA-based biosensors that the author's group has developed by utilizing bacterial and eukaryotic cell-free translation systems.

Keywords Suppressor tRNA, biosensor, cell-free translation, amber suppression, gene regulation

(Received August 3, 2020; Accepted September 24, 2020; Advance Publication Released Online by J-STAGE October 2, 2020)

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1 Introduction

A nonsense suppressor tRNA (sup-tRNA) is an atypical elongator whose anticodon is complementary to one of the nonsense codons (ochre: UAA, amber: UAG, or opal: UGA).¹ This unique tRNA allows an amino acid charged thereon to be incorporated into a nascent polypeptide at the corresponding nonsense codon in mRNA during translation, as typical

elongators do at sense codons. In other words, a nonsense codon can be read through (*i.e.*, suppressed) by the corresponding sup-tRNA with an amino acid as if it was a sense codon. Although sup-tRNAs compete against release factors (RFs), which also recognize and bind to the nonsense codons but only in order to terminate translation, the “read-through” or “suppression” takes place efficiently if a competent sup-tRNA is charged with an amino acid again and again by an aminoacyl-tRNA synthetase (aaRS), preferably in an RF-deactivated or RF-free translation system.

Sup-tRNAs were utilized initially to elucidate various aspects of tRNA functions but lately are used more for protein engineering and gene regulation.²⁻⁷ In recent protein engineering, a sup-tRNA is used as a carrier not of a natural but of a non-natural amino acid, in combination with an artificial aaRS that can orthogonally add the non-natural amino acid to the sup-tRNA.⁵ To site-specifically incorporate non-natural amino acids into proteins during translation, various orthogonal sup-tRNA/aaRS pairs have been developed. In contrast, a sup-tRNA that is recognized by a natural aaRS and thus charged with a natural amino acid is applicable to gene regulation. The sup-tRNA/aaRS pair is not necessarily orthogonal to natural pairs in this application. When one sense codon in a gene of interest is mutated to a nonsense codon (different from the true termination



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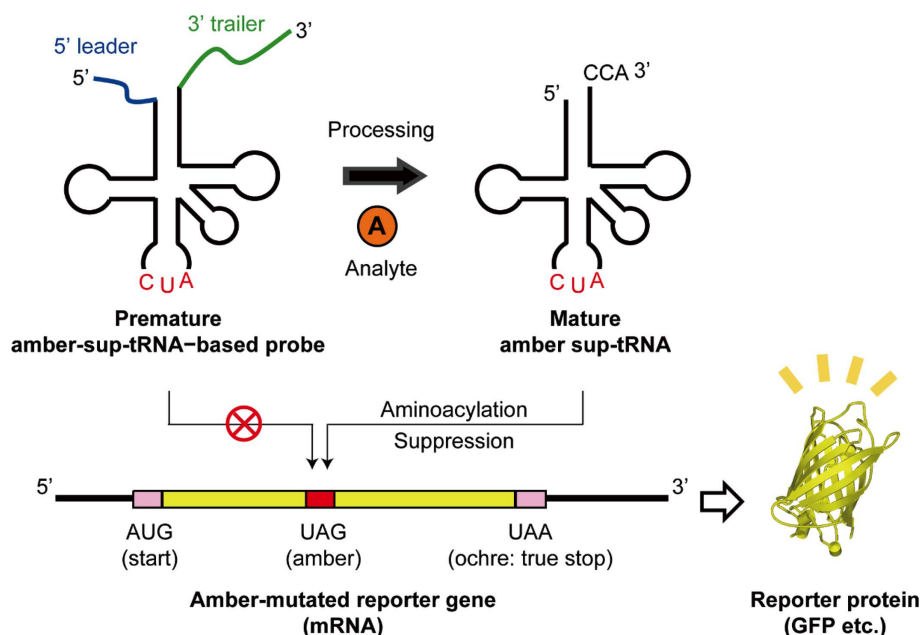


Fig. 1 Schematic illustration of sup-tRNA-based biosensors. An analyte induces the processing of a premature amber-sup-tRNA-based probe into a mature amber sup-tRNA, which suppresses the amber codon in the middle of a reporter gene (mRNA) to express the full length of an easily detectable reporter protein. In contrast, the probe has no suppression ability due to the prematurity in the absence of its analyte.

codon), the full-length protein can be expressed from the mutated gene with the corresponding sup-tRNA that is charged with a natural amino acid assigned to the original sense codon. Therefore, this type of sup-tRNA has been expected to be useful for treating premature termination codon disorders.^{6,7}

