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A diversity-oriented library of fluorophore-modified receptors constructed from a chemical library of synthetic fluorophores

Shun Nakano, Tomoki Tamura, Raj Kumar Das, Eiji Nakata, Young-Tae Chang, and Takashi Morii*

Abstract: The practical application of biosensors can be determined by evaluation of the sensing ability of fluorophore-modified derivatives of a receptor with appropriate recognition characteristics for target molecules. One of the key determinants to successfully obtain a useful biosensor is wide variation of fluorophores attached to a given receptor. Thus, use of a larger fluorophore-modified receptor library provides a higher probability of obtaining a practically useful biosensor. However, no effective method has yet been developed for constructing such a diverse library of fluorophore-modified receptors. Herein, we report a method for constructing fluorophore-modified receptors using a chemical library of synthetic fluorophores with a thiol-reactive group. This library was converted into a library of fluorophore-modified adenosine-binding ribonucleopeptide (RNP) receptors by introducing the fluorophores to the Rev peptide of the RNP complex via alkylation of the thiol group. This method enabled the construction of 263 fluorophore-modified ATP-binding RNP receptors and allowed for the selection of suitable receptor-based fluorescent sensors targeting ATP.

Fluorescent biosensors that allow for the specific detection and quantification of a wide range of target molecules are important tools in the fields of therapeutics and diagnostics.[1] In general, fluorescent biosensors consist of a substrate-binding or substrate-reacting module and a fluorescent reporter module. The substrate-binding module is a key component for determining the affinity and selectivity of a biosensor, while the fluorophore in the reporter module is responsible for converting the ligand-binding event to measurable fluorescent signal responses. Optimal arrangement between the binding module and reporter module is necessary to exert a high fluorescence intensity change that facilitates detection. Naturally occurring protein or nucleic acid receptors (aptamers) have generally been used as the substrate-binding module for receptor-based fluorescent biosensors, owing to their superior substrate selectivity.[2] When a receptor with appropriate affinity and selectivity to a given target molecule is available, the receptor of interest is next modified with an appropriate reporter such as a fluorescent dye. The process of obtaining a practically useful fluorescent sensor generally requires the modification of a given receptor using several fluorophores tested at various locations, which is a laborious task. Although there have been numerous successful reports on the construction and application of receptor-based fluorescent biosensors, useful biosensors are not always obtained from the limited numbers of fluorophore-modified receptors available. The probability to select suitable biosensors would increase by preparing a large diversity of fluorophore-modified receptors, but an effective method to construct such a library of fluorophore-modified receptors is yet to be developed. Moreover, direct chemical modifications or genetic mutations introduced near the ligand-binding site of the receptor tend to alter or reduce the function of the parent receptor. These issues set a limitation for their application to the substrate-binding module. Therefore, a convenient method remains to be developed for construction of receptor-based fluorescent sensors for a target molecule.

Toward this end, we constructed receptors using the structurally well-characterized Rev responsive element (RRE)[3] ribonucleopeptide (RNP) complex.[4] RNP libraries with structural diversity were constructed by introducing randomized nucleotide sequences into the RNA subunit. Because RNP receptors are obtained by in vitro selection,[5] they have been applied to the detection of a wide range of biologically important targets such as ATP,[4,6] a phosphorylated peptide,[7] and dopamine.[8] The variation in the nucleotide sequences of the in vitro-selected RNA subunit generally confers the RNP receptors with various affinities to the given target and overall structures. Such a group of diverse RNA subunits can be used to construct a fluorophore-modified RNP (F-RNP) library by complexing with a fluorophore-modified Rev peptide (F-Rev).[6-8] Simple screening of the F-RNP library in the presence and absence of the target will induce high relative fluorescence intensity changes of the F-RNPs, which are suitable as fluorescent sensors to detect the target at a particular detection wavelength. Most importantly, these F-RNP receptors retain the parent ligand-binding characteristics.

