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Selection of Turning-on Fluorogenic Probe as Protein-Specific Detector Obtained via the 10BASE$_d$-T

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Abstract. In order to obtain a molecular probe for specific protein detection, we have synthesized fluorogenic probe library of vast diversity on bacteriophage T7 via the gp10 based-thioetherification (10BASE$_d$-T). A remarkable turning-on probe which is excitable by widely applicable visible light was selected from the library.

INTRODUCTION

Antibodies are frequently used because they can specifically detect target-proteins in the medical front. However complicated operations, such as enzyme-linked immunosorbent assay (ELISA) or Western blot, are needed. Therefore, rapid and readily interpretable detection of a specific protein is crucial for broad range of disciplines, such as disease diagnostics [1]-[3] and environmental/cellular monitoring [3]-[5]. For this purpose, microenvironmentally sensitive solvatochromic probes [3] [6] are frequently used. In particular, those that result in a turning-on fluorescence (i.e., increase in light intensity on target recognition) are desirable because of the ease of implementation for practical use and the readily interpretable readout [7]. To obtain antibody-like specific binders with remarkable turning-on ability, we have evolved a solvatochromic fluorophore (i.e., Prodan) on the basis of a modified T7 phage display system, namely gp10 based-thioetherification (10BASE$_d$-T) [8] [9]. A major drawback of using Prodan is that it is designed to be excited by ultraviolet light sources, which often causes high background fluorescence when crude biological samples are used as analytes. To obtain a target-specific binder with low background fluorescence, here we evolved superior turning-on fluorophores, 4-*N,N*-dimethylamino-1,8-naphthalimide (4-DMN) [10], and 4-*N,N*-dimethylaminosulfonyl-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DBD) [11] [12]; these turning-on probes can be excitable by visible light sources such as widely applicable 458 and 488 nm lasers (Table 1).

TABLE 1. Photophysical properties of the fluorogenic cores of bromoacetamide state in DMSO.

Fluorophore core	$\lambda_{max, abs}$ (nm)	ε ($M^{-1}cm^{-1}$)
4-DMN	455	8.8×10^3
DBD	442	8.6×10^3
Prodan	391	2.0×10^4

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RESULTS AND DISUSSION

4-*N,N*-dimethylamino-1,8-naphthalimide-bromoacetamide (4-DMN-BA) and 4-*N,N*-dimethylaminosulfonyl-7-(2-aminoethylamino)-2,1,3-benzoxadiazole-bromoacetamide (DBD-BA) were reacted with T7 phage-displayed randomized peptide library (-SGGG-X_3-C-$X_{5\text{-}7}$-C-X_3; where X represents any amino acids) [8] [13] independently via the $10BASE_d$-T as shown in Figure 1, to make fluorogenic libraries with vast diversity (i.e., 10^9).

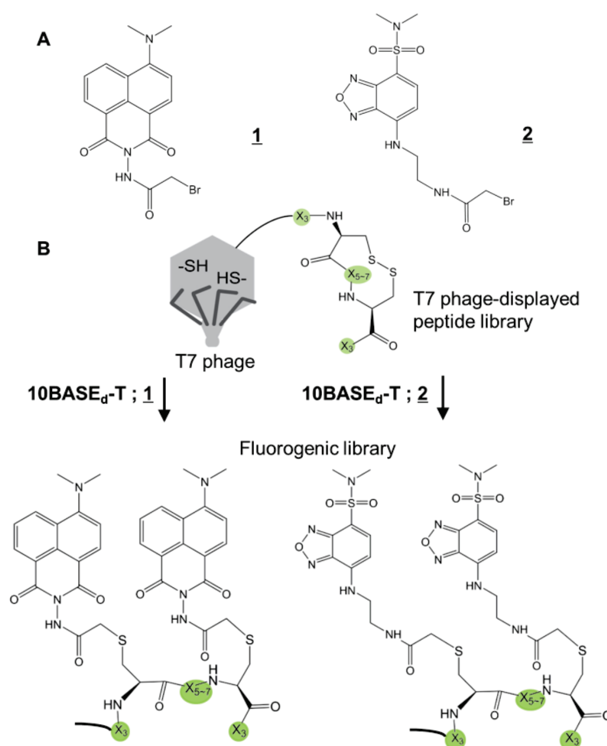

FIGURE 1. (A) Structures of fluorescent cores (**1**; 4-DMN-BA, **2**; DBD-BA). (B) Construction of fluorogenic libraries through the $10BASE_d$-T. X represents randomized amino acids.

