

**Functionalization of ribonucleopeptide receptors
for sensing and catalytic activities**

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Preface and Acknowledgements

The studies in this thesis have been carried out under the direction of Professor Takashi Morii at the Graduate School of Energy Science, Kyoto University, during April 2011 to March 2017. The study focuses on the development of the methods for functionalization of RNP receptor for sensing and catalytic activity.

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Chapter 1

General introduction

1-1. Introduction

Since the industrial revolution, people have utilized fossil fuels to build a highly developed society. Today, fossil fuels are indispensable for maintaining many elements of modern lifestyle, both as raw materials and as energy sources. Since fossil fuels are a finite resource, their high consumption in our society inevitably forces us to face an energy problem. In order to solve this problem, methods for both obtaining new energy sources and efficiently using energy have been extensively studied. Among the latter, bio-inspired energy utilization systems attracted our attention.

Living organisms are extremely efficient in using energy to transport and convert substances necessary for maintaining life activities. In the living body, functional biopolymers specifically interact with their target molecules to promote transport and chemical reactions, allowing highly efficient conversions of target molecules to be carried out in a complicated environment, in the presence of various molecules. Many research efforts have been made to mimic the systems that convert and utilize materials and energy in living organisms. For achieving this goal, researchers have been trying to develop a methodology for the preparation of highly functional biopolymers. However, even though various functional biopolymers have been isolated and their amino acid sequences, three-dimensional structures, and mechanisms of function have been determined, it is still difficult to artificially prepare functional biopolymers with a specific desired function. Thus, it is required to develop novel methods in order to construct artificial biopolymers with tailor-made functions.

1-1-1. Importance of molecular recognition of functional biopolymers

A crucial feature of natural functional biopolymers is their high molecular recognition ability toward their target substrates. Due to this high selectivity, for which the “lock and key” model¹ is often used, functional biopolymers can only act on their target substrates, even in an intracellular environment that is highly crowded and inhomogeneous. This selectivity of the recognition of the substrate comes from complicated three-dimensional structure of biopolymers based on their monomer sequences (i.e., their primary structures). Proteins are composed of twenty types of amino acids, and nucleic acids contain four types of nucleotides. These monomers provide a huge number of the interactions in them to stabilize complicated three-dimensional structures. By taking advantages of the complicated structures, some of

biomacromolecules have a cavity to work as a binding pocket for the target substrate, which is held by noncovalent interactions such as hydrogen bonds, hydrophobic interactions, electrostatic interactions, π - π , sigma- π , and cation- π interactions (Figure 1-1). For example, an enzyme, which is one of the functional biopolymers catalyzing the reaction *in vivo* with high selectivity, has the binding pocket for its target substrate. A catalytic group is located around the binding pocket so that the target reaction is accelerated due to the proximity effect between the substrate and the catalytic group. The selectivity is based on the substrate recognition by the binding pocket of the enzyme. Thus, molecular recognition is a most important for expression of the function with high selectivity *in vivo*.

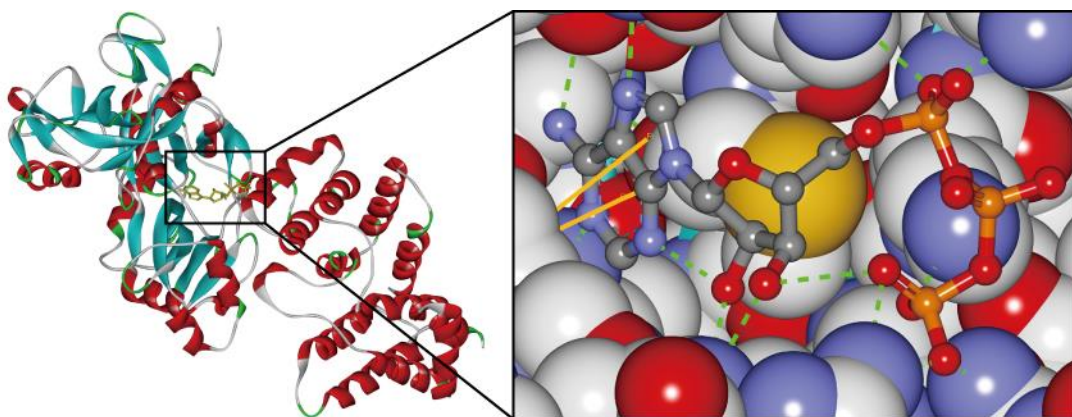


Figure 1-1. Three-dimensional structure of an ATP-dependent DNA ligase from *Sulfolobus solfataricus* bound to ATP (PDB ID: 2HIX).² An image was created using the Discovery Studio v3.1 software (Accelrys, San Diego, CA, USA). In the overall structure (left), the protein and the ATP molecule are displayed using solid ribbon and a yellow-colored stick model, respectively. The right panel displays a detailed view of the structure in the area of the binding pocket, using stick models for both the protein and the ATP molecule. Orange lines and green dashed lines indicate π - π /sigma- π interactions and hydrogen bonds, respectively, connecting the protein and the ATP molecule.

1-1-2. Methods for construction of artificial receptors

The ability to specifically bind their targets is the most essential function of biopolymers. Thus, construction of functional molecules by functionalization of receptors is one of the most rational approaches. However, naturally occurring receptors do not always provide the desired molecular recognition characteristics. Methods to prepare receptors for the desired target substrates are described below.

Structure-based design of naturally occurring receptor

Site-specific mutagenesis or modification of a protein based on its three-dimensional structure is the most traditional and rational approach for understanding the mechanism underlying the molecular recognition ability of receptors. Since genetic mutation or chemical modification of the substrate-binding site of a receptor is expected to change the selectivity and affinity for the target substrate, the successive mutation/modification of residues and evaluation of the resulting mutants can help us determine which amino acids are important for target recognition. The same approach, i.e., site-specific mutagenesis based on structure, can be used to modulate/fine-tune the selectivity and affinity of a receptor (Figure 1-2).³⁻⁸



Figure 1-2. Schematic illustration of the structure-based design of protein. Specific amino acid residues (AA2 and AA3 in left) at the active site are replaced by other amino acid residues AA4 and AA5.

In this method, only a part of the protein is converted. This is a rational and convenient approach because it does not require the challenging task of constructing the whole structure of a protein. However, it is a laborious trial-and-error process that necessitates repeated cycles of conversion and evaluation of the function of the protein. Moreover, the design relies on existing information on the three-dimensional structure of an original protein receptor, which is not always available in the three-dimensional structure database, the Protein Data Bank (PDB) (<http://www.rcsb.org/>).⁹

Design of host molecule in host-guest chemistry

Based on the crystal structures of functional biomolecules and knowledge of the marvelous specificity, many chemists have tackled designing and synthesizing simpler organic compounds mimicking the features or functions of naturally occurring biopolymers. The name “host-guest chemistry” was given to this approach.¹⁰⁻¹² Examples of host molecules are cyclodextrins, natural products composed of six to eight glucopyranoside units bound together

in a ring (Figure 1-4). The hydrophobic “hole” is used to bind the hydrophobic part of a substrate molecule in water solutions. Apart from cyclodextrins, crown ethers and supramolecules have also been used as host molecules.^{12–16} However, the selectivity of small organic host compounds is lower than that of biopolymers, because biopolymeric receptors recognize numerous sites on the target molecules (Figure 1-1), which remains difficult to achieve using synthetic receptors.

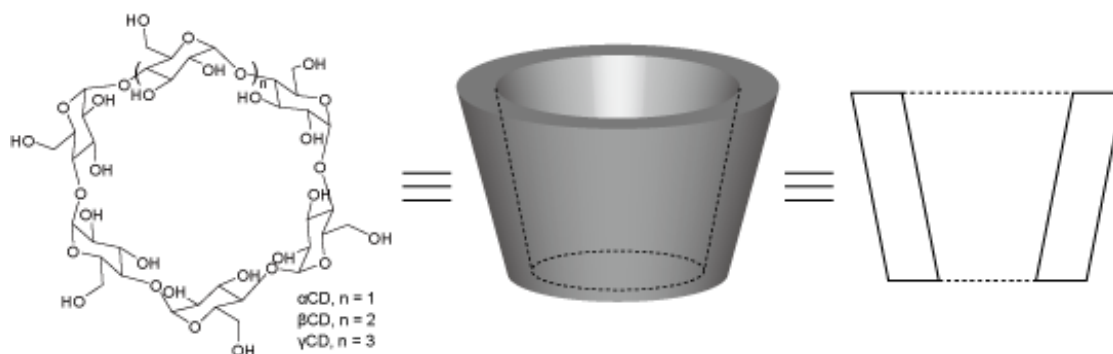


Figure 1-4. Various representations of cyclodextrins

Construction of library and screening

Selection from combinatorial libraries is now an established methodology of mainstream chemical biology, with new applications of developed combinatorial strategies continuing to emerge every year. Handling large diversities by physically linking the phenotype to the encoding genotype allows the simultaneous physical separation of a huge number of elements, depending on certain binding properties in an iterative enrichment process.

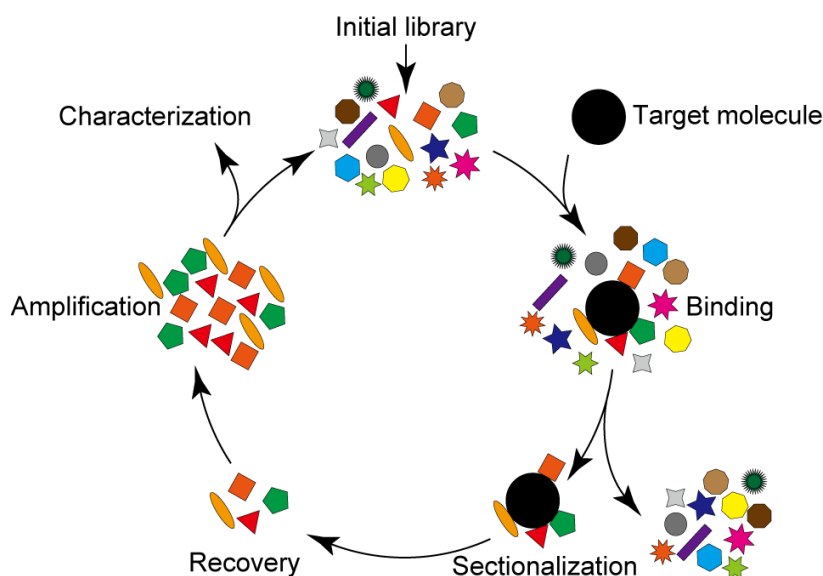


Figure 1-5. Schematic illustration of the selection from a randomized combinatorial library¹⁷

The selection from a randomized combinatorial library according to binding properties follows the process displayed in Figure 1-5. First, the initial library is incubated with the target molecule. The unbound variants are removed by a washing step, while the bound variants are eluted, amplified, and fed into the next selection round. The process is repeated until a specific variant is observed.

Examples of technologies that rely on this library approach to provide a variety of receptors include phage display,^{18,19} ribosome display,²⁰ *in vitro* selection,^{21,22} mRNA display,^{23,24} *in vitro* compartmentalization,²⁵ and cDNA display.²⁶ Library construction for target-receptor selection has to be optimized for diversity screening.

De novo design

De novo design relies on physical principles to generate new proteins with sequences unrelated to those found in nature.^{27–29} This approach is based on the hypothesis that proteins fold into the lowest energy states that are accessible to their amino acid sequences.³⁰ First, modular structures are predicted based on information derived from the analysis of natural biopolymers. Molecular dynamics calculations are then carried out to obtain the overall structures (Figure 1-6). To date, the efforts to design novel proteins based on the understanding of the fundamental principles governing amino acid sequence, protein structure, and protein folding have only managed to yield small-molecule binding proteins.

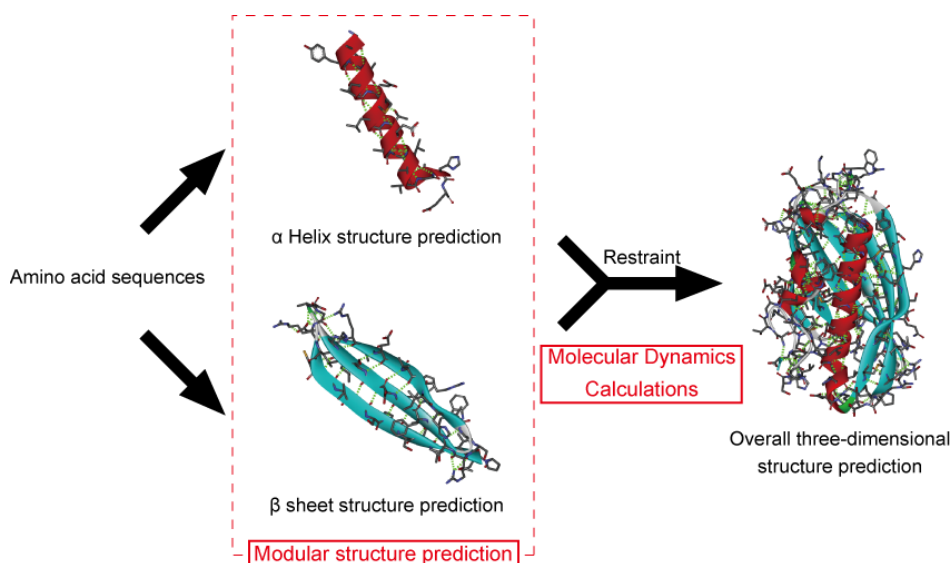


Figure 1-6. Schematic illustration of the *de novo* protein design

This approach is in its initial stages, and it currently faces three issues to overcome: (1) the energy of a system cannot be computed with absolute accuracy, (2) the space of possible structures and sequences is very large and, therefore, difficult to search comprehensively, and, (3) only a fraction of protein designs adopt stable folded structures when produced in *Escherichia coli* because of insolubility and the formation of unintended oligomeric states.

1-1-3. Functionalization of receptors

Modification of receptors is an efficient method to construct biomolecules displaying both high substrate selectivity and the ability to perform the desired function. Lots of studies have reported the construction of biosensors through modification of protein and nucleic acid receptors with appropriate fluorophores³¹⁻³⁴ and artificial enzymes possessing a structure that allows a proximity effect between the substrate and their catalytic group.^{35,36}

Structure-based design of natural and unnatural receptors

Structure-based design of receptors is the most rational method for functionalizing a receptor that displays satisfactory binding affinity for the desired target. When we have an access to information on the three-dimensional structure of the receptor of interest, we can determine the atoms, groups, or motifs located near the target. Afterwards, we use site-specific chemical modification or mutagenesis to replace them with the appropriate functional atom, group, or motif, in order to bestow the desired function on the receptor (Figure 1-6). For example, a fluorescent protein was fused to a protein receptor to create a fluorescent biosensor,³⁷ and scientists have been able to alter the catalytic activity of natural enzymes by introducing a mutation in the proximity of the active center.⁷ C-S bond formation of cysteine and thiol-reactive site, and complex formation of metal-chelator also give us the new functional biopolymers.^{38,39} However, information on the three-dimensional structure of a protein receptor, which is required by this method, is not always available. In addition, the binding affinity of the original receptor for the substrate may be disrupted by the chemical modifications or mutations that are applied to enable the receptor to perform a new function in the substrate binding site of the receptor. Finally, the actual production of a designed receptor (i.e., successfully performing the required changes) often comes with its own set of difficulties.

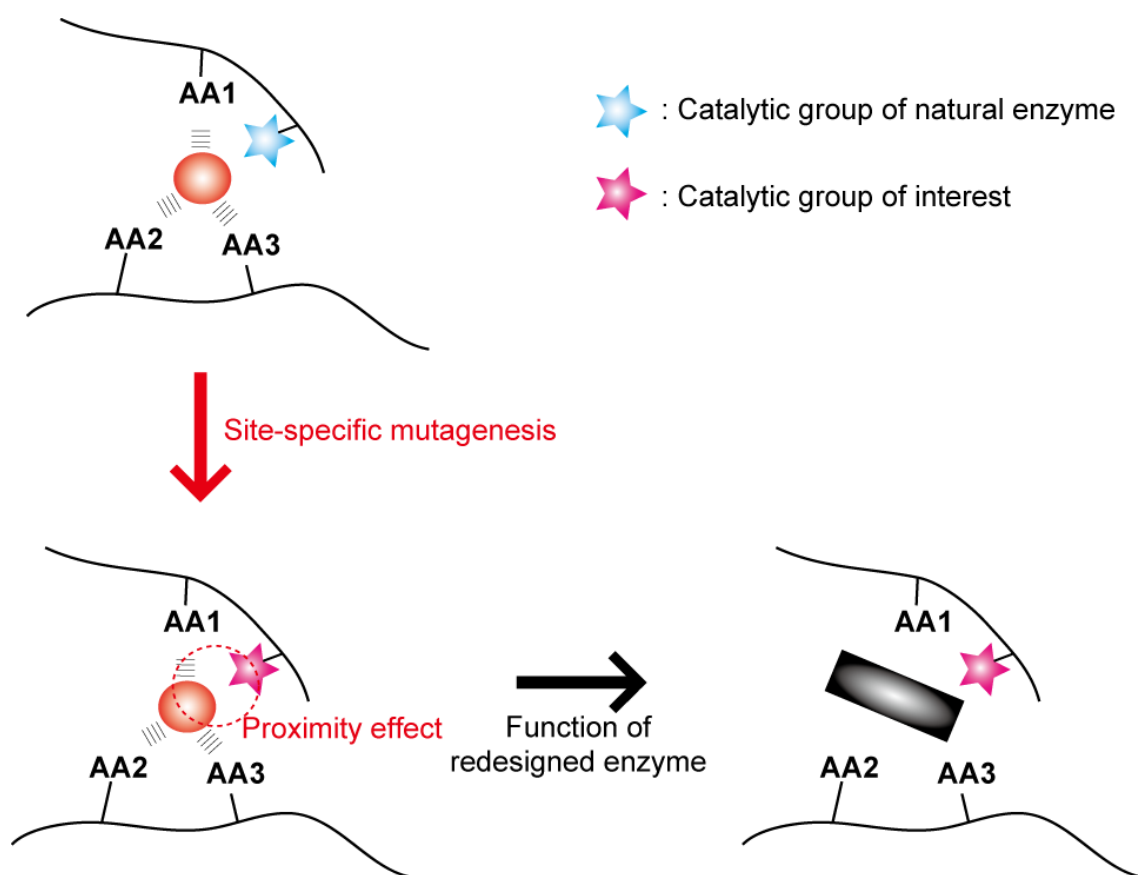


Figure 1-6. Schematic illustration of the structure-based design of a natural enzyme to create an enzyme with the ability to perform the function of interest. The original catalytic group near the binding pocket is converted into a group catalyzing the desired reaction.

Modification of synthetic host molecule with a functional group

“Artificial enzymes” are functional molecules constructed utilizing the ability of synthetic host molecules to bind targets of interest, by attaching catalytic groups.^{12–14,16,40} For example, a metal complex with an effective and selective hydrolytic activity was attached to the face of cyclodextrin, creating an artificial enzyme capable of hydrolyzing substrates that cannot directly bind to the metal complex. As seen in Figure 1-7, the cyclodextrin-bound substrate is held next to the metal ion, allowing these two to interact. Various functional groups can be attached to cyclodextrins because of their three-dimensional structure. One of the disadvantages of this method is that unless cyclodextrins are extensively modified, their complexes with substrates can be rather flexible with, perhaps, an unpredictable preferred geometry. Moreover, as this method utilizes synthetic receptors, it suffers from their low selectivity.

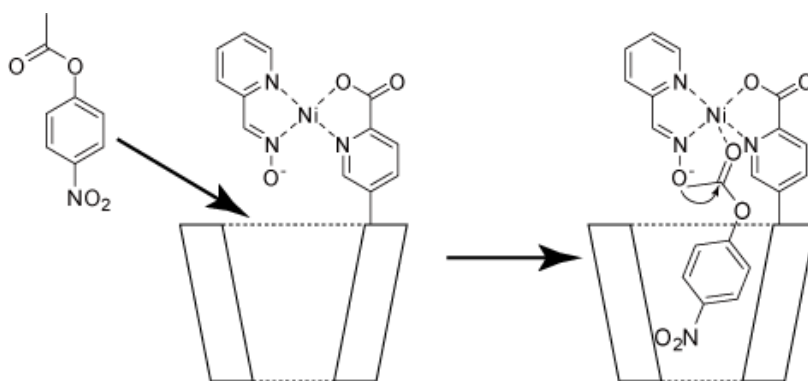


Figure 1-7. Cleavage of bound esters by a metal complex/cyclodextrin artificial enzyme⁴⁰

Construction of library and screening

This method does not require information on the three-dimensional structure of the original receptor. We can select biopolymers with a desired function by screening a diverse library using a variety of methods. One of these methods is directed evolution, which is useful in receptor functionalization. First, a diverse biopolymer library is created by introducing, using error prone PCR or DNA shuffling techniques, random mutations into the gene coding for a receptor possessing the ability to recognize the desired target (Figure 1-8). Screening for the desired function allows us to select the variants that possess this function. By repeating the cycle of library construction and screening, a biomolecule that possesses a binding pocket for the target of interest and performs the desired function is finally isolated.

Libraries based on biological systems have two advantages. First, the biopolymer can be amplified, thus multiple rounds of selection can take place. Second, they have the ability to generate new diversity during the amplification stage. On the other hand, it is difficult to use these libraries with unnatural compounds because of the latter's incompatibility with enzyme-mediated biopolymer amplification systems.

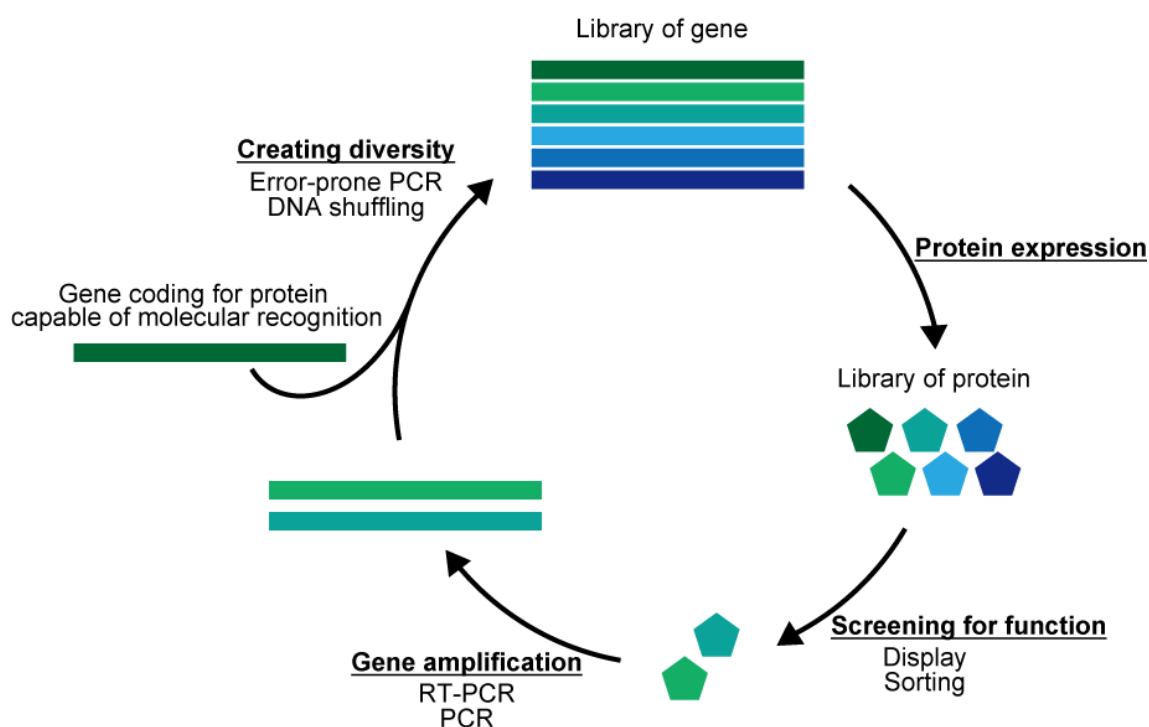


Figure 1-8. Schematic illustration of the directed evolution method.

1-1-4. Construction of functional molecules by ribonucleopeptides

Each of the methods of receptor functionalization described above has its own advantages and disadvantages. Although the construction of functional biopolymers from single molecules based on these methods has brought significant advantages, we are still unable to prepare tailor-made functional molecules. One of the possible reasons is that single-molecule receptors to be functionalized have one function already, i.e., the ability to bind the target molecule. Thus, the addition of the second desired function must be done in a way that will not compromise the binding ability.

An alternative approach uses complexes made of more than one biopolymer. There are several functional biopolymer complexes in nature. Nevertheless, currently there are only a few methods for constructing new functional polymer complexes. An example is the stepwise molding method that was used for creating a functional ribonucleopeptide (RNP). Human immunodeficiency virus type-1 (HIV-1) Rev peptide and the RRE (*Rev Response Element*) RNA, whose three-dimensional structure has been solved by solution NMR spectroscopy (Figure 1-9),⁴¹ was chosen as a RNP scaffold.

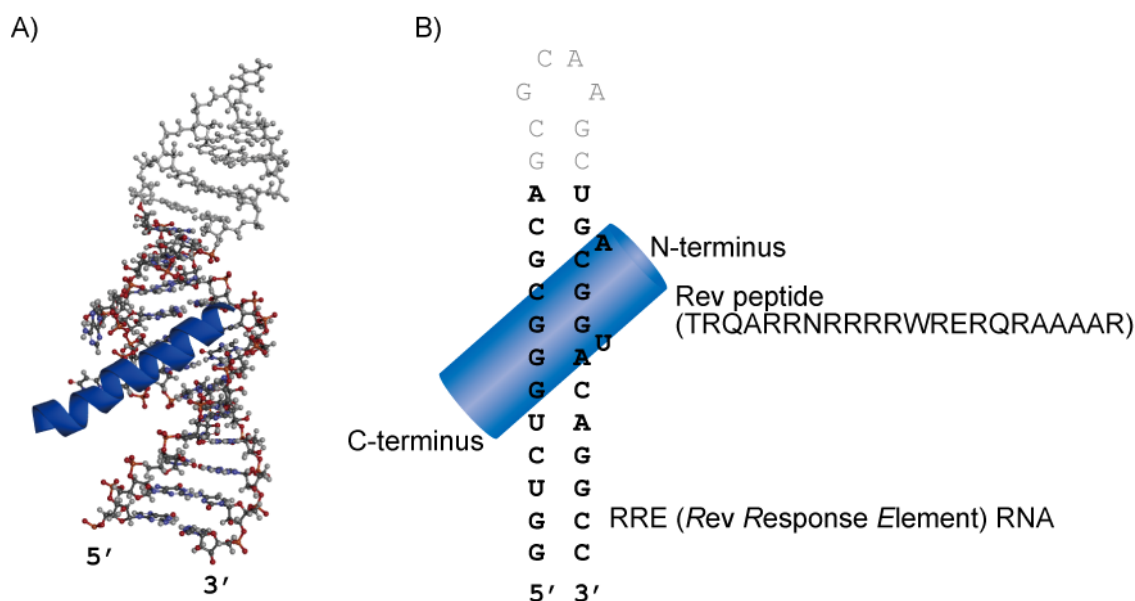


Figure 1-9. (A) Three-dimensional structure of the Rev peptide-RRE RNA complex. The Rev peptide is shown by a ribbon model, while the RRE RNA is displayed with a ball-and-stick model in which balls and sticks are used to display atoms and bonds, respectively. The atoms forming the part of the RRE RNA that was replaced by randomized ribonucleotide sequences to form an RNP library are indicated by gray color. (B) Sequences of the RRE RNA and the Rev peptide. The ribonucleotides that were replaced by a randomized sequence to construct the RNP library are indicated by gray color.

In the first step, *in vitro* selection method was applied to construct an RNP receptor with a binding site for the given target molecule such as ATP, which is one of the most important energy carrier molecules *in vivo*, from an RNP pool originating from an RNA library and the Rev peptide (Figure 1-10A).^{42,43} Afterwards, a peptide library was created by introducing a random amino acid sequence into the peptide subunit of this RNP receptor, and a new round of selection could be performed. The end result was an RNP receptor with improved substrate selectivity (Figure 1-10B).⁴⁴ Furthermore, the introduction of a fluorophore into the peptide subunit allows the creation of a fluorescent RNP sensor with sensing ability, of which fluorescence intensity changes upon substrate binding (Figure 1-10B).⁴⁵ The change in fluorescence intensity of the fluorescent RNP sensor is caused by the structural change that takes place upon binding of the target substrate.⁴³ Thus, by using RNP as a basic framework, it is possible to create artificial receptors for various target molecules, and easily bestow new functions without losing the substrate binding ability inherent to the receptor. The ability of this stepwise RNP functionalization method to be used in multiple systems was confirmed by the

construction of receptors and sensors for phosphotyrosine in specific amino acid sequences,⁴⁶ as well as for histamine⁴⁷ and dopamine.⁴⁸

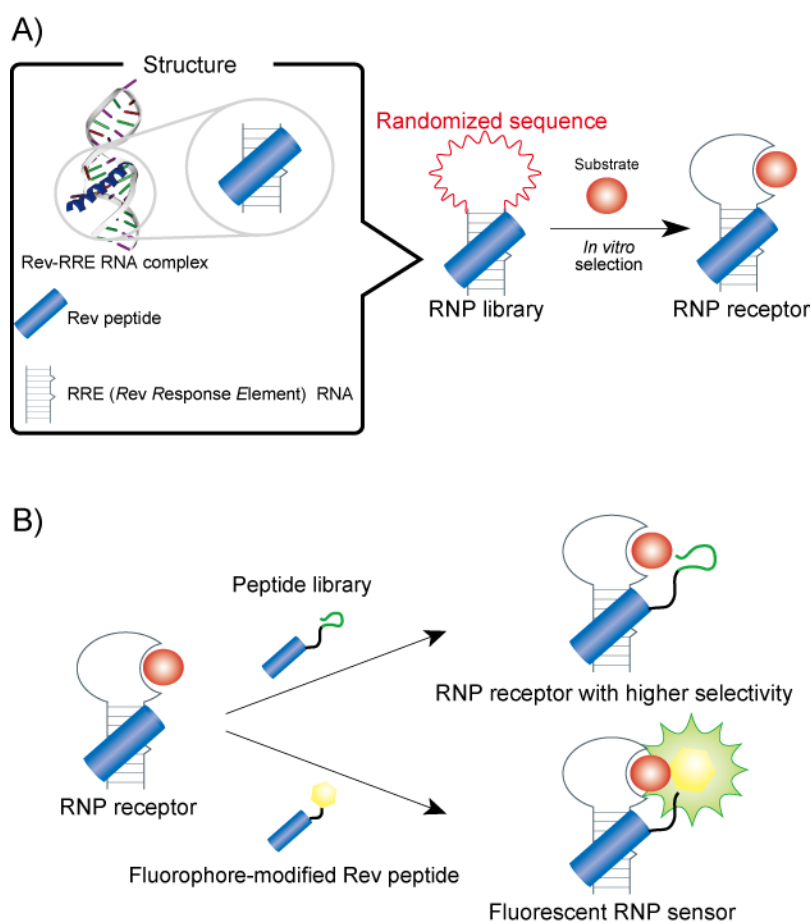


Figure 1-10. Stepwise molding method for the creation of a novel ribonucleopeptide (RNP). (A) Construction of RNP receptor by in vitro selection. (B) Functionalization of the RNP receptor.

It is also able to create novel RNP receptors without *in vitro* selection, by directly constructing an RNA subunit that can bind the target substrate.⁴⁹ The new RNA subunit was constructed by conjugation of the RRE RNA with a GTP-binding RNA aptamer (Figure 1-11), and is able to both bind GTP and interact with the Rev peptide to create an RNP complex. As was also the case with the *in vitro* selection method described above, a fluorophore can be introduced to the Rev subunit. As the binding of GTP causes a change in structure that in turn modifies the fluorescence intensity, the complex acts as a fluorescent GTP sensor. To summarize, this method allows the easy (as it does not require *in vitro* selection from an RNP pool originating from an RNA library) creation of a fluorescent sensor by converting an aptamer into an RNA subunit for a new RNP receptor.

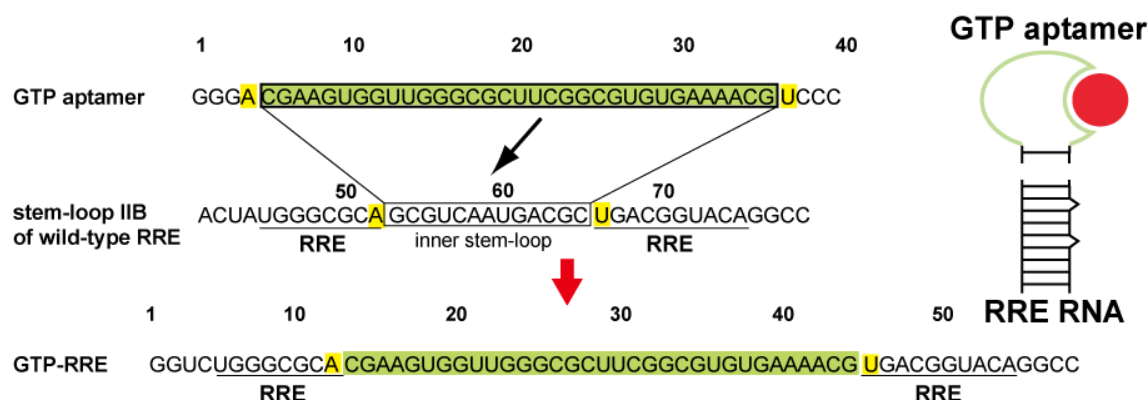


Figure 1-11. Construction of the RNA subunit of a new RNP receptor by utilizing a previously reported GTP-binding RNA aptamer. The inner stem-loop of the RRE RNA (the area between the two yellow-colored nucleotides) is replaced by the green-colored sequence of the GTP aptamer.

The aforementioned methods have managed to deliver only few functional RNPs, new RNP receptors displaying higher molecular recognition ability compared to the original receptors and some fluorescent sensors. Hopefully, further development and improvement of the functionalization and library formation of the peptide subunit will allow bestowing unprecedented sensing ability or catalytic activity to RNP receptors. Thus, it is necessary to continuously explore methods for creating new functional RNP receptors while taking advantage of the high substrate specificity of these complexes.

1-2. Aims of the research in this thesis

We already have the method to construct the binding pocket for the molecule of interest on RNP scaffold. We already know that peptides of interest or unnatural molecules can be introduced into RNP scaffold by mixing the RNA subunit and the peptide subunit that is modified with peptides of interest or unnatural molecule. Taken together, it is natural to think that RNP is useful scaffold to construct the receptor for desired molecule with desired function. In this thesis, I would like to expand the method of stepwise functionalization of RNP in order to show the possibility of the construction of tailor-made functional molecule by using RNP. This thesis describe the functionalization of RNP receptor by using a methodology for library construction and screening or structure-based design, as well as incorporating a high sensing ability and catalytic activity to RNP receptors.

First, the application of functionalization of RNP receptor for sensing ability was

expanded. Modular fluorescent RNP sensors were constructed by introducing the fluorophore that change its emission intensity at single wavelength corresponding to the local environment around the fluorophore into N-terminus of Rev peptide. However, some of the fluorophore-modified RNPs that were constructed by above-described method were difficult to use in practice as fluorescent sensors because of displaying only small changes in emission intensity upon substrate binding. In order to enhance the signal, a ratiometric fluorophore, which changes the emission wavelength due to the micro environment, was used as a signal transducer in order to enhance the change (Chapter 2; Figure 1-12A). A ratiometric fluorophore SNARF was attached to N-terminus of Rev peptide of RNP. Emission wavelength was shifted upon binding event, so that we could recognize the change.

Second, a method of screening of a fluorophore library for the creation of RNP sensors was developed (Chapter 3; Figure 1-12B). Previously we use only several fluorophores to convert RNP receptors to RNP sensors. However, fluorophore-modified RNPs do not always show the large intensity change. If we would like to convert RNP receptor of interest to fluorescent RNP sensor, we should choose the RNP sensor from only several fluorophore-modified RNP receptors due to the limitation of the number of fluorophore-modified Rev peptides. In this library method described in chapter 3, libraries of 4 types of fluorophore were utilized for construction of fluorophore-modified RNP for the screening.

Moreover, covalently linking between RNA and peptide subunits were developed (Chapter 3; Figure 1-12C). We could prepare fluorescent RNP sensors by the methods described above. One of the problems of RNP sensors is instability of noncovalent complex formation in the condition of low concentration. Also it is difficult to use different RNP sensors simultaneously due to the subunits exchange between the RNP complexes. So the method of covalent linkage between RNA and peptide subunits to stabilize RNP was established.