Recently, the author's group has artificially evolved the latter type of sup-tRNA (for the amber codon: amber sup-tRNAs) to develop biosensors.⁸⁻¹⁰ In these advanced applications, a mature amber sup-tRNA is generated from its engineered precursor (*i.e.*, a premature amber-sup-tRNA-based probe) through processing in response to a specific analyte, and then suppresses the amber codon introduced into a reporter gene to express a full-length, easily detectable reporter protein, such as GFP or luciferase (Fig. 1). In this review, the author focuses on these sup-tRNA-based biosensors and compares bacterial and eukaryotic ones.

2 Cell-free Translation Systems

Sup-tRNA-based biosensors of course require a translation system for expressing a reporter gene with the amber codon in the middle. For this purpose, *in vitro* cell-free translation systems are more beneficial than living cell-based ones in the following aspects: (1) much easier to handle; (2) almost no bioethical and biohazard concerns; (3) available for analytes that inhibit cell growth.^{11,12} We thus sought to develop sup-tRNA-based sensors by utilizing an *in vitro* cell-free translation system.

2.1 Bacterial cell-free translation system

Although an *E. coli* S30 extract has been widely used for *in vitro* translation of bacterial mRNAs, a reconstituted translation system (the PURE system) is more suitable for creating bacterial sup-tRNA-based sensors.¹³ This system is literally reconstituted by mixing individually purified *E. coli*.

factors involved in protein synthesis such as aaRSes, initiation factors (IFs), elongation factors (EFs), and RFs, so that a custom system free of a certain factor can be easily prepared by not adding that factor. In addition, its productivity is comparable to that of an *E. coli* extract.¹⁴ We thus decided to use a custom-reconstituted system devoid of RF1, which exclusively recognizes the amber codon in bacterial translation, to enhance the efficiency of amber suppression by amber sup-tRNAs. Incidentally, another RF, RF2, terminates translation at the other two nonsense codons in the RF1-free system.

2.2 Eukaryotic cell-free translation system

To date, no eukaryotic fully-reconstituted efficient translation system comparable to the PURE system has been available. However, there is an excellent cell-free system based on a eukaryotic cell extract, wheat germ extract (WGE).¹⁵ Its productivity has been enhanced, mainly by improving the preparation method, so that it is much higher than those of other eukaryotic cell-free systems. In addition, its cost is relatively low, which contrasts with the expensive, bacterial RF1-free reconstituted system. Therefore, we selected WGE as a foundation for eukaryotic sup-tRNA-based sensors.

3 Optimization of Sup-tRNA Sequences

A competent sup-tRNA cannot be obtained just by altering the anticodon of a natural tRNA into a sequence complementary to a nonsense codon. Because most aaRSes recognize their corresponding tRNAs at both the acceptor stem and the anticodon,^{16,17} the anticodon alteration generally decreases a tRNA's ability to be aminoacylated. In addition, an *in vitro*-transcribed tRNA does not have modified bases, which are considered important for tRNAs to form active tertiary structures,¹⁸ though some modifications may occur in WGE.

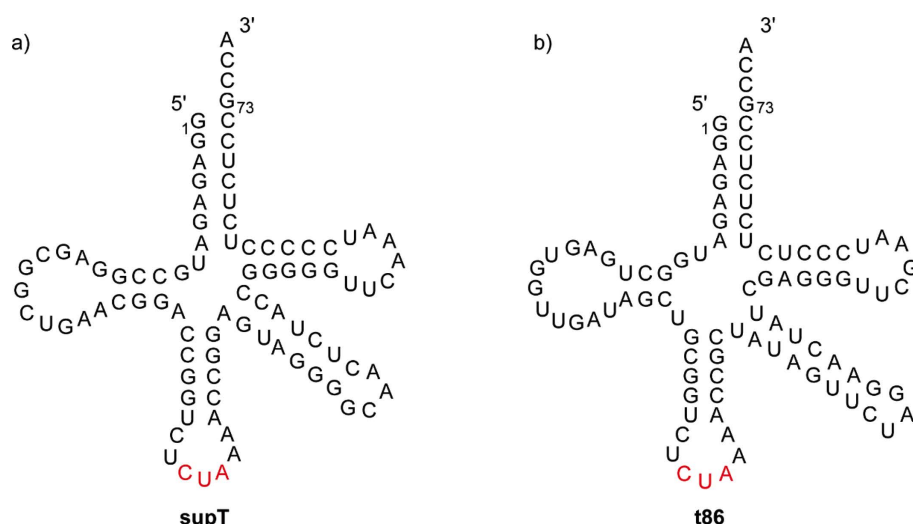


Fig. 2 The sequences of the optimized sup-tRNAs for the RF1-free bacterial reconstituted translation system, supT (a), and for the WGE-based translation system, t86 (b).