As a target protein for the proof-of-concept study, we chose glutathione S-transferase (GST) because we have already evolved tetramethylrhodamine (TMR) and Prodan to GST-specific binders [8] [9]. By using each fluorogenic library of 4-DMN or DBD, six rounds of biopanning were performed against biotinylated-GST and enrichment of GST binders was assessed by ELISA. During the biopanning, increasing stringent conditions such as shortening the binding time and increasing the washing frequency were applied stepwise to each round. After the biopanning, 4-DMN and DBD-conjugated polyclones on T7 phage showed the strongest binding to GST, whereas ones lacking the 4-DMN and DBD core structures did not. Among 20 randomly chosen single T7 phage plaques, 10 and 13 clones of the 4-DMN and DBD conjugates had positive signals on ELISA, respectively. The clones gave consensus displaying-peptide sequences as shown in Figure 2. They are categorized in two different groups in 4-DMN; ZXZC*ZXDGZ and LNYC*DGW (C* and Z represent 4-DMN-conjugated Cys and hydrophobic amino acids, respectively), and in DBD; C*DZZ and ZZC*DGZ (C* represents DBD-conjugated Cys), respectively. They showed consensus peptide sequences of GZ (underlined) in the C-terminal region, which are also identical with TMR-evolved one (i.e., ZC*XDGZ; C* represents TMR-conjugated Cys) [8] and Prodan-evolved ones (i.e., NXVSCXGZ, and NPCTGZ; C* represents Prodan-conjugated Cys) [9]. Excluding the Prodan evolvers, aspartic acid (D) was also found as the consensus sequence at the next to the C-terminal penultimate residue. When bigger fluorogenic cores with different structures were evolved (i.e., TMR and 4-DMN), the obtained peptide sequences were almost identical (ZC--DGZ). When the size of the fluorogenic core was getting smaller, the location of the fluorogenic core in the evolved peptide was gradually shifted closer to the C-terminus. These suggest that different

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structural motifs in the peptide region could be optimized against the very same target protein by independent evolution of different artificial cores.

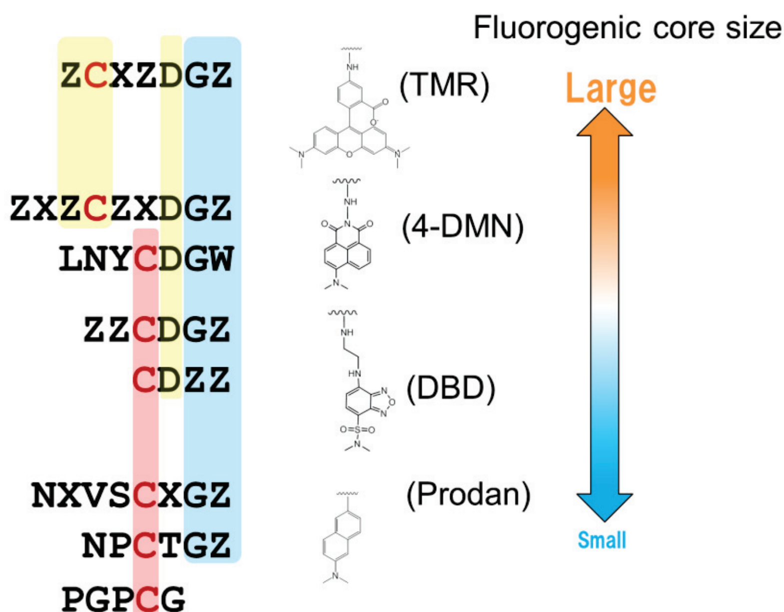

FIGURE 2. Sequence alignment of GST-binders. Cysteine is alkylated with the fluorogenic core (i.e., TMR, 4-DMN, DBD, and Prodan). Z and X represent hydrophobic and any amino acids, respectively. Consensus sequences between the groups of the different fluorogenic cores were hatched in yellow, blue, and red.