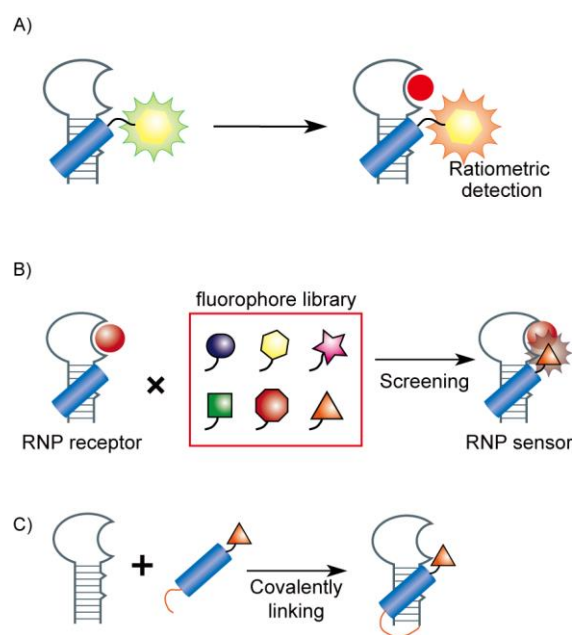


Figure 1-12. Schematic illustration of the construction of RNP sensors by (A) using a ratiometric fluorophore, (B) screening a fluorophore library and (c) covalently linking between RNA and peptide subunits.

Next, we developed methods for functionalization of RNP receptors for catalytic activity. Both natural and unnatural molecules can be added to the N-terminus of the Rev peptide by solid-phase synthesis, so that we can introduce a catalytic group to the RNP receptor. When the geometry of the RNP receptor is such that a catalytic group is located near the substrate binding pocket, the target reaction would be accelerated due to the proximity effect. As information on the three-dimensional structure of an RNP receptor is often unavailable, it is critical to develop a method for constructing a structure suitable for catalytic activity. In order to construct RNP receptors with catalytic activity without structural information, we used library method. A structurally diverse RNP receptor library with a catalytic group was constructed and screened (Chapters 4 and 5, respectively; Figure 1-13A).

In chapter 6, I have designed RNP receptors with catalytic activity based on the three-dimensional structures of Rev/RRE complex and ATP aptamer. The three-dimensional structures of only a few RNP receptors are available. We might design a possible structure of RNP with catalytic activity by utilizing structural motif of RNA or RNP. Three motifs that named receptor module, catalytic module and geometry adjusting module were selected from reported structures, and some of the RNPs were designed and their catalytic activity was evaluated (Chapter 6; Figure 1-13B).

The methods for the receptor-based preparation of biopolymer with function of interest were discussed in this thesis. Finally, they were summarized in conclusion.

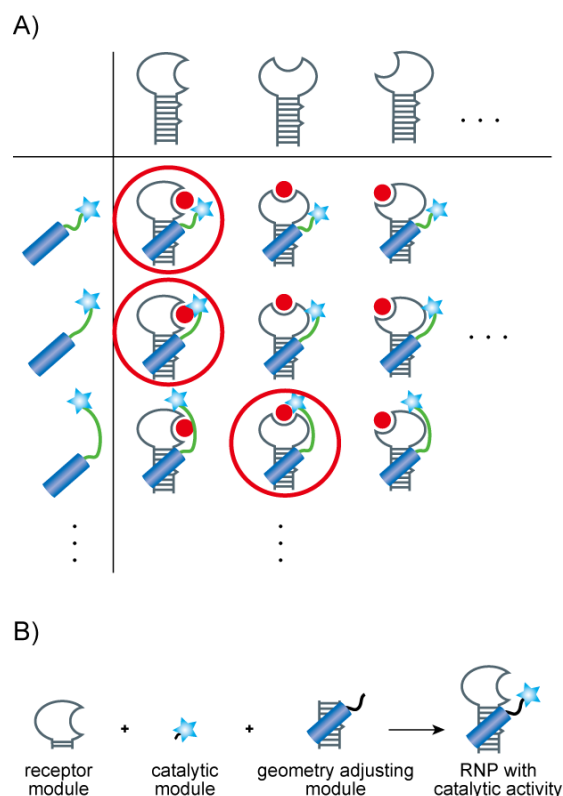


Figure 1-13. Schematic illustration of RNP receptor functionalization for catalytic activity by (A) screening of a structurally diverse library, and (B) structure-based design. The RNPs of which the substrate binding pocket located near the catalytic site will be selected by screening of structurally diverse RNP library based on the catalytic activity.

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Chapter 2

Construction of ratiometric fluorescent sensors from RNP receptor

2-1. Introduction

Fluorescent sensors are important tools in the fields of therapeutics and diagnostics due to the superior physical properties of fluorescence detection.¹⁻⁷ Fluorescent sensors consist, essentially, of a recognition module for the target and a fluorophore-modified signal transduction module that affords the optical signal output. Despite the fact that various sensors have been developed to date, a lengthy and laborious trial-and-error procedure is usually required to obtain a sensor with optimized functions since the interplay between recognition and signal transduction events is unique for each individual sensor.

One of the promising strategies to overcome this drawback is the assembly of fluorescent ribonucleopeptide (RNP) sensors consisting of an RNA subunit that binds to a target of interest and a fluorophore-labeled peptide subunit that specifically binds to the RNA subunit.⁸⁻¹⁷ A stable complex between the Rev peptide and RRE (*Rev Responsive Element*) RNA, for which the three-dimensional structure and the interaction mode have been established,¹⁸ was used as a scaffold for constructing fluorescent RNP receptors. RNP derivatives were generated by replacing the stem loop region of RRE RNA with target specific receptor or aptamer sequences and subsequently converted into target-specific fluorescent RNP sensors by a simple modification of the Rev peptide with a fluorophore at its N-terminus in a stepwise manner.¹⁵ The RNP sensor was found to undergo structural changes upon substrate binding.¹⁶ As a result, a change in fluorescence intensity of the RNP sensor was observed due to micro-environmental changes of the attached fluorophore. As for fluorescence detection, ratiometric sensing presents several advantages, such as enhanced dynamic range, precise correction without being affected by environmental variables, and convenient visual monitoring that make it suitable for bio-analytical measurements.¹⁹ For the purpose of expanding the suitability of this technique, I describe here an innovative strategy for obtaining universally applicable ratiometric fluorescent sensors with modular RNP structure by using targets specific RNA aptamers as a receptor module and ratiometrically detectable fluorophore-attached Rev as a reporter module.

2-2. Results and discussions

2-2-1. Design of ratiometric RNP sensor by using SNARF

Seminaphthorhodafluor (SNARF)¹⁹⁻²¹ is a representative red emitting fluorophore that

shows pH dependent dual-emission properties. RNPs that tether SNARF through the Rev peptide were designed in order to sensitively detect micro-environmental changes associated with substrate binding. Two types of SNARF-attached Rev peptide, which incorporate linkers of different length, were designed. The first one (SNARF-Rev) was obtained by directly modifying the N-terminus of the Rev peptide with the 6-carboxy-SNARF isomer (Figure 2-1B). The second one (SNARF-abu-Rev) contemplated the insertion of an aliphatic linker (γ -aminobutyric acid) between the N-terminus of the Rev peptide and SNARF (Figure 2-1B). These Rev peptides were characterized by mass spectrometry.

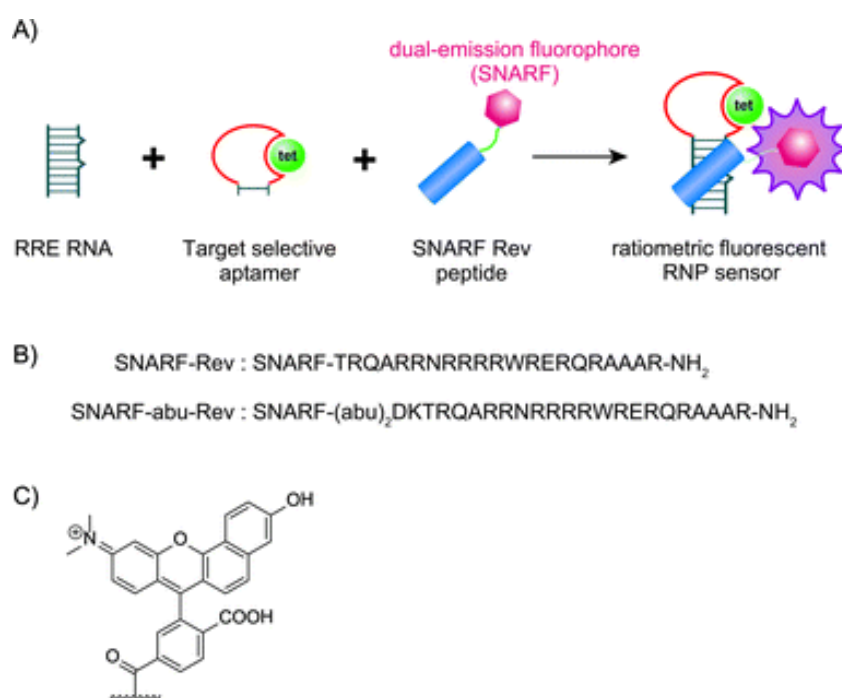


Figure 2-1. (A) Schematic illustration of a ratiometric fluorescent sensor based on RNP. (B) Amino acid sequences of the SNARF-Rev derivatives used in this study. (C) Structure of SNARF (6-isomer).

2-2-2. Construction of a ratiometric fluorescent RNP sensor for tetracycline

As a first trial, tetracycline selective ratiometric fluorescent sensors were designed. Tetracycline is an antibiotic that inhibits prokaryotic translation by interfering with binding of the aminoacyl-tRNA to the ribosomal A-site. It is a widely used therapeutic agent of low toxicity, active against most common pathogens. It has also been used at sub-therapeutic levels in animal feed to stimulate weight gain, as well as for prophylactic disease control.^{22,23} To construct a tetracycline-binding RNA receptor module of RNP, tetracycline binding aptamer cb28, isolated by Berens *et al.*,²⁴ via *in vitro* selection, was fused to RRE RNA (tet-RRE) (Figure 2-2B).

7-methoxycoumarin-3-carboxy acid (7mC) and 5-carboxyfluorescein (5FAM) are the fluorophores that have been used in previous RNP sensors.^{10–17} Rev peptides modified by 7mC and 5FAM, 7mC-Rev and 5FAM-Rev, respectively, in Figure 2-3B, were complexed with tet-RRE RNA. The RNPs were evaluated by fluorescent titration experiments with tetracycline (Figure 2-3C).

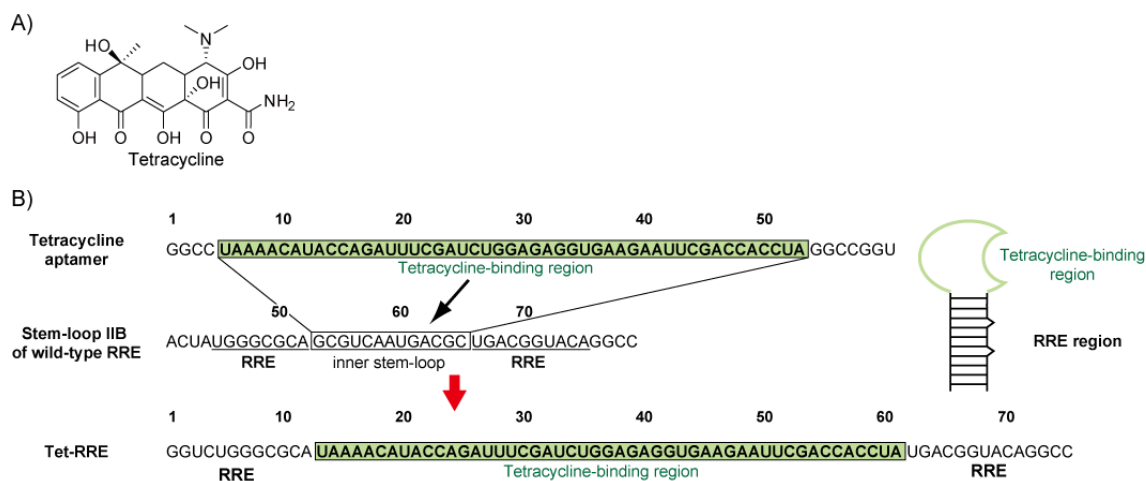


Figure 2-2. (A) Structure of tetracycline. (B) Construction of RNA subunit of RNP receptor by utilizing reported tetracycline aptamer. Nucleotide sequences of the tetracycline aptamer, RRE RNA, and modular tetracycline-binding RNP (tet-RRE RNA) were shown. The tetracycline aptamer fused to the RRE sequence (tet-RRE) was complexed with 6-carboxy-SNARF-modified Rev (SNARF-Rev and SNARF-abu-Rev).

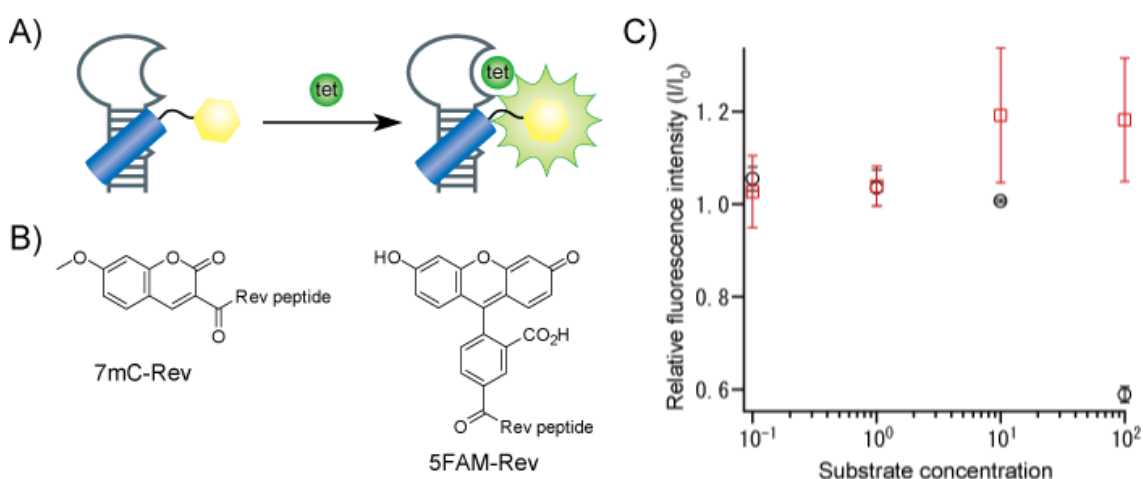


Figure 2-3. (A) Schematic illustration of substrate detection by a fluorescent RNP sensor. (B) Structure of 7mC-Rev and 5FAM-Rev. (C) Fluorescence titration plots of the emission intensity of tet-RRE/7mC-Rev (black circle) and tet-RRE/5FAM-Rev (red square) vs tetracycline.

Due to existence of the absorption of tetracycline around 355 nm, 7mC-Rev (Ex. 355nm, Em. 455 nm) was not suitable as a reporter module of RNP sensor for tetracycline. When the concentration of tetracycline was increased to 100 μ M, the fluorescent intensity was suddenly decreased (Figure 2-3c), possibly due to the shadowing in the excitation path by the strong absorption of tetracycline. The RNP tet-RRE/5FAM-Rev (Ex. 485 nm, Em. 535 nm) was capable to use to this concentration range, however, the reliability of its fluorescence signal was insubstantial because of its low fluorescence intensity. These results indicate that the combination of target substrate and reporter module is an important subject for the fluorescence sensors.

Therefore, I applied SNARF as a reporter group. SNARF modified Rev peptides were then complexed with tet-RRE to provide fluorescent RNPs, tet-RRE/SNARF-Rev and tet-RRE/SNARF-abu-Rev) In order to confirm applicability of RNP with SNARF, fluorescence spectral changes of tet-RRE/SNARF-Rev in the absence or presence of tetracycline were first evaluated in aqueous buffered conditions (pH 7.6) As shown in Figure 2-4A, a typical ratiometric fluorescence change was observed. A decrease in the emission at 600 nm with increasing tetracycline concentration is due to the acidic phenol form of SNARF, and simultaneous increment of the emission at 645 nm is due to its basic phenolate form (excitation wavelength 534 nm). A clear change in the fluorescence color of tet-RRE/SNARF-Rev was detected in the presence of tetracycline (Figure 2-4A, inset). The results indicate that the SNARF-modified RNP receptor responses to the existence of its target substrate.

The sensing capability and selectivity of tet-RRE/SNARF-Rev and tet-RRE/SNARF-abu-Rev were evaluated by fluorescence titration experiments with tetracycline (Figures 2-4C and 2-4D). The emission intensity ratio R ($I_{645\text{ nm}}/I_{600\text{ nm}}$) plot of tet-RRE/SNARF-Rev and tet-RRE/SNARF-abu-Rev showed good saturation behavior (differential $R = 0.7$) and afforded a dissociation constant much lower than 1 μ M (Figures 2-4E and 2-4F). No significant spectral change was observed when tet-RRE/SNARF-Rev was titrated by streptomycin or chloramphenicol. These results indicate that the ratiometric fluorescence change successfully read out the binding of tetracycline to tet-RRE/SNARF-Rev with substrate specificity.

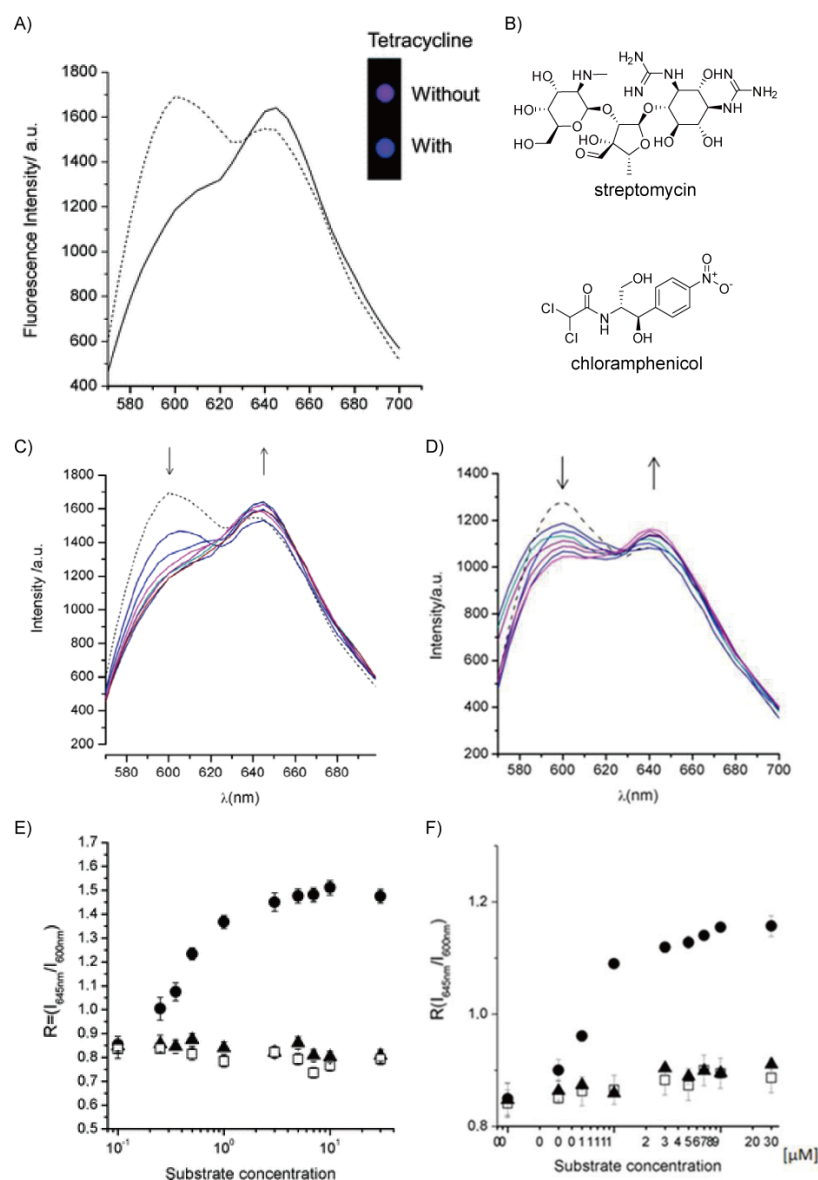


Figure 2-4. (A) Comparison of the fluorescence spectra of tet-RRE/SNARF-Rev with (solid line) or without (dashed line) tetracycline (30 μM). (inset) Merged fluorescent images of tet-RRE/SNARF-Rev with or without tetracycline (30 μM) filtered at 580 $\pm$ 25 nm (red; acidic phenol form) and 640 $\pm$ 20 nm (blue; basic phenolate form). (B) Structures of streptomycin and chloramphenicol. (C) Fluorescence spectral changes of tet-RRE/SNARF-Rev upon the addition of dopamine (0-300 μM). (D) Fluorescence spectral changes of tet-RRE/SNARF-abu-Rev upon the addition of dopamine (0-300 μM). (E) Fluorescence titration plots of the emission intensity ratio (645 nm/600 nm) of tet-RRE/SNARF-Rev against antibiotics concentrations: tetracycline (black filled circle), streptomycin (black filled triangle), and chloramphenicol (black open square). (F) Fluorescence titration plots of the emission intensity ratio (645 nm / 600 nm) of tet-RRE/SNARF-abu-Rev against antibiotics concentrations: tetracycline (black filled circle),

streptomycin (black filled triangle), and chloramphenicol (black open square).

2-2-3. Mechanistic study of the ratiometric detection

In order to clarify the mechanism of the ratiometric fluorescence change, pH titration of the SNARF unit on tet-RRE/SNARF-Rev in the absence or presence of tetracycline was monitored by its fluorescence intensity changes. The pH-dependent change in the ratio of fluorescence emission for tet-RRE/SNARF-Rev in the presence of tetracycline was larger than that for tet-RRE/SNARF-Rev alone (Figure 2-5).

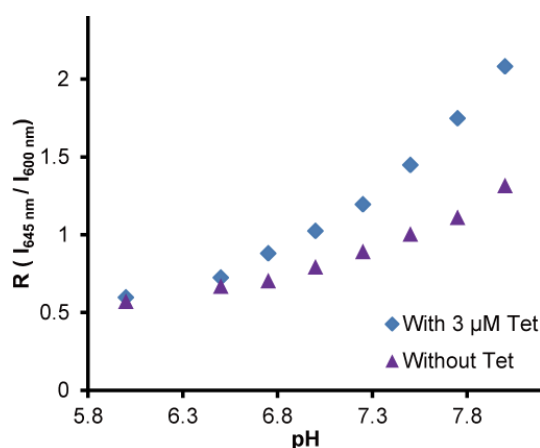


Figure 2-5. A pH titration (pH 6.00 to 8.00) of tet-RRE/SNARF-Rev RNP in the absence (filled purple triangle) or presence (filled blue diamond) of tetracycline (3 μ M)

In case above pH titration experiments suggest a pK_a shift of SNARF to acidic pH regions, the results were interpreted that the basic phenolate form of the SNARF unit was stabilized. The ratiometric fluorescent emission change of tet-RRE/SNARF-Rev upon the tetracycline binding is likely ascribed to the pK_a shift of the SNARF unit as a result of the micro-environmental change of SNARF, which is induced by the conformational change of RNA upon tetracycline binding.^{21,24} Another interesting consideration comes from the observation of the better response of tet-RRE/SNARF-Rev RNP compared to Tet-RRE/SNARF-abu-Rev, in which the linker was attached between SNARF and the Rev peptide (Figures 2-4E and 2-4F). The result indicates that the proximity of SNARF to the binding pocket is an important factor for the efficiency of these sensors.

2-2-4. Construction of a ratiometric fluorescent RNP sensor for dopamine

The applicability of the design strategy of modular fluorescent RNP was further tested by using dopamine aptamers DHc65 and DH05 previously isolated by the Morii group (Figures 2-6A and 2-6B).¹³ Because dopamine plays important role in brain,²⁵ it is of particular interest to prepare a suitable analytical detecting tool that would provide a clear understanding of the relationship between the function and the structure of each biologically active catecholamine.¹³ Both DHc65-RRE/SNARF-Rev and DH05-RRE/SNARF-Rev complexes showed changes in the fluorescence intensity ratio R ($I_{645\text{ nm}}/I_{600\text{ nm}}$) upon addition of dopamine (Figures 2-6E and 2-7D).

The emission intensity of the basic phenolate form (645 nm) increased concomitantly with a decrease in the emission intensity of the acidic phenol form at 600 nm (differential $R = 0.1$) (Figures 2-6B, 2-6C, 2-7B and 2-7C). These results indicate that the modular RNP constructed from the induced fit-type aptamer¹⁶ and the fluorescent RRE/Rev module successfully transduces the dopamine-binding event into measurable fluorescence signals. Each catecholamine has a distinct structure of a benzene ring with two hydroxyl groups and a terminal aminoethyl group. The selectivity of DHc65-RRE/SNARF-Rev and DH05-RRE/SNARF-Rev for dopamine against other catecholamine derivatives was also evaluated to ask a question of whether the subtle difference in their structures could be selectively recognized by SNARF-RNP sensors (Figures 2-6E and 2-7D). The dissociation constants for the complexes of DHc65-RRE/SNARF-Rev and DH05-RRE/SNARF-Rev with dopamine and the catecholamine derivative norepinephrine were obtained from the fluorescence titration and summarized in Table 2.

The affinity of both RNPs to tyrosine was much lower than that to dopamine. However, both DHc65-RRE/SNARF-Rev and DH05-RRE/SNARF-Rev showed marginal discrimination of dopamine against norepinephrine, as observed for the parent RNP.¹³ This was also the case for SNARF-abu-Rev containing RNPs (Figures 2-6F and 2-7E).

These results support that the conversion of the RNP receptor into ratiometric fluorescent RNP sensor maintains the affinity and selectivity of parent RNP.

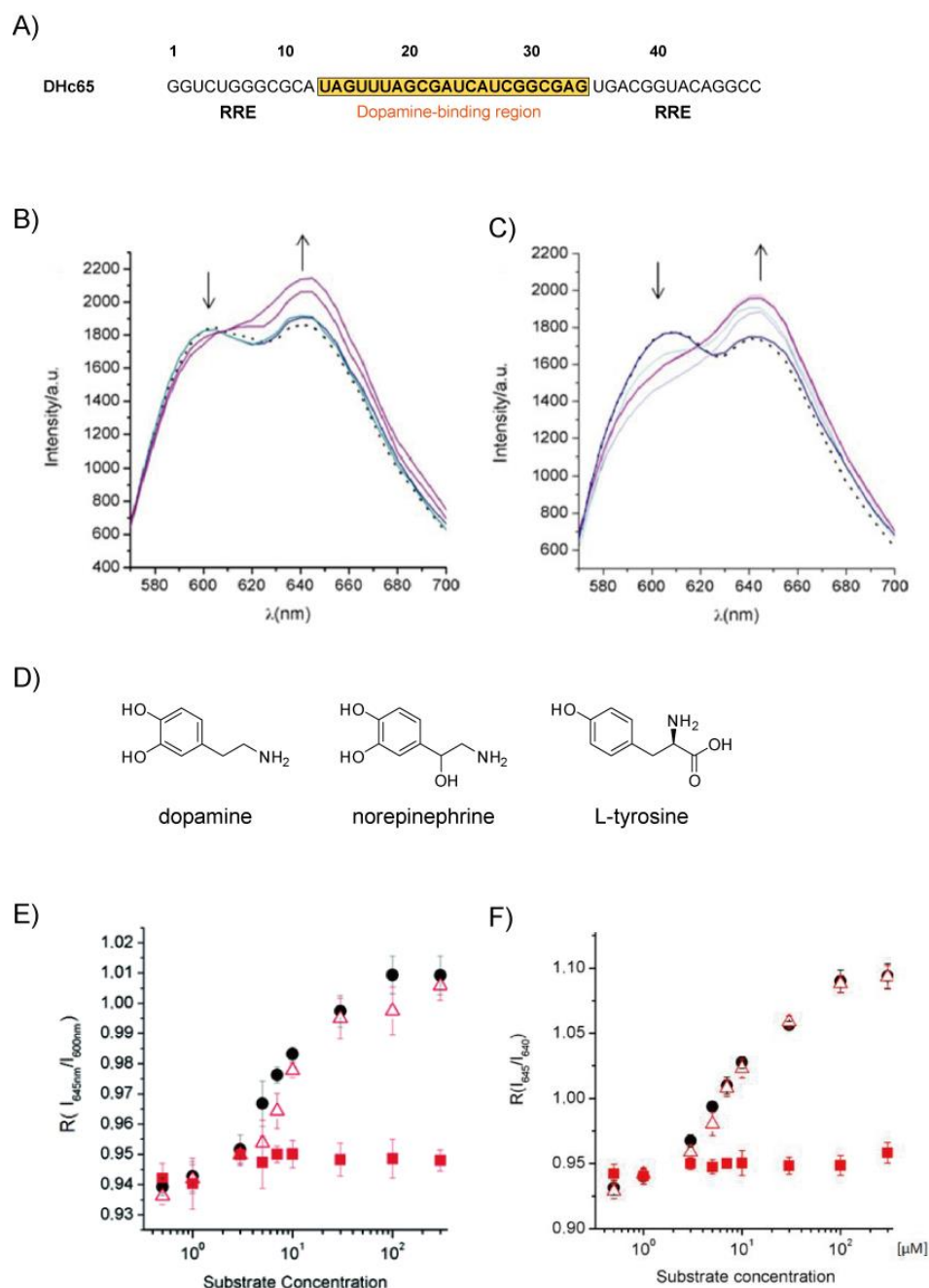


Figure 2-6. (A) Nucleotide sequence of DHc65.¹³ (B) Progressive fluorescence spectral changes of DHc65-RRE/SNARF-Rev upon the addition of dopamine (0-300 μ M). (C) Progressive fluorescence spectral changes of DHc65-RRE/SNARF-abu-Rev upon the addition of dopamine (0-300 μ M). (D) Structures of dopamine, norepinephrine and L-tyrosine. (E) Fluorescence titration plots of the emission intensity ratio (645 nm / 600 nm) for DHc65-RRE/SNARF-Rev with dopamine (filled black circle), norepinephrine (open red triangle), and L-tyrosine (filled red square). (F) Fluorescence titration plots of the emission intensity ratio (645 nm / 600 nm) for DHc65-RRE/SNARF-abu-Rev with dopamine (filled black circle), norepinephrine (open red

triangle), and L-tyrosine (filled red square).

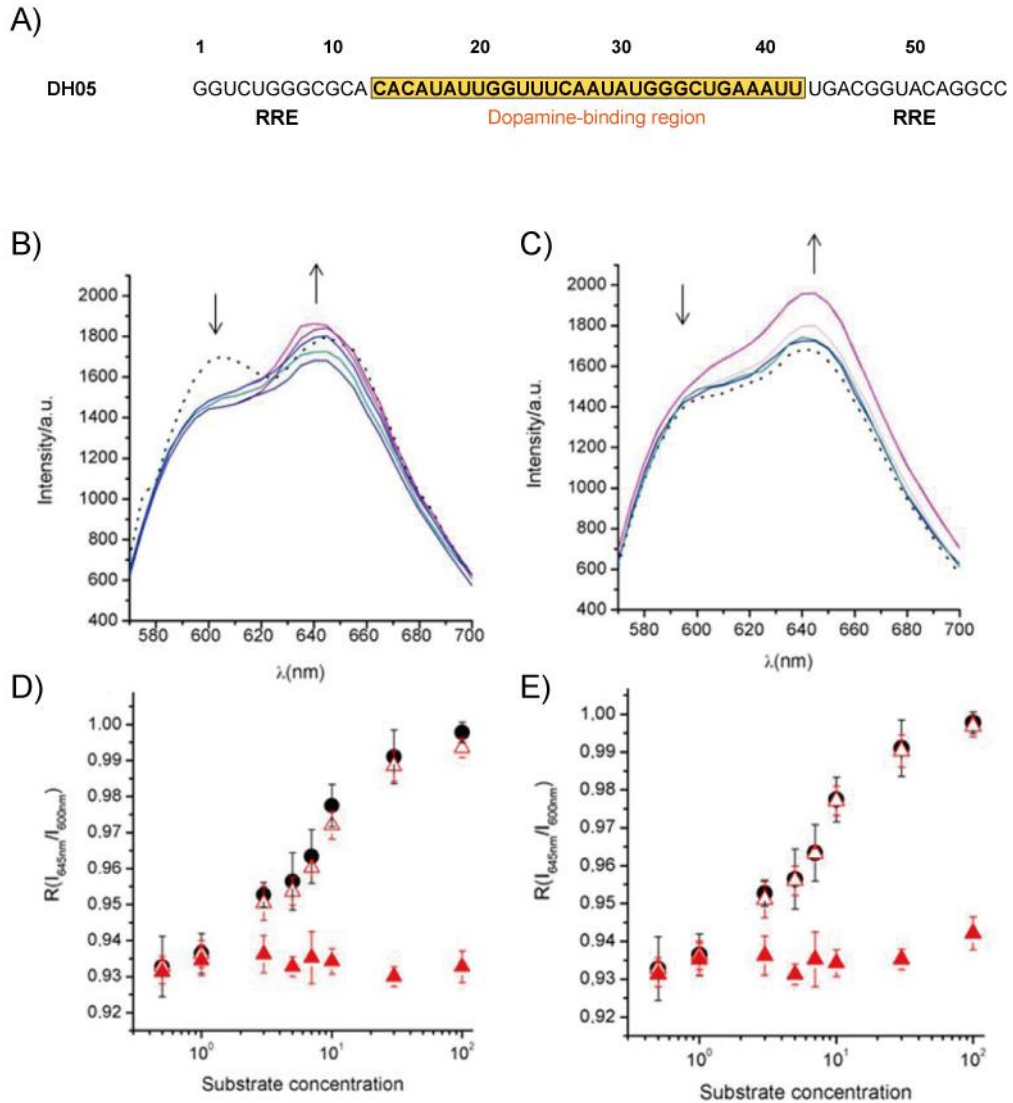


Figure 2-7. (A) Nucleotide sequence of DH05.¹³ (B) Progressive fluorescence spectral changes of DH05-RRE/SNARF-Rev upon the addition of dopamine (0-300 μ M). (C) Progressive fluorescence spectral changes of DH05-RRE/SNARF-abu-Rev upon the addition of dopamine (0-300 μ M). (D) Fluorescence titration plots of the emission intensity ratio (645 nm / 600 nm) for DH05-RRE/SNARF-Rev with dopamine (filled black circle), norepinephrine (open red triangle), and L-tyrosine (filled red square). (E) Fluorescence titration plots of the emission intensity ratio (645 nm / 600 nm) for DH05-RRE/SNARF-abu-Rev with dopamine (filled black circle), norepinephrine (open red triangle), and L-tyrosine (filled red square).

Table 2. Equilibrium dissociation constants K_D (μM) of dopamine-binding RNPs

Ligand	K_D (μM)			
	DHc65		DH05	
	SNARF-Rev	SNARF-abu-Rev	SNARF-Rev	SNARF-abu-Rev
Dopamine	1.1	1.2	1.1	1.5
Norepinephrine	1.9	1.3	2.0	1.5
Tyrosine	>100	>100	>100	>100

2-2-5. Construction of a ratiometric fluorescent RNP sensor for streptomycin

The modular RNP structure was also applied to construct a ratiometric fluorescent sensor for streptomycin by using streptomycin aptamer15.²⁶ SNARF-Rev peptides were complexed with streptomycin aptamer15-modified RRE (str-RRE) (Figure 2-8). As observed for the tetracycline detection by tet-RRE/SNARF-Rev, str-RRE/SNARF-Rev showed spectral changes upon addition of streptomycin that were characterized by a decrease of the emission derived from the phenol form at 600 nm and a simultaneous increase of the emission derived from the phenolate form at 645 nm with an isosbestic point at 625 nm (Figure 2-9).

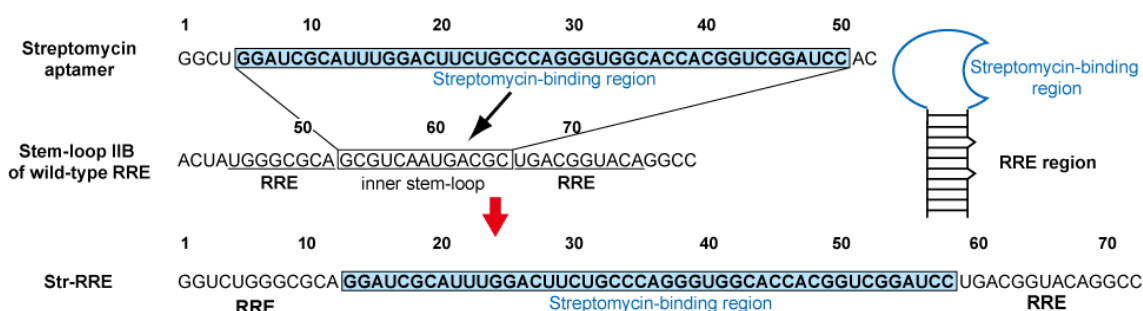


Figure 2-8. Nucleotide sequences of the streptomycin aptamer, RRE RNA, and the streptomycin aptamer fused to the RRE (str-RRE RNA) of the streptomycin-binding modular RNP. The RNA subunit str-RRE was complexed with the 6-carboxy-SNARF-modified Rev peptides SNARF-Rev and SNARF-abu-Rev.