Synthetic fluorophores by themselves are also widely applied as fluorescent probes for biological targets. Although the target-oriented rational design and synthesis of fluorescent probes are relatively straightforward, this approach requires detailed empirical knowledge on the interaction and reactivity between the target of interest and the candidate probe.[9] Therefore, diversity-oriented synthesis has been applied for the efficient construction of fluorescent sensors and probes. In particular, the diversity-oriented fluorescence library (DOFL) approach was successfully applied for selection of fluorescent probes targeting several types of biological targets, including proteins, nucleic acids, and organelles.[10] In this approach, libraries of small molecules consisting of fluorescent scaffolds with structural diversity are efficiently prepared using a solid-phase synthesis technique. The constructed libraries can then

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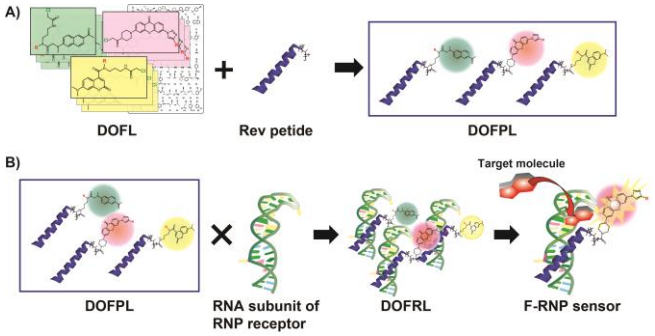
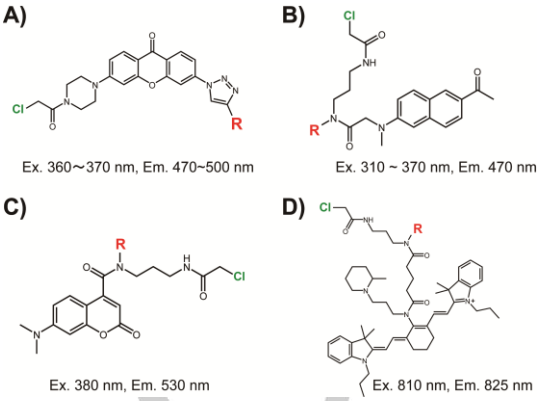


Figure 1. A schematic illustration for the screening of fluorophore-modified RNPs (F-RNP). (A) A diversity-oriented fluorophore library (DOFL) was converted to a diversity-oriented fluorophore-modified Rev (F-Rev) peptides library (DOFPL). (B) A diversity-oriented F-RNP library (DOFRL) was constructed by complexation of an RNA subunit of an ATP-binding RNP receptor and the DOFPL. The DOFRL was then screened by evaluation of the relative fluorescence intensity in the absence and presence of the target (Ado) to allow for the selection of F-RNP sensors for Ado and ATP with high relative fluorescence intensity changes.



Scheme 1. Structures of (A) CXCA-, (B) TPGCA-, (C) CORCA-, and (D) CyRCA-based libraries. The bold R in red indicates the position of each fluorescent scaffold at which the different building blocks are introduced (see also Figures S2–S5).

be utilized for high-throughput screenings of various targets of interest.

A DOFL is also quite suitable for the construction of a diversity-oriented F-RNP library (DOFRL), which could then be used to screen for a receptor-based fluorescent biosensor with desired detection characteristics. The high ligand-binding ability of RNPs would help to improve the probability for the selection of 'hit' biosensors with optimal sensing of target molecules. However, before such a combinatorial approach can be established, a novel method is required for the chemical modification of RNPs based on the DOFL without diminishing the ligand-binding ability of parent RNPs. Chemical conversion of a DOFL to a diversity-oriented fluorescence peptide library (DOFPL) of Rev peptides would provide a DOFRL by complexing with RNA subunit of target-binding RNP receptor. Based on this concept, we here report the construction of an F-RNP library using an ATP-binding RNP and fluorophore libraries (Figure 1). Screening of the DOFRL derived from DOFLs and a given Ado-binding RNP enabled the selection of suitable fluorescent sensors that show detectable fluorescence intensity changes upon binding to ATP and Ado.