Therefore, to obtain a competent *in vitro*-transcribed sup-tRNA that can be used as the mother body of a sensor probe in cell-free systems, sequence optimization is likely to be required so that the sup-tRNA can be efficiently aminoacylated with an amino acid by an endogenous aaRS and then transferred into the ribosome by an EF-Tu (in bacterial systems) or eEF1A (in eukaryotic systems). In any case, the sequences of natural tRNAs should be referred to for the initial design of sup-tRNAs. We thus started by lining up natural tRNA candidates that were possibly applicable as a sup-tRNA backbone in each translation system.

3-1 Bacterial sup-tRNA

Potential candidates for a bacterial sup-tRNA backbone were *E. coli* tRNAs with long variable arms for Leu and Ser, because Leu-RS and Ser-RS, respectively, had not been found to recognize their anticodons for aminoacylation.^{16,17} In addition, their *in vitro* transcripts had been reported to be efficiently aminoacylated by the corresponding aaRSes, despite the absence of any modified bases therein.^{19,20} We thus altered their anticodons into a CUA triplet complementary to the amber codon and investigated their effects on suppression efficiency, which is the ratio of the translation efficiency of an amber-mutated mRNA to that of the original wild-type mRNA, in the RF1-free reconstituted system. The results showed that two *in vitro*-transcribed amber sup-tRNAs derived from tRNA^{Leu5}_{UAA} and tRNA^{SerU}_{CGA} suppressed well the amber codon (mutated from a sense codon for each amino acid) in the middle of the mRNA encoding a reporter protein. In particular, the latter (tRNA^{SerU}_{CUA}), named supT (Fig. 2a), exhibited considerably high suppression efficiency of almost 100% (slightly higher than that by the former, 80%).^{8,21,22} This means not only that supT is aminoacylated (probably by the corresponding Ser-RS) and transferred into the ribosome as well as natural tRNAs in the RF1-free system, but also that no further optimization is needed. In fact, although we tried *in vitro* selection of bases in the anticodon loop of supT by using the combination of read-through ribosome display and *in vitro* compartmentalization techniques, we found the original bases to be the best for amber suppression.²² Incidentally, the suppression efficiency decreased in an *E. coli* extract due to the endogenous RF1.²³

3-2 Eukaryotic sup-tRNA

Because not all of the sequences of wheat tRNAs had been completely revealed, we attempted to construct eukaryotic amber sup-tRNAs based on tRNAs from rice (*Oryza sativa*) by altering their anticodons into CUA for amber suppression in WGE.²⁴ Although it was also unknown which wheat aaRS does not recognize the anticodon and aminoacylates *in vitro*-transcribed tRNAs, we selected tRNAs for Ala, Leu, and Ser as backbones of the amber sup-tRNAs, on the basis of reports regarding eukaryotic aaRSes in other organisms.^{16,17,25-27} As a result of comprehensive analyses of 18 candidates of *in vitro*-transcribed amber sup-tRNAs, 3 from rice tRNAs for Ser were found to be the most competent, but they only moderately (15 – 25%) suppressed the amber codon in WGE. We thus next sought to improve suppression efficiency by optimizing the sequences of these moderately competent amber sup-tRNAs.²⁴ Several optimization steps, including chimerization, successfully gave rise to a much more competent amber sup-tRNA (named S2-G₂₇C₄₃-G₇₃ or t86, Fig. 2b), which exhibited suppression efficiency as high as 60 – 85% in WGE despite the existence of a eukaryotic RF, eRF1. Although t86 has some mutated bases different from those in the original natural rice tRNAs, we confirmed that it is charged with Ser by endogenous Ser-RS in WGE. Incidentally, some knowledge obtained through the optimization in these experiments afterwards helped us to rationally improve a sup-tRNA for efficient incorporation of a non-natural amino acid into protein in WGE.²⁸