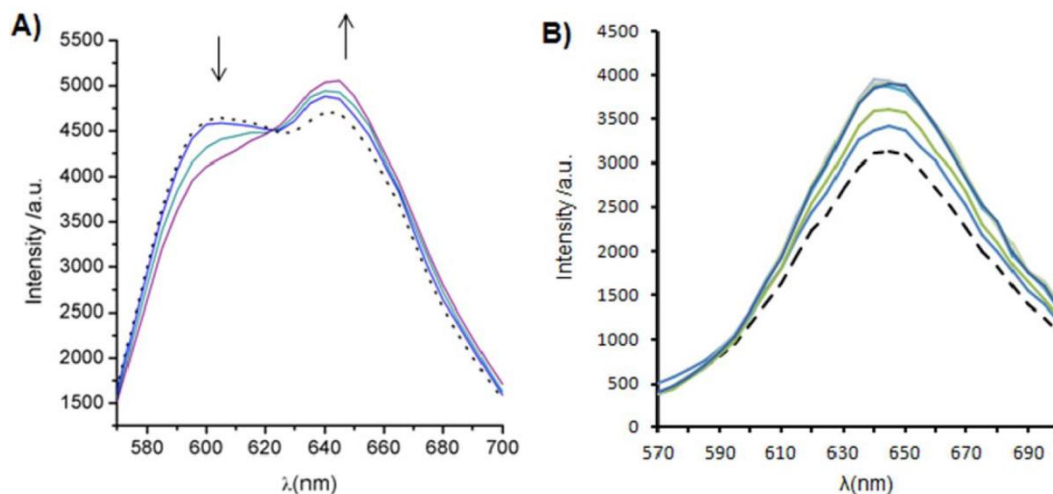


Figure 2-9. A) Progressive fluorescence spectral changes of str-RRE/SNARF-Rev upon the addition of streptomycin (0-300 μ M). B) Progressive fluorescence spectral changes of str-RRE/SNARF-abu-Rev upon the addition of streptomycin (0-300 μ M).

It is likely that a micro-environmental pH change around the fluorophore, which is caused by structural changes in the receptor module upon substrate binding, is a prerequisite for effective communication between the substrate binding event at the receptor module and the fluorescence changes at the reporter module. This notion is strongly supported by the observation that the efficiency and selectivity of detection decrease when a spatial linker is inserted at the fluorophore-labeling position as observed for the fluorescent RNPs formed with the SNARF-abu-Rev peptide.

2-3. Conclusion

I have developed a versatile ratiometric fluorescence sensing system by using a fluorescent RNP complex of an RNA subunit that fuses a target specific aptamer to RRE RNA and a SNARF-modified Rev peptide. The mechanism of the dual emission sensing is most likely ascribable to the pK_a shift of the SNARF chromophore in the reporter module that is induced by a ligand-binding event at the binding pocket of the receptor module. Further tuning of this system would enable the quantitative detection of an analyte not only *in vitro*, but also *in vivo* environment.

2-4. Materials and methods

2-4-1. Materials

Tetracycline, dopamine, norepinephrine and tyrosine were purchased from Sigma Aldrich. Chloramphenicol, gel electrophoresis grade acrylamide and bisacrylamide was provided by Wako Chemicals GmbH. Streptomycin was obtained from Nacalai Tesque. N-Fmoc-protected amino acids, Fmoc-NH-SAL-PEG resin (0.23mmol/g), HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HOBt (1-hydroxybenzotriazole), DIEA (*N,N*-diisopropylethylamine), TFA (trifluoroacetic acid) and distilled DMF (*N,N*-dimethylformamide) were purchased from Watanabe Chemical Industry. All other chemicals were reagent grade and used without further purification.

2-4-2. Nucleic acids preparation

The double-stranded DNA templates for RNA transcription were constructed by PCR amplification to add the promoter for T7 RNA polymerase using PrimeSTAR DNA polymerase (TaKaRa) with 5'-DNA primer (5'-TCTAATACGACTCACTATAGGTCTGGGCGCA-3': T7 RNA promoter is underlined) and 3'-DNA (5'-GGCCTGTACCGTC-3'). RNA transcription was performed using an AmpliScribeT7 kit (Epicenter) for 3 h at 37 °C, according to the supplier's recommended protocols. The resulting RNA was phenol/chloroform extracted, precipitated with ethanol, and pelleted by centrifugation. The RNA was purified by denaturing polyacrylamide gel electrophoresis and eluted. Concentrations of RNAs were determined by UV spectroscopy.

2-4-3. SNARF-Rev and SNARF-Abu-Rev preparation

The procedures for the synthesis of fluorophore-labeled Rev peptides were previously reported.¹⁰ Briefly, the two Rev peptides SNARF-Rev and SNARF-Abu-Rev were synthesized on a Shimadzu PSSM-8 peptide synthesizer according to the Fmoc chemistry protocols. For SNARF-Abu-Rev two residues of γ -aminobutyric acid were manually attached to the N-terminus of the peptide by using HOBt, HBTU as activators, and DIEA as base. SNARF was synthesized according to the previous report.²⁷ SNARF was directly coupled to the N-terminal deprotected residue of Rev peptide on the resin by succinimidyl ester. SNARF-Rev and SNARF-Abu-Rev were then deprotected, cleaved from the resin and purified by using RP-HPLC. Purified SNARF-Rev and SNARF-Abu-Rev were characterized by MALDI-TOF MS spectrometry (AXIMA-LNR, Shimadzu Biotech) [6-carboxy-SNARF-Rev+H]⁺ calc. m/z 3314.12 obs. m/z 3313.35; [6-carboxy-SNARF-abu-Rev+H]⁺ calc. m/z 3729.38 obs. m/z 3728.23.

2-4-4. Fluorescence measurements on the microplate

The 96-well fluorescence measurements were performed on an Infinite M200PRO instrument (TECAN co. ltd.). For tetracycline titration, a binding solution (100 μ l) containing 1 μ M of fluorescent RNP in 10mM Tris HCl (pH 7.0), 250 mM NaCl, 5 mM MgCl₂, 0.005% Tween 20 was prepared. For dopamine and other catecholamine derivatives, a binding solution (100 μ l) containing 1 μ M fluorescent RNP in 10mM Tris HCl (pH 7.60), 300 mM NaCl, 5 mM MgCl₂, 0.005% Tween 20 was prepared. For streptomycin, a binding solution (100 μ l) of 1 μ M concentration of fluorescent RNP was prepared in 50mM Tris-HCl (pH 7.6), 250 mM NaCl, 10 mM MgCl₂, 0.005% Tween 20 buffer conditions. Well-mixed samples with different concentrations of substrates were incubated at 25 °C for 30 min followed by the measurement of emission spectra (measured emission spectrum range: 570-700 nm; excitation wavelength utilized: 534 nm).

2-4-5. Determination of equilibrium dissociation constants

An equilibrium dissociation constant for the complex of fluorescent RNP and its substrate was obtained from the following equation:

$$F_{\text{obs}} = \frac{A([RNP]_T + [\text{substrate}]_T + K_D)([RNP]_T + [\text{substrate}]_T + K_D)^2 - (4[RNP]_T [\text{substrate}]_T)^{1/2}}{2[RNP]_T}$$

Where A is the increase in fluorescence at the saturating substrate concentration ($R_{\text{sat}}-R_0$), K_D is an equilibrium dissociation constant, $[RNP]_T$ and $[\text{substrate}]_T$ are the total concentrations of RNP and the substrate, respectively. Titration of DHc65-RRE/SNARF-Rev by dopamine provided an I/I_0 value of 0.85 and a K_D of 1.1 μ M. Titration by norepinephrine provided a K_D value of 2.0 μ M. Titration of DH05-RRE/SNARF-Rev by dopamine and norepinephrine provided K_D values of 1.1 μ M for dopamine and 1.9 μ M for norepinephrine. These values are consistent with the results previously reported.¹³

2-4-6. Fluorescence pH titration

The 96-well fluorescence measurements were performed on an Infinite M200 PRO instrument (TECAN co. ltd.). Samples were prepared in buffers containing 10mM Tris HCl, acetate, and Mes to adjust the pH of buffers to 6.00, 6.25, 6.50, 6.75, 7.00, 7.25, 7.50, 7.75, and 8.00 at 25 °C. To the buffer solution containing 1 μ M of fluorescent RNP, 250 mM NaCl, 5 mM MgCl₂, and 0.005% Tween 20, 3 μ M tetracycline was added. Well-mixed samples at different pH in the presence and in the absence of tetracycline were incubated at 25 °C for 30 min followed by the measurement of emission spectra (measured emission spectrum range: 570-700 nm; excitation

wavelength: 534 nm).

2-4-7. Fluorescent image evaluation

Fluorescent images were taken in the presence and in the absence of tetracycline using Pharos FX Imager. Buffers with a variety of pH (6.00, 6.25, 6.50, 6.75, 7.00, 7.25, 7.50, 7.75, and 8.00) were utilized (adjusted by Tris HCl, acetate, or Mes). A buffered solution (10 μ l) at the indicated pH containing 1 μ M RNP, 250 mM NaCl, 5 mM MgCl₂, and 0.005% Tween 20 in a glass plate (S117814, Matsunami glass int. ltd.) was incubated for 30 min at room temperature, then scanned (excitation wavelength 534 nm; filters at 580 ± 25 nm and 640 ± 20 nm) by the imager.

2-5. References

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Chapter 3

Screening of fluorescent RNP sensors by using fluorophore library and construction of covalently linked RNP sensors

3-1. Introduction

Fluorescent biosensors that allow the specific detection and quantification of a wide range of target molecules are important tools in the fields of therapeutics and diagnostics.¹⁻⁴ The preparation of receptor-based fluorescent sensors is an efficient strategy to successfully provided various biosensors.⁵⁻⁸ In general, biosensors consist of a substrate-binding pocket and a reporter molecule. The binding pocket for the substrate usually determines the affinity and the selectivity of the sensor, and the reporter molecule is responsible for converting the ligand-binding event to optical signals. Naturally occurring receptors have been used for the substrate-binding pocket of fluorescent sensors.⁵⁻⁸ However, an appropriate receptor for a given target molecule does not necessarily exists, and the chemical modifications or genetic mutations of receptors with reporter molecules introduce the possibility of the loss of original function of the receptor. The rational design of a receptor with a desired molecular recognition is still a challenging task, and laborious trial and error experimentation is needed to achieve and optimize sensors with the function of interest. Therefore, an alternative convenient method is required to construct receptor-based fluorescent sensors for specific targeted molecules. Combinatorial methods that require the construction of specificity, diversity library and a screening system according to the intended purpose is an effective method to avoid these problems.

Morii, *et al.* have constructed receptors for targets of interest from ribonucleopeptide (RNP) complexes⁹ by applying the *in vitro* selection method.^{10,11} Because RNP is a noncovalent complex of RNA and peptide, the complex formation of RNP is conveniently achieved by mixing an RNA and a peptide subunits in aqueous solution. The RNP library with structural diversity was constructed by the introduction of randomized sequences into the RNA subunit of the Rev-RRE (Rev Response Element) complex, the three-dimensional structure of which has been established,¹² and RNP receptors that can specifically bind to the targeted small molecules were selected from this RNA-oriented RNP library by applying an *in vitro* selection method. Furthermore, the fluorescent sensors that exert the fluorescence intensity changes upon binding the substrate can be produced by the modification of the N-terminal of the peptide subunit of the RNP receptor with fluorophores.¹³⁻¹⁷ The RNP receptors selected by *in vitro* selection include various RNA subunits with different affinities for the substrate and different overall structures (Figure 3-1A). Fluorophore-modified RNP (F-RNP) library was produced by complexing the RNA subunit library and a Rev peptide modified by a fluorophore of interest. The RNP complex

that showed the highest relative fluorescence intensity was selected as the optimal sensor for use with the fluorophore at the particular target detection wavelength (Figure 3-1B).¹³ Screening of the different combinations of RNA subunits having various affinities for the target molecule with the fluorophore-modified Rev peptide allowed various RNP sensor candidates with different dynamic ranges and fluorescent responses.

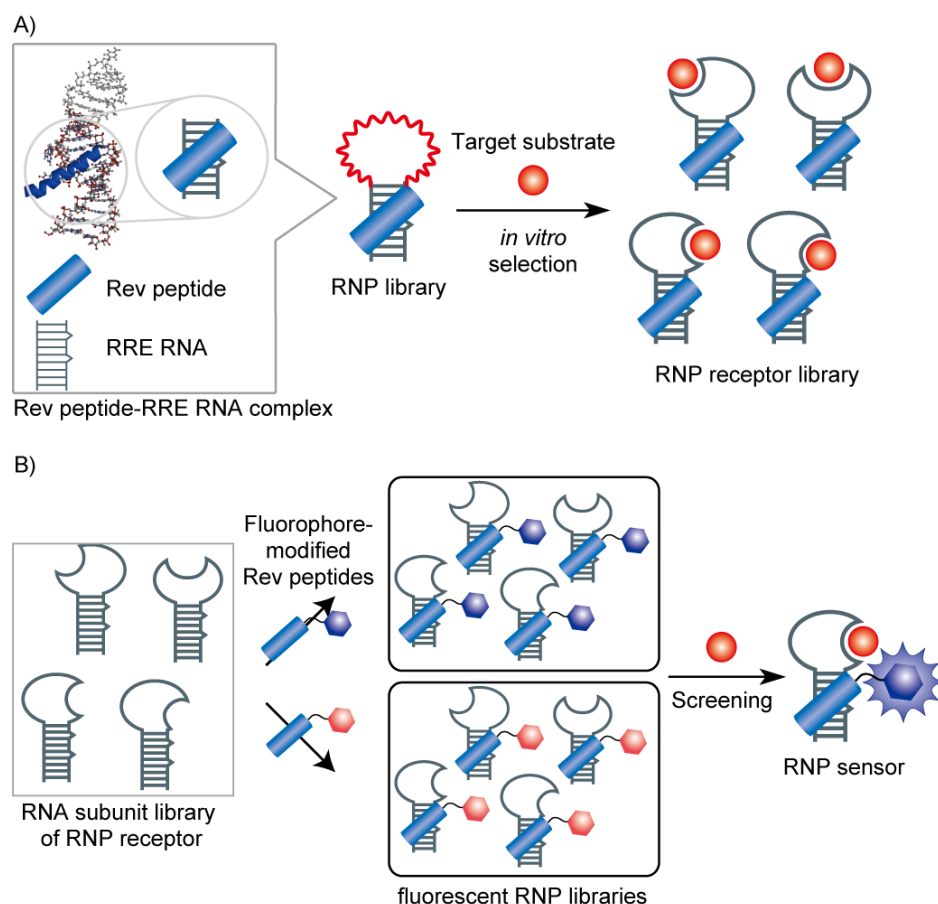


Figure 3-1. Schematic illustration of the stepwise functionalization of RNP.¹³ (A) *In vitro* selection based on the selective binding to target was applied to an RNP library consisting of the Rev peptide and a library of RRE RNAs containing a randomized region. RNP receptors with various RNA sequences and affinity for the target substrate were isolated and characterized. (B) In the second step, a high-through-put selection based on fluorescence response to the target was applied to a F-RNP library. Various RNA subunits derived from the RNP receptors and fluorophore-modified Rev peptides afford F-RNPs by only mixing the two subunits based on the specific noncovalent interaction between them. Among the F-RNPs, ones showed high relative fluorescence intensity changes on substrate binding were selected as RNP sensors.

However, a specific RNP receptor with properties of interest, such as the selectivity and affinity to the target, does not always show the high relative fluorescence intensity change. When the number of RNA subunit library is limited, an F-RNP receptor with the desired functionality may not exist with the fluorophores applied. Therefore, to improve the versatility of this method, a novel strategy is needed, where the diversity of F-RNP is not solely dependent on the RNA library but also on a fluorophore-modified peptide library.

In this study, I first established a method for construction of an F-RNP library with a specific RNA derived from an RNP receptor by using a fluorophore library (Figure 3-2). A convenient method for modification of the RNP receptor with a variety of fluorophores was needed for the practical screening of sensors. Multiple F-RNP sensors showing high relative fluorescence intensity changes would be obtained from a F-RNP library that includes various types of fluorophores by measuring the actual fluorescence intensity in the presence and absence of the substrate on a microplate in the high-through-put manner.

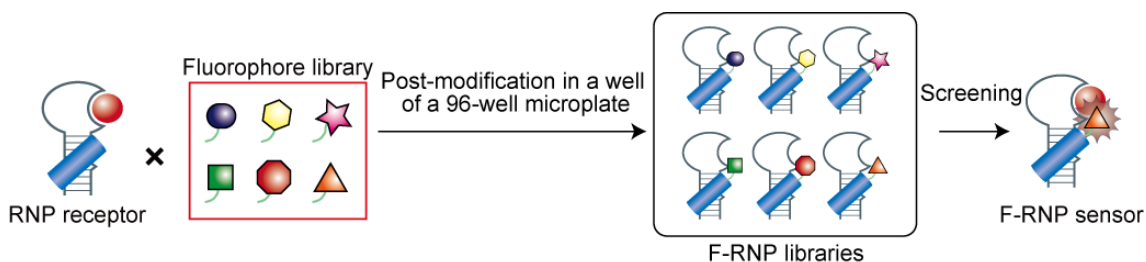


Figure 3-2. Schematic illustrations of a F-RNP library and a screening strategy used to obtain specific and functional fluorescent biosensors.

The noncovalent RNP complex is an ideal scaffold to construct a library of diverse F-RNPs. However, the noncovalent complex formation of the RNP sensors becomes a disadvantage for practical measurements, when one would like to reduce the sensor concentration or to apply multiple sensors in the same solution. The covalent linking strategy would allow a development of F-RNP sensors for detection with low concentration of sensors and/or a simultaneous detection of multiple ligands by monitoring each wavelength corresponding to the respective ligand.

I developed a synthetic method of stable F-RNP sensors by covalently linking the RNA and the fluorophore-modified Rev peptide to demonstrate that the covalently linked RNP sensors were capable of detecting the target substrate with low sensor concentrations (Figure 3-3).

These two methods will expand the application of F-RNP sensors with desired target detection characteristics.

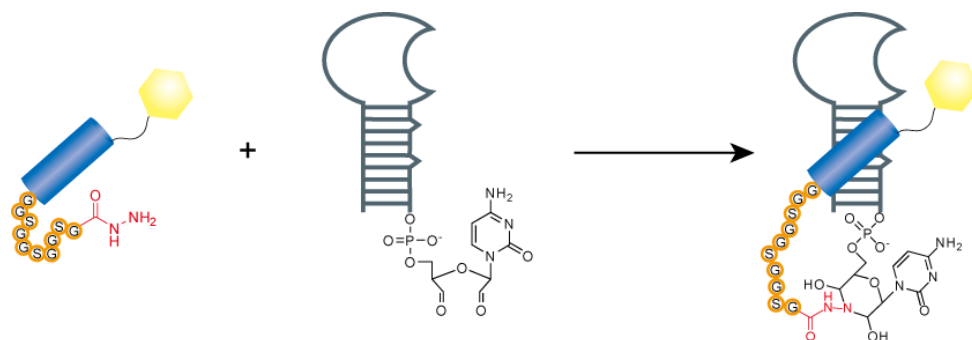


Figure 3-3. Schematic illustration of the construction of a covalently linked F-RNP sensor. The covalent complex consisting of a fluorophore-modified peptide subunit with hydrazine linker at its C-terminal and an RNA subunit of the RNP receptor of interest was constructed through a coupling of the RNA and peptide subunit via formation of hydrazone bond.

3-2. Results and discussion

3-2-1. Design and evaluation of the applicability of peptide subunits of the RNP sensors by derivatization of cysteine modified Rev peptide with thiol-reactive fluorophores

In order to prepare F-RNP sensors with given RNA subunits, we prepared a library of fluorophore-modified Rev peptides. Since individual solid-phase synthesis of fluorophore-modified Rev peptides was laborious and impracticable for the preparation of an extensive library, we designed a facile modification method to attach the fluorophore to the peptide subunit. The Rev peptide (TRAQRRNRRRRWRERQRAAAAR) contains no cysteines and, therefore, we introduced a single cysteine residue at the N-terminal as a site for thiol reactive modification. The chloroacetyl (CA) group is a reactive group to form a C-S bond.¹⁸ A library of fluorophores equipped with the CA group should react with a cysteine-modified Rev peptide (CRev) to give a fluorophore-modified C-Rev peptide (F-CRev) library (Figure 3-4).

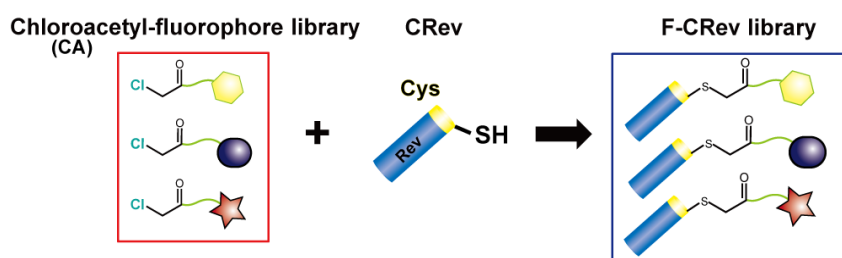


Figure 3-4. Schematic illustration of a construction of fluorophore-modified peptide subunit library

Initially, we prepared 5FAM-CRev (shown in Figure 3-5B) as an F-CRev by using 5-iodoacetamidofluorescein (5-IAF) and CRev. Based on the previous work,¹⁶ the N-terminal of Rev peptide was chosen as the modification site for introduction of the fluorophores to ensure that the fluorescent intensity change would be caused by the structural change of the RNA subunit upon binding the substrate. The micro-environmental change of the attached fluorophore strongly affected the response of the RNP sensors. Hence, the location of the cysteine residue and the reaction site of the fluorophore with the cysteine side chain were critical to determining a useful change of fluorescent intensity upon substrate binding. An16 RNA¹⁶ was used as a representative RNA subunit of ATP-binding RNP receptor for evaluation of the applicability of F-CRev for the F-RNP sensor. An RNP consisting of An16 and peptide subunit was known to recognize the adenine base and to bind ATP and adenosine (Ado) with similar equilibrium dissociation constants (K_D) of 1.1 μ M and 2.3 μ M, respectively.¹⁶ A combination of An16 RNA and 5FAM-CRev produced an F-RNP (An16/5FAM-CRev). Titration of the An16/5FAM-CRev complex with the substrate Ado was performed and compared with the K_D value of the previous F-RNP sensor (An16/5FAM-Rev) in order to evaluate the effect of this derivatization method on the relative fluorescence intensity change and substrate affinities of the RNP sensors (Figure 3-5). The K_D values for the complexes of Ado with An16/5FAM-Rev and An16/5FAM-CRev were, 4.6 μ M and 1.0 μ M, respectively (Figures 3-5C and 3-5D). Relative fluorescence intensity changes were almost identical for the two sensors. In order to confirm the generality of the usage of F-CRev, pyrene and coumarin groups derivatized with the thiol reactive group were reacted with CRev (pyrene-CRev and coumarin-CRev, respectively) (Figures 3-5E and 3-5F). Upon formation of the RNP complexes with An16, both F-RNPs showed relative fluorescence intensity change by responding the substrate binding event (Figures 3-5G and 3-5H). The RNP with pyrene-CRev showed a relative fluorescence intensity change to opposite direction, the fluorescence intensity was decreased upon the substrate binding, compared to the previous results of pyrene-modified F-RNP.^{13,14,16}

These results indicated that F-CRev could also act as the peptide subunit of the F-RNP sensor. Furthermore, the affinity of the RNP receptor was not affected in these experiments. Taken together, these results demonstrated that the RNP receptor could be converted to an F-RNP sensor by using F-CRev in a manner similar to that used for F-Rev in the previous research.¹³⁻¹⁷ Thus, I concluded that modification of the cysteine modified Rev peptide would be successfully applied to construct an F-CRev peptide library.

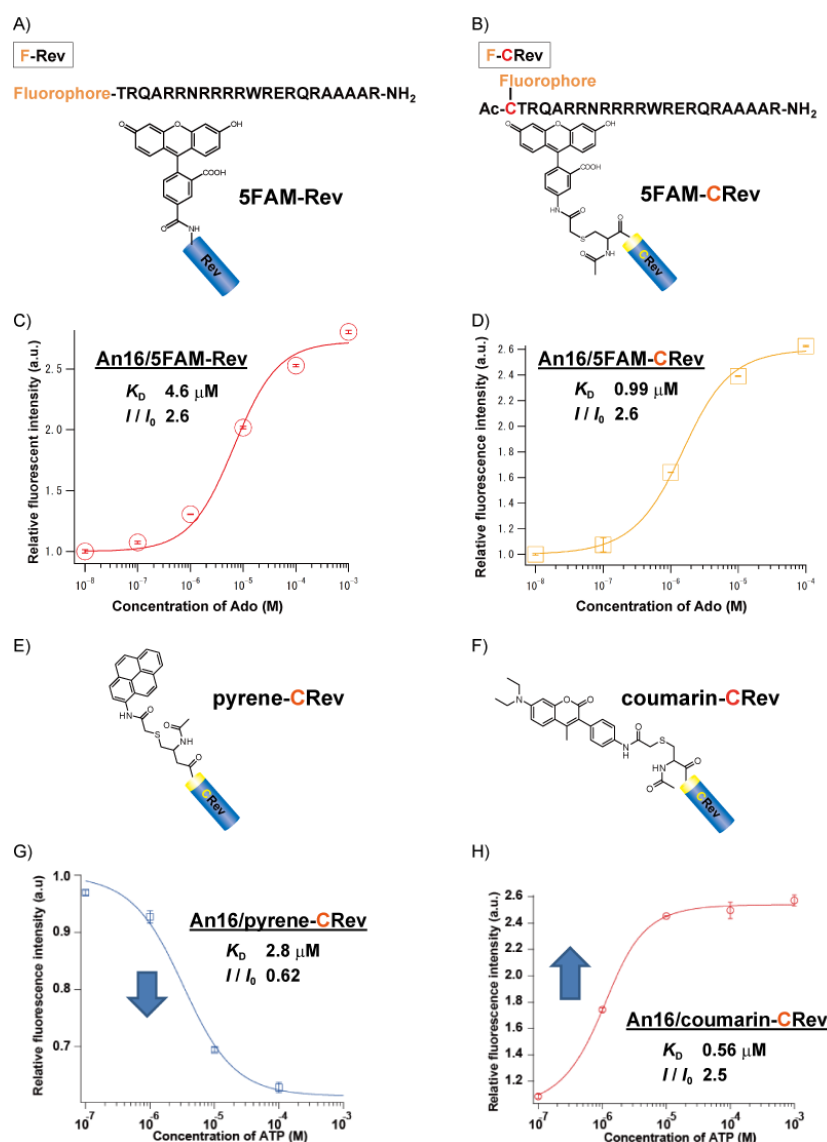


Figure 3-5. (A) An amino acid sequence and an illustrative structure of F-Rev prepared by modification of the N-terminal of Rev peptide with 5-carboxy fluorescein (5FAM). (B) An amino acid sequence and an illustrative structure of F-CRev prepared by modification of cysteine of CRev peptide with 5-IAF. (C) Titration curve for relative fluorescence intensity changes of An16/5FAM-Rev with increasing concentration of Ado in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM MgCl₂, and 0.005% Tween 20 at 4 °C. (D) Titration curve for the relative fluorescence intensity changes of An16/5FAM-CRev under the similar conditions. (E) An illustrative structure of pyrene-CRev. (F) An illustrative structure of coumarin-CRev. (G) Titration curve for relative fluorescence changes of An16/pyrene-CRev. (F) Titration curve for relative fluorescence changes of An16/coumarin-CRev. The dissociation constant (K_D) and the maximum relative fluorescence intensity for the ATP complex of each RNP are shown in the inset.

3-2-2. Optimization of the conditions for modification of CRev peptide with chloroacetylated fluorophores

A model compound, CLACA124 (Figure 3-6A), was first used to optimize the conditions for synthesis of the F-CRev library. I evaluated the reaction yield of F-CRev (CLACA124-CRev) in a buffer containing 10 mM sodium phosphate (pH 8.0) and acetonitrile ranging from 5 to 55% (v/v) by HPLC analysis of the product. Based on the analysis of peak area of chromatogram, the expected covalent bond formation (at a one to one molar ratio of CLACA124 to CRev) was barely observed under these conditions with a CRev concentration ranging from 50 to 200 μ M. The reaction did not proceed even after seven days in the 55% buffer (pH 8.0) and 45% acetonitrile (Figure 3-6B). In order to increase the reactivity for C-S bond formation between the fluorophore and CRev, I investigated iodination of the CA group of the fluorophore to convert to an iodoacetyl (IA) group. CLACA124 and 0.95 equivalent of NaI in 50% acetonitrile and 50% acetone (v/v) were incubated at 50 °C in a well of a 96-well microplate. After two hours, quantitative conversion of CLACA124 to its derivative with the IA group, CLAIA124, was confirmed by disappearance of a peak of CLACA124 in the chromatogram (Figure 3-6C). CLAIA124 was then used to alkylate the thiol group of the cysteine residue of CRev (Figure 3-6D). The iodinated fluorophore was mixed with CRev in 25 mM sodium phosphate buffer (pH 8.3) and 50% acetonitrile, then allowed to react at ambient temperature for four hours; under these conditions a yield of 74% was obtained (Figure 3-6D). These results demonstrated that the formation of F-CRev through the IA derivatized fluorophore, converted from the CA derivative of fluorophore with NaI, could be used to modify the CRev peptide in a 96-well microplate with the total yield of 74%.

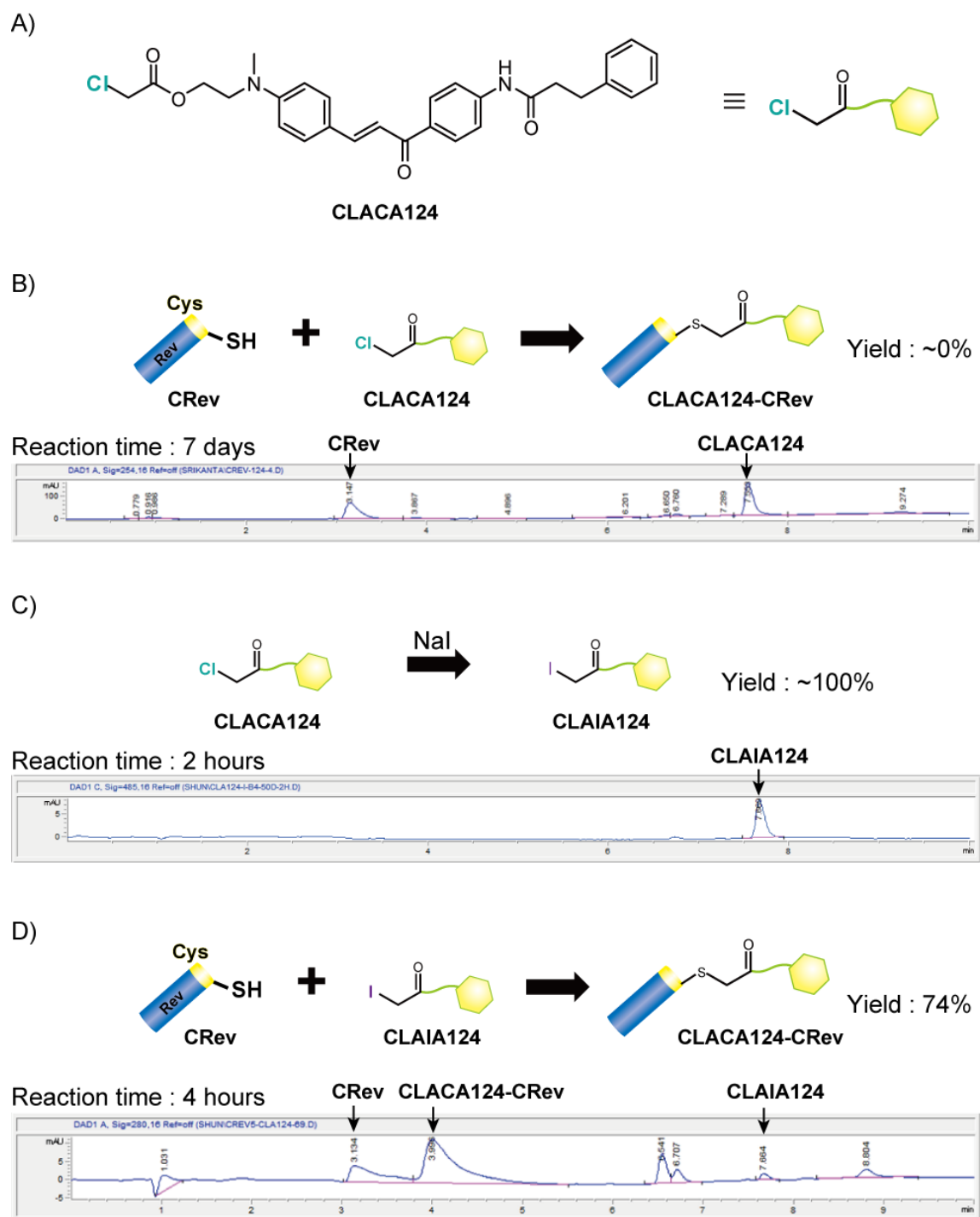
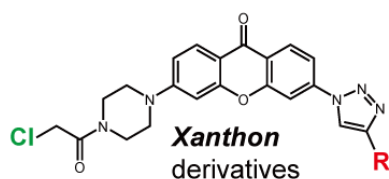
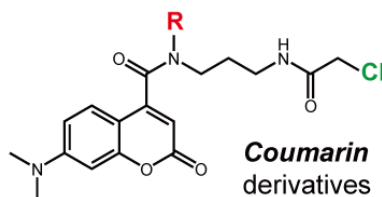


Figure 3-6. (A) Structure of the fluorophore CLACA124. (B) Reaction of CRev and the CA derivatized fluorophore. Chromatogram of the crude reaction mixture at ambient temperature for seven days is shown below. The peaks corresponding to CRev and CLACA124 are indicated by arrows. (C) Iodination of the CA derivatized fluorophore with sodium iodide. (D) Covalent bond formation between CRev and the IA derivatized fluorophore.

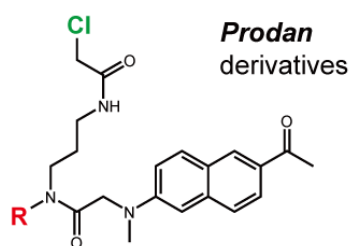
CXCA library



CORCA library



TPGCA library



CyRCA library

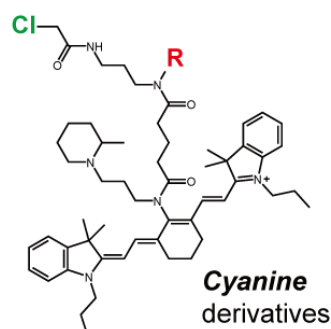


Figure 3-7. Fluorophore libraries constructed from four fluorophore scaffolds: the CXCA library¹⁹ based on xanthone ; the TPGCA library²⁰, CORCA, and CyRCA²¹ libraries based on prodan, coumarin, and cyanine, respectively. R (red) indicates the varied side chains within each library (e.g., building blocks for the CXCA, TPGCA, CORCA and CyRCA libraries are shown in Figures 3-8, 3-9, 3-10 and 3-11, respectively).

Next, I applied the optimized modification conditions to modify the fluorophore libraries derived from four base molecular scaffolds (Figure 3-7) with CRev. All compounds assigned by the position number of the well on the 96 well plates. Iodination and modification by CRev were performed for multiple compounds at once. In a well of a 96-well microplate, yields of the iodination ranged from 50 to 100% (Figure 3-6B) and the C-S bond formation of each scaffold (Figure 3-6D) ranged from 10 to 75%. The F-CRev was roughly purified by an extraction with diethyl ether to remove unreacted fluorophore, then mixed with the RNA subunit of ATP-binding RNP receptor, An16,¹⁶ to form F-RNP. Thus, an F-RNP library having various fluorophores in the peptide subunit could be constructed in a same manner as described for the model fluorophore.