Because individual solid-phase synthesis of F-Rev is impractical for the preparation of a large number of F-Rev peptides, a simple but reliable reaction was applied for coupling a fluorophore and the Rev peptide, which allowed for the conversion of the DOFL to the DOFPL of Rev peptides (Figure S1). DOFLs have previously been developed by modification of several fluorescent scaffolds with chemically diverse building blocks,[10] and certain fluorophore libraries were equipped with a chloroacetyl (CA) group (Scheme 1).[11] CA is a haloacetyl group, which can selectively react with thiol groups in a manner that can be quantitatively assessed.[12] Therefore, the library of fluorophores with the CA group can be readily converted into the library of F-Revs using a cysteine-modified Rev peptide (CRev) through selective reaction with the thiol group of cysteine. Four types of fluorophore scaffolds, CXCA,[13a] TPGCA,[13b] CORCA, and CyRCA[13c] classified as derivatives of xanthone, acedan, coumarin, and cyanine, respectively, were selected for the construction of CA-containing DOFLs (Scheme 1 and Figures S2–S5). A library of fluorophores with the same scaffold would show a similar fluorescence property such as the excitation and emission wavelengths, which is useful for simplifying the screening protocol. Since these four DOFLs vary with respect to the sub-chemical group, they will exert different fluorescent responses of the F-RNPs toward the target molecule.

To construct these libraries, the fluorophores (10 nmol each) at the defined positions in the microplates for CXCA, TPGCA, CORCA, and CyRCA were modified with the Rev peptide bearing an N-terminal Cys residue (CRev, 5 nmol). The CA group in each fluorophore was first converted to an iodoacetyl (IA) group to enhance the reactivity toward the thiol group, and was then reacted with CRev (see SI text for details). The yields for the conversion of the CA group of fluorophores to the IA group ranged from 80% to 100%, and the yields of F-CRev ranged from 10% to 75% (Figures S6 and S7). The resulting F-CRev in each well of the microplate was washed with diethyl ether to remove the unreacted fluorophores, and was then mixed with the RNA subunit (An16) of ATP or Ado-binding RNP[6c] to obtain 1.0 µM or 1.25 µM of the F-RNP complex.

Because its ligand binding properties and structural aspects have been well characterized,[6c] the ATP-binding RNP receptor is a good candidate to validate the present strategy of using an F-RNP library. The ATP-binding receptor An16/Rev binds not only ATP but also Ado.[6c] Therefore, Ado was used as a substrate in the preliminary screening of F-RNPs to ensure stable results of screening. Each F-RNP library was screened to select F-RNPs that showed apparent fluorescence intensity changes in response to the addition of 500 µM Ado. In total, 263 F-RNPs were constructed by complexation of An16 RNA and the F-CRev peptides. The change in the fluorescence emission intensity of each F-RNP upon binding to Ado, was measured in a screening buffer containing 10 mM sodium-phosphate (pH 7.6), 100 mM NaCl, and 10 mM $MgCl_2$. Based on the equilibrium dissociation constant of parent An16 RNP and Ado (K_D = 2.3