4 *In situ* Generation of Mature Sup-tRNAs

In a sup-tRNA-based sensor, a sup-tRNA must be activated only in the presence of its specific analyte. One of the best strategies for the activation is the processing of a premature sup-tRNA into a mature one. The process of tRNA processing in cells is roughly divided into three steps: (1) end processing, (2) intron splicing, and (3) nucleotide modification.^{29,30} Since both the optimized bacterial and eukaryotic sup-tRNAs (supT and t86, respectively) do not require nucleotide modification (though t86 possibly undergoes some modifications in WGE) and the genomes of their original natural tRNAs do not have introns, we

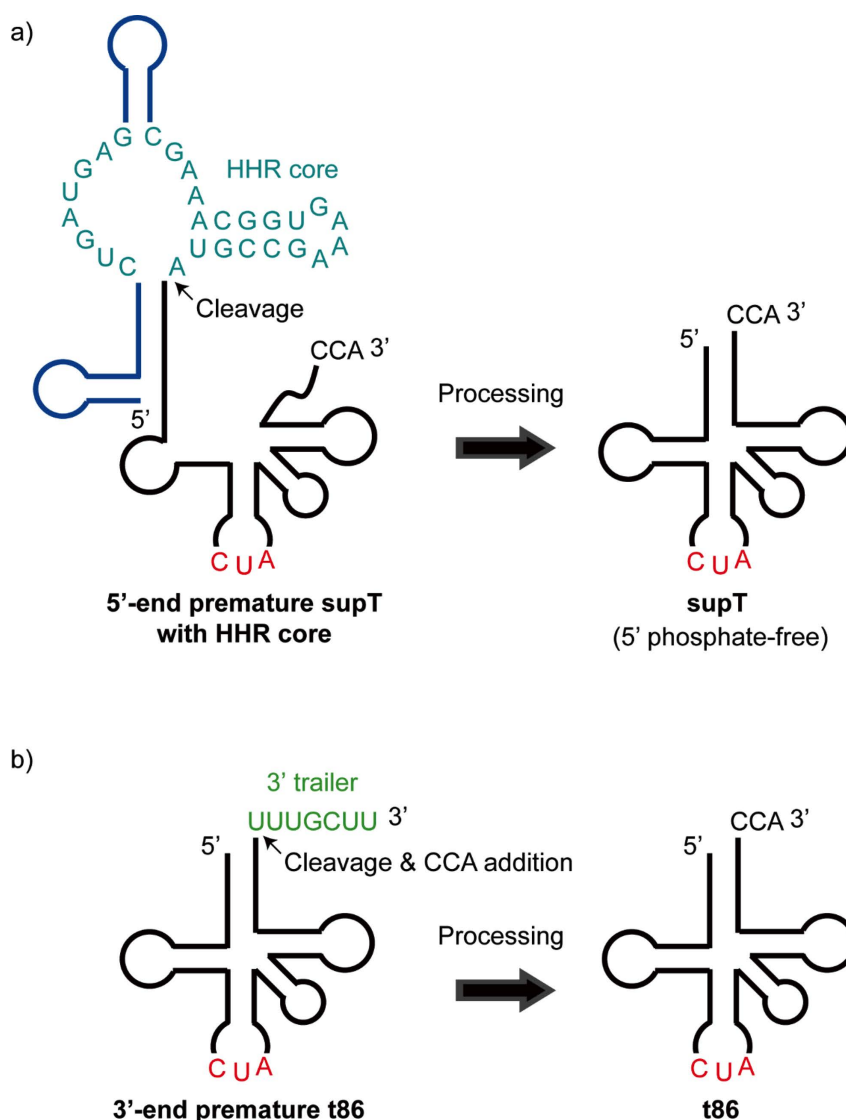


Fig. 3 *In situ* generation of the mature sup-tRNAs through the end processing of their precursors. (a) Maturation of a 5'-end premature supT by the HHR-catalyzed self-cleavage in the RF1-free system. Note that the resulting mature supT has a 5' phosphate-free terminus. (b) Maturation of a 3'-end premature t86 by endogenous enzymes in WGE.

focused on end processing to generate an active, end-mature sup-tRNA *in situ* (i.e., in each cell-free translation system).