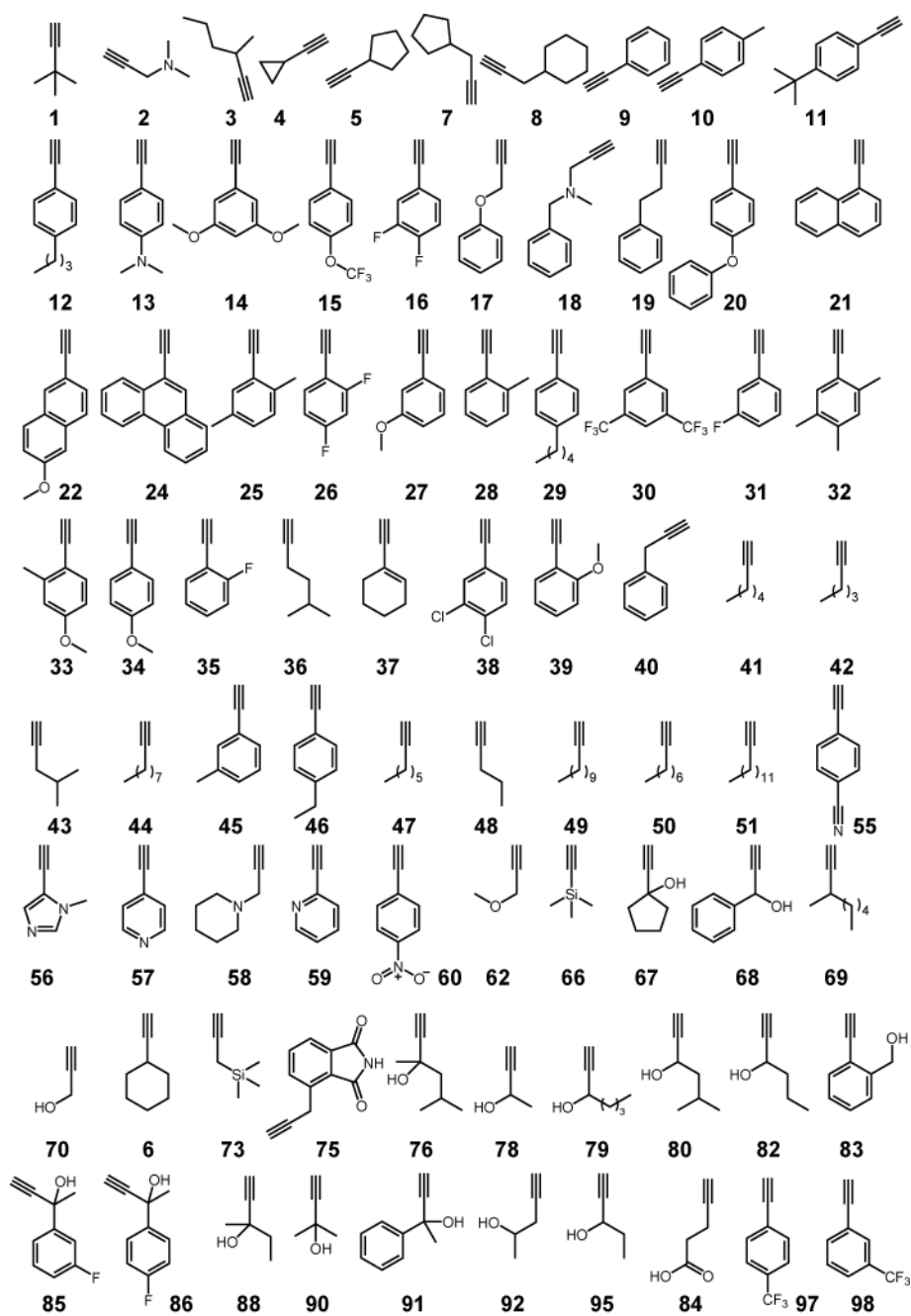


Figure 3-8. Alkyne building blocks for the CXCA library¹⁹

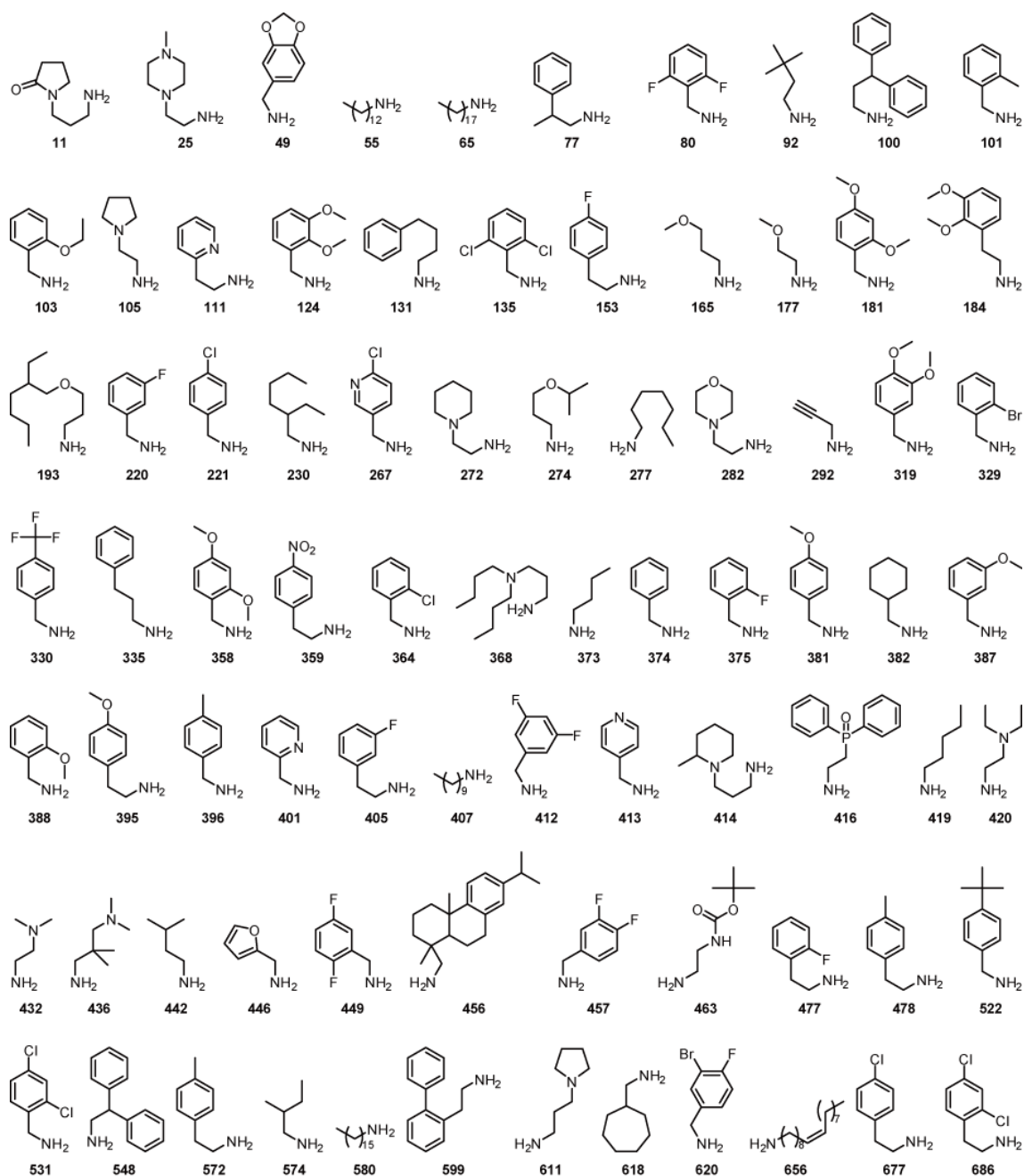


Figure 3-9. Amine building blocks for the TPGCA library²⁰

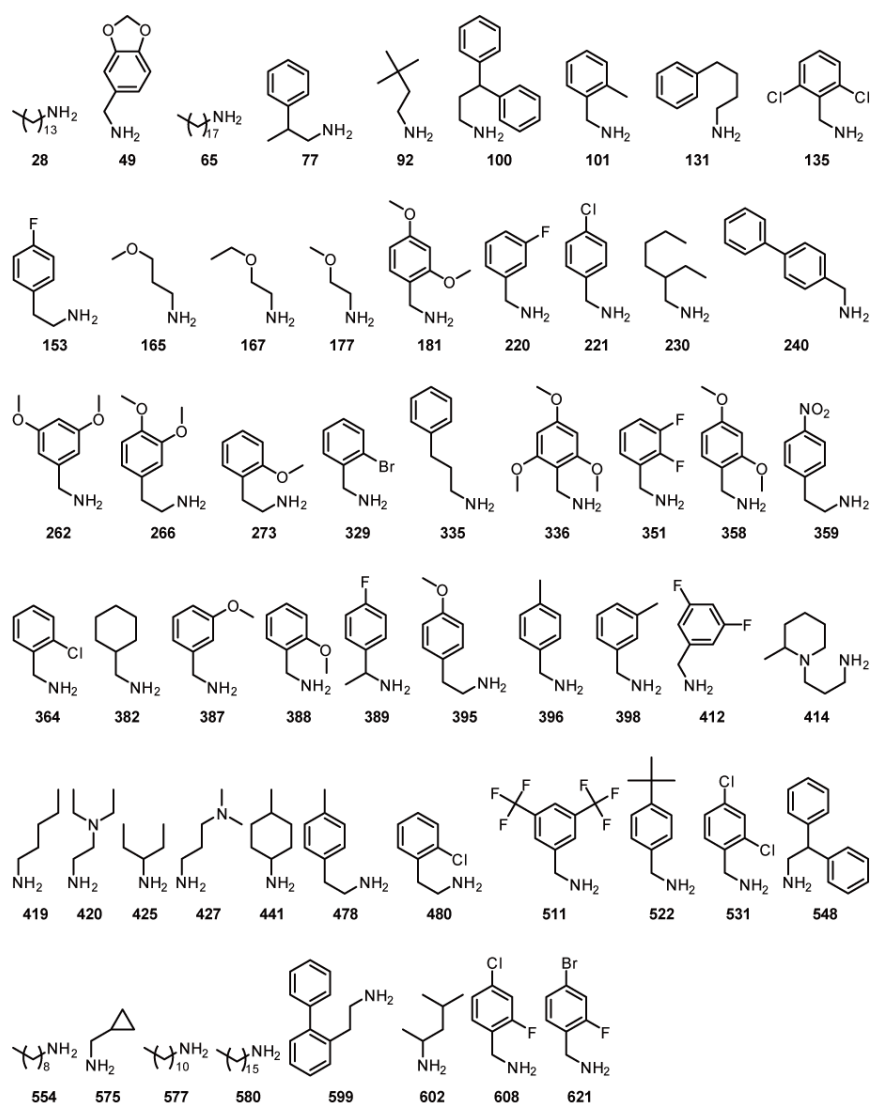


Figure 3-10. Amine building blocks for the CORCA library

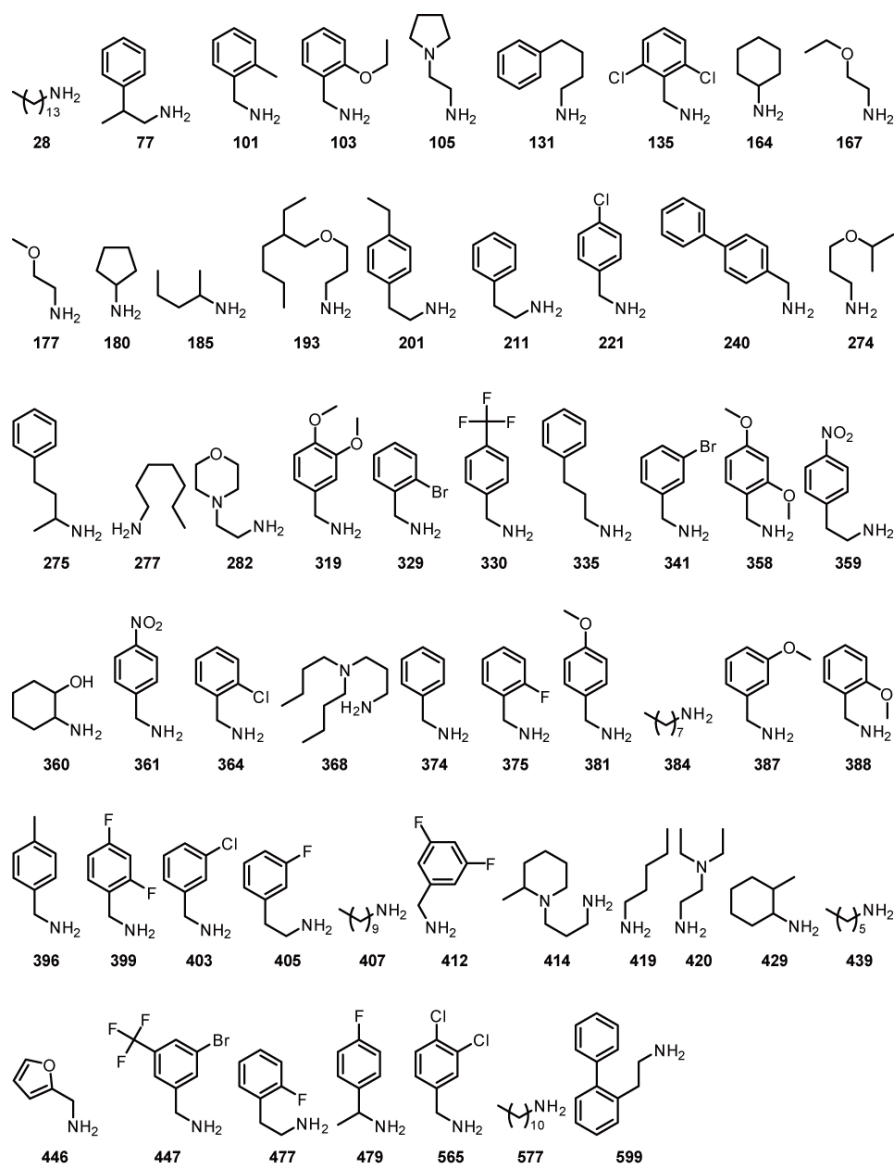


Figure 3-11. Amine building blocks for the CyRCA library²¹

3-2-3. Preliminary screening based on the relative fluorescence intensity change of F-RNP libraries

Preliminary screening was performed with An16 RNA and roughly purified F-CRev peptides to determine conditions suitable for the high-through-put screening. RNA was purified by denaturing PAGE after transcription, and each synthesized peptide was purified by extraction with diethyl ether after modification. In total, 259 F-RNPs were constructed by complexation between An16 RNA and F-CRev. In order to select the F-RNP with the high relative fluorescence intensity change upon binding to the substrate Ado by using a 96-well microplate reader, the fluorescence emission intensity of each F-RNP was determined, with and without Ado, in screening buffer containing 10 mM sodium-phosphate (pH 7.6), 100 mM NaCl and 10 mM MgCl₂. The concentration of Ado was 500 μ M, at which it is expected that the fluorescence intensity has reached its maximum value for original An16 RNP ($K_D \approx 1$ μ M)¹⁶.

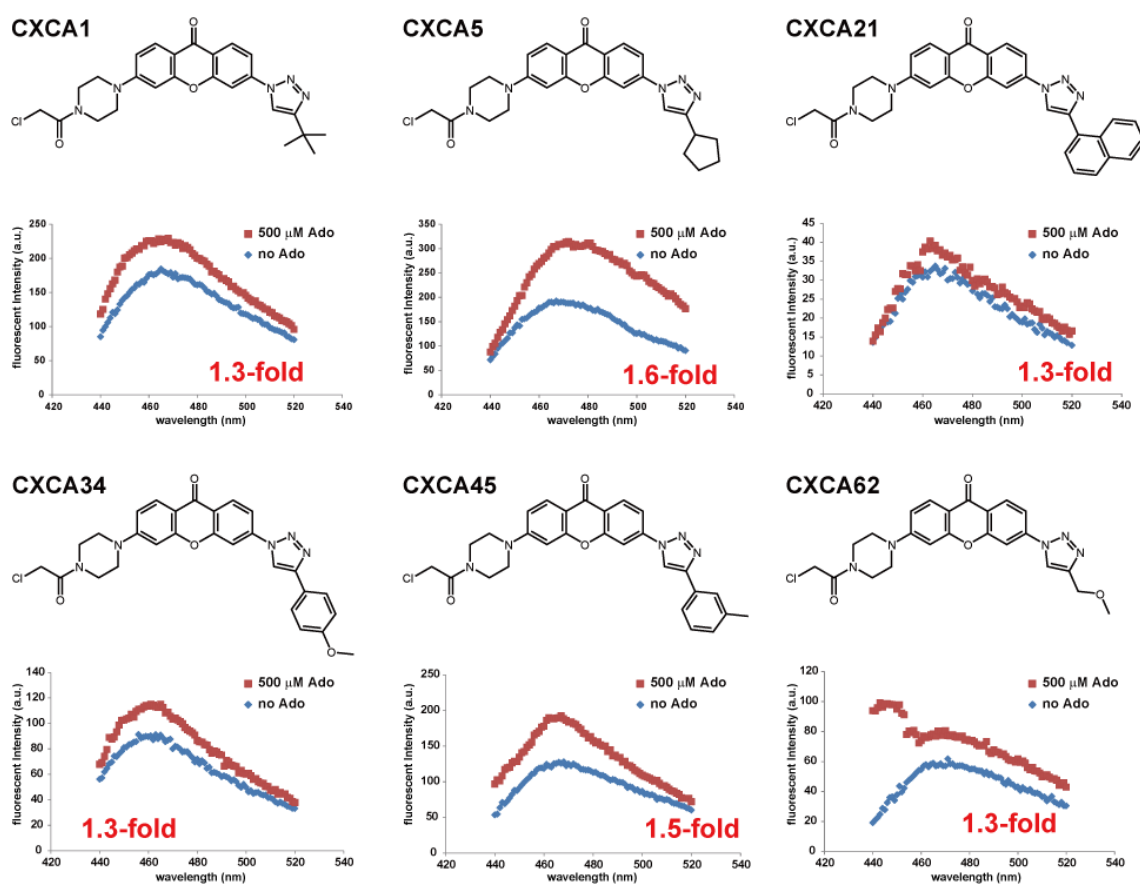


Figure 3-12. Fluorescence spectra (λ_{Ex} = 370 nm) of selected An16/CXCA-modified Rev with (red) and without (blue) Ado; preliminary screening in binding buffer (10 mM Na-phosphate, pH 7.6, 100 mM NaCl and 5 mM MgCl₂ at 20 °C).

Seventy-five CXCA derivatives were produced for the F-RNP library. Relative ratios of fluorescence intensity in the absence (I_0) and in the presence (I) of Ado were monitored at 470 nm ($\lambda_{\text{Ex}} = 370$ nm). Six RNPs showed > 1.3 -fold enhancement of I/I_0 in the first screening (Figure 3-12).

Seventy-six TPGCA derivatives were evaluated for their fluorescent properties as RNP sensors, and eight compounds showed > 1.3 -fold enhancement of I/I_0 at 460 nm ($\lambda_{\text{Ex}} = 360$ nm) in the first screening (Figure 3-13).

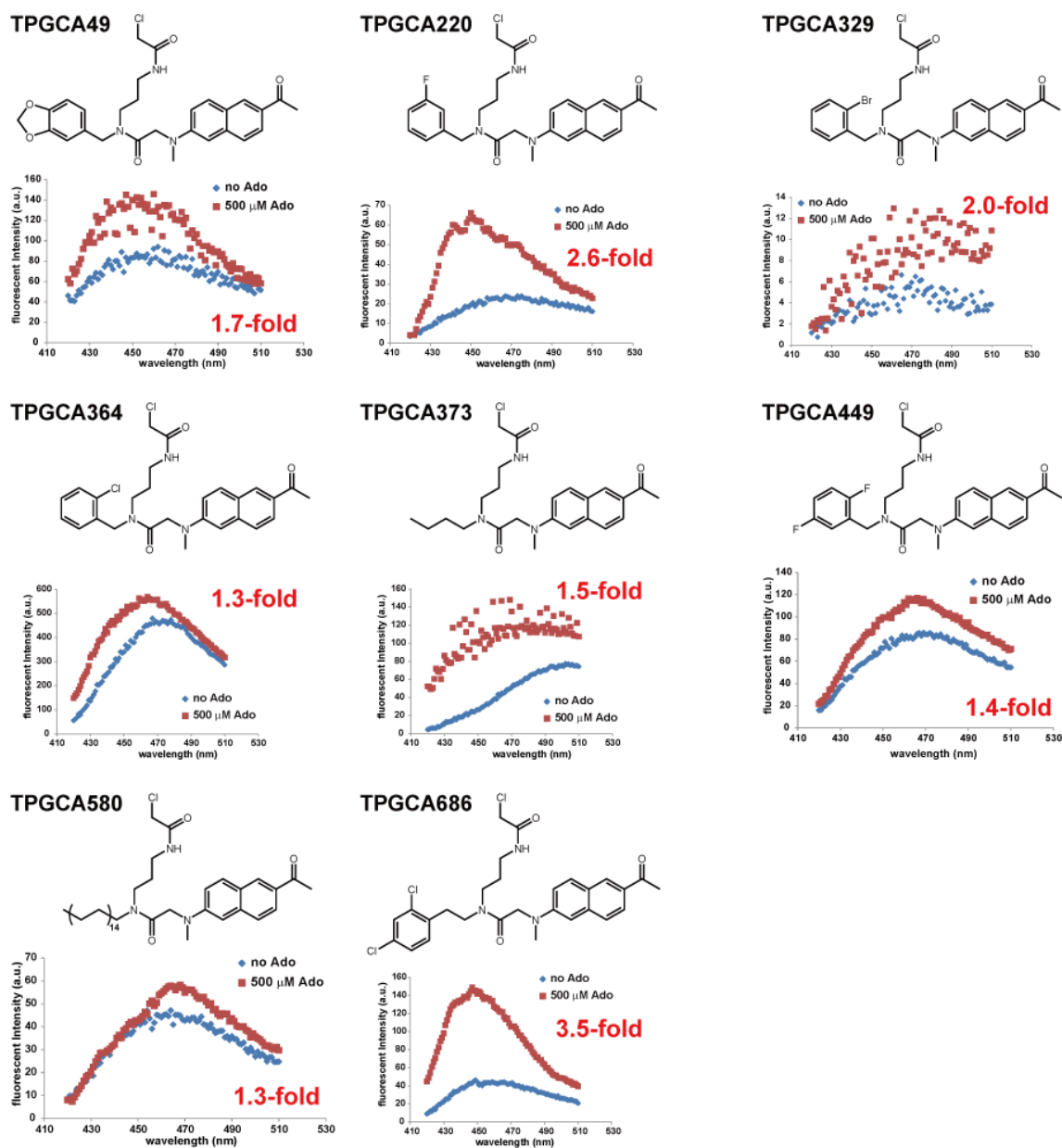


Figure 3-13. The fluorescent spectra ($\lambda_{\text{Ex}} = 360$ nm) of selected An16/TPGCA-modified Rev with (red) and without (blue) Ado

In the same way, two from fifty-six compounds of the CORCA library ($\lambda_{\text{Ex}} = 380 \text{ nm}$) (Figure 3-14) and two from fifty-six compounds of the CyRCA library ($\lambda_{\text{Ex}} = 800 \text{ nm}$) (Figure 3-15) showed > 1.3 -fold enhancement of I/I_0 . The F-RNPs constructed from the CORCA library tended to show the decrease in fluorescent intensity upon addition of Ado. In almost all cases, the F-RNPs constructed from the CyRCA library did not show consistent values of fluorescent intensity changes. This tendency might originate from the difficulty in attaching CyR derivatives to CRev due to their poor solubility in the reaction buffer.

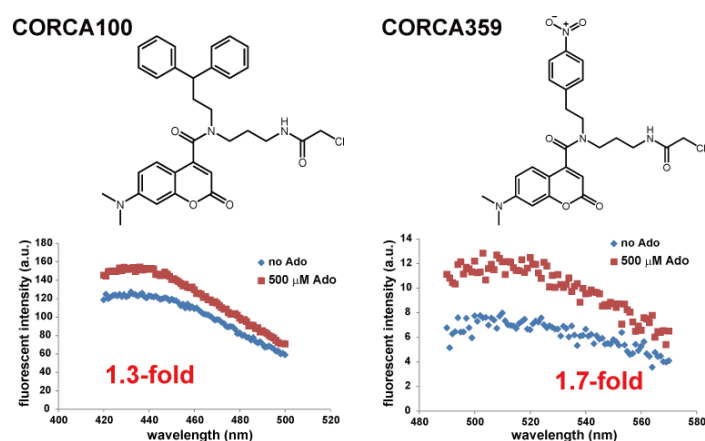


Figure 3-14. Fluorescence spectra ($\lambda_{\text{Ex}} = 380 \text{ nm}$) of selected An16/CORCA-modified Rev with (red) and without (blue) Ado

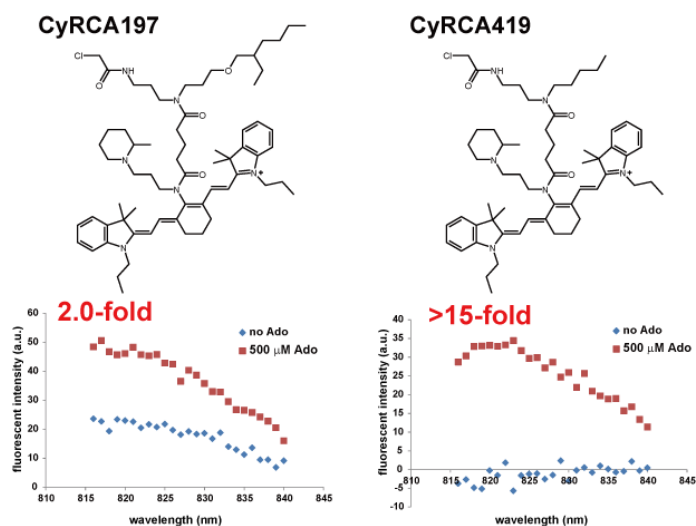


Figure 3-15. Fluorescent spectra ($\lambda_{\text{Ex}} = 800 \text{ nm}$) of selected An16/CyRCA-modified Rev with (red) and without (blue) Ado

In summary, 259 compounds were evaluated for their fluorescence properties and eighteen candidates (Figures 3-12, 3-13, 3-14 and 3-15) were selected to show > 1.3-fold enhancement of relative fluorescence intensities on first screening.

3-2-4. Second screening of the selected F-RNP after further purification of F-CRev

In the preliminary screening, F-CRev peptides of the F-RNP library were used as crude compounds by purification with simple extraction with diethyl ether. Impurities and unreacted CRev may decrease the relative fluorescence intensity change of the RNP sensor in the absence and presence of Ado by increasing the background fluorescence intensity, which might cause difficulty in the reproducibility of screening. Moreover, impurities might affect the fluorescence spectrum by changing the environment around F-RNPs. In order to confirm the relative intensity changes observed in the preliminary screening and to confirm reproducibility, thirteen selected peptides (CXCA1-CRev, CXCA5-CRev, CXCA34-CRev, CXCA45-CRev, CXCA62-CRev, TPGCA49-CRev, TPGCA220-CRev, TPGCA364-CRev, TPGCA373-CRev, TPGCA449-CRev, TPGCA580-CRev, TPGCA686-CRev, and CORCA100-CRev) were purified by HPLC. Other five peptides (CXCA21-CRev, TPGCA329-CRev, CORCA359-CRev, CyRCA197-CRev and CyRCA419-CRev), of which corresponding F-RNPs showed > 1.3-fold changes in relative fluorescence intensities for the preliminary screening, were not used in the second screening due to their low fluorescence intensities. The fluorescence spectra were measured under the same conditions as those used for the preliminary screening.

Results are shown in Figure 3-16. The relative fluorescence intensity changes of An16/CXCA1-CRev increased from 1.3-fold in the preliminary screening to 2.2-fold in second screening. An16/CXCA1-CRev and eight F-RNPs (An16/CXCA5-CRev, An16/CXCA62-CRev, An16/TPGCA49-CRev, An16/TPGCA220-CRev, An16/TPGCA364-CRev, An16/TPGCA373-CRev, An16/TPGCA449-CRev and An16/TPGCA686-CRev) also showed >1.3-fold changes in relative fluorescence intensities. However, An16/CXCA34-CRev, An16/CXCA45-CRev and An16/TPGCA580-CRev were not suitable for sensors owing to the small changes in relative fluorescence intensities. An16/CORCA100-CRev showed a decreasing fluorescence change upon substrate binding, despite the fact that the preliminary screening showed a promising result as a sensor with enhancement of I/I_0 response.

These results indicate that not only positive but also false-positive F-RNPs were selected in the first screening, and that the results of the second screen after further purification elucidated reliable fluorescence properties of F-RNPs. Hence, the chromatographic purification step is required prior to second screening so that the RNP sensors with the binding ability of interest have a high probability for usage. Taken together, a given RNP receptor was converted into F-RNP sensors by utilizing fluorophore library with two-step screening.

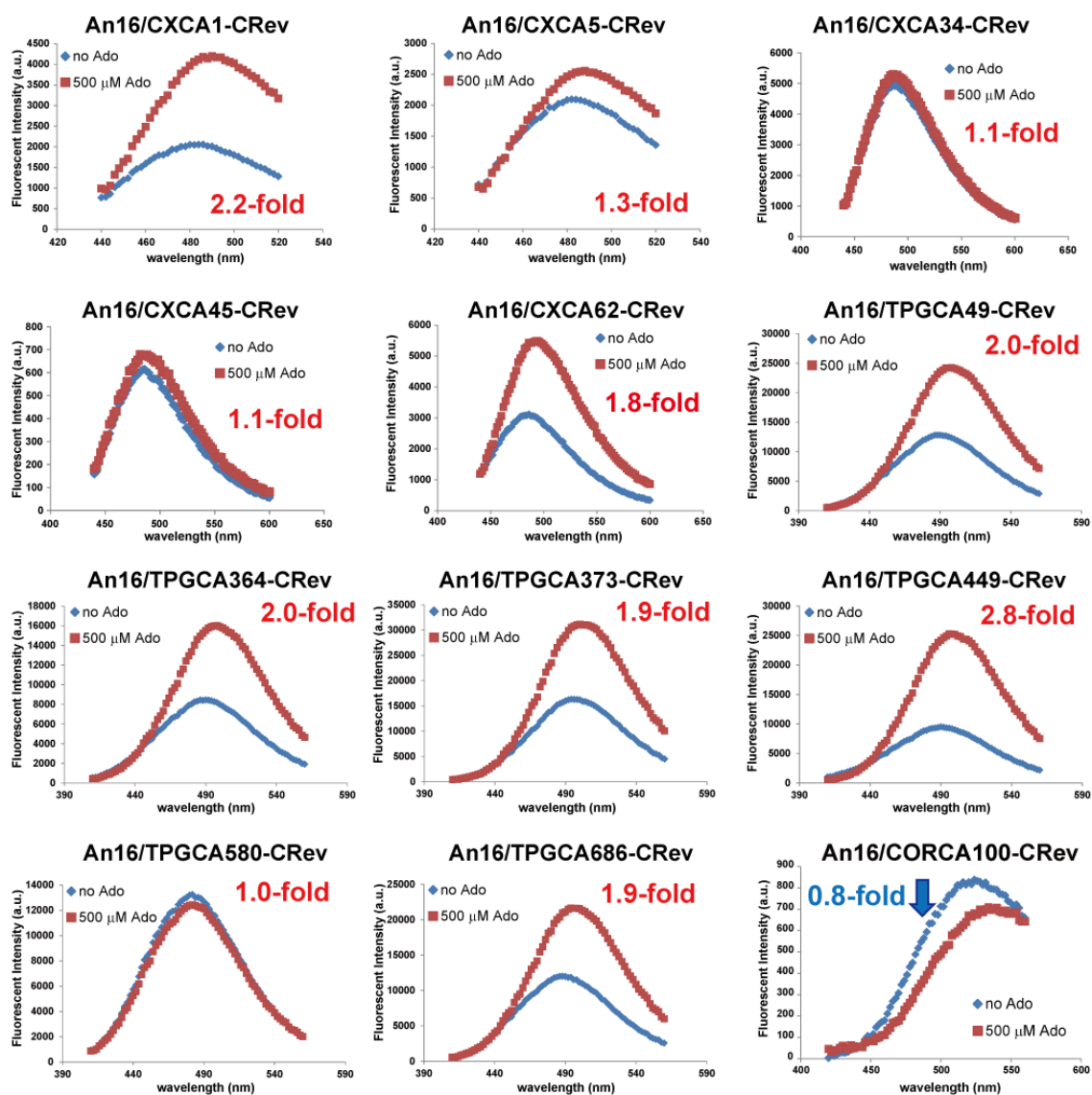


Figure 3-16. Fluorescence spectra of purified F-RNP with (red) and without (blue) Ado in the second screening after purification of F-CRev peptides.

3-2-5. Evaluation of the function of selected F-RNP sensor An16/CXCA1-CRev

In order to evaluate the function of the resulted RNP for the targeted ligand, An16/CXCA1-Rev was evaluated by titration with Ado. Titration of the changes in fluorescence intensities of An16/CXCA1-CRev by addition of increasing concentrations of Ado gave a K_D value of 21 μ M for the Ado-An16/CXCA1-CRev complex with the maximum relative fluorescence intensity (I/I_0) of 2.2, which was in good agreement with the result in the second

screening. This demonstration showed that the conversion of the focused RNP receptor to F-RNP sensor by utilizing fluorophore library was successfully accomplished.

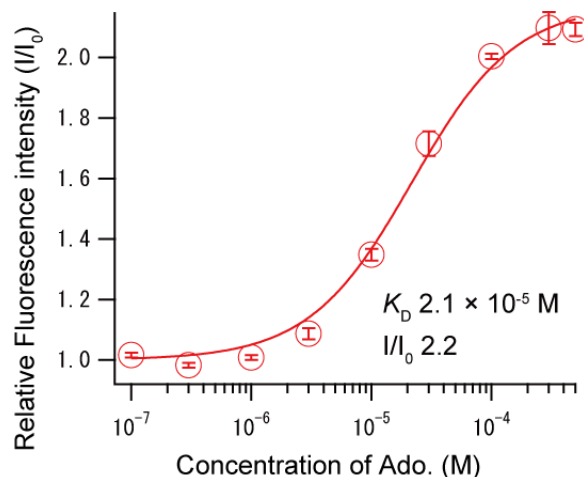


Figure 3-17. A saturation curve for the relative fluorescence intensity changes of An16/CXCA1-CRev (1 μ M) by titration with Ado in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM MgCl_2 , and 0.005% Tween 20 at 4°C. The dissociation constant (K_D) and the maximum relative fluorescence intensity for the Ado complex of RNP are shown in the inset.

3-2-6. Construction of covalently linked F-RNP sensors

Other issue needs to be improved on the construction of RNP sensor is the stability of RNP complex for the detection with low concentration of RNP sensors and/or the multiple usages of RNP sensors. Noncovalent F-RNP sensors with the binding ability of interest were constructed by screening of F-RNP library constructed by post-modification of a library of fluorophores. One of the advantages of noncovalent F-RNP sensors is simplicity of the complex formation. We can conveniently construct an F-RNP library owing to this advantage. On the other hand, it is difficult to use different RNPs in the same solution due to a possibility of the replacement of subunits of RNPs, and to use the RNP sensor in low concentrations owing to the low RNP complex formation. In order to construct a stable F-RNP sensor, the 3'-terminal of RNA subunit and the C-terminal of Rev peptide were covalently tethered by a hydrazone bond formation (Figure 3-18).

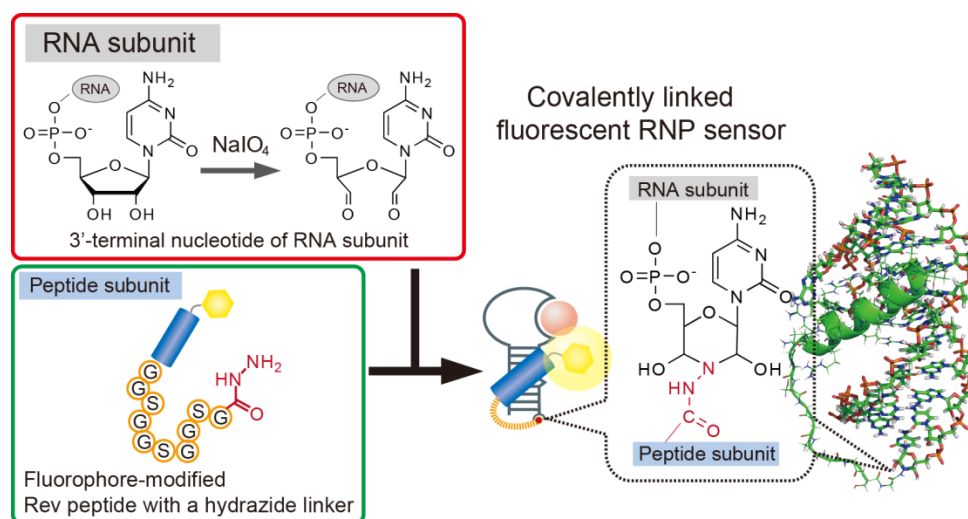


Figure 3-18. Schematic illustration of the construction of covalently linked F-RNP sensor. 3'-Ribose of the RNA subunit was converted to 3'-dialdehyde by treating with sodium periodate. A flexible peptide linker (GGSGGSGGSG) with a carboxyhydrazine was introduced at the C-terminal of the fluorophore-modified Rev peptide. A covalently linked F-RNP sensor was synthesized through a coupling of these modified RNA and peptide subunits via hydrazone bond formation.