Next, we chemically synthesized peptides representing these two groups of 4-DMN and one group of DBD, and the target-binding property was investigated by fluorescence titration experiment excited at 470 ± 10 nm against GST. Most remarkable fluorescent intensity increasing was observed when 4-DMN-conjugated VSYC*LEDGY was titrated against GST (Figure 3A). The GST-binding ability was almost equal to that of the previously discovered Prodan and TMR evolved one; the dissociation constant (K_D) was estimated to be 3.1 ± 0.3 µM by relative fluorescence intensity change at 547 nm (Figure 3B).

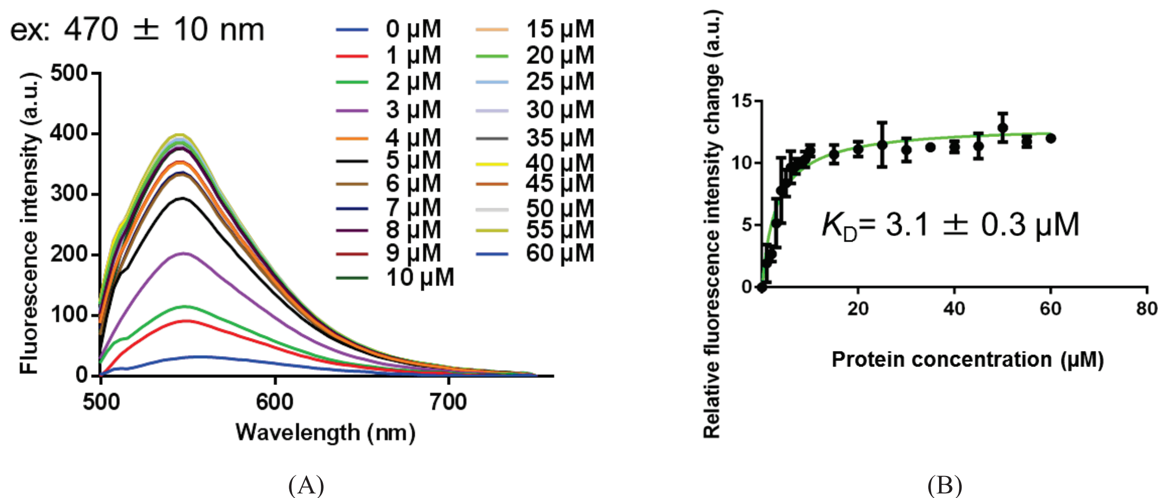

FIGURE 3. (A) Fluorescence emission titration upon GST-binding by using the best turning-on probe (VSYC*LEDGY; C* represents 4-DMN-conjugated Cys) excited at 470 ± 10 nm. (B) Determination of GST-binding affinity (dissociation constant; K_D) of the turning-on probe by relative fluorescence intensity change at 547 nm.

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To obtain insight of binding of VSYC*LEDGY to GST, ligand-based epitope mapping was performed by saturation transfer difference nuclear magnetic resonance (STD-NMR). Saturation time was varied in each measurement, and the relative STD intensities of these protons of interest were plotted against the saturation time [9] [14]. Exponential curve fitting of the plot gave us relative saturation rate constant of each proton (k) which indicated the importance of these atoms upon binding. As summarized in figure 4, valine and leucine strongly interacted with GST, whereas other residues moderately and weakly, respectively. In contrast to the Prodan evolver reported previously [9], most of the hydrogen atoms in 4-DMN core seems not very proximate to those in the GST, presumably because the size of the core is too large to fit in the deep inside of the GSH-binding pocket. In the 4-DMN case, the evolved peptide moiety looks more dominant contribution for the preferential molecular recognition. Nevertheless, the 4-DMN evolver possessed expected turning-on property with moderate binding affinity.