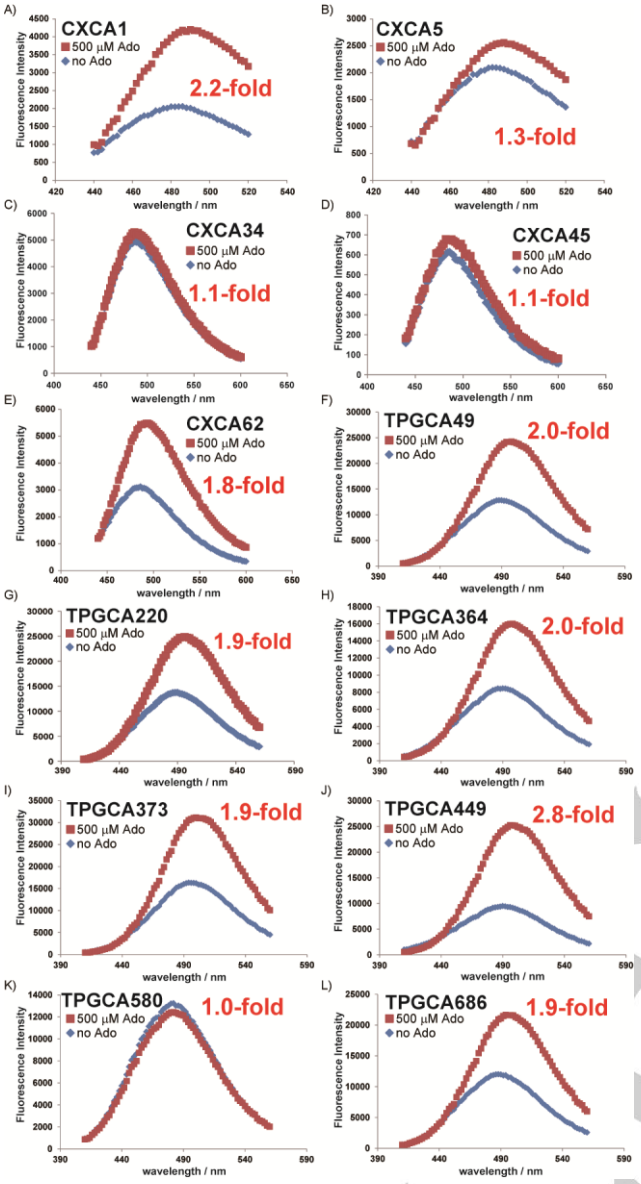


Figure 2. Fluorescence spectra of the complexes of an Ado-binding RNA subunit (An16) and fluorophore-modified CRev (F-CRev) in the absence (blue) or presence (red) of Ado (500 μM). Purified F-CRev peptides were used. (A) An16/CXCA1-CRev, (B) An16/CXCA1-CRev, (C) An16/CXCA5-CRev, (D) An16/CXCA34-CRev, (E) An16/CXCA45-CRev, (F) An16/CXCA62-CRev, (G) An16/TPGCA49-CRev, (G) An16/TPGCA220-CRev, (H) An16/TPGCA364-CRev, (I) An16/TPGCA373-CRev, (J) An16/TPGCA449-CRev, (K) An16/TPGCA580-CRev, and (L) An16/TPGCA686-CRev.

μM),[6c] the fluorescence intensity of the F-RNP was expected to reach its maximum value in the presence of 500 μM Ado. The relative ratios of fluorescence intensity in the absence (I_0) and presence (I) of Ado were monitored at 470 nm (λ_{Ex} = 370 nm). For the fluorophores with the CXCA scaffold, 75 derivatives were used to construct the F-RNP library. An I/I_0 value of 1.3 was set as a threshold to select roughly one tenth of the candidates in the initial screening. Among the 75 derivatives, 6 F-RNPs showed I/I_0 values over 1.3 in the screening (Figure S8). Similarly, 8 of 76, 2 of 56, and 2 of 56 F-RNPs derived from the

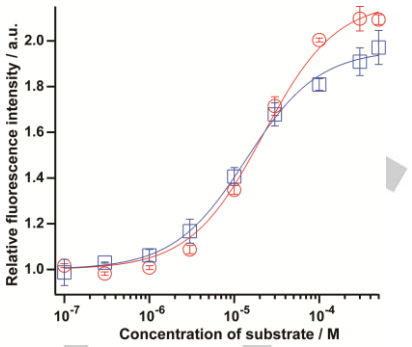


Figure 3. Saturation curves for the relative fluorescence intensity changes of An16/CXCA1-CRev (1 μM) by titration with Ado (red) or ATP (blue) in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween 20 at 4 °C.

TPGCA (λ_{Ex} = 360 nm), CORCA (λ_{Ex} = 380 nm), and CyRCA (λ_{Ex} = 800 nm) libraries, respectively, showed I/I_0 values over 1.3 at 460 nm (Figures S9–S11).

The F-RNPs constructed from the CXCA, TPGCA, and CyRCA libraries revealed enhancement in the I/I_0 values, whereas those from the CORCA library tended to show a decrease in the fluorescence intensity upon the addition of Ado. In almost all cases, the F-RNPs constructed from the CyRCA library did not show reproducible I/I_0 values in the screening with Ado. This tendency might be due to the difficulty in attaching the CyRCA derivatives to CRev because of their poor solubility in the reaction solvent. In total, this preliminary screening selected 18 candidates showing I/I_0 values over 1.3 among the 263 F-RNP derivatives converted from four types of chemical libraries of synthetic fluorophore.