This reaction has been utilized as an immobilization method of nucleic acids or proteins to solid supports^{22,23} or as a crosslinking method of nucleic acids and proteins.²⁴ The RNA subunit of ATP-binding RNP was treated with sodium periodate to form a 3'-dialdehyde group. A flexible linker with ten amino acid residues (GGSGGSGGSG) was introduced at the C-terminal of the Rev peptide, and a hydrazine group was introduced to the C-terminal. The length of the linker GGSGGSGGSG was determined by reference to the three-dimensional structure of the complex of the Rev peptide and RRE RNA.¹²

The noncovalent complex of F-RNP sensors for ATP and GTP were converted to covalently linked RNP to confirm the above strategy. Among the ATP-binding RNP receptors with various affinity to ATP selected from the RNP library, A26 RNP was chosen as a representative RNP for the first trial of construction of a covalently linked RNP sensor because A26 RNP shows measurable changes in fluorescence intensities upon binding to the targets with many types of fluorophore (Pyr, NBD, Cy5 etc.).¹³ In a similar manner, G23 RNP was chosen.¹⁴ Purified RNA subunits of these RNP, A26 RNA and G23 RNA, was treated with freshly prepared sodium periodate to modify the 3'-terminal ribose to 3'-dialdehyde by periodate oxidation. The peptide subunit for the covalent linkage was prepared by ante-modification method. A peptide linker (GGSGGSGGSG) was introduced at the C-terminal of Rev peptide.

Then a fluorophore was modified to the N-terminal, and the peptide was derivatized to hydrazide by hydrazine treatment of the resin. 5-carboxyfluorescein and 1-pyrenesulfonyl chloride were conjugated to the N-terminal of the Rev peptide to give 5FAM-Rev-HZ and Pyr-Rev-HZ.

Subsequently, the modified RNA and peptide subunits were covalently linked together. Coupling reaction of A26 RNA 3'-dialdehyde and 5FAM-Rev-HZ was performed in sodium acetate buffer (pH 5.2) at ambient temperature. The resulting covalently linked RNP complex was purified by phenol-chloroform extraction, ethanol precipitation, and denaturing PAGE to remove the unreacted RNA and peptide. The isolated yield of covalently linked RNPs of A26 RNA with 5FAM-Rev-HZ (c-A26/5FAM-Rev) was 36%. The purities of the covalently linked RNP sensors were checked by denaturing PAGE (Figure 3-19). In the same manner, c-G23/Pyr-Rev was prepared.



Figure 3-19. A PAGE image shows purity of the covalently linked RNP sensor. The purity of each sample was confirmed by the denaturing gel electrophoresis (6 M Urea, 15% PAGE). Each lane shows A26 RNA (Lane 1) and c-A26/5FAM-Rev (Lane 2), respectively.

3-2-7. Fluorescent Responses of the Covalent RNP Complexes

The fluorescence responses of the covalently linked RNP sensor (c-A26/5FAM-Rev and c-G23/Pyr-Rev), and corresponding noncovalent derivative (A26/5FAM-Rev and G23/Pyr-Rev) were evaluated in the presence of increasing concentrations of ATP and GTP (Figures 3-20 and 3-21, respectively). Relative fluorescence intensities were plotted against the ATP concentration to calculate the dissociation constant (K_D) for the complex between the RNP sensor and ATP. The saturation value of the change in relative fluorescence intensity was found to be 1.6 for c-A26/5FAM-Rev (100 nM) (Figure 3-20A), which was similar to the value of 1.7 for A26/5FAM-Rev (1000 nM) (Figure 3-20B). The binding affinity of covalently linked

c-A26/5FAM-Rev was higher than that of noncovalent A26/5FAM-Rev.

The K_D value of the Rev-RRE complex has been shown to be a few nanomolar at similar buffer conditions to those used in this study. The covalently linked sensor c-A26/5FAM-Rev provided distinct fluorescent responses, even at 1 nM (Figure 3-20A). On the other hand, the noncovalent sensor A26/5FAM-Rev exhibited almost no fluorescent response when the RNP concentration was reduced to 100 nM (Figure 3-20B), indicating that the noncovalent RNP complex was mostly dissociated into each subunit. Furthermore, the fluorescence response of the covalently linked sensor c-A26/5FAM-Rev showed higher tolerance to increasing temperatures, even at a sensor concentration of 100 nM (Figure 3-20C), than that of A26/5FAM-Rev (1000 nM; Figure 3-20D).

The covalently linked GTP sensor c-G23/Pyr-Rev showed a higher affinity to GTP than that of the noncovalent sensor G23/Pyr-Rev (Figure 3-21A). The I/I_0 value for c-G23/Pyr-Rev and GTP at the saturation was 3.1, which was significantly higher than that of the parent noncovalent GTP sensor G23/Pyr-Rev (1.8). Again, the covalently linked sensor c-G23/Pyr-Rev showed higher tolerance to increasing temperatures (Figure 3-21B) than that of G23/Pyr-Rev (Figure 3-21C).

These results indicated that the noncovalent RNP sensors for ATP or GTP were converted to a covalently linked RNP sensor without diminishing sensing functions for ATP or GTP, respectively. The covalently linked RNP sensor exhibited much higher stability at low concentrations and maintained the fluorescence response of individual RNP sensors.

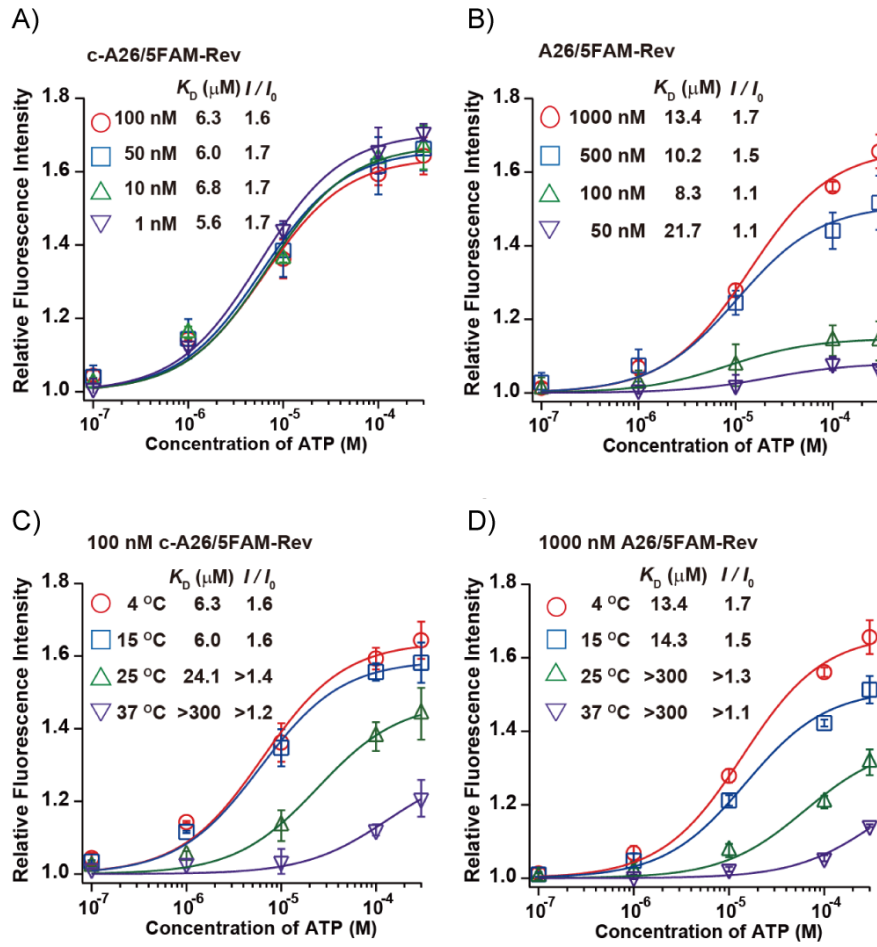


Figure 3-20. Saturation curves for the relative fluorescence intensity changes of c-A26/5FAM-Rev and A26/5FAM-Rev by titration with ATP in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween 20. (A) Titration of the ATP-binding F-RNP complexes c-A26/5FAM-Rev (1 nM, purple reverse triangles; 10 nM, green triangles; 50 nM, blue squares; 100 nM, red circles) with ATP at 4 $^{\circ}$ C and (B) A26/5FAM-Rev (50 nM, purple reverse triangles; 100 nM, green triangles; 500 nM, blue squares; 1000 nM, red circles) with ATP at 4 $^{\circ}$ C are shown. (C) Titration profiles of the ATP-binding F-RNP complexes c-A26/5FAM-Rev and (D) A26/5FAM-Rev with ATP at different temperatures (4 $^{\circ}$ C, red circles; 15 $^{\circ}$ C, blue squares; 25 $^{\circ}$ C, green triangles; 37 $^{\circ}$ C, purple reverse triangles) are shown. The dissociation constant (K_D) and the maximum relative fluorescence intensity for the ATP complex of each RNP are shown in the inset.

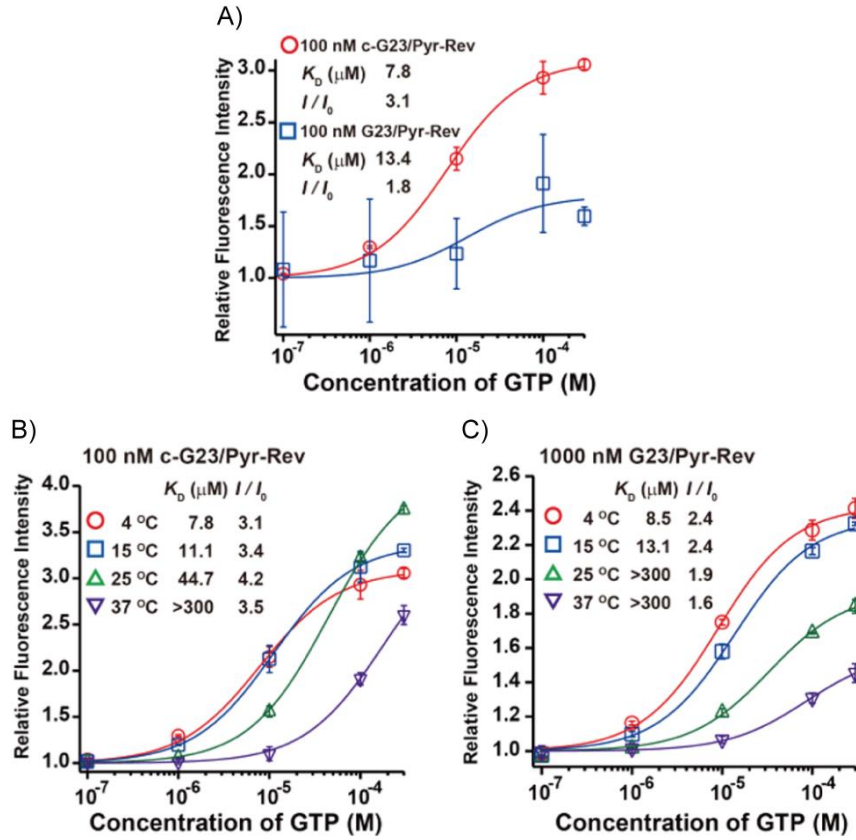


Figure 3-21. (a) Saturation curves for the relative fluorescence intensity changes of c-G23/Pyr-Rev (100 nM, red circles) and G23/Pyr-Rev (100 nM, blue squares) by titration with GTP in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM MgCl₂, and 0.005% Tween 20 at 4°C. (b) Titration profiles of c-G23/Pyr-Rev and (c) G23/Pyr-Rev with GTP at different temperatures (4°C, red circles; 15°C, blue squares; 25°C, green triangles; 37 °C, purple reverse triangles) are shown. The dissociation constant (K_D) and the maximum relative fluorescence intensity for the GTP complex of each RNP are shown in the inset.

3-2-8. Simultaneous Detection of ATP and GTP by F-RNP Sensors

Because the fluorophore-modified Rev peptide binds to a common RRE RNA sequence, a unique noncovalent RNP sensor must be applied for each separate sample solution to reduce the possible exchange between different subunits of multiple RNP species. In contrast, the covalently linked RNP sensor forms an intramolecular complex that prevents such an exchange between the subunits, even when several types of RNP sensors co-exist in the solution.

To test this, the covalently linked sensors c-A26/5FAM-Rev and c-G23/Pyr-Rev were applied to monitor the concentrations of ATP and GTP in the same solution at different wavelengths. The ATP sensor c-A26/5FAM-Rev shares the same consensus sequence with the ATP-binding RNP A26/5FAM-Rev, which selectively binds ATP over GTP. The parent RNP of the covalent GTP sensor, G23/Pyr-Rev, has been shown to distinguish GTP from ATP. When monitored at 535 nm, the ATP sensor c-A26/5FAM-Rev responded to concentration changes in ATP, but not to changes in the concentration of GTP (Figure 3-22A), and showed a similar response that was observed in the absence of the GTP sensor c-G23/Pyr-Rev (Figure 3-21A). Similarly, the GTP sensor c-G23/Pyr-Rev specifically detected GTP at 390 nm without any interference from ATP and the ATP sensor c-A26/5FAM-Rev in the same solution (Figure 3-22B). In contrast, the solution containing both the noncovalent sensors A26/5FAM-Rev and G23/Pyr-Rev showed fluorescence responses to both ATP and GTP at the detection wavelengths of 5FAM and Pyr (Figures 3-22C and 3-22D). This result suggests that exchange of 5FAM-Rev and Pyr-Rev has occurred for the noncovalent RNP sensors in the solution. These results indicate that ATP and GTP in the same solution are specifically detected by c-A26/5FAM-Rev and c-G23/Pyr-Rev, respectively, in the presence of other substrate and/or the other sensor.

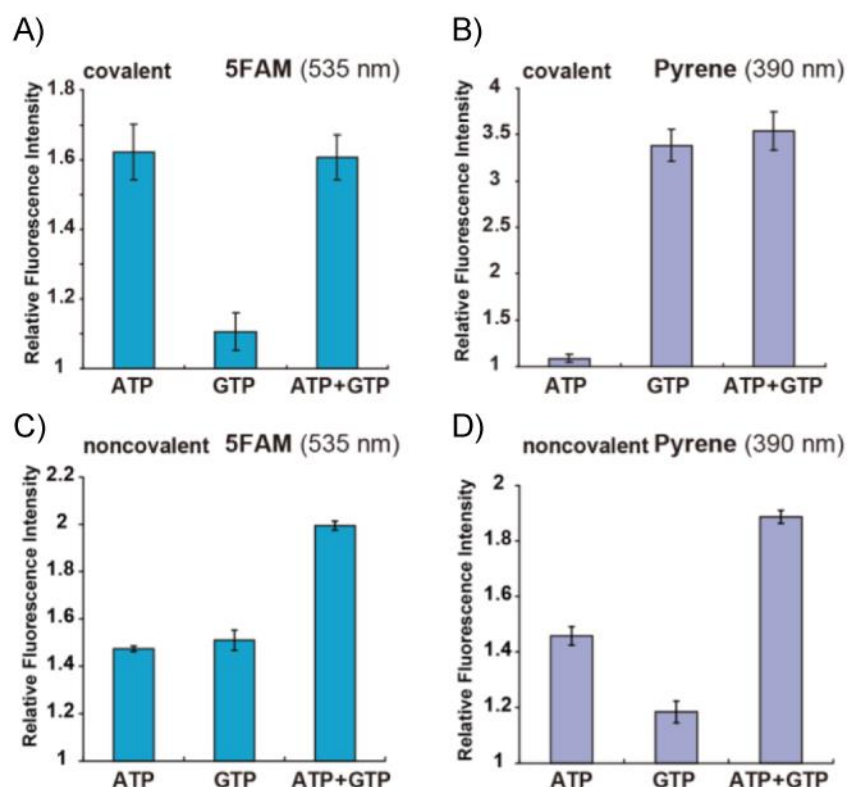


Figure 3-22. (A, B) Simultaneous detection of ATP and GTP by covalently linked ATP and GTP sensors at different wavelengths in the same solution. A solution containing both the ATP sensor c-A26/5FAM-Rev (100 nM) and the GTP sensor c-G23/Pyr-Rev (100 nM) in the presence of 100 μ M ATP and/or 100 μ M GTP was measured at (A) 535 nm or at (B) 390 nm at 4°C to selectively detect ATP or GTP. (C, D) Fluorescence responses of noncovalent ATP and GTP sensors at different wavelengths in the same solution in the presence of ATP, GTP, or both ATP and GTP. A solution containing both the ATP sensor A26/5FAM-Rev (1000 nM) and the GTP sensor G23/Pyr-Rev (1000 nM) in the presence of 100 μ M ATP and /or 100 μ M GTP was measured at (C) 535 nm or at (D) 390 nm at 4°C under the mixing condition.

These results demonstrate that simultaneous detection of multiple ligands is possible by applying covalently linked RNP sensors that sense their respective ligands at different detection wavelengths. In addition, a variety of sensors that respond at the target concentration range with the desired detection wavelength would be prepared by the combination of an appropriate RNA subunit and a fluorophore-labeled Rev peptide.

3-3. Conclusion

A screening method for fluorescent ribonucleopeptide (RNP) sensors was developed in this study. Efficient conversion of CA-modified fluorophore libraries to CRev-modified fluorophore libraries prior to the complexation with the RNA component of a given RNP receptor (An16) allowed successful construction of an F-RNP (F-RNP) library. Preliminary screening of the library of roughly purified F-RNP yielded 18 candidates from more than 250 fluorophores tested. Further evaluation of the relative fluorescence intensity of purified F-RNP candidates demonstrated that the majority of the candidates showed higher performance compared to that expected from the results of preliminary screening, but in one case, a false positive result was identified. Thus, a screening method employed to obtain a particular RNP receptor-based fluorescent sensor was successfully established.

Furthermore, covalently linked RNP sensors showed enhanced stability and enabled the detection of ligands at lower concentrations of sensors. The covalently linked ATP and GTP sensors enabled the simultaneous optical detection of ATP and GTP in the same buffer. Because the combination of RNA and a fluorophore-modified peptide library afforded fluorescence responses in various concentration ranges, this strategy provided an appropriate set of fluorescent sensors for the detection of multiple ligands in the desired concentration range. Once an F-RNP sensor with a desired property is obtained, the covalent linking strategy reported here allows development of F-RNP sensors to detect in the low concentration of RNP and for simultaneous detection of multiple ligands by monitoring each wavelength corresponding to the respective ligand.

3-4. Materials and methods

3-4-1. Materials

All fluorophore libraries were provided from Prof. Chang, Y. T. (Department of Chemistry, National University of Singapore, Singapore). 10 nmol of all compounds were isolated into 96-well plate. PrimeSTAR HS DNA polymerase for PCR reactions was obtained from TaKaRa Bio Inc. (Shiga, Japan). AmpliScribe T7 Kit for RNA preparation was obtained from Epicentre (Madison, USA). *N*- α -Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), SP grade *N,N*-dimethylformamide (DMF), trifluoroacetic acid (TFA) and 4-[(2,4-Dimethoxyphenyl)-*N*-(9-fluorenylmethoxycarbonyl)aminomethyl]phenoxy-acetamido polyethyleneglycol resin (Fmoc-NH-SAL-PEG resin) were obtained from Watanabe Chemical Industries (Hiroshima, Japan). Dichloromethane (DCM), was purchased from Nacalai Tesque (Kyoto, Japan). *N,N*-diisopropylethylamine (DIEA), HPLC-grade acetonitrile and

N,N-dimethyl-4-aminopyridine (DMAP) were obtained from Sigma-Aldrich (St. Louis, USA). Adenosine, was purchased from Tokyo Chemical Industry (Tokyo, Japan). 4-hydroxymethylbenzoic acid PEGA resin (HMBA-PEGA resin) was obtained from Merck Millipore (Darmstadt, Germany). Reversed-phase C18 columns (4.6 × 150 mm, Ultron VX-ODS; Shinwa Chemical Industries, Kyoto, Japan and 10 × 150 mm, 5C18-ARII; Nacalai Tesque, Kyoto, Japan) were used for purification of peptides for preparative purposes. Sodium azide, succinic anhydride, gel electrophoresis grade acrylamide, bisacrylamide, phenol, thioanisol, 1,2-ethandithiol, 10% palladium on carbon, distilled organic solvents and other compounds were purchased from Wako Chemicals (Tokyo, Japan).

3-4-2. Preparation of RNA subunit of RNP receptors

The double-stranded DNA templates for RNA transcription were constructed by PCR amplification to add the promoter for T7 RNA polymerase using PrimeSTAR DNA polymerase (TaKaRa) with 5'-DNA primer (5'-TCTAATACGACTCACTATAGGTCTGGGCGCA-3': T7 RNA promoter is underlined) and 3'-DNA (5'-GGCCTGTACCGTC-3'). RNA transcription was performed using an AmpliScribeT7 kit (Epicenter) for 3 h at 37 °C, according to the supplier's recommended protocols. The resulting RNA was purified by denaturing PAGE and eluted. Concentrations of RNAs were determined by UV spectroscopy with excitation coefficients 439,500 for An16 and 541,600 for A26.

Sequences of RNAs:

An16 : GGUCUGGGCGCACGUAGUGGUGUGUGUGUGGUGACGGUACAGGCC

A26 : GGUCUGGGCGCAUCCGGUAGUGGUUGUGUGUGUGCGGUUUUUGACGGUACAGGCC

3-4-3. Synthesis of cysteine-modified peptide library

The peptide was synthesized as follows. Fmoc-NH-SAL-PEG resin was placed in a dry flask, and a sufficient amount of DMF was added to soak the resin; this mixture was allowed to swell for 30 min. The resin was loaded onto an automated peptide synthesizer (Liberty; CEM / PSSM-8; Shimadzu), and the subsequent synthesis was performed according to Fmoc chemistry protocols using protected Fmoc-amino acids and HBTU. The peptide was cleaved from the resin and deprotected by a solution containing phenol (0.75 g), distilled water (0.5 mL), thioanisol (0.5 mL), 1,2-ethandithiol (0.25 mL), and TFA (10 mL) for 3 h. The peptide was washed by diethylether, gel-filtrated with a G-10 resin, purified by reversed-phase HPLC and characterized by MALDI-TOF-MS. CRev calculated for [MH⁺] 3023.5 found 3022.9.

Sequences of CRev : Ac-CTRQARRNRRRRWRERQRAAAAR-NH₂

3-4-4. Iodination of fluorophore-CA

Chloro-acetylated fluorophore libraries were provided from Prof. Chang, Young-Tae in National University of Singapore. 10 μ L of 1 mM fluorophore-CA (10 nmol, 1 eq.) was moved into the well in the 96 well plate. After dried up the solvent, 10 μ L of 0.95 mM NaI (9.5 nmol, 0.95 eq.) in acetone were added. The plate was covered by silverseal (griner bio one, platesealer, silverseal, aluminium, 80 x 140) and incubated in an oven at 50 °C. After 2.5 h, the solvent was evaporated and colored solid (iodinated fluorophore, fluorophore-CI) was obtained in each well. 100 μ L of acetonitrile was added to each well and an aliquot (40 μ L) was evaluated by LC-MS. The samples were used in next step without purification.

3-4-5. Covalent bond formation between fluorophore and CRev

Dried fluorophore-CI solid in the well was dissolved in 10 μ L of acetonitrile and transferred to a 1.5 mL tube. Then 5 μ L of 1 mM C-Rev (5 nmol, 0.5 eq.) and 5 μ L of 100 mM Na-phosphate buffer (pH 8.3) were added into the tube and mixed. Water (90 μ L) was added and the resulting solution was washed with ether (150 μ L) two times to purify F-CRev. An aliquot (35 μ L) was used for LC-MS to characterize and check the purity of F-CRev. The sample was used for first screening without further purification. The F-Rev peptides affording the relative fluorescence intensity changes of corresponding F-RNP > 1.3 were purified by HPLC before second screening.

3-4-6. Fluorescence measurement

A solution containing An16 (1 μ M), F-CRev (1 μ M), 10 mM sodium phosphate buffer (pH 7.6), 100 mM NaCl, and 10 mM MgCl₂ was loaded into 96 well plate with or without 500 μ M Ado. For the preliminary screening, the plate was measured by SpectraMax M2 spectrophotometer (Molecular Devices) using F-CRev with rough purification. Excitation wavelengths were 370 nm, 360 nm, 380 nm and 800 nm for CXCA library, TPGCA library, CORCA library and CyRCA library. For the second screening, the plate was measured by Infinite 200 Pro (Tecan) using purified F-CRev.

3-4-7. Construction of RNA subunit for the covalent linkage

RNA subunits of RNP receptors were purified by denaturing 8% PAGE. The purified RNA subunit was treated with freshly prepared sodium periodate to convert the cis-diol of the 3'-terminal ribose to 3'-dialdehyde by periodate oxidation. Freshly prepared 50 eq. of 10 mM sodium periodate (10 μ L; 100 nmol) was added to 100 μ M RNA (20 μ L; 2 nmol) in 32 μ L of 0.01 M sodium phosphate (pH 7.5), and the reaction mixture was incubated for 30 min at 37 °C in the dark. After the reaction, an excess of sodium periodate was quenched by adding 1 M

glycerol, and the resulting oxidized RNA was purified by ethanol precipitation.

3-4-8. Construction of F-Rev for the covalent linkage

The peptide subunit for the formation of a covalent linkage was synthesized by introducing a fluorophore to the N-terminal and the peptide linker (GGSGGSGGSG) with a hydrazide group at the C-terminal of the Rev peptide TRQARRNRRRRWRERQRGGSGGSGGSG. The modified peptide was synthesized as follows. A 4-hydroxymethylbenzoic acid PEGA resin was placed in a dry flask, and a sufficient amount of DMF was added to soak the resin; this mixture was allowed to swell for 30 min. N-a-Fmoc-glycine (10 eq. relative to resin loading) was dissolved in dry DCM with 1 or 2 drops of DMF and then mixed with a solution of DIC (5 eq. relative to resin loading) in dry DMF on ice. After incubating for 20 min, DCM was removed by evaporation under the reduced pressure. The residue was dissolved by minimum volume of DMF and added to the resin prepared above. A DMF solution of DMAP (0.1 eq. relative to resin loading) was added to the resin/amino acid mixture and incubated at room temperature for 1 h with occasional swirling. This first attachment procedure was repeated twice. The resin with the first amino acid was used for solid-phase synthesis according to Fmoc chemistry protocols and 5FAM was attached by ante-modification. The fluorophore-labeled peptide was cleaved from the resin with 0.1 M hydrazine hydrate in DMF for 30 min at room temperature and purified by HPLC.

3-4-9. Construction of stable F-RNP sensor by the covalent linking method

A coupling reaction between the 3'-modified RNA (50 μ M) and fluorophore-modified Rev-HZ (75 μ M) was performed in 0.02 M sodium acetate (pH 5.2) containing 0.01 M NaCl (total 40 μ L) at 37 °C in the dark. After 5 h, the reaction mixture was extracted by phenol-chloroform and then purified by ethanol precipitation. The precipitate was dissolved in 0.01 M sodium phosphate (pH 7.5) containing 0.1 M NaCl (40 μ L). The sample solution was treated with a cation-exchange resin (SP Sepharose Fast Flow, GE healthcare) for 30 minutes at room temperature to remove the excess peptide, and the unbound fraction was collected to obtain a covalently linked RNP complex. The sample solution was purified by 6 M urea 15% denaturing PAGE. As a result, about 50 μ L of 15 μ M covalently linked RNP complex, c-A26/5FAM-Rev, was recovered (yield, 36%).

3-4-10. Simultaneous Detection of ATP and GTP in the Same Solution

Samples were prepared by mixing c-A26/5FAM-Rev (100 nM), c-G23/Pyr-Rev (100 nM) or mixing of A26/5FAM-Rev (1000 nM), G23/Pyr-Rev (1000 nM) and the indicated substrates in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM MgCl₂, and 0.005% Tween 20. The samples

were incubated for 30 minutes at 4°C. When two noncovalent sensors were applied in the same solution, each noncovalent sensor was incubated in an independent tube for 30 minutes at 4°C to secure the complex formation, then the solutions containing the noncovalent sensors were added to the assay solution and incubated for 30 min at 4°C. Fluorescence intensities were recorded on a Tecan Infinite M200 plate reader. The excitation wavelengths were 485 nm for 5FAM-Rev and 350 nm for Pyr-Rev. The emission wavelengths were 535 nm for 5FAM-Rev and 390 nm for Pyr-Rev, and were used for the determination of relative fluorescence intensity.

3-5. References

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Chapter 4

Construction of a structurally diverse ribonucleopeptide receptor library with a catalytic group

4-1. Introduction

Biomacromolecular receptors provide useful frameworks for constructing sensors with high specificity for a particular ligand. Efforts have been taken to construct biosensors through modification of proteins and nucleic acids receptors with appropriate fluorophores¹⁻⁵ or artificial enzymes with a catalytic group to achieve proximity effect between the substrate and the catalytic group.^{6,7} However, naturally occurring receptors do not always provide the recognition characteristics for the molecule of interest, and rational design of a receptor with desired specificity is still a challenging task. A library of RNA molecules that differ in their three-dimensional structures by means of randomized nucleotide sequences has been applied for the selection of receptors for the target ligands.⁸⁻¹⁰ Although the library-based selection offers one of the promising methods to obtain aptamers with the specific recognition characteristics for the molecule of interest, further modification of the selected aptamers to exert a newly desired function is often difficult due to the lack of their structural information. In addition, the original substrate binding characteristics of the aptamer could be impaired by the introduction of a new functional group. Synthetic functional groups were incorporated to biomacromolecules in the library by modification of proteins or nucleic acids via chemical modification or genetic mutation.^{11,12} These chemical or genetic methods required laborious tasks to generate a library with limited size. Therefore, an alternative method is required to construct a large size library to increase the possibility of selecting a "hit" biomacromolecule with the function of interest.

Ribonucleopeptide (RNP) is one of the appropriate scaffolds to construct a structurally diverse library of RNA-peptide complexes. Morii, *et al.* have reported a method to obtain ATP-binding RNP receptors by *in vitro* selection of a library of RNP with randomized RNA sequences.¹³ Because the RNA subunit works as a receptor for the substrate, the selected ATP-binding RNP receptor was further converted into a new RNP library by complexation of the RNA subunit and a library of Rev peptides, in which a peptide loop with randomized amino acid residues was incorporated at the N-terminal of Rev peptide. An ATP-binding RNP receptor selected from this peptide-based RNP library exerted higher ATP-binding specificity than the original RNP (Figure 4-1a).¹⁴ A fluorescent RNP library was also constructed by combining the RNA subunit library and a library containing fluorophore-modified Rev peptides (Figure 4-1a).¹⁵⁻²¹ The original substrate-binding ability of the RNP receptor was maintained in the selected RNP sensor even upon modification of the Rev peptide by a fluorophore. By taking

advantage of the noncovalent complex formation for RNP, combination of the RNA library and the peptide library would dramatically increase the size of the RNP library. Therefore, RNP is a good candidate of scaffolds to construct a library of structurally diverse biomolecular assemblies containing a substrate-binding pocket and a synthetic functional group.

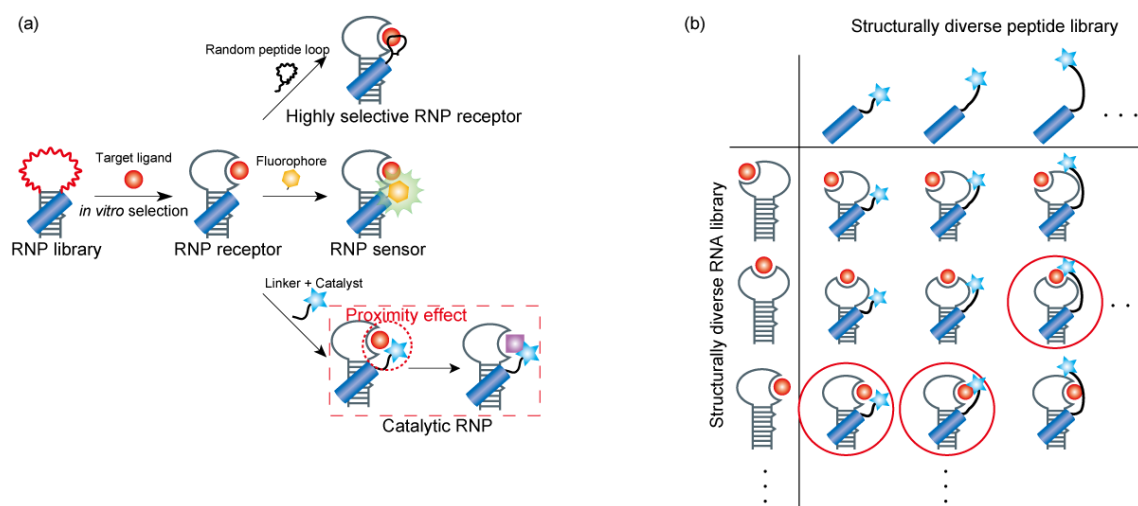


Figure 4-1. (a) Schematic illustration of stepwise molding method of RNP receptor. RNP receptor was first selected by in vitro selection of the RNP library. The resulting RNP receptor was converted into more selective RNP receptors by a suitable peptide loop containing randomized amino acid residues¹⁴ or to fluorescent RNP sensors by fluorophore modification.^{15–21} RNP receptor with catalytic activity would be constructed by introducing catalyst to the peptide subunit in this study. A target reaction would proceed by a proximity effect between the substrate in the binding pocket and the catalytic group on the peptide. (b) Combination of a structurally diverse RNA library and a structurally diverse peptide library affords an RNP library with various orientation of between the substrate-binding pocket and the catalytic group.

In this study, I have constructed of a library of structurally diverse RNP receptors equipped with a synthetic functional group. A series of RNA subunits obtained by the *in vitro* selection of ATP-binding RNP receptors^{15,20} afforded a library of structurally diverse RNAs consisting of the ATP-binding region and the RRE (*Rev Responsive Element*) nucleotide sequence. A library of peptides was constructed by modification of the Rev peptide with catalytic groups through various peptide linkers (Figure 4-1b). Combination of these two types of libraries by the complex formation between the RRE RNA and the Rev peptide generated a library of functionalized and structurally diverse RNPs, in which the catalytic group would align with various orientations against ATP binding pocket of RNA subunits. The functionalized and structurally diverse RNPs were expected to exhibit the ATP-binding ability. An adenosine derivative with an ester group was utilized to select the RNPs that could accelerate the ester hydrolysis reaction by the proximity effect between the substrate and the catalytic group (Figure 4-1a). Screening of the RNP library was successfully performed for the hydrolysis reaction of the ester derivative of adenosine to obtain catalytic RNPs.

4-2. Results and discussion

4-2-1. Construction of structurally diverse RNA subunit library

A structurally diverse RNP library with various geometries between the substrate binding pocket and the catalytic group was constructed by combination of the RNA subunit of ATP-binding RNP and a library of Rev peptide modified with catalytic groups. From the RNA-oriented RNP library, a series of ATP-binding RNP receptors was obtained through *in vitro* selection with a variation in the RNA sequences (Figure 4-1a).^{15,20} The nucleotide sequence of ATP-binding RNP consisted of three segments, the RRE, consensus, and variable regions (Figure 4-2). The consensus region of the ATP-binding RNP receptors forms the binding pocket for the substrate adenosine. The consensus and the RRE regions were connected through the variable region that differ in various length and sequences of the nucleotides. The structural diversity in the variable region affords diverse structures of RNPs, which in turn exhibits a variety of geometries between the ATP-binding pocket and the Rev peptide that is bound to the RRE sequence.