4.1 End processing of bacterial premature sup-tRNA

In bacteria, the end processing of a premature tRNA with a 5' leader and a 3' trailer is achieved by some nucleases that cleave the unnecessary sequences out.³⁰ However, the reconstituted translation system includes no nucleases. Although it is in principle possible to add end-processing nucleases to the system, it is difficult to achieve that. Even only for the simpler 5' end processing, the 5' end-processing nuclease, RNase P, which is an RNA-protein complex and thus hard to prepare in the active form, is required.³¹ Therefore, we decided to utilize, as an alternative, a simple hammer-head ribozyme (HHR) core, which can cleave its own RNA, for the end processing.³² This selection was reasonable because the HHR core had been used in the 5' leader region of *in vitro*-transcribed tRNAs to obtain 5' non-G tRNAs by the site-specific self-cleavage at the 5' end of the N₁ base.³³ Although the resulting 5' terminus has a hydroxyl group,

unlike those of mature tRNAs generated through the natural 5' end processing with RNase P (monophosphate) or *in vitro* transcription (triphosphates), a 5' phosphate-free tRNA had been reported to only slightly decrease translation efficiency.³⁴ In fact, a premature supT with the HHR core at the 5' terminus showed relatively high amber suppression efficiency in the RF1-free system, approximately 50% of the efficiency shown by the *in vitro*-transcribed mature supT (unpublished result), whereas an uncleavable 5'-end premature supT exhibited no suppression ability.⁸ This indicates that the premature HHR-supT was successfully processed and that the generated 5' phosphate-free mature supT was acceptably competent for amber suppression (Fig. 3a).

4.2 End processing of eukaryotic premature sup-tRNA

In contrast to the bacterial reconstituted system, WGE should contain enzymes required for the end processing of premature tRNAs. However, as the activities thereof had not been elucidated, we investigated the tRNA end processing in WGE

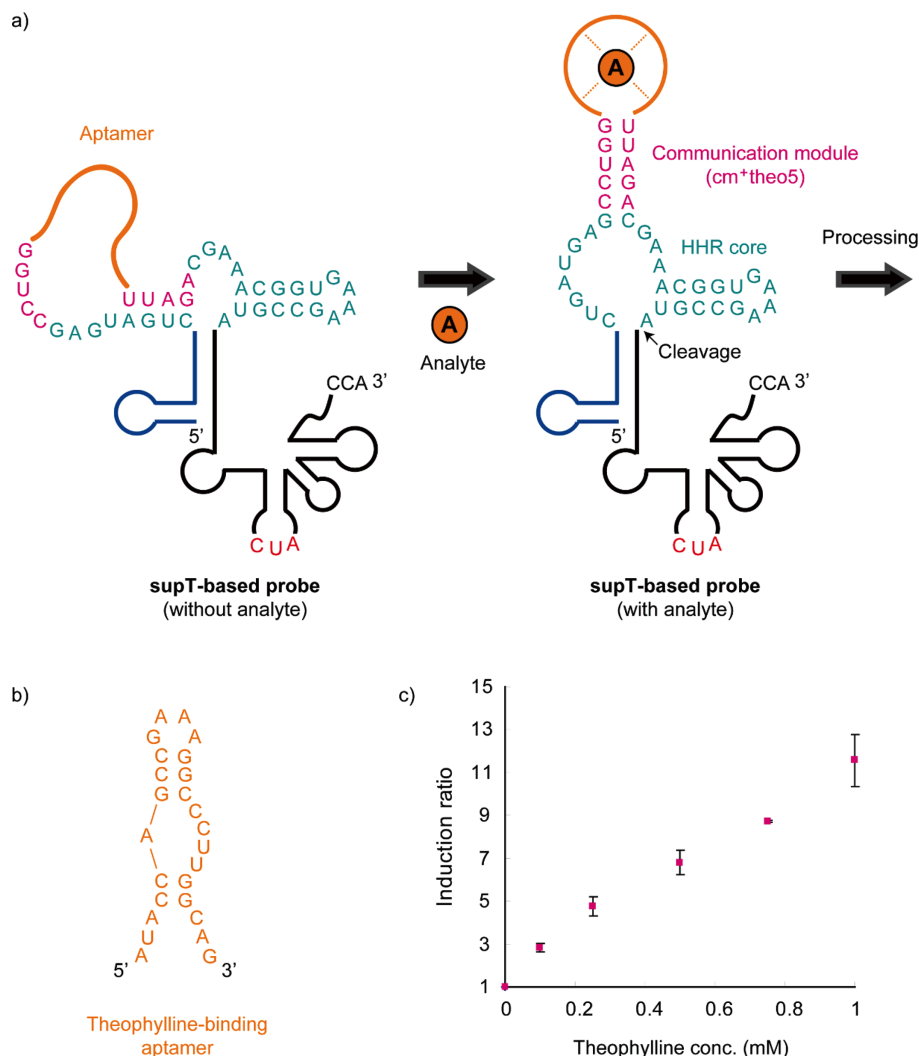


Fig. 4 Bacterial supT-based biosensors. (a) Predicted analyte-dependent conformational changes of a supT-based probe. (b) The sequence of the theophylline-binding aptamer. (c) The induction ratios of amber suppression by AST4m at various concentrations of theophylline in the RF1-free reconstituted translation system. Adapted from Ref. 8 with permission from Wiley.