In this preliminary screening described above, we used crude F-CRevs obtained by the simple extraction of the fluorophore-CRev reaction mixture with diethyl ether for the construction of the F-RNP libraries. To confirm the relative fluorescence intensity changes observed in the preliminary screening step, 13 peptides of the selected F-RNPs (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 high-performance liquid chromatography and then complexed with An16 RNA to form F-RNPs. The fluorescence spectra of F-RNPs were measured under the same conditions as those used for the preliminary screening. Among the 18 F-RNPs selected in the preliminary screening, five were excluded from the second screening because of their low fluorescence emission intensities The change in the relative fluorescence intensity for An16/CXCA1-CRev upon Ado addition increased from 1.3 in the preliminary screening step to 2.2 in this second step (Figure 2). The other 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 over 1.3-fold changes in the fluorescence intensity upon the addition of Ado. An16/CORCA100-CRev showed a decrease

in the fluorescence intensity upon binding to Ado (Figure S12), despite the increase of I/I_0 detected in the preliminary screening. The other two F-RNPs showed an I/I_0 value below 1.3 in the second screening in contrast to the results for the preliminary screening. In total, 9 of 13 F-RNPs showed high relative fluorescence intensity changes. The remaining F-RNPs showed unsatisfactory fluorescence responses compared to those observed in the preliminary screening.

The dissociation constants (K_D) of An16/CXCA1-CRev to Ado and ATP were determined to evaluate the affinity of the selected F-RNPs to the substrates (Figure 3). Titration of the changes in the fluorescence intensity of An16/CXCA1-CRev in response to Ado or ATP at various concentrations provided K_D values of 21 μM and 13 μM, respectively. Maximum relative fluorescence intensity (I/I_0) values were 2.2 and 2.0, respectively, to Ado and ATP. An16/CXCA1-CRev retained the selectivity of parent RNP receptor to other nucleotide triphosphates (Figure S13), which is consistent with our previous results for the fluorescent ATP sensors constructed from ATP-binding RNP.[6c] Although the K_D value of An16/CXCA1-CRev to ATP (13 μM,) was slightly increased from that of An16 RNP (1 μM), these results showed that a given RNP receptor was successfully converted to an F-RNP sensor by screening a DOFL, thereby validating this approach for application of the facile construction of receptor-based fluorescent sensors.

In summary, a novel method was developed for the construction of a library of fluorophore-modified RNP receptors converted from a DOFL, which was successfully applied for screening ATP-binding An16 RNP receptors with various modifications as effective fluorescent ATP sensors, yielding 18 candidates in a preliminary screen. When the F-RNPs were constructed with purified F-CRevs, the majority showed expected or higher responses compared to those observed in the preliminary screen. Thus, the DOFPL strategy effectively converted an ATP-binding RNP receptor with a given ligand-binding property to a fluorescent ATP sensor. It should be noted that DOFPL strategy can be applied to many other RNP receptors targeting various ligands[4,6-8], because the F-RNP library can be prepared by just complexing the RNA subunit of RNP and the diverse fluorophore-modified Rev peptide library. Furthermore, the DOFPL size can be easily expanded using other building blocks or scaffolds of the base DOFL, which would increase the probability of obtaining RNP sensors with a desired fluorescent property and binding ability to the target molecule. This method is expected to facilitate the versatile and facile construction of receptor-based sensors with good binding ability for a target of interest to achieve optimal fluorescent responses.

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Keywords: diversity-oriented fluorophore library • fluorescent probes • receptors • ribonucleopeptide • thiol alkylation

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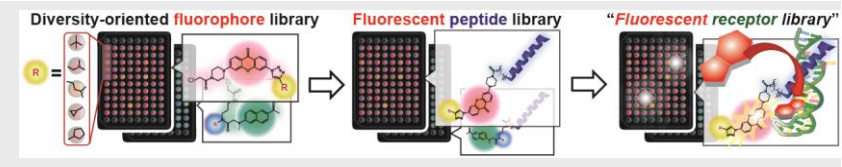

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A diversity-oriented library of fluorophore-modified receptors constructed from a chemical library of synthetic fluorophores

Conversion of a diversity-oriented fluorophore library to a library of fluorophore-modified peptide subunits of a ribonucleopeptide enabled construction of a library of fluorophore-modified receptors to a given target. Fluorescent biosensors with appropriate recognition characteristics for adenosine were screened from a library of fluorophore-modified adenosine-binding ribonucleopeptides.