No.	RRE	Variable region	Consensus Region	Consensus Region	Variable region	RRE
A02	GGUCUGGGCGCA	UUGCAU	GUUGUGG	GUA	UGUGUGUG	AUGUAU
A08	-----	UUUUCAUGGCC	CUUGUGG	GAAGGAU	UGUG	A
A09	-----	CUGGUGU	GUAGUGG	GC	UGUGUGUG	AUGCCG
A14	-----	UACUGC	GUAGUGG	UU	GGUGUGUG	GCGGUUU
A15	-----	GCAGU	GUAGUGG	UU	UGCGUGUG	AUUGCUUA
A17	-----	UUAGAU	GUAGUGG	GU	AGUGUGUG	UUUUCUG
A24	-----	UGAUUGC	GUAGUGG	UU	UGUGUGUG	GUUGUC
A25	-----	UGCUG	GUAGUGG	GUA	UGUGUGUG	CGGCAUU
A26	-----	UUCCG	GUAGUGG	UUG	UGUGUGUG	CGGUUUU
A30	-----	UAUACC	GUAGUGG	UU	UCUGUCGG	GGUUGG
A33	-----	GUUUGCUGUUGCCGU	GUAGUGG	UU	UGUGUG	-----
A34	-----	U	GUAGUGG	UGCCUGUGAUGGC	UGUGAUGG	A
A36	-----	-----	GUGGUGG	UUCG	UCUGGGUG	CGCUGGUUUU
An02	-----	CAUAUC	GUAGUGG	UGA	GGUGUGUG	GAUAU
An03	-----	UU	GUAGUGG	UUUC	UCUGUCGG	AA
An08	-----	AU	GUAGUGG	UUU	UGUGUGUG	AUU
An09	-----	AC	GAAGUGG	UUU	UGAGUGUG	GU
An10	-----	-----	UUAGUGG	UUU	UGUGUGUG	U
An15	-----	AU	GUAGUGG	UU	UGUGUGUG	AU
An16	-----	C	GUAGUGG	UG	UGUGUGUG	G
An17	-----	-----	UUAGUGG	UU	UGUGUGUG	-----
An18	-----	AC	GUAGUGG	GU	UGAGUGUG	GU
An19	-----	-----	UUAGUGG	UUU	UGUGUGUG	-----
An22	-----	U	GCAGUGG	GUA	UGUGUGUG	A
An29	-----	GCU	GUAGUGG	UU	UGAGUGUG	AGU
An30	-----	AGUCAAUC	GUAGUGG	AC	UGUGUGUG	GAU
An33	-----	C	GUAGUGG	AA	UGUGUGUG	G
An34	-----	AC	UUAGUGG	UU	UGUGUGUG	GU
An35	-----	AUCAC	GUAGUGG	AU	UGUGUGUG	GUGGU

Figure 4-2. RNA sequences of RNA subunit of ATP-binding RNP receptor.^{15,20} Nucleotides in the consensus region forms the binding pocket to the substrate ATP. The red squared regions indicate the variable region that determines the location binding pocket to the peptide subunit.

4-2-2. Construction of structurally diverse peptide subunit library

The N-terminal of Rev peptide in RNP can be chemically modified without diminishing the binding affinity of Rev peptide to the RRE sequence.¹⁴⁻²⁰ Judging from the structure of Rev-RRE complex,²² the N-terminal of Rev peptide in RNP should locate closer to the binding-pocket than its C-terminal. Therefore, the N-terminal of Rev peptide is a suitable position for the modification by a catalytic group. *N,N*-Dimethyl-4-aminopyridine (dmap), 4,4'-dimethyl-2,2'-bipyridine (bpy) and histidine were chosen as possible catalytic groups for the ester hydrolysis reaction. In addition, peptide linkers with various amino acid sequences were introduced between the catalytic groups and the N-terminal of Rev peptides. Four types of

short peptide motifs were selected from known RNP structures²³⁻²⁶ (Figure 4-3) to achieve a structural diversity (Figure 4-4). The motif pa (Figure 4-3b) traverse the phosphate backbone as found in the eukaryotic ribosome structure (Figure 4-3a), the motif pb (Figure 4-3d) snugly fits in the minor major groove of RNA duplex in the Tav2b/siRNA complex (Figure 4-3c), the motif pc (Figure 4-3f) binds along the phosphate backbone in the complex of Mss16p DEAD-box helicase domain 2/RNA duplex (Figure 4-3e), and the motif pd (Figure 4-3h) passes over the phosphate backbone from the major groove to the minor groove as found in the small RNA methyltransferase HEN1 complex (Figure 4-3g).

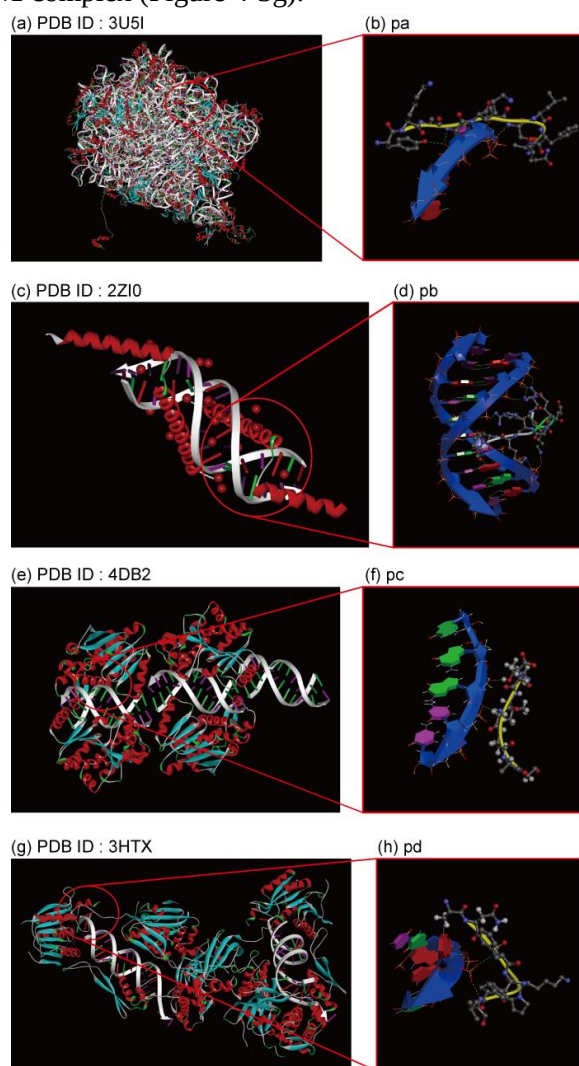
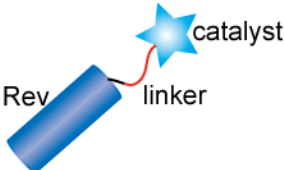


Figure 4-3. Structural models of natural RNPs and partially motifs for the peptide linker design. The natural RNP structures of (a) eukaryotic ribosome (PDB ID: 3U5I),²³ (c) Tav2b/siRNA complex (PDB ID: 2ZI0),²⁴ (e) Mss116p DEAD-box helicase domain 2/RNA duplex (PDB ID: 4DB2)²⁵ and (g) small RNA methyltransferase HEN1 (PDB ID: 3HTX)²⁶ were chosen and the partially structure (b) pa, (d) pb, (f) pc and (h) pd were selected as the linkers on peptide subunit of RNP, respectively.



Peptide Name	Catalyst-linker	Rev
dmap-Rev		dmap-TRQARRNRRRRWRERQRAAAAR
dmap-pa1-Rev	dmap-YKAGKASLFAQ	GGTRQARRNRRRRWRERQRAAAAR
dmap-pa2-Rev	dmap-YKAGKASLFAQ	SGGTRQARRNRRRRWRERQRAAAAR
dmap-pb1-Rev	dmap-NRKERGHKSP	GGGTRQARRNRRRRWRERQRAAAAR
dmap-pb2-Rev	dmap-NRKERGHKSP	GGGTRQARRNRRRRWRERQRAAAAR
dmap-pc1-Rev	dmap-TDVGA	SGGTRQARRNRRRRWRERQRAAAAR
dmap-pc2-Rev	dmap-TDVGA	SGGTRQARRNRRRRWRERQRAAAAR
dmap-pd1-Rev	dmap-ANAFFKPPL	GGGTRQARRNRRRRWRERQRAAAAR
dmap-pf1-Rev		dmap-SGGTRQARRNRRRRWRERQRAAAAR
dmap-pf2-Rev		dmap-SGGSGGTRQARRNRRRRWRERQRAAAAR
dmap-pf3-Rev		dmap-SGGSGGSGGTRQARRNRRRRWRERQRAAAAR
bpy-Rev		bpy-TRQARRNRRRRWRERQRAAAAR
bpy-pa1-Rev	bpy-YKAGKASLFAQ	GGTRQARRNRRRRWRERQRAAAAR
bpy-pb2-Rev	bpy-NRKERGHKSP	GGGTRQARRNRRRRWRERQRAAAAR
bpy-pc2-Rev	bpy-TDVGA	SGGTRQARRNRRRRWRERQRAAAAR
bpy-pd1-Rev	bpy-ANAFFKPPL	GGGTRQARRNRRRRWRERQRAAAAR
H-Rev		HTRQARRNRRRRWRERQRAAAAR
H-pe1-Rev		HGTRQARRNRRRRWRERQRAAAAR
H-pe3-Rev		HGSGTRQARRNRRRRWRERQRAAAAR
H-pe5-Rev		HGGSGGTRQARRNRRRRWRERQRAAAAR
H-pe8-Rev		HGGSGGSGGTRQARRNRRRRWRERQRAAAAR

Figure 4-4. Amino acid sequences of Rev peptide derivatives. Short peptide motifs are introduced through a flexible linker (GGS: orange) to the N-terminal of Rev peptide (TRAQRRNRRRRWRERQR). Dmap, bpy and histidine were modified at the N-terminal of peptide as the catalytic group for hydrolysis.

Synthetic flexible linkers containing multiple repeating sequences (GGS) were also incorporated between the N-terminal of Rev peptide and the catalytic group. Combination of the RNA subunit library and the peptide subunit library afforded a structurally diverse RNP library with catalytic group, in which the substrate ATP resided in various orientation and distances to the catalytic group (Figure 1b). All peptides were synthesized by solid-phase synthesis according to Fmoc chemistry protocols and roughly purified by a diethyl ether extraction after deprotection and cleavage for the first screening.

4-2-3. Complex formation of a structurally diverse RNP library

By complex forming of RNA subunit library and the peptide subunit library, the binding pocket for ATP and catalytic groups were arranged with different length, angle and orientation depends on the variety of two libraries (Figure 4-1b). Thus, the structurally diverse RNP receptor library with binding pocket and catalytic group was prepared. In order to confirm the formation of the complex, the RNP was evaluated by native polyacrylamide gel electrophoresis (PAGE). An15 RNA was used as a representative RNA from structurally diverse RNA subunit library and dmap-pa1-Rev was chosen as representative of peptide from peptide subunit library. For the purpose of evaluation of complex formation by PAGE, ³²P-labeled An15 and dmap-pa1-Rev were purified by 8M denatured PAGE and HPLC, respectively.

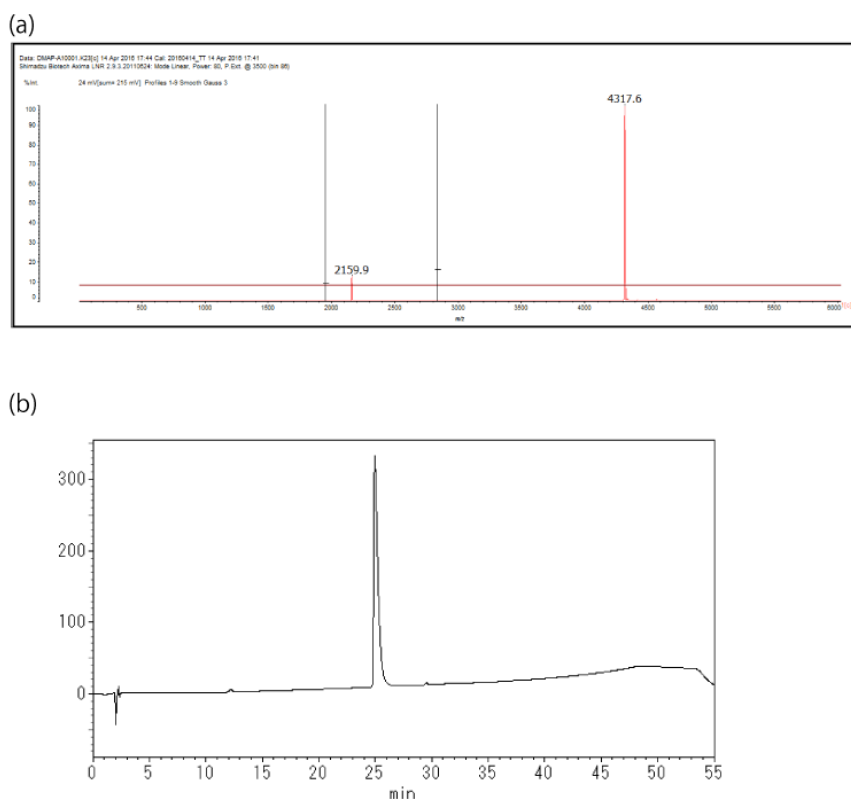


Figure 4-5. (A) MALDI-TOF-MS chart of purified dmap-pa1-Rev ($C_{183}H_{306}N_{78}O_{45}$: (MH^+) calcd: 4320.0, found 4317.6). (B) HPLC chromatogram of dmap-pa1-Rev after purified by HPLC.



Figure 4-6. The purity of 5'-³²P-labeled An15 RNA (1000 and 2000 cpm) was checked by denatured PAGE.

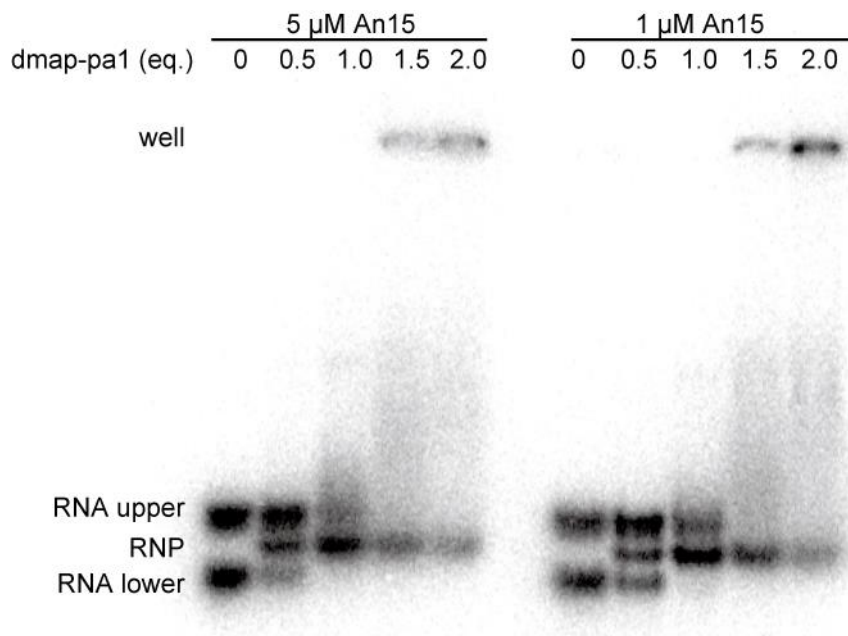


Figure 4-7. Autoradiograms of the gel shift assay showed (a) 5 μM and (b) 1 μM An15 RNA forms a complex with the dmap-pa1 peptide in a buffer containing 30 mM Bis Tris-HCl (pH6.0), 100 mM NaCl, 5 mM MgCl₂, 0.005% Tween 20, and 6% sucrose at 20 °C. Chemical equivalents of the peptide to RNA were 0, 0.5, 1.0, 1.5, and 2.0, respectively.

4-3. Conclusion

The structurally diverse library of RNP with catalytic group was successfully prepared by complexing RNA library that has a binding pocket to adenosine derivative with different structures and structurally diverse peptide library that was modified with catalytic group via various peptide linkers. This is the first example for constructing RNP library with structural diversity, and the library is easy to expand by increasing RNA library or peptide library.

4-4. Materials and methods

4-4-1. Materials

PrimeSTAR HS DNA polymerase for PCR reactions was obtained from TaKaRa Bio Inc. (Shiga, Japan). AmpliScribe T7 Kit for RNA preparation was obtained from Epicentre (Madison, USA). *N*- α -Fmoc-protected amino acids, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), SP grade *N,N*-dimethylformamide (DMF), trifluoroacetic acid (TFA) and 4-[(2,4-Dimethoxyphenyl)-*N*-(9-fluorenylmethoxycarbonyl)aminomethyl]phenoxy-acetamido polyethyleneglycol resin (Fmoc-NH-SAL-PEG resin) were obtained from Watanabe Chemical Industries (Hiroshima, Japan). Dichloromethane (DCM) was purchased from Nacalai Tesque (Kyoto, Japan). *N,N*-diisopropylethylamine (DIEA), HPLC-grade acetonitrile and dmap were obtained from Sigma-Aldrich (St. Louis, USA). Reversed-phase C18 columns (4.6 $\times$ 150 mm, Ultron VX-ODS; Shinwa Chemical Industries, Kyoto, Japan and 10 $\times$ 150 mm, 5C18-ARII; Nacalai Tesque, Kyoto, Japan) were used for purification of peptides for preparative purposes. Gel electrophoresis grade acrylamide, bisacrylamide, phenol, thioanisol, 1,2-ethandithiol and distilled organic solvents were purchased from Wako Chemicals (Tokyo, Japan).

4-4-2. Preparation of RNA subunit library

The plasmid DNA templates were prepared as described.¹³ The double-stranded DNA templates for RNA transcription were constructed by PCR amplification to add the promoter for T7 RNA polymerase using PrimeSTAR DNA polymerase (TaKaRa) with 5'-DNA primer (5'-TCTAATACGACTCACTATAGGTCTGGGCGCA-3': T7 RNA promoter is underlined) and 3'-DNA (5'-GGCCTGTACCGTC-3'). RNA transcription was performed using an AmpliScribeT7 kit (Epicenter) for 3 h at 37 °C, according to the supplier's recommended protocols. The resulting RNA was phenol/chloroform extracted, precipitated with ethanol, and pelleted by centrifugation. For the first screening, the RNA was used without further purification. For the second screening, the RNA purified by denaturing polyacrylamide gel electrophoresis and eluted. Concentrations of RNAs were determined by UV spectroscopy.

4-4-3. Synthesis of catalyst-modified peptide library

The peptides were synthesized as follows. Fmoc-NH-SAL-PEG resin was placed in a dry flask, and a sufficient amount of DMF was added to soak the resin; this mixture was allowed to swell for 30 minutes. The resin was loaded onto an automated peptide synthesizer (Liberty; CEM / PSSM-8; Shimadzu), and the subsequent synthesis was performed according to Fmoc chemistry protocols using protected Fmoc-amino acids and HBTU. Unnatural catalytic groups (3-(Methyl-4-pyridylamino)propionic acid, 4'-Methyl[2,2'-bipyridine]-4-butanoic acid) were directly coupled to N-terminus deprotected Rev peptides on the resin in DMF containing WSC·HCl. Acetylated histidine was introduced by usual Fmoc chemistry protocol and acetylation. The synthesized peptides were used as crude for the first screening and purified by HPLC before carrying out the second screening.

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Chapter 5

Screening of structurally diverse RNP library based on catalytic activity

5-1. Introduction

Biomacromolecules whose three-dimensional structures are suitable for expression of the function of interest would be obtained from a structurally diverse library of biomacromolecules. A structurally diverse library consisted of various three-dimensional structures of biomacromolecules equipped with synthetic functional groups would be obtained by applying the combinatorial method.

Ribonucleopeptide (RNP) is one of the appropriate scaffolds to construct a structurally diverse library of RNA-peptide complexes. The structurally diverse RNP receptor library with catalytic group was already prepared in chapter 4 (Figure 4-2 and 4-4). I hypothesized that RNP receptor with catalytic activity was able to be selected from the library with structural diversity.

Catalytic activity-based screening is one of major methods for the screening of catalytic molecules.¹⁻³ Spectroscopic monitoring of the reaction is rapid, sensitive and quantitative way to evaluate the catalytic activity. In the case of performing spectroscopic measurement, it is very important to realize the condition for the detection with high S/N ratio to accurate evaluation of the “hit” compounds. The deference of signals between in the existence and the absence of RNPs should be significant as much as possible.

In this chapter, I established a catalytic activity based screening method by using novel substrate for the hydrolysis reaction whose conversion could be detected by fluorescence intensity change (Figure 5-1).

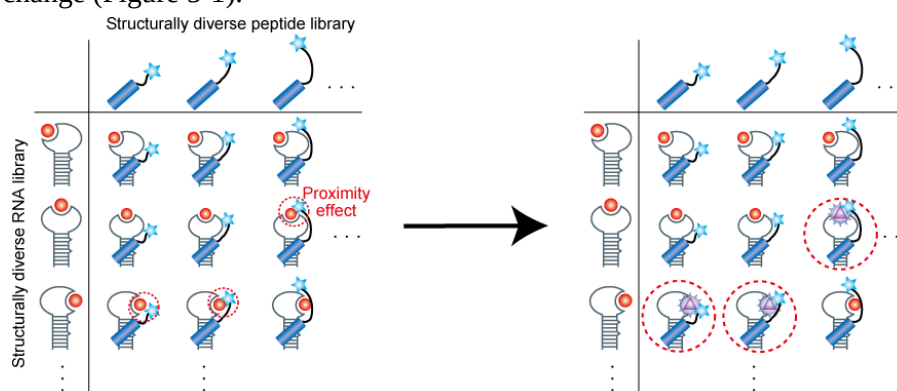


Figure 5-1. Schematic illustration of the catalytic activity-based screening of structurally diverse RNP library.

5-2. Result and discussion

5-2-1. Design and synthesis of the substrate ester for the convenient screening of catalytic RNP

Screening of a library of molecules based on the catalytic activity is one of the major methods for the selection of catalytic biomacromolecules.¹⁻⁴ Spectroscopic monitoring of the ester hydrolysis reaction is a rapid, sensitive and quantitative way to evaluate the catalytic activity. For such measurements, it is very important to optimize the condition for the detection with high S/N ratio to evaluate the “hit” molecules accurately. The signals resulting from the reaction in the presence of RNPs should be distinguishable from that in the absence of RNP. Accordingly, I designed an adenosine derivative as the substrate with a reporter motif for monitoring an ester hydrolysis reaction of umbelliferyl ester derivative by fluorescent measurements (Figure 5-2).

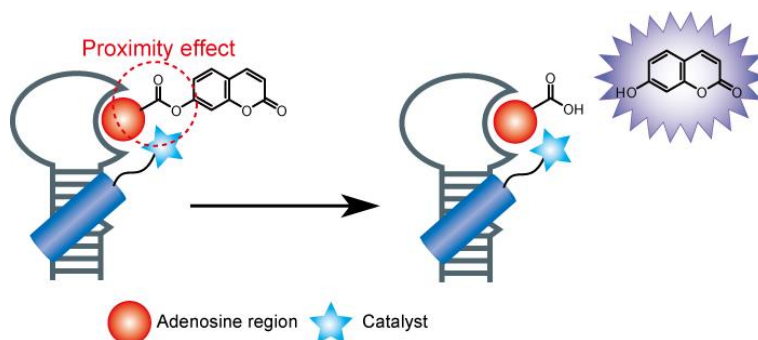


Figure 5-2. Schematic illustration of expression of catalytic activity of RNP receptor. The proximity effect between substrate and catalytic group would accelerate the hydrolysis reaction and the resulted umbelliferone could be detected by fluorescence measurement.

In order to evaluate the artificial enzymes for ester hydrolysis,⁵⁻⁷ *p*-nitrophenyl ester derivatives have been widely used as the reporter motif of substrate because *p*-nitrophenyl ester derivatives show little or no absorbance of around 400 nm in water but show the specific absorbance of *p*-nitrophenolate upon hydrolysis of the ester.⁵⁻⁸ Generally, autohydrolysis of the *p*-nitrophenyl ester is occurred in aqueous condition and accelerated in the basic condition, which causes to increase the background noise. Though the reaction in acidic condition is desirable to avoid the autohydrolysis of *p*-nitrophenyl ester, the reaction in the acidic condition results no significant absorbance of *p*-nitrophenolate due to the high pK_a value of the *p*-nitrophenol (7.15 at 25 °C).⁹ The extinction coefficient at 400 nm (ϵ_{400}) of *p*-nitrophenol at pH 6.2 is only 2800 (Table 5-1), and the quantitative determination of the conversion of the hydrolysis is difficult by the high-through-put UV measurement by the plate reader.

Table 5-1. pH dependence of extinction coefficient ($\epsilon_{400\text{nm}}$) of *p*-nitrophenol

pH	$\epsilon_{400\text{nm}}$	pH	$\epsilon_{400\text{nm}}$
6.24	2800	7.25	9900
6.36	3200	7.3	12100
6.41	3700	7.48	13500
6.49	4300	7.51	14300
6.55	4800	7.65	15000
6.65	5400	7.76	15500
6.72	6100	7.86	15900
6.82	6900	7.91	16400
6.91	7700	7.97	16700
7.02	7600	8.11	17000

In contrast, the fluorescent emission of umbelliferone is detectable by plate reader even in slightly acidic conditions because of its high quantum yield, because of its high quantum yield ($\Phi_f = 0.79$),¹⁰ and the fluorescence intensity of an umbelliferyl ester is quite low ($\Phi_f = 0.0011$ for 7-acetoxycoumarin).¹⁰ Also the fluorescence detection has an advantage in reducing the background noise caused by aggregation or bubble formation in the wells on the plate compared to that in the UV absorbance detection. These advantages of umbelliferyl ester prompted us to utilize umbelliferone as the reporter motif of the ester derivative of adenosine for screening the catalytic activity of structurally diverse RNP library.

The ATP-binding RNP receptor binds to the adenine base of ATP by forming the Hoogsteen U:A:U triple (Figure 5-3) which has been determined by NMR measurements.¹¹ Due to this recognition mechanism, modification of the ribose moiety of adenosine would not significantly affect the substrate binding ability of RNP receptors. I designed an umbelliferyl ester derivative of adenosine (Umb-Ado) (7) as the substrate of hydrolysis reaction by structurally diverse RNPs.

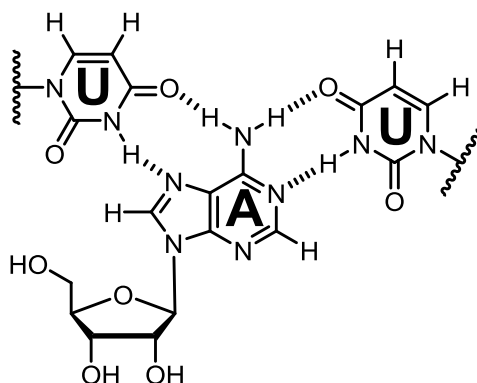
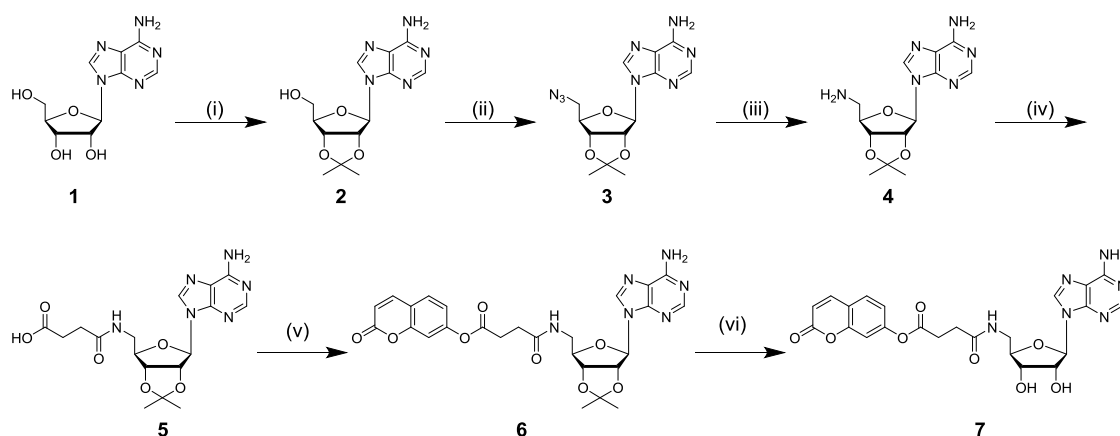


Figure 5-3. Binding mode of the ANP-binding RNP receptor with the substrate adenosine¹¹



Scheme 1. Synthetic scheme of Umb-Ado. Reaction conditions: (i)TsOH, acetone; (ii)dppa, DBU, 15-crown 5-ether, NaN₃, 1,4-dioxane; (iii)H₂, Pd/C, MeOH; (iv) Succinic anhydride, pyridine; (v) WSCI·HCl, umbelliferone, DMAP, CH₂Cl₂; (vi) 50% TFA/H₂O

For the synthesis of Umb-Ado 7, 2',3'-isopropylideneadenosine²⁸ 2 was converted to an azide compound 3, then to 5'-deoxyamino-2',3'-isopropylideneadenosine 4 upon reduction under hydrogen atmosphere.²⁹ Condensation of 4 with succinic acid anhydride gave 5'-calboxylic acid 5, which was further converted to the umbelliferyl ester 6 by WSC. Deprotection of the ester 6 gave the designed substrate Umb-Ado 7.

5-2-2. Determination of the condition for the screening of catalytic RNP receptors

To evaluate the availability of Umb-Ado as the substrate for screening of the ester hydrolysis activity of RNPs, time dependent spectral change of Umb-Ado was evaluated at pH 6 (Figure 5-4). Time dependent increment of the fluorescence emission of Umb-Ado ($\lambda_{\text{Ex}} = 355$ nm/ $\lambda_{\text{Em}} = 455$ nm) were observed, indicating that ester hydrolysis process of Umb-Ado could be monitored by fluorescence intensity change. To decide the screening condition, next, the time course profiles of the background hydrolysis reaction at 20 °C were monitored at different pH (Figure 5-5).

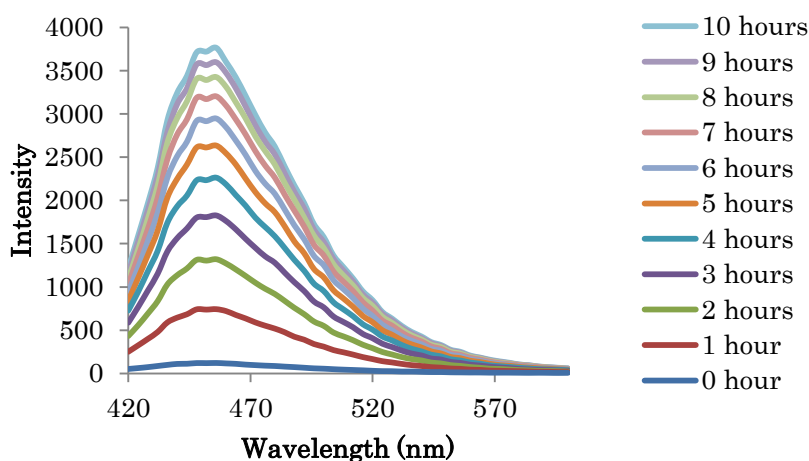


Figure 5-4. Time dependent spectra of umbelliferyl ester (6) in the buffer containing 30 mM Bis-Tris-HCl (pH 6.0) with 100 mM NaCl, 5 mM MgCl₂, and 0.005% Tween 20 at 20 °C.

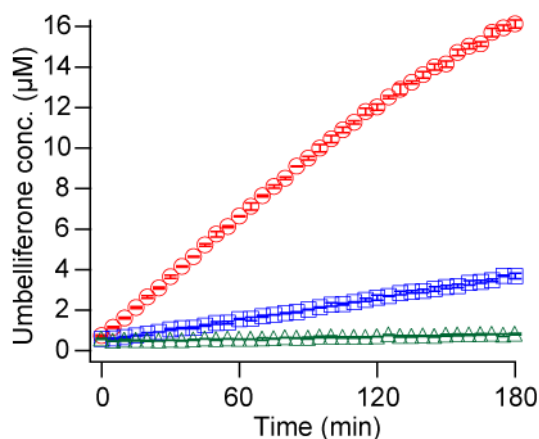


Figure 5-5. Time course plot of autohydrolysis reaction of Umb-Ado in pH 7 (red circle), pH 6 (blue square) and pH 5 (green triangle) at 20 °C.

The same concentrations of the salts and detergent (100 mM NaCl, 5 mM MgCl₂, 0.005% Tween 20) as described previously for the ligand-binding studies of ATP-binding RNP were applied.^{11,12} For the condition of pH 7, sodium phosphate buffer was used. A Bis-Tris buffer and an acetate buffer were, respectively, used for the conditions of pH 6 and pH 5. Autohydrolysis of Umb-Ado was observed in the buffer at pH 7, and the conversion was 80% after 3 hours at 20 °C (Figure 5-5). At pH 6, the conversion was around 10% after 3 hours. Umb-Ado showed almost no hydrolysis at pH 5 as judged from the emission intensity, but the quantum yield for the fluorescence emission of the umbelliferone at pH 5 was too low to determine the accurate concentration. These results taken together, the buffer containing 30 mM Bis-Tris-HCl (pH 6.0), 100 mM NaCl, 5 mM MgCl₂ and 0.005% Tween 20 was suitable to use for screening the hydrolytic activity of RNP library at 20 °C.

Complex formation of RNP at 20 °C in this buffer was confirmed by gel shift assay using 8% native polyacrylamide gel electrophoresis (PAGE) for several concentrations of RNPs (in chapter 4). Formation of a 1:1 complex of RNA and peptide was confirmed in this buffer condition. Complex formation of the ATP-binding RNP and Umb-Ado was analyzed by using a 5FAM-labeled Rev peptide (5FAM-Rev)¹¹ and An15 RNA to give the dissociation constant (K_D) of 2.6 ± 0.2 μ M in this buffer condition at 20 °C (Figure 5-6). This result indicated that the designed substrate for the hydrolytic reaction, Umb-Ado, maintained the binding ability to the ATP-binding RNP receptor^{11,12} in this condition.

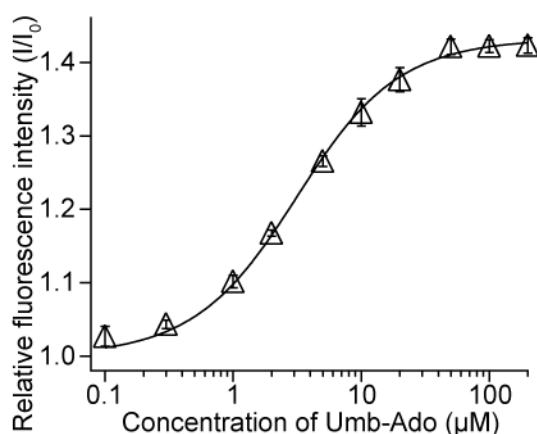


Figure 5-6. Titration curve for the relative fluorescence intensity changes of An15/5FAM-Rev with increasing concentrations of Umb-Ado (7) in a buffer containing 30 mM Bis-Tris-HCl (pH 6.0) with 100 mM NaCl, 5 mM MgCl₂, and 0.005% Tween 20 at 20 °C. The calculated dissociation constant (K_D) of An15/5FAM-Rev for the target (7) ester was 2.6 ± 0.2 μ M.

5-2-3. Screening of the structurally diverse RNP library for selecting catalytic RNPs

The first screening of structurally diverse RNP library with catalytic group was performed with roughly purified RNAs and peptides to test a condition suitable for the high-through-put screening. RNA was purified with size-exclusion after the transcription reaction and each synthesized peptide was purified by only a diethyl ether extraction after deprotection and cleavage from the resin. In total, 609 structurally diverse RNPs were obtained by combination of 29 RNAs and 21 peptides (Table 5-2). Each RNP of the library was mixed with Umb-Ado in the screening buffer and time course changes of the fluorescence emission intensity (455 nm) were monitored. Conversion of the ester hydrolysis was calculated by using the standard curve of the umbelliferon emission (Figure 5-7).

Table 5-2. Results of the acceleration ratio of rate of the reaction (k_{obs}) in 1st screening. Time dependent fluorescent intensities of the samples containing 20 μ M Umb-Ado, 5 μ M RNA, 7.5 μ M peptide, 30 mM Bis-Tris-HCl (pH 6.0) with 100 mM NaCl, 5 mM MgCl₂, and 0.005% Tween 20 were measured at 20 °C and compared with the sample without RNP in same condition. Colored cells were indicated the 1.2 times or more acceleration of the reaction.