by using premature tRNAs based on the optimized eukaryotic sup-tRNA, t86.³⁵ Specifically, we added a typical length of a 5' leader or a 3' trailer to each end of t86 or to that devoid of the 3' terminal CCA, respectively, and then evaluated the end processing by measuring suppression efficiency. The results showed that, whereas the 5' processing takes place slowly, the 3' processing including the CCA addition proceeds rapidly in WGE (Fig. 3b): the 3'-end premature t86 exhibited relatively high suppression efficiency, which is 70% of that exhibited by t86. It was also elucidated that the premature tRNAs undergo *exo* degradation from their ends in WGE at a speed slower than the 3' processing but faster than the 5' processing, in contrast to the highly stable mature tRNA, t86. This instability is favorable for a sup-tRNA-based sensor probe, which is a premature sup-tRNA, because the false-positive rate can be reduced through the degradation in the absence of its analyte.^{9,10} Incidentally, although general *in vitro* transcripts also degrade rapidly in WGE, we found from these and other experiments that they can be well protected by adding a simple stem-loop and a t86-derived rigid structure to the 5' and 3' ends, respectively.^{35,36}

5 Sup-tRNA-based Sensor Probes

To construct a sup-tRNA-based probe for detecting an analyte, an analyte-recognizing domain is required in the 5' leader or 3' trailer region of a premature sup-tRNA. This domain is called an aptamer. A well-established method named SELEX, or *in vitro* selection, enables us to obtain an aptamer that binds to a user-defined target analyte with high affinity and specificity.^{37,38} However, it is not highly likely that just adding an *in vitro*-selected aptamer to premature sup-tRNAs will make them responsive to the analyte, even though the aptamer is well minimized. An aptamer should be inserted so that the resulting probes induce their conformational changes through hybridization switches upon binding to the analyte and then undergo end processing to mature. We thus used reported, analyte-responsive hybridization switches or rationally designed switches to obtain bacterial or eukaryotic sup-tRNA-based sensor probes, respectively.

5-1 Bacterial sup-tRNA-based probes

Some communication modules that organically connect the HHR core and *in vitro*-selected aptamers had been obtained also through *in vitro* selection to implement analyte responsiveness into the HHR core; in other words, to induce the self-cleavage of HHR in response to an analyte.^{39,40} In an aptazyme, which is an aptamer-HHR conjugate with a communication module, the active (or inactive) HHR structure is induced by analyte-aptamer complex formation (through hybridization switches). We thus designed a pattern of supT-based probes by adding an HHR-based aptazyme with one of the activating modules, cm⁺theo5, to the 5' terminus of supT (Fig. 4a). Because this module had been originally selected for an aptamer binding to a small molecule analyte, theophylline (Fig. 4b),⁴¹ we prepared some probes with the theophylline-binding aptamer as a model, according to the probe pattern we designed. We then optimized a duplex sequence linking the aptazyme and supT in these probes to increase the theophylline-dependent induction ratio of amber suppression in the RF1-free translation system.⁸ Consequently, the optimized probe (named AST4m) exhibited an induction ratio of 11.8 at 1 mM theophylline (Fig. 4c). The ratio linearly increased depending on the theophylline dose, indicating that the probe functioned as designed. The probe also showed high specificity: it was unresponsive to a theophylline analogue, caffeine (unpublished result). Although the communication module used in this probe may not be available for other aptamers, *in vitro* selection allows us to obtain an appropriate module for each of them.^{39,40} In addition, the sensing ability would be improved by implanting an aptamer/communication module pair obtained under the translation conditions and/or by using a highly active, full-length HHR with loop-loop interactions instead of the HHR core only.⁴²