	Peptide library																					
	dmap											bpy				H					no peptide	
	Rev	GS1	GS2	GS3	pa1	pa2	pb1	pb2	pc1	pc2	pd1	Rev	pa1	pb2	pc2	pd1	Rev	pe1	pe3	pe5		pe8
RNA library	A02	1.0	1.2	1.2	1.0	1.2	1.0	1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	0.9	1.0	1.0
	A08	1.1	1.1	1.0	1.0	1.1	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.1	1.0	1.0	0.9	0.9	1.1
	A09	1.1	1.1	1.0	1.1	1.1	1.1	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.1	1.1	0.9	1.3	1.0	1.0	0.9	1.1
	A14	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.1	1.0	1.0	1.0	1.0	1.1	0.9	1.1	1.0	1.0	0.9	1.0
	A15	1.0	1.1	1.0	1.0	1.4	1.1	1.4	1.0	1.1	1.0	1.0	1.0	1.1	1.0	1.2	0.9	1.1	1.1	1.0	1.0	1.0
	A17	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.1	1.0	1.0	1.0	1.0	1.1	0.9	1.0	1.1	0.9	0.9	1.0
	A24	1.0	1.1	1.0	1.0	1.1	1.0	1.1	1.1	1.1	1.0	1.1	1.0	1.1	1.1	1.1	0.9	1.1	1.1	1.0	0.9	1.0
	A25	1.1	1.1	1.0	1.1	1.2	1.0	1.0	1.0	1.2	1.1	1.0	1.0	1.0	1.0	1.1	0.9	1.1	1.1	1.0	0.9	1.0
	A26	1.1	1.0	1.0	1.0	1.3	1.1	1.0	1.0	1.1	1.0	1.0	1.0	1.1	1.2	1.2	0.9	1.1	1.1	1.0	1.0	1.0
	A30	1.0	1.1	1.0	1.0	1.0	1.1	1.1	1.0	1.1	1.0	1.1	1.0	1.1	1.1	1.1	1.0	1.1	1.0	1.0	1.0	1.0
	A33	1.0	1.0	1.1	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	0.9	1.0	0.9	1.1	1.0	0.9	1.0
	A34	1.0	1.1	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0
	A36	1.0	1.1	1.1	1.0	1.1	1.0	1.0	1.0	1.1	1.0	1.1	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	0.9	1.0
	An02	1.0	1.1	1.1	1.0	1.1	1.0	1.1	1.0	1.1	1.1	1.0	1.0	0.9	0.9	1.0	1.0	1.0	1.1	1.0	0.9	1.0
	An03	1.0	1.1	1.1	1.0	1.1	0.9	1.1	1.0	1.1	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	0.9	1.0
	An08	1.1	1.0	1.0	1.0	1.0	1.1	1.0	0.9	1.1	1.1	1.0	1.0	1.0	1.0	1.1	0.9	1.1	1.1	1.1	0.9	1.0
	An09	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	0.9	1.1	1.0	1.1	0.9	1.0
	An10	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.1	1.0	1.1	0.9	1.0
	An15	1.0	1.0	1.0	1.0	1.2	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.1	0.9	1.1	1.0	1.1	0.9	0.9
	An16	1.2	0.9	1.0	1.1	1.1	1.1	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.1	0.9	1.1	1.1	1.1	0.9	1.0
An17	1.1	1.0	1.0	1.1	1.0	1.1	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	0.9	1.1	1.1	1.0	1.0	1.1	
An18	1.1	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.0	1.0	1.0	1.0	1.1	0.9	1.1	1.1	1.1	0.9	1.0	
An19	1.0	0.9	1.0	1.0	1.0	1.1	1.0	1.0	1.1	1.0	1.0	1.1	1.0	1.0	1.1	0.9	1.0	1.0	1.0	0.9	1.0	
An22	1.1	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.1	0.9	1.0	1.0	1.0	0.9	1.0	
An29	1.2	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	0.9	1.1	1.1	1.0	0.9	1.0	
An30	1.1	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.1	1.0	1.0	1.0	1.0	
An33	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.1	1.0	1.0	0.9	1.0	
An34	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.1	1.0	1.1	1.0	1.0	1.0	1.0	1.1	0.9	1.0	1.0	1.0	1.0	1.0	
An35	1.0	1.1	1.1	1.0	1.1	1.0	1.0	0.9	1.1	1.1	1.0	1.0	1.0	1.0	1.1	0.9	1.1	1.0	1.0	0.9	1.0	
no RNA	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.1	0.9	1.0	

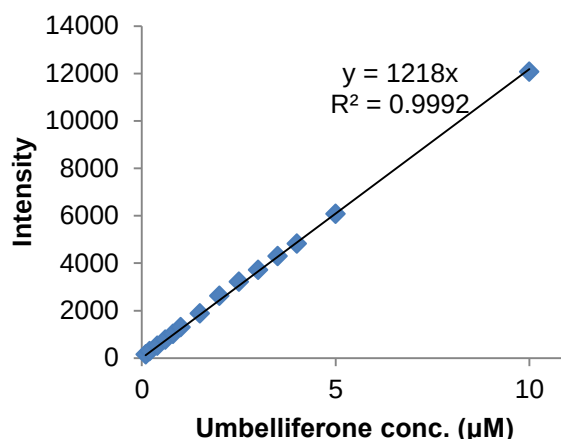


Figure 5-7. Standard curve for the fluorescent intensity (Ex. 355nm/ Em. 455 nm) of umbelliferone in a buffer containing 30 mM Bis-Tris-HCl (pH 6.0) with 100 mM NaCl, 5 mM MgCl_2 , and 0.005% Tween 20 at 20 °C.

In the first screening of the hydrolytic activity, RNPs showed acceleration of the reaction rate over 1.2 times higher than that in the condition without RNP (Table 5-2). These RNPs were selected for the second screening, in which the RNA and the peptide components were purified by denaturing PAGE and HPLC, respectively. In the second screening, a combination of An15 RNA and dmap-pa1-Rev peptide showed the highest acceleration of the hydrolysis (Figure 5-8a). An15 RNA or dmap-pa1-Rev peptide by itself showed no acceleration of the ester hydrolysis reaction (Figure 5-9), indicating that the RNP complex formation by An15 and dmap-pa1-Rev is necessary to accelerate the reaction. The proximity effect between the target ester in the binding pocket of An15 and dmap in dmap-pa1-Rev upon the complex formation of An15/dmap-pa1-Rev RNP likely accounts for the observed acceleration of the ester hydrolysis. To support this, hydrolysis of 7-acetoxycoumarin, an umbelliferyl ester without the adenosine moiety, did not show any acceleration in the presence of An15/dmap-pa1-Rev RNP (Figure 5-10). The pseudo-first order kinetics constants (k_{obs}) of the hydrolysis of Umb-Ado with and without An15/dmap-pa1-Rev were 4.0 and $3.4 \times 10^{-4} \text{ min}^{-1}$, respectively. The k_{obs} with RNP is 1.2-fold higher than that without RNP (Figure 5-8b). Thus, the facile screening method for the construction of the catalytic RNPs by using structurally diverse RNP library was developed.

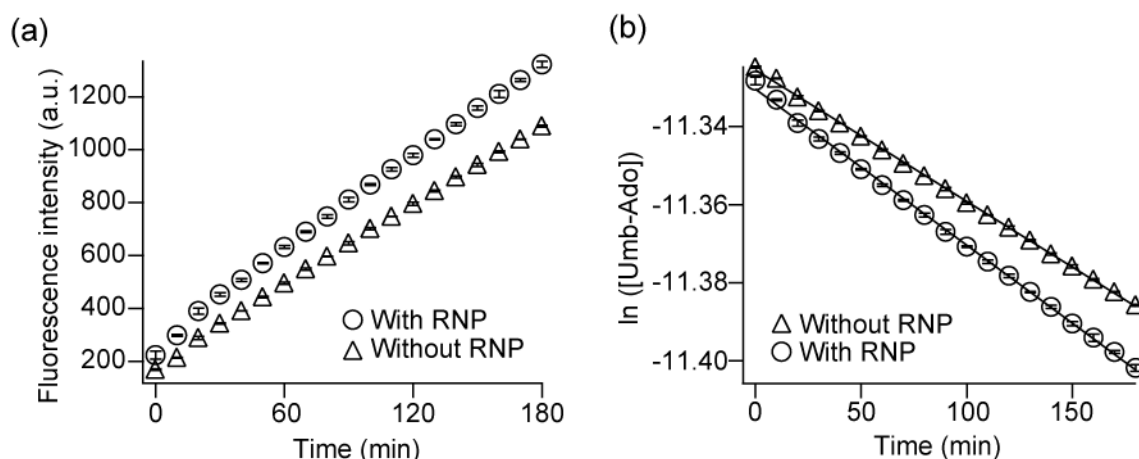


Figure 5-8. (a) Time course plot of the intensity of the sample containing Umb-Ado with (circle) and without (triangle) RNP (An15/dmap-pa1-Rev); (b) Time course plot of $\ln([Umb-Ado])$ of the sample containing ester (7) with (circle) and without (triangle) RNP (An15/dmap-pa1). The absolute values of the slopes indicate the pseudo-first order kinetics constant (k_{obs}). All samples were measured in a buffer containing 30 mM Bis-Tris-HCl (pH 6.0) with 100 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20 at 20 °C.

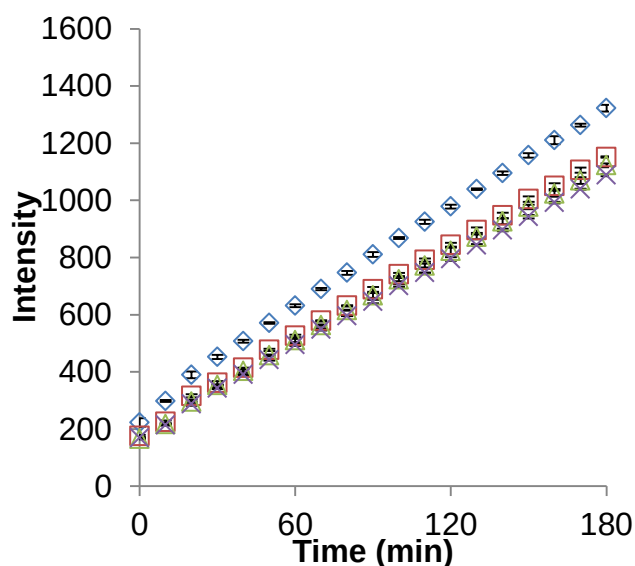


Figure 5-9. Time course plot of the intensity of the sample containing the ester (7) with RNP (An15/dmap-pa1-Rev) (blue diamond), with peptide only (red rectangle), with RNA only (green triangle) and without any peptide and RNA (purple cross) in a buffer containing 30 mM Bis Tris-HCl (pH 6.0) with 100 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20 at 20 °C.

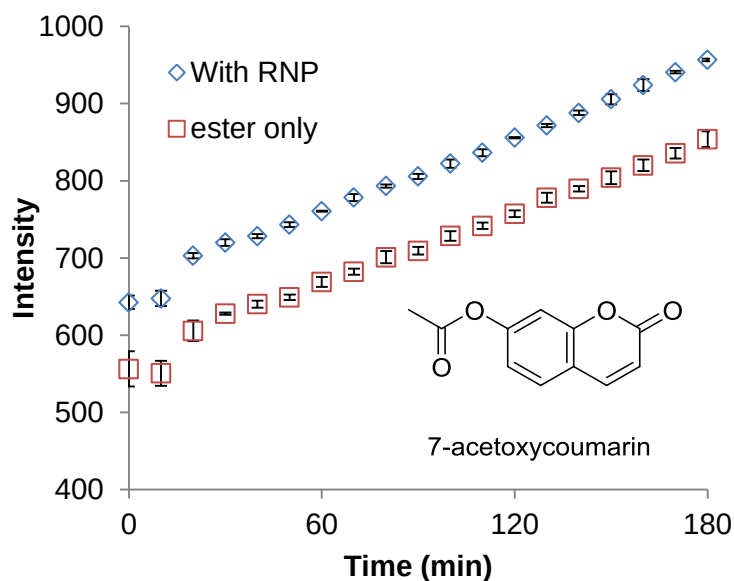


Figure 5-10. Time course plot of the intensity of the sample containing 7-acetoxycoumarin with (blue diamond) and without (red rectangle) RNP (An15/dmap-pa1-Rev) in a buffer containing 30 mM Bis-Tris-HCl (pH 6.0) with 100 mM NaCl, 5 mM MgCl₂, and 0.005% Tween 20 at 20 °C.

5-3. Conclusion

The designed ester, Umb-Ado, could bind to the RNP receptor and could be used for the fluorescence detection in acidic condition. The library was screened to select the RNP that has suitable structure to show catalytic activity for ester hydrolysis of Umb-Ado. As the result of screening, the combination of An15 RNA and dmap-pa1 peptide clearly showed the acceleration of the hydrolysis of Umb-Ado. Thus, I could get RNP that has a catalytic activity for ester hydrolysis in this demonstration. It is notable that the catalytic activity of RNP screened from the small size of library less than 1000 in this study would be improved by the expansion of the size of the library. Because each RNA and peptide subunit is easy to be prepared and the combination of two subunits affords to expand the size of library conveniently, this method can be utilized for the further exploration of catalytic receptors.

5-4. Materials and experiments

5-4-1. Materials

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCl·HCl) and trifluoroacetic acid (TFA) were obtained from Watanabe Chemical Industries (Hiroshima, Japan). Dichloromethane (DCM), 1,8-diazabicyclo[5,4,0]-7-undecene (DBU) and umbelliferon were purchased from Nacalai Tesque (Kyoto, Japan). *N,N*-diisopropylethylamine (DIEA), HPLC-grade acetonitrile and dmap and *p*-toluenesulfonic acid monohydrate were obtained from Sigma-Aldrich (St. Louis, USA). Adenosine, 15-crown 5-ether and diphenylphosphoryl azide (dppa) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Reversed-phase C18 columns (4.6 × 150 mm, Ultron VX-ODS; Shinwa Chemical Industries, Kyoto, Japan and 10 × 150 mm, 5C18-ARII; Nacalai Tesque, Kyoto, Japan) were used for purification of peptides for preparative purposes. Sodium azide, succinic anhydride, 10% palladium on carbon and distilled organic solvents were purchased from Wako Chemicals (Tokyo, Japan).

5-4-2. Synthesis of 5'-deoxy-5'-(umbelliferyl butylate)amidoadenosine

Synthesis of 2',3'-O-isopropylideneadenosine (2)¹³

A mixture of compound 1 (4.96 g, 18.56 mmol), *p*-toluenesulfonic acid monohydrate (3.53 g, 18.56 mmol) and acetone (150 mL) was stirred at r.t. under open vessel. After 46 h from starting reaction, 5.10 g of the acid were added. After 2h, 5.21g of the acid were added (total 13.94g, 73.3 mmol). After 2h from adding the acid, the spot of compound 1 disappeared. The mixture became yellow solution. The reaction solution was quenched by adding 12.3 mL of triethylamine (88.4 mmol, 4.73 eq.). The mixture were evaporated and purified by column chromatography (chloroform / methanol 20 : 1) to get 2 (4.50 g, 14.6 mmol, 79 %). ¹H NMR (300 MHz, DMSO-d₆) 1.33 and 1.55 (2s, 6H, CMe₂), 3.52-3.56 (m, 2H, H5'), 4.19-4.23 (m, 1H, H4'), 4.95-4.98 (m, 1H, H 3'), 5.25 (vbs, 1H, OH), 5.33-5.36 (m, 1H, H 2'), 6.11-6.12 (m, 1H, H 1'), 7.34 (bs, 2H, NH₂), 8.15 (s, 1H, H2), 8.34 (s, 1H, H8); ESI-MS for C₁₃H₁₇N₅O₄: (MNa⁺) calcd: 330.12, found: 330.03

Synthesis of 5'-deoxy-5'-azido-2',3'-O-isopropylideneadenosine (3)¹⁴

Compound 2 (1.0 g, 3.25 mmol, 1.0 eq.), dppa (1.4 mL, 6.51 mmol, 2.0 eq) and DBU (1.47 mL, 9.77 mmol, 3.0 eq.) were dissolved in 1,4-dioxane 10 mL in nitrogen atmosphere. The mixture was stirred at room temperature for 2 hours followed by the addition of sodium azide (1.0 g, 16.3 mmol, 5.0 eq.) and 15-crown 5-ether (6.5 μL, 33 μmol, 0.01 eq.). The mixture was refluxed for 1 hour and filtered. The filtrate was evaporated and purified by column

chromatography (chloroform/methanol 29 : 1) to give the compound 3 (0.87 g, 2.6 mmol, 80%). ¹H NMR (300 MHz, CDCl₃): 1.39 and 1.62 (2s, 6H, CMe₂), 3.52-3.64 (m, 2H, H5'), 4.36-4.41 (m, 1H, H4'), 5.06 (dd, 1H, J = 3.3, 6.3 Hz, H3'), 5.46 (dd, 1H, J = 2.5, 6.5 Hz, 2H'), 5.89 (br, 2H, NH₂), 6.11 (d, 1H, J = 2.5 Hz, H1'), 7.93 (s, 1H, H8), 8.36 (s, 1H, H2). ESI-MS for C₁₃H₁₆N₈O₃: (MNa⁺) calcd: 355.12, found: 355.08.

Synthesis of 5'-deoxy-5'-amino-2',3'-O-isopropylideneadenosine (4)¹⁴

Compound 3 (1.0 g, 3.3 mmol), 10% Pd/C (200 mg) and methanol (50 mL) were stirred in room temperature at hydrogen atmosphere. After 4h, the reaction mixture was filtered. This resulting compound was used to next step without further purification. ¹H NMR (300 MHz, CDCl₃) 1.41 and 1.64 (2s, 6H, CMe₂), 3.01-3.07 (m, 2H, H5'), 4.30-4.31 (m, 1H, H4'), 5.05 (dd, J = 6.3, 3.3 Hz, 1H, H3'), 5.48 (dd, J = 6.3, 3.0 Hz, 1H, H2'), 6.12 (d, J = 3.0 Hz, 1H, H1'), 8.18 (s, 1H, H2), 8.82 (s, 1H, H8); ESI-MS for C₁₃H₁₆N₈O₃: (MNa⁺) calcd: 307.14, found: 307.11

Synthesis of 5'-deoxy-5'-(3''-carboxypropyl)amido-2',3'-O-isopropylideneadenosine (5)

The compound 4 (313 mg, 1.02 mmol) and succinic anhydride (795 mg, 7.5 mmol) were stirred in distilled pyridine (20 mL) in the nitrogen atmosphere at 0 °C. The temperature was increased to room temperature over 30 min. After 12 hours, the organic layer was washed with HCl_{aq}, conc. NaHCO₃, brine and dried by sodium sulfate to give compound 5 (230 mg, 0.57 mmol, 56%). ¹H NMR (300 MHz, CDCl₃) 1.35 and 1.69 (2s, 6H, CMe₂), 2.78-2.84 (m, 4H, (CH₂)₂), 3.23 (d, J = 14.7 Hz, 1H, H5'), 3.64-3.76 (m, 2H, NH₂), 4.20-4.28 (m, 1H, H5'), 4.52 (d, J = 2.1 Hz, 1H, H4'), 4.78 (dd, J = 6.3, 1.8 Hz, 1H, H3'), 5.30 (m, 1H, H2'), 5.78 (d, J = 5.4 Hz, 1H, H1'), 7.87 (s, 1H, H2), 8.31 (d, J = 9.6 Hz, 1H, NH), 8.38 (s, 1H, H8); ESI-MS for C₁₇H₂₂N₆O₆: (MH⁺) calcd: 407.17, found: 407.09

Synthesis of 5'-deoxy-5'-(umbelliferyl butylate)amido-2',3'-O-isopropylideneadenosine (6)

The compound 5 (28 mg, 0.07 mmol), and umbelliferone (57 mg, 0.35 mmol) were stirred in DCM (15 mL) in nitrogen atmosphere at 0 °C. DMAP (0.86 mg, 0.007 mmol) and WSCI·HCl (16 mg, 0.084 mmol) were added and the reaction temperature was increased to room temperature over 30 min. After 24 hours, the resulting solution was evaporated and purified by HPLC (column : 5C18-AR-II 20 mm(ID) × 150 mm(L)/mobile phase 5% AcOH with 24 to 48% MeCN) to give compound 6 (0.011 mmol, 16%). ¹H NMR (300 MHz, CDCl₃) 1.18 and 1.58 (2s, 6H, CMe₂), 2.69-2.75 (m, 1H, H5'), 2.86-2.95 (m, 2H, H8'), 3.16-3.25 (m, 2H, H9'), 4.24-4.32 (m, 1H, H5'), 4.51 (d, J = 1.9 Hz, 1H, H4'), 4.78 (dd, J = 6.2, 1.9 Hz, 1H, H3'), 5.31 (t, J = 5.7 Hz, 1H, H2'), 5.74-5.78 (b, 3H, H1' and NH₂), 6.34 (d, J = 9.4 Hz, 1H, Ar-H), 7.06 (dd, J = 8.5, 2.2 Hz, 1H, Ar-H), 7.13 (d, J = 2.2 Hz, 1H, Ar-H), 7.44 (d, J = 8.5 Hz, 1H,

Ar-H), 7.67 (d, J = 9.6 Hz, 1H, Ar-H), 7.84 (s, 1H, H8), 8.36 (s, 1H, H2), 8.79 (d, J = 9.3 Hz, 1H, NH); ESI-MS for C₂₆H₂₆N₆O₈: (MH⁺) calcd: 551.18, found: 551.20

Synthesis of 5'-deoxy-5'-(umbelliferyl butylate)amidoadenosine (Umb-Ado) (7)

The solution containing compound 6 (20 mg, 0.036 mmol) was stirred in 50% TFA/H₂O and after 48 hours it was purified by HPLC (column : 5C18-AR-II 20 mm(ID) × 150 mm(L)/mobile phase 5% AcOH with 24 to 48% MeCN) to give compound 7 (0.008 mmol, 24%). ¹H NMR (300 MHz, CDCl₃) 2.75-2.81 (m, 2H, H9'), 2.95-3.06 (m, 2H, H8'), 3.34-3.38 (m, 3H, H4' and H5'), 4.29 (d, J = 2.57, 1H, H3'), 4.75 (t, J = 6.2 Hz, 1H, H2'), 5.81 (d, J = 6.6 Hz, 1H, H1'), 6.42 (d, J = 9.4 Hz, 1H, Ar-H), 7.10 (dd, J = 8.4, 2.2 Hz, 1H, Ar-H), 7.17 (d, J = 1.8 Hz, 1H, Ar-H), 7.55 (d, J = 8.4 Hz, 1H, Ar-H), 7.79 (d, J = 9.5 Hz, 1H, Ar-H), 8.01 (s, 1H, H8), 8.30 (s, 1H, H2), 8.82 (d, J = 7.0 Hz, 1H, NH); ESI-MS for C₂₃H₂₂N₆O₈: (MH⁺) calcd: 511.16, found: 511.09

5-4-3. Fluorescence measurements on the microplate

The fluorescence measurements were performed using 96-well plate on a Wallac ARVOsx 1420 multilabel counter or Tecan infinity pro. A binding solution (100 µL) containing 1 µM of fluorescent RNP (An15/5FAM-Rev) in 30 mM Bis-Tris-HCl (pH 6.0), 100 mM NaCl, 5 mM MgCl₂, and 0.005% Tween 20 with indicated concentration of substrate was gently swirled for a few minutes and allowed to sit for 30 min at 20 °C. Emission spectra were measured with an appropriate filter set for each fluorophore. Excitation and emission wavelengths for 5FAM-Rev were 485 and 535 nm, respectively.

5-4-4. Determination of binding affinity by fluorescence titrations

The binding affinity of fluorescent RNP to Umb-Ado was obtained by fitting the substrate titration data using the equation:

$$F_{obs} = \frac{A([FRNP]_T + [substrate]_T + K_D) - (([FRNP]_T + [substrate]_T + K_D)^2 - 4[FRNP]_T[substrate]_T)^{1/2}}{2[FRNP]_T}$$

where A is the increase in fluorescence at saturating substrate concentrations (F_{max} - F_{min}), K_D is the equilibrium dissociation constant, and [FRNP]_T and [substrate]_T are the total concentrations of fluorescent RNP and the substrate, respectively.

5-4-5. Screening of RNP library

Fluorescence measurements in 96-well plates were performed on a Wallac ARVOsx 1420 multilabel counter. A solution (100 μ L) containing 5 μ M of each combination of RNPs and 20 μ M ester (7) in reaction buffer containing 30 mM Bis-Tris-HCl (pH 6.0), 100 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20 was gently swirled for 30 seconds and the time dependent emission intensity of the umbelliferone, which is a product of the hydrolysis, was measured every 20 min for 3 hours. Excitation and emission wavelengths for umbelliferone were 355 and 455 nm, respectively. The calculation of the concentration of the umbelliferone was done by using standard curve.

5-4-6. Calculation of the pseudo-first order kinetics constant (k_{obs})

The pseudo-first order kinetics constant (k_{obs}) was obtained from the slope of the log plot of the time-dependent concentration of umbelliferone.

5-5. References

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Chapter 6

Structure-based design of a ribonucleopeptide receptor with a catalytic group

6-1. Introduction

Modification of naturally occurring receptors is one of the promising methods to construct functional biomolecules with high substrate selectivity. Efforts have been reported to construct biosensors through modification of protein and nucleic acid receptors with appropriate fluorophores,¹⁻⁴ or artificial enzymes with a catalytic group to achieve the proximity effect between the substrate and the catalytic group.^{5,6} In order to modify a receptor to exert a function of interest, we must perform trial-and-error experiments of substitution or insertion of functional groups by chemical modification or genetic mutation even with the three-dimensional structure of the receptor. These chemical or genetic methods include laborious tasks. In addition, when a new functional group is introduced near the substrate-binding pocket of the receptor, there is a possibility of losing the original substrate binding ability. If the binding pocket and the newly introduced functional group locate within the same domain of receptor, the possibility of losing the original function would increase because even a modification of partial structure could disturb the stability of whole domain.

We have reported a modular method for constructing a biomolecular complex, ribonucleopeptide (RNP).⁷⁻¹³ The three-dimensional structure based on the NMR study of Rev peptide-RRE (Rev Response Element) RNA complex and interaction mode between Rev peptide and RRE RNA have been established.¹⁴ By connecting an RNA aptamer, for which the 3D structure is known, as a receptor module and the Rev-RRE complex, in which a fluorophore has been modified as a reporter module, we succeeded in preparing a fluorescent RNP sensor that responds to the substrate binding event at the binding pocket as changes in fluorescence intensity (Figure 6-1).¹⁵

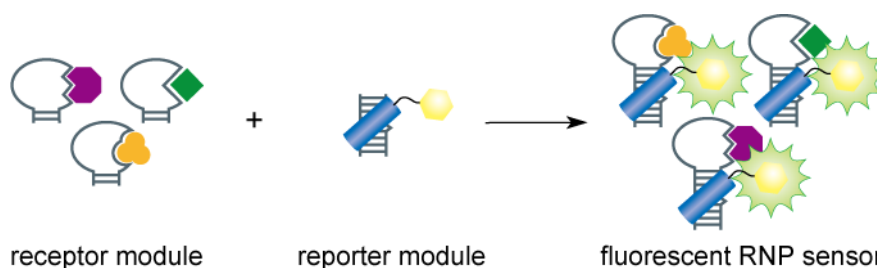


Figure 6-1. Schematic illustration of modular strategy for construction of a fluorescent RNP sensor. A receptor module consists of an RNA aptamer with a binding pocket for the target substrate. A reporter module consists of a complex of fluorophore-modified Rev and RRE RNA. The receptor and reporter modules were connected at the duplex region of each RNA. Structural changes that occur during the binding event between the RNP and target substrate affect the micro-environment around the fluorophore to change the fluorescence intensity.

In this modular method for construction of a fluorescent RNP sensor, the communication between the structural change at the receptor module and the micro-environment of the fluorophore in the reporter module is most important.¹⁵ These results indicate that the RNP is an appropriate scaffold to perform modular design and to allow communication between the designed modules to express the function of interest. This modular strategy would be applicable to construct a catalytic RNP. The catalytic module has already been developed for the library of peptides (Chapters 4 and 5). We hypothesized that an RNP with catalytic activity could be constructed by the modular design based on the three-dimensional structure to achieve the proximity effect between the substrate and the catalytic group. Three modules were connected to enable communication: the substrate-binding module consisting of an RNA aptamer; the catalytic module consisting of the catalytic group; and a geometry-adjusting module consisting of RNP (Figure 6-2). In this research, the three modules were designed to construct an RNP with catalytic activity.

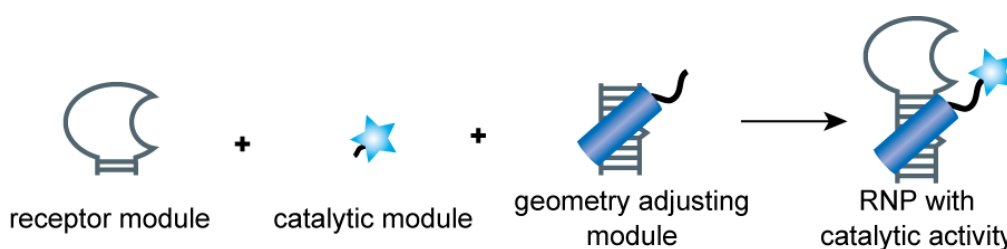


Figure 6-2. Schematic illustration of a modular strategy based on three-dimensional structures to construct an RNP with catalytic activity. A receptor module consists of an RNA aptamer with a binding pocket for the target substrate. The catalytic module consists of a catalytic group for the reaction of interest. The geometry-adjusting module consists of an RNP where the N-terminus of the Rev peptide and the RNA subunit are located in close proximity.

6-2. Results and discussion

6-2-1. Three-dimensional structure models of RNP receptors

One of the most important structural features to drive formation of a stably folded RNA structure is the stacking of aromatic rings of nucleotides. Thus, the duplex is the most frequent type of stable three-dimensional RNA structure because it can maximize the numbers of base stacking interactions and hydrogen bonds, which are also an important driving force. We can predict the secondary structure of RNA based on these features.

Substitution of a base-pair of RNA duplex to another can generally be achieved because the substitution does not change the number of stacking interactions. Using this duplex-substitution, we have already designed an RNP sensor by connecting an RNA aptamer to an RNP with a fluorophore-modified Rev peptide by substituting the duplex at the edge of RNA subunit of RNP with the duplex at the edge of RNA aptamer. The binding affinity of the resulting RNP sensor was comparable to that of the parent RNA aptamer.¹⁵ This indicated that connecting two modules by substitution of their duplex region is a powerful method for enabling structural communication between multiple RNAs.

Three-dimensional structure of an ATP aptamer has been reported by an NMR-molecular dynamics calculation approach (PDB ID: 1RAW)¹⁶ (Figure 6-3A). This aptamer contains a duplex next to the substrate binding pocket. In addition, a stable complex between the Rev peptide and RRE RNA (PDB ID: 1ETF)¹⁴ (Figure 6-3B) contains an RNA duplex next to the peptide-binding region. Using the structural information of the RNA aptamer and the RRE-Rev RNP, three-dimensional structure models of an ATP-binding RNP can be predicted by substituting the duplexes of the aptamer and of the RRE RNA (Figure 6-3C). The structural models of the ATP aptamer and the Rev-RRE RNA complex were first superimposed at their RNA duplex regions (colored yellow in Figure 6-3A and 6-3B). After removing overlapping regions in the ATP aptamer and RRE RNA, the single bond between the 5'

phosphorous atom and 3' oxygen atom was created to form phosphate, then the resulting ATP aptamer-appended RNP (ATP-RRE/Rev) was energy minimized using the modeling software (Discovery Studio 3.1, Accerlys) (Figure 6-3C).

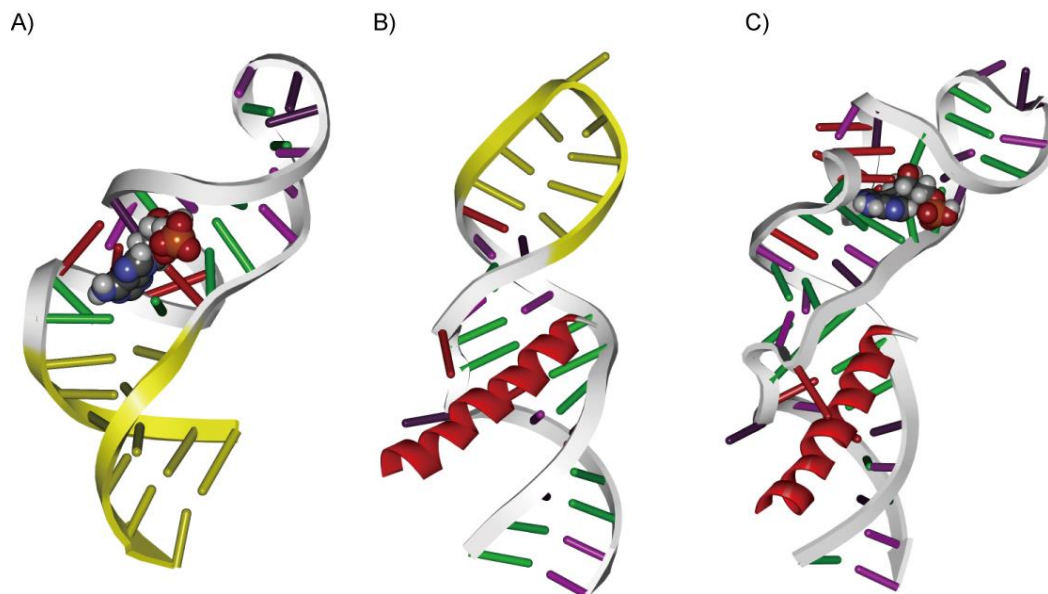


Figure 6-3. Three-dimensional structures of (A) the ATP-binding RNA aptamer selected by Szostak *et al.*¹⁷ (PDB ID: 1RAW),¹⁶ (B) the Rev peptide-RRE RNA complex (PDB ID: 1ETF)¹⁴ and (C) a predicted structure of ATP-RRE/Rev.¹⁵ RNAs are shown by ladders and arrows, peptides are shown by red ribbons, and the ligand AMP is shown by the CPK model. The aptamer and RNP were connected at the yellow colored duplex regions.

6-2-2. Introduction of a three-dimensional structural RNA motif into the RNP receptor

To achieve wide application of the construction of a fluorescent RNP sensor from the RNP receptor, the structural model of the RNP receptor was combined with the RNA aptamer and Rev-RRE complex directly in the stem region.¹⁵ To construct an RNP receptor with catalytic activity, it is important to locate the catalytic module close to the substrate bound to the receptor module. Such an arrangement of the catalytic module and the ligand is at least required to exert the proximity effect between them. A possible method to construct such an RNP structure to exert a catalytic activity is to adjust the geometry between the receptor module and the catalytic module by using another stable three-dimensional structure (Figure 6-4). For this purpose, I chose an RNA structure motif as a geometry-adjusting module in Figure 6-2 because it did not require synthesis of a new peptide motif.

Multiway junctions are representative structural motifs of nucleic acids in natural ribozymes. One of the multiway junctions is the three-way junction (TWJ) that has three

helices.

The *Neurospora* Varkud Satellite (VS) RNA is one of a family of small nucleolytic ribozymes. VS ribozymes contain two TWJs and a 47-nucleotide J345 RNA has been studied and a three-dimensional structure model derived from NMR measurement was reported (PDB ID: 2MTJ)¹⁸ (Figure 6-5A). Two helices associate each other to extend to one direction, and the other helix extends to the opposite direction. From this structural feature, it is possible to use the VS ribozyme TWJ structure to design a three-dimensional structure where the binding pocket of RNP is located close to the N-terminal of Rev peptide.

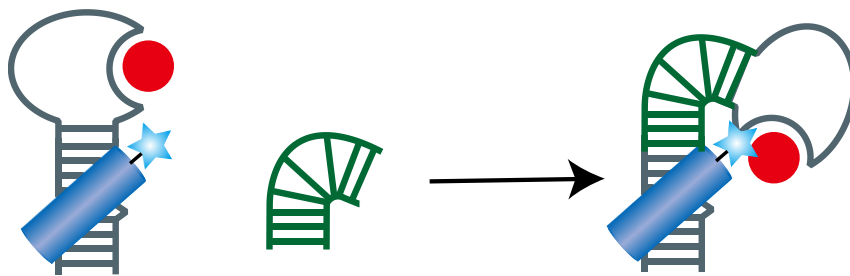


Figure 6-4. Schematic illustration of constructing an RNP by using an RNA structure motif to locate the ATP-binding pocket and the N-terminal of Rev in close proximity.

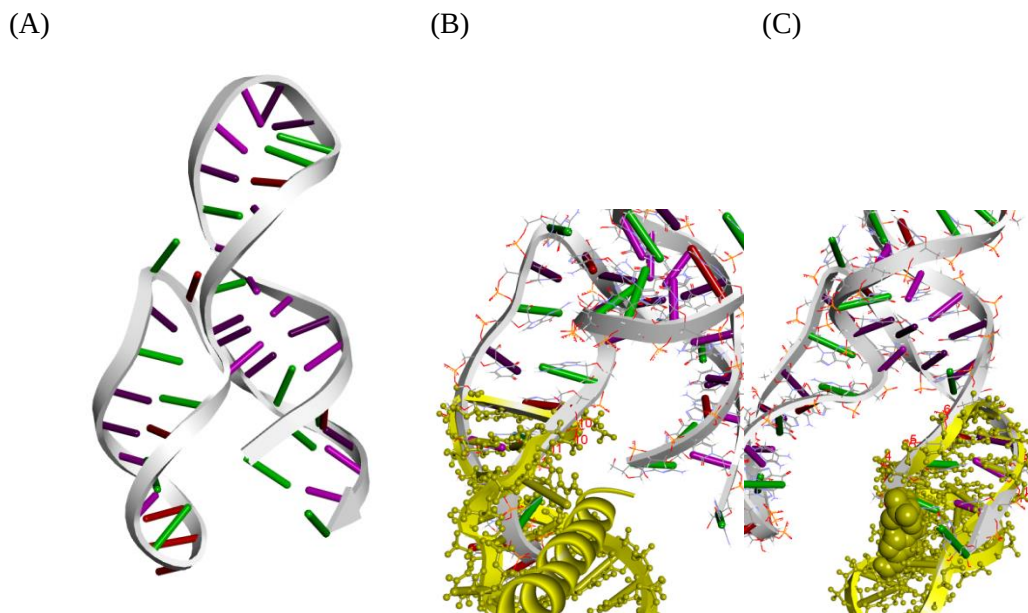


Figure 6-5. (A) The 3D structure of three-way junction (TWJ) RNA (PDB ID: 2MTJ). (B) Superimposition of RRE and TWJ at their duplex regions. The RRE region is colored in yellow. (C) Superimposition of the aptamer and TWJ at their duplex regions. The aptamer is colored in yellow.

Based on the design of catalytic RNP utilizing the VS ribozyme TWJ, four structures of RNPs were generated by substitution of their duplexes (Figure 6-5) with variation in the lengths of the duplex region. The nucleotide sequences and expected secondary structures calculated by the mfold software are shown in Figure 6-6. From the secondary structure prediction, all RNAs maintained the secondary structure of the ATP-binding region marked by the green rectangle in Figure 6-6. This indicates that all four RNPs designed should bind to ATP. The RRE region maintained its original secondary structure in all the constructs except for ATP-TWJ-RRE02. Therefore, ATP-TWJ-RRE01, ATP-TWJ-RRE03, and ATP-TWJ-RRE04 were expected to form RNP with the Rev peptide. All of the four RNA constructs showed suitable structures from the modeling software for expression of catalytic activity based on the distance between the substrate in the binding pocket of RNA and the catalytic group in the peptide (Figure 6-7). These results indicate that the TWJ structure is beneficial for construction of a geometrically constrained RNP from the Rev-RRE RNA complex and the ATP aptamer.

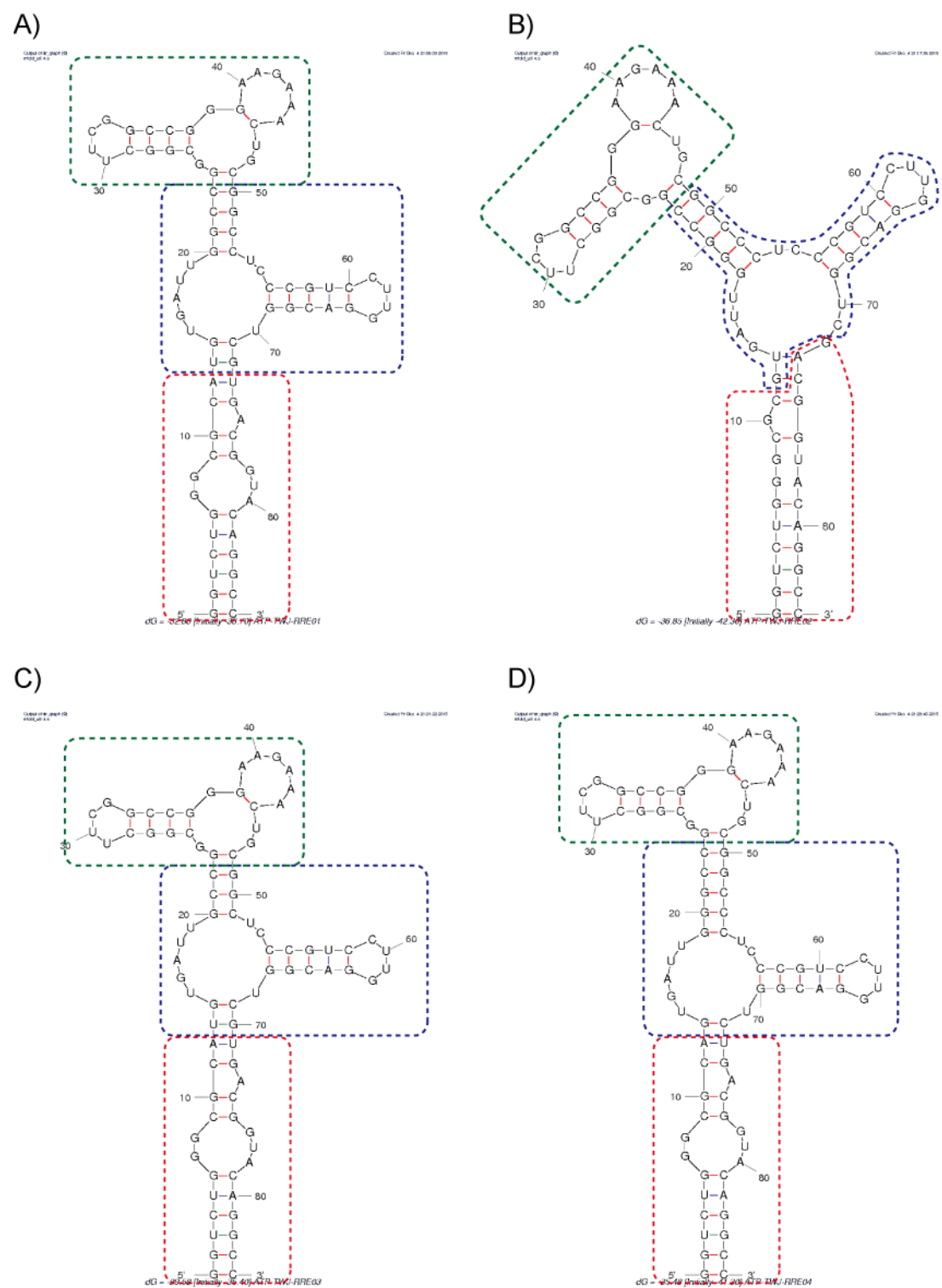
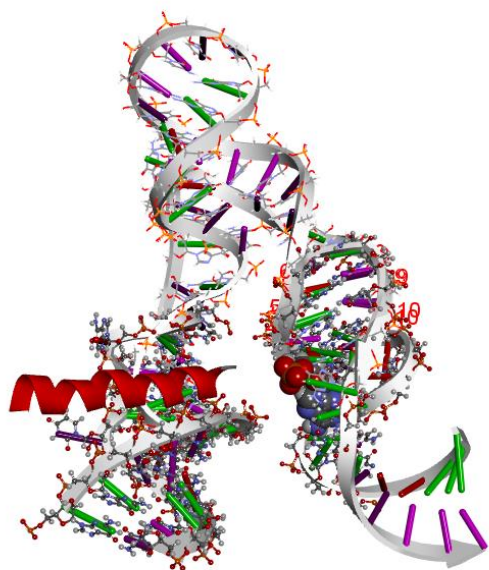
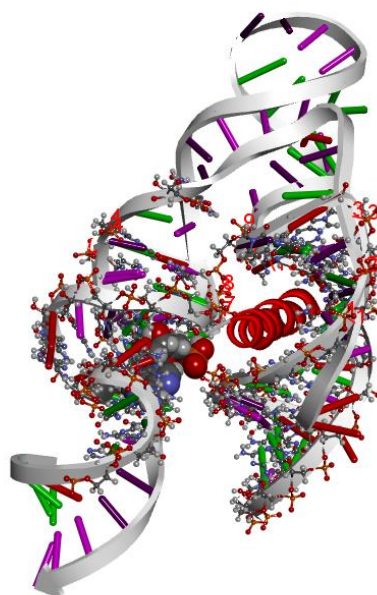


Figure 6-6. Secondary structures of RNA subunits of RNPs designed for exerting a catalytic activity. (A) ATP-TWJ-RRE01, (B) ATP-TWJ-RRE02, (C) ATP-TWJ-RRE03 and (D) ATP-TWJ-RRE04. Green boxes indicate the binding region of ATP. Blue and red boxes indicate TWJ and the RRE region, respectively.

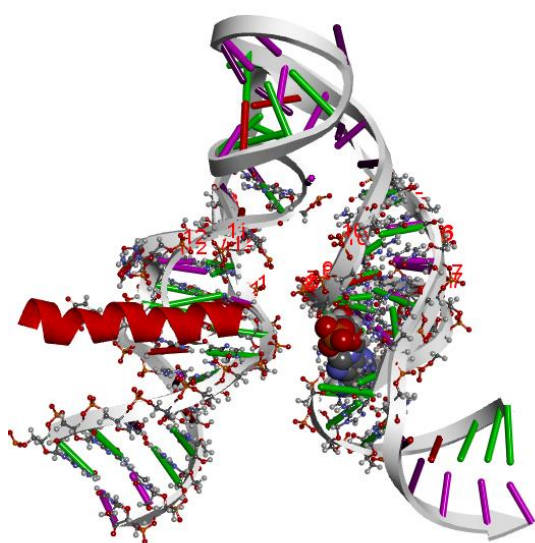
(A)



(B)



(C)



(D)

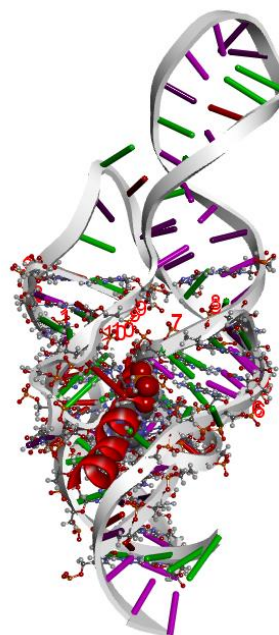


Figure 6-7. Superimposed structures of (A) ATP-TWJ-RRE 01, (B) ATP-TWJ-RRE 02, (C) ATP-TWJ-RRE 03 and (D) ATP-TWJ-RRE 04

6-2-3. Introduction of a structural peptide motif to RNP receptor

As mentioned above, a suitable geometry between the substrate and catalytic group is required to construct a three-dimensional structural model of an RNP with catalytic activity. To achieve this geometrical requirement by modification of the Rev peptide, a structural motif should be introduced between the N-terminal of the Rev peptide and the substrate in the binding pocket, because the substrate ATP and the N-terminal of Rev peptide of ATP-RRE are too far apart to allow catalytic activity (Figure 6-3C).

A linker peptide is required to position the N-terminal of Rev close to the substrate ATP in the binding pocket in the RNA subunit of RNP. I hypothesized that the probability to locate the N-terminal of Rev near the substrate binding site would increase if the linker region between the N-terminus of Rev and the catalytic group could interact with RNA (Figure 6-8). Based on this hypothesis, two peptide motifs found in the natural RNA-protein or RNA-peptide complexes were utilized. The partial three-dimensional structure model extracted from the large model does not necessarily maintain its original structure. However, the possibility of the formation of the desired structure would be larger for these peptide motifs than the case of using a random peptide sequence.

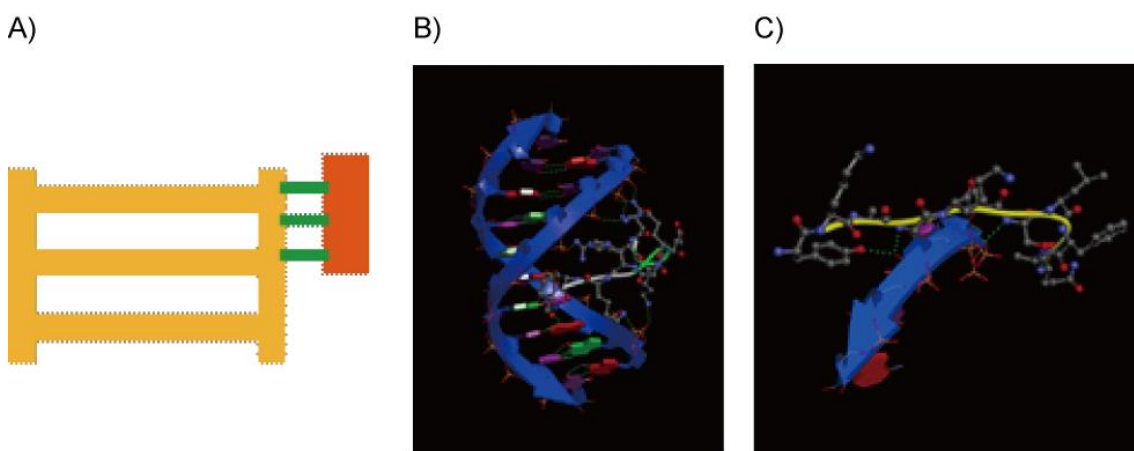


Figure 6-8. (A) Schematic illustration of the RNP complex. The yellow ladder represents the RNA duplex; the red bar indicates the Rev peptide; and the green lines denote the interaction between RNA and peptide. (B) A partial 3D structure of Tav2b/siRNA complex (PDB ID: 2ZIO)¹⁹. (C) A partial 3D structure of eukaryotic ribosome (PDB ID: 3U5I)²⁰

RNP models were designed in a stepwise manner (Figure 6-9). After designing the ATP-binding RNP, the RNA stem region of the small structural motif from the natural RNA/protein complex was incorporated into the stem region of the ATP-binding RNP. The C-terminus of the linker peptide and the N-terminal of the Rev peptide were connected through a flexible GGS repeat linker, and the N-terminus of the conjugated peptide would be modified with a catalytic group. Based on this strategy, a compatible three-dimensional structure model was designed by using the partial motif from a eukaryotic ribosome and the ATP-binding RNP receptor (Figure 6-10).

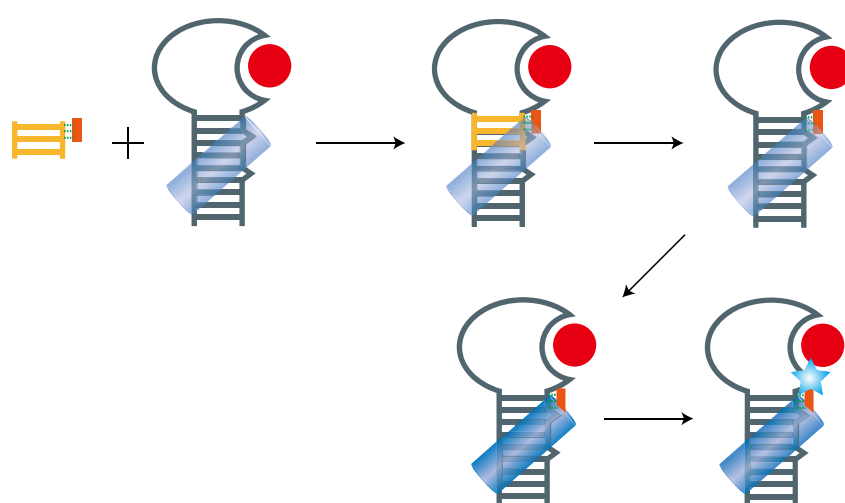


Figure 6-9. Schematic illustration of constructing an RNP with close proximity between the substrate and the N-terminal of peptide by using an RNP structure motif. First, three-dimensional structures of an RNA duplex of a small structural motif from a natural RNA/protein complex and the RNA duplex of ATP-binding RNP receptor were superimposed. Second, an overlapped RNA region was deleted. Then, the C-terminus of a peptide of the structural motif and the N-terminal of Rev peptide were connected by a flexible GGS repeat linker. After modification of the N-terminal of conjugated peptide with a catalytic group, the RNP was energy minimized to estimate its stable three-dimensional structure.

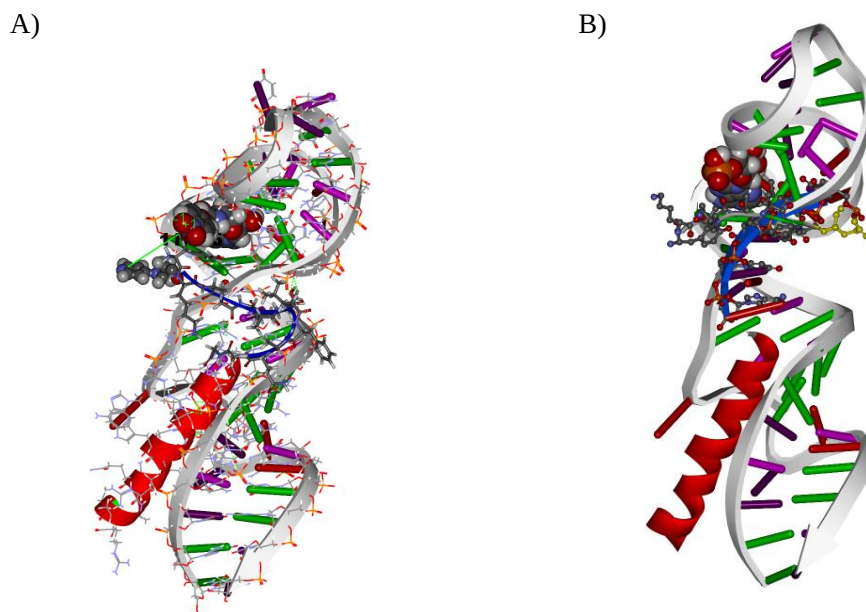


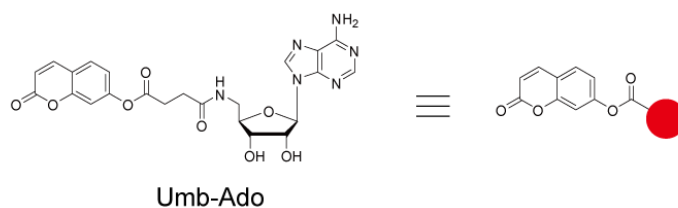
Figure 6-10. (A) Superimposed structures of AMP/ATP-RRE/DMAP-modified Rev and a partial motif of the eukaryotic ribosome (Blue RNA: RNA subunit of partial motif of eukaryotic ribosome) (ATP-RRE/dmap-pa1-Rev). (B) Superimposed structures of AMP/ATP-RRE/DMAP-modified Rev and a partial motif of the Tav2b/siRNA complex (Blue RNA: RNA subunit of partial motif of eukaryotic ribosome) (ATP-RRE/dmap-pb1-Rev).

6-2-4. Evaluation of the catalytic activity of designed RNPs

The ester hydrolysis was chosen as the target reaction. We have already developed the convenient detection method of the hydrolysis of umbelliferyl ester derivative of adenosine (Umb-Ado) in chapter 5 (Figure 6-11). This substrate was used in here for a rapid and convenient evaluation of the catalytic activity of designed RNP.

All designed RNP (ATP-TWJ-RRE01/DMAP-Rev, ATP-TWJ-RRE02/DMAP-Rev, ATP-TWJ-RRE03/DMAP-Rev, ATP-TWJ-RRE04/DMAP-Rev, ATP-RRE/dmap-pa1 and ATP-RRE/dmap-pb1) were prepared by the complex formation of the RNA subunit constructed by T7 transcription and the peptides constructed by solid-phase synthesis as described previously.⁷ All RNAs were purified by denatured PAGE, and all peptides were purified by HPLC.⁷ In order to evaluate the catalytic activity of RNPs, each RNP was mixed with Umb-Ado in a buffer containing 30 mM Bis-Tris-HCl (pH 6.0), 100 mM NaCl, 5 mM MgCl₂ and 0.005% Tween 20 at 20 °C and time course changes of the fluorescence emission intensity (455 nm) were monitored (Figure 6-12).

A)



B)

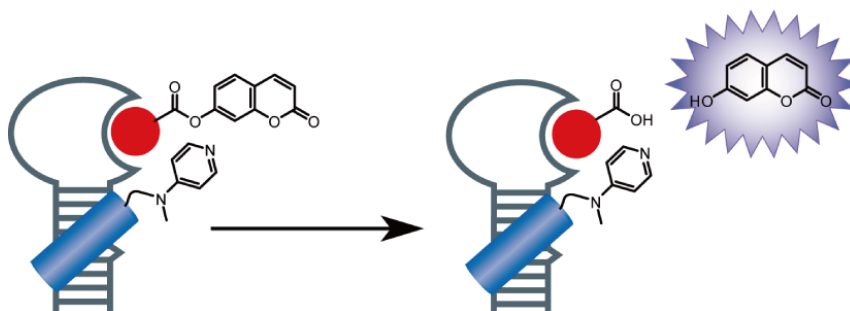


Figure 6-11. (A) Structure of Umb-Ado. (B) Schematic illustration of the hydrolysis reaction accelerated by RNP. The catalytic activity is exerted by a proximity effect between the ester group and the catalytic group DMAP.

None of these six designed RNPs showed catalytic activity in the hydrolysis of Umb-Ado ester. A possible explanation is that the structural motifs appended in these RNPs did not maintain the original structures. In the structure-based design of the functional biopolymers, trial-and-error is usually required to obtain a molecule with the function of interest. Further trial in the design of RNP with catalytic group and binding pocket is required.

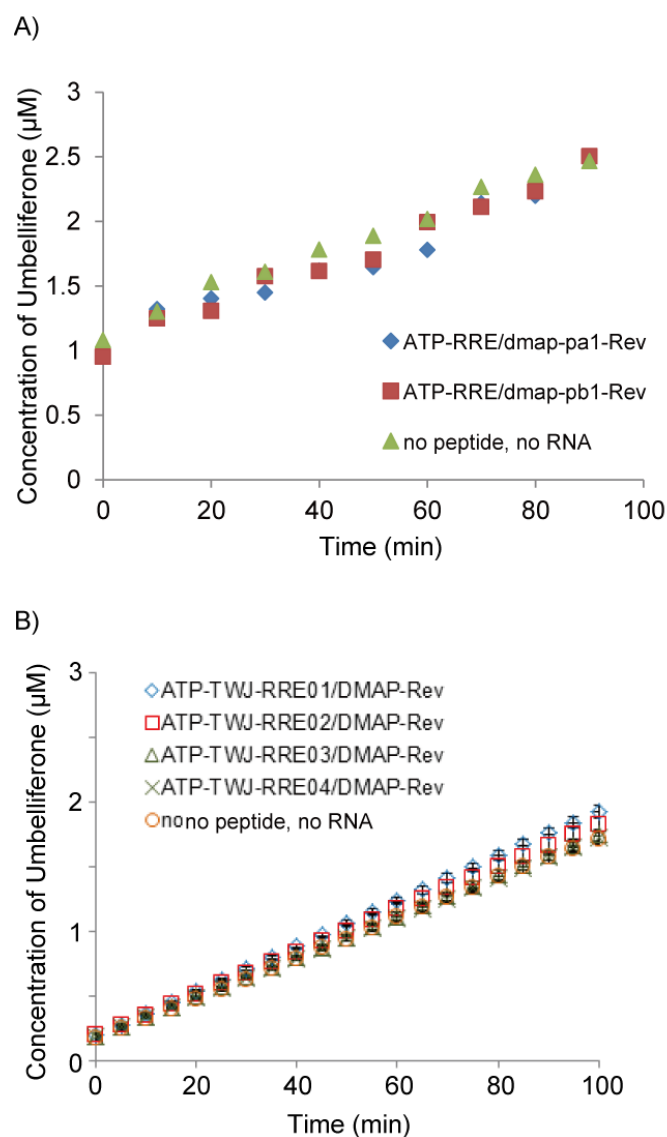


Figure 6-12. Time course plots of the concentration of umbelliferone in the reaction mixtures containing Umb-Ado with (A) RNP (ATP-RRE/dmap-pa1-Rev (blue diamond) and ATP-RRE/dmap-pb1-Rev (red rectangle)) and without any RNA and peptide (green triangle) and (B) RNP (ATP-TWJ-RRE01/DMAP-Rev (blue diamond), ATP-TWJ-RRE02/DMAP-Rev (green triangle), ATP-TWJ-RRE03/DMAP-Rev03 (green triangle) and ATP-TWJ-RRE04/DMAP-Rev (purple x-mark)) and without any peptide and RNA (purple cross) in a buffer containing 30 mM Bis Tris-HCl (pH 6.0) with 100 mM NaCl, 5 mM MgCl₂, and 0.005% Tween 20 at 20 °C.

6-3. Conclusion

ATP-binding RNPs were designed to achieve a suitable 3D structure for exerting the proximity effect between the substrate ATP in the binding pocket and the catalytic group at the N-terminal of peptide. Structure-based design of RNP was investigated by using a structural motif of RNA or a partial structural motif of large RNP structure. The structural design in this chapter was based on the hypothesis that two stem regions could be superimposed. Out of six RNPs designed with DMAP as the catalyst of hydrolysis, no RNP exerted the catalytic activity. These results indicated that further variations of RNPs should be designed to obtain RNP with a function of interest. However, modeling simulation studies indicated no contradiction between the hypothesis and the energy calculations. The three-dimensional structures of designed RNPs indicated that incorporating structural motifs would be a powerful tool to construct structural model based on the RNP receptor that contain functional group.

6-4. Methods

6-4-1. Design of the three-dimensional structure models of the RNP receptors

Three-dimensional structure of the RNP receptor was obtained as follows by using Discovery Studio 3.1 software (Accelrys, San Diego, CA, USA). From the Protein Data Bank (PDB), the NMR-structures of the ATP-binding aptamer-AMP complexes were received (structures associated with PDB code 1RAW). RNA duplexes of ATP-binding aptamer and RNA subunit of Rev peptide-RRE RNA complex structure that was derived from 1ETF.pdb was superimposed on the software. After removing overlapping nucleotides of the duplex of 1RAW.pdb and 1ETF.pdb, the two structures were connected by single bond formation between the 5' phosphor atom and 3' oxygen atom to form phosphate. The *in silico* generated conjugate was subjected to a structure minimization simulation (force field : CHARMM, partial charges : Momany Rone, algorithm : Smart Minimizer, max step : 10000, RMS gradient : 0.01, energy change : 0, implicit solvent model : Generalized Born with a simple Switching, other settings : default); the final refined structures are shown. In the same manner, other three-dimensional structures were design by connecting each module at RNA duplex.

6-4-2. RNA Secondary Structure Prediction

Prediction of the secondary structures of ATP-TWJ-RRE 01, ATP-TWJ-RRE 02, ATP-TWJ-RRE 03 and ATP-TWJ-RRE 04 RNA by using mfold v3.2 algorithm (offered at <http://mfold.rna.albany.edu/>) were done at 37 °C with 1 M NaCl, constraining that the RRE region formed the reported secondary structure.

6-4-3. Evaluation of the catalytic activity of the designed RNP

Fluorescence measurements in 96-well plates were performed on a TECAN infinity pro multilabel counter. A solution (100 μ L) containing 5 μ M of each combination of RNPs and 20 μ M Umb-Ado in reaction buffer containing 30 mM Bis-Tris-HCl (pH 6.0), 100 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20 was gently swirled for 30 seconds and the time-dependent emission intensity of the umbelliferone, which is a product of the hydrolysis, was measured every 5 or 10 min for 100 min. Excitation and emission wavelengths for umbelliferone were 355 and 455 nm, respectively.

6-5. References

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General conclusions

In this thesis, methods for appending a function of interest to RNP receptors were developed in order to accomplish the preparation of RNPs with tailor-made function. Three different approaches for the construction of fluorescent RNP sensors and two for exerting a catalytic activity of RNP were described.

In chapter 2, a ratiometric fluorescent dye SNARF that changes the fluorescence intensity at two wavelengths was used as a reporter instead of a dye which changes the emission intensity at single wavelength used in previous reports. SNARF was introduced to RNP receptors targeting tetracycline, streptomycin and dopamine to construct fluorescent RNP sensors for these targets. A shift in the maximum emission wavelength of fluorescence was observed depending on the substrate binding. Tetracycline-binding RNP receptor, which was difficult to convert to sensors, can be converted to a fluorescence sensor with high sensitivity.

As discussed in chapter 3, two methods were developed to improve the versatility of the previous method for the construction of fluorescent RNP sensors, and the properties of the sensors regarding stability of the RNP complex formation. In order to expand the constructing method of RNP sensors, RNP receptors were converted into fluorescence sensors by utilizing fluorophore library. Previously, RNP sensors that showed high relative fluorescence intensities upon binding to a targeted molecule were selected from an RNP library produced by a combination of several fluorescent molecules and a library of RNA from the RNP with various affinities for the substrate. In this method, a specific RNA subunit that has the desired selectivity and affinity for the specific substrate does not necessarily show a dramatic change of fluorescence intensity upon binding the substrate. Therefore, a "fluorescent molecule-modified Rev peptide library", in which a fluorophore library was converted to a Rev peptide modified library, was prepared, then complexed with an RNA subunit of interest to construct a fluorophore-modified RNP library. Screening of the fluorescent RNP libraries prepared from four kinds of fluorophore library and the RNA subunit of ATP-binding RNP indicated that certain fluorophores in the original library were capable of converting RNP receptors into fluorescence sensors. Next, a method to construct covalently linked RNP sensors was developed. The covalently linked RNP sensors, in which the 3'-terminal of RNA subunit and the C-terminal of Rev peptide were connected by a hydrazone bond formation, exhibited much higher stability than noncovalent RNP sensors and enabled the detection of the ligands at lower concentrations of sensors. The covalently linked ATP and GTP sensors further enabled simultaneous optical detection of ATP and GTP in the same buffer, while it was not possible by noncovalent RNP sensors due to the exchanging each subunits of ATP and GTP sensors.

In chapter 4, in order to develop a method to construct an RNP with catalytic activity,

a structurally diverse RNP library with catalytic group was constructed for catalytic activity-based screening. In order to achieve the construction of RNP with catalytic activity, the catalytic group should locate near the binding pocket for acceleration of the target reaction. Such a orientation between the catalytic group and the substrate is expected to exert the proximity effect. One of the approaches to construct such an RNP is screening of the structurally diverse RNP library. The structurally diverse RNP library was prepared by combination of an RNA library that has a binding pocket to adenosine derivatives with a variety of overall structures and a Rev peptide library that contained catalytic group through various peptide linkers. The noncovalent complex formation of RNA carrying the ATP-binding region and the Rev peptide derivative enable facile expansion of the library size.

RNPs with catalytic activity for the ester hydrolysis reaction were selected from the structurally diverse RNP library in chapter 5. ATP derivative containing umbelliferyl ester, for which fluorescence properties are changed upon the hydrolysis reaction, were synthesized as a substrate in order to select RNPs with catalytic activity conveniently and quickly. As a result of screening using this substrate as an indicator of hydrolysis activity, RNPs with catalytic activity for the ester hydrolysis were successfully obtained.

In chapter 6, RNPs expected to exert catalytic activity were designed by molecular modeling based on the three-dimensional structures of RNA aptamers and RNPs. In order to achieve proximity effect between catalytic group and substrate, the catalytic group and the binding pocket for the substrate should be brought close together. An ATP-binding RNA aptamer and a three-way junction motif derived from natural RNP were utilized as basic scaffolds. Six RNPs were designed and constructed for evaluation of the ester hydrolysis activity. However, none of the designed RNPs show the catalytic activity. The results indicate that further trial-and-error is required to obtain the RNP with catalytic activity.

In summary, the methods for constructing functional RNPs by utilizing the RNP receptors were developed by the construction of new libraries and the establishment of screening systems based on fluorescent responses or catalytic activity. Especially, for the construction of catalytic RNPs, the construction and screening of structurally diverse RNP library enabled to obtain the first example of RNPs with catalytic activity based on the RNP receptor. These results were important to verify the feasibility of the construction of catalytic RNP from RNP receptors.

Finally, these techniques established here would be practical methods for construction of biomacromolecular complex with the function of interest. *In vitro* selection of RNP library provides the RNP receptor not only for the target substrate shown in this thesis but also for the molecule of interest for example bioactive molecule, unnatural molecule, and transition state analog. The functionalized diverse library of RNP receptor would be quite useful for exploring

other functional RNPs not only for the application described in this thesis. The achievement of this work would serve as the basis of the technology to construct tailor-made enzymes with high catalytic activity and selectivity for the target of interest that are comparable to those of natural enzymes.

List of publications

1. Construction of ratiometric fluorescent sensors by ribonucleopeptides

Chiara Annoni; Eiji Nakata; Tomoki Tamura; Fong Fong Liew; Shun Nakano; Maria Luisa Gelmi; Takashi Morii

Org. Biomol. Chem. **2012**, *10*, 8767.

(Chapter 2)

2. Simultaneous detection of ATP and GTP by covalently linked fluorescent ribonucleopeptide sensors

Shun Nakano; Masatora Fukuda; Tomoki Tamura; Reiko Sakaguchi; Eiji Nakata; Takashi Morii

J. Am. Chem. Soc. **2013**, *135*, 3465.

(Chapter 3)

3. Screening of fluorescent sensors by using fluorophore library and ribonucleopeptide receptor

In preparation

(Chapter 3)

4. Construction of a library of structurally diverse ribonucleopeptides with catalytic groups

Tomoki Tamura; Shun Nakano; Eiji Nakata; Takashi Morii

Bioorg. Med. Chem. **2017**, *in press*. DOI information: 10.1016/j.bmc.2017.02.007

(Chapter 4, 5)

Other publications

5. A peptide nucleic acid (PNA) heteroduplex probe containing an inosine–cytosine base pair discriminates a single-nucleotide difference in RNA

Katsuhiko Matsumoto; Eiji Nakata; Tomoki Tamura; Isao Saito; Yasunori Aizawa; Takashi Morii

Chem. Eur. J. **2013**, *19*, 5034

List of presentations

International conference

1. Facile conversion of RNA aptamers to modular fluorescent sensors

Tomoki Tamura; Shun Nakano; Eiji Nakata; Takashi Morii

The 38th International Symposium on Nucleic Acids Chemistry, November 9-11, 2011, Sapporo, Hokkaido, Japan

2. Ribonucleopeptides with catalytic functional groups

Tomoki Tamura; Kenta Ariyama; Shun Nakano; Takashi Morii

The 41th International Symposium on Nucleic Acids Chemistry, November 5-7, 2014, Kitakyushu, Fukuoka, Japan

3. Construction and screening of the RNP library with catalytic functional group

Tomoki Tamura; Kenta Ariyama; Shun Nakano; Takashi Morii

The 2015 International Chemical Congress of Pacific Basin Societies, December 15-20, 2015, Honolulu, Hawaii, USA

4. Construction of a structurally diverse library of ribonucleopeptides

Tomoki Tamura; Shun Nakano; Takashi Morii

The 43th International Symposium on Nucleic Acids Chemistry, September 27-29, 2016, Kumamoto, Kumamoto, Japan

Domestic conference

5. RNA アプタマーを用いた機能性リボスクレオペプチドの段階的構築

田村友樹・仲野 瞬・劉 芳芳・中田栄司・森井 孝

2012 年 3 月 25 日 ~ 28 日、日本化学会第 92 回春季年会、慶應義塾大学 日吉キャンパス、矢上キャンパス

6. RNA-ペプチド複合体を基本骨格とした蛍光性バイオセンサーの合理的設計法の開発

田村友樹・劉 芳芳・仲野 瞬・中田栄司・森井 孝

2012 年 5 月 19 日、第 59 回日本生化学会近畿支部例会、京都大学 宇治キャンパス

7. 反応活性中心を導入したリボヌクレオペプチド

田村友樹・藤川祐典・仲野 瞬・中田栄司・森井 孝

2013 年 3 月 22 日 ~ 25 日、日本化学会第 93 回春季年会、立命館大学 びわこ・くさつ
キャンパス

8. 反応活性中心を導入したリボヌクレオペプチドの触媒活性

田村友樹・吉村祐輝・仲野 瞬・中田栄司・森井 孝

2014 年 3 月 27 日 ~ 30 日、日本化学会第 94 回春季年会、名古屋大学 東山キャンパス

9. 非天然活性中心を導入したリボヌクレオペプチドの触媒活性

田村友樹、有山健太、仲野 瞬、森井 孝

2014 年 9 月 11 日 ~ 13 日、第 8 回バイオ関連化学シンポジウム、岡山大学 津島キャンパス

10. 触媒分子を導入したリボヌクレオペプチドライブラリーの活性スクリーニング

田村友樹、有山健太、仲野 瞬、森井 孝

2015 年 3 月 26 日 ~ 29 日、日本化学会第 95 春季年会、日本大学理工学部船橋キャンパス／薬学部

11. リボヌクレオペプチドによるエステル加水分解反応

田村友樹、有山健太、仲野 瞬、森井 孝

2016 年 3 月 24 日 ~ 27 日、日本化学会第 96 春季年会、同志社大学 京田辺キャンパス

12. 構造多様性を有する RNP リセプターライブラリーの構築

田村友樹、仲野 瞬、森井 孝

2016 年 9 月 7 日 ~ 9 日、第 10 回バイオ関連化学シンポジウム、石川県立音楽堂・もてなしドーム地下イベント広場