5-2 Eukaryotic sup-tRNA-based probes

To create an efficient 3'-end premature probe based on t86, the rapid 3' processing in WGE must be suppressed in the absence of its analyte. We thus started by adding a stem-loop structure to the 3' end of t86 (devoid of the 3' CAA) in such a way that the stem includes the cleavage site in the middle to sequester it from RNase Z,⁹ which is the eukaryotic 3' trailer-removing enzyme.⁴³ After optimizing the stem length to sufficiently inhibit the 3' processing, we rationally designed hybridization switches for analytes to dose-dependently induce the 3' processing. Specifically, an aptamer and a modulator sequence (MS) were added to the 3' end of the inhibitory stem sequence (IS). In this design, analyte-aptamer complex formation induces a duplex of MS/IS through hybridization switches, thus releasing the cleavage site and in turn promoting the 3' end processing (Fig. 5a). We first prepared theophylline-sensing probes by using the theophylline aptamer (Fig. 4b) and optimized the MS length towards a higher theophylline-dependent induction ratio of amber suppression in WGE.⁹ The optimized probe (named theo(th1)-MS(4)) exhibited a high theophylline responsiveness and specificity comparable to those of the bacterial supT-based probe for theophylline (Fig. 5b).⁸ In addition, since the aptamer domain is independent from IS and MS, it was replaceable with other *in vitro*-selected aptamers for sensing their target analytes. In this regard, it was found that implanting a longer aptamer was preferable because it provided more-sensitive detection, which can be attributed to the fact that a premature tRNA probe with a longer 3' trailer is more susceptible to degradation in WGE and thus more efficiently suppresses the false-positive rate in the absence of its analyte. For instance, a tetracycline-sensing probe (named tc(th1)-MS(4)), prepared just by replacing the theophylline aptamer with a much longer tetracycline-

binding aptamer (Fig. 5c), exhibited much higher sensitivity: the induction ratio at 100 μ M tetracycline was 81 and the detection limit was 100 nM (approx. 50 ppb) with an induction ratio of 1.5 (Fig. 5d). Incidentally, we also developed another type of eukaryotic sup-tRNA-based sensor for detecting a specific sequence of DNA with a similar design strategy.¹⁰

6 Conclusions and Future Perspectives

In this review, the author introduced bacterial and eukaryotic sup-tRNA-based biosensors that his group has developed. Both types are composed of a premature amber sup-tRNA probe, an amber-mutated reporter gene, and a cell-free translation system. An analyte binds to the probe to induce the end processing of the latter, and then the resulting mature sup-tRNA suppresses the amber codon in the mRNA to express an easily detectable reporter protein with the help of the cell-free system. These biofunction-assisted sensors have the advantage of not requiring the use of either special detectors or labeling probes that are generally used in aptamer-based sensors. The detection limits of sup-tRNA-based sensors for the model analyte, theophylline, are slightly (~one order) higher than that of an electrochemical sensor requiring expensive equipment and expertise,⁴⁴ but are comparable to those of other label-free aptameric sensors.⁴⁵⁻⁴⁷ In addition, sup-tRNA-based probes would be available as *trans*-acting, ligand (analyte)-responsive gene regulators both *in vitro* and *in vivo* to control the expression of a gene of interest into which an amber mutation is inserted. A sharper or more complex regulation could be achieved by combining them with sup-tRNA-based probes for another nonsense codon (ochre or opal) or with artificial riboswitches, namely *cis*-acting ligand-responsive gene regulators.⁴⁷⁻⁵² Therefore, sup-tRNA-based sensor systems are promising also as gene circuit elements in cell-free and cell-based synthetic biology.

In a comparison between the bacterial and eukaryotic sup-tRNA-based sensors that we developed, the latter are superior in cost, versatility, and sensitivity. The former require a self-cleaving HHR-based aptazyme for analyte-dependent probe maturation in the costly custom-reconstituted translation system. The communication module (cm⁺theo5) that connects the HHR core and an aptamer might not work properly, depending on the aptamer. In that case, an appropriate one must be obtained through *in vitro* selection. In addition, the minimum HHR core is not likely to sufficiently function *in vivo* due to its requirement for high concentrations of magnesium ions for efficient self-cleavage,⁴⁹ though this problem can be addressed by using a highly active HHR.⁴² In contrast, the latter (eukaryotic sup-tRNA-based sensors) use endogenous end-processing enzymes in a low-cost cell extract for probe maturation, which allows for a probe design with high versatility. In fact, the analyte specificity can be easily altered just by replacing the aptamer domain with the modulator sequence unchanged. The analyte-dependent maturation (3' processing) of the probes would take place efficiently also in other eukaryotic extracts^{53,54} and even in the nucleoplasm in eukaryotes. In addition, sensitivity can be increased by using a probe with a long aptamer (*i.e.*, a long 3' trailer) and/or by adding an eRF1-binding aptamer (preferably a stabilized one with end protectors).^{24,36,55} The weakness of eukaryotic sensors is that an efficient coupled transcription/translation is not available, in contrast to bacterial sensors: not DNA templates but transcripts (not only for an amber-mutated mRNA but also for a sup-tRNA-based probe) must be added directly to the extract. Lower-cost sensors with higher sensitivity can be obtained by solving this defect; for example, by

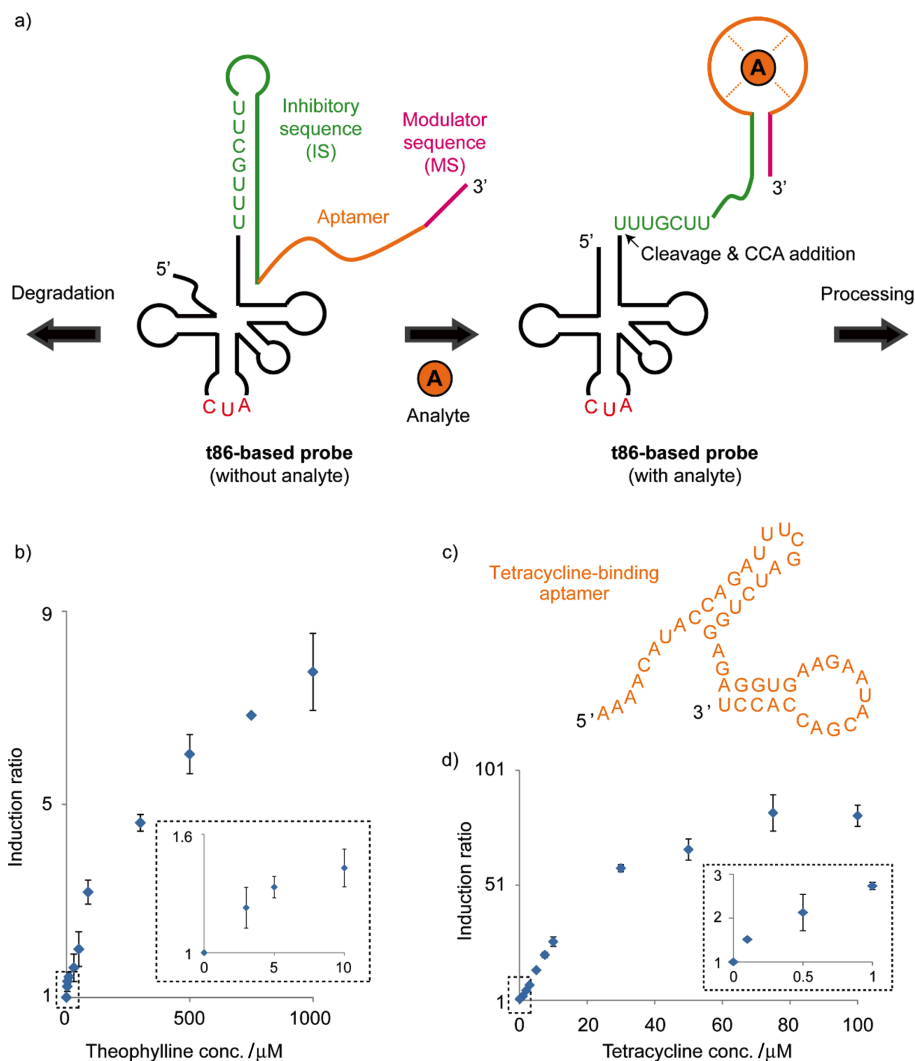


Fig. 5 Eukaryotic t86-based biosensors. (a) Predicted analyte-dependent conformational changes of a t86-based probe. This type of probe undergoes *exo* degradation by endogenous nucleases in WGE in the absence of its analyte, thereby decreasing the false-positive rate. (b) The induction ratios of amber suppression by theo(th1)-MS(4) at various concentrations of theophylline in WGE. (c) The sequence of the tetracycline-binding aptamer. (d) The induction ratios of amber suppression by tc(th1)-MS(4) at various concentrations of tetracycline in WGE. Adapted from Ref. 9 with permission from The Royal Society of Chemistry.

developing an RNA polymerase that functions efficiently at low magnesium concentrations at low temperature for *in situ* transcription in eukaryotic cell-free translation systems.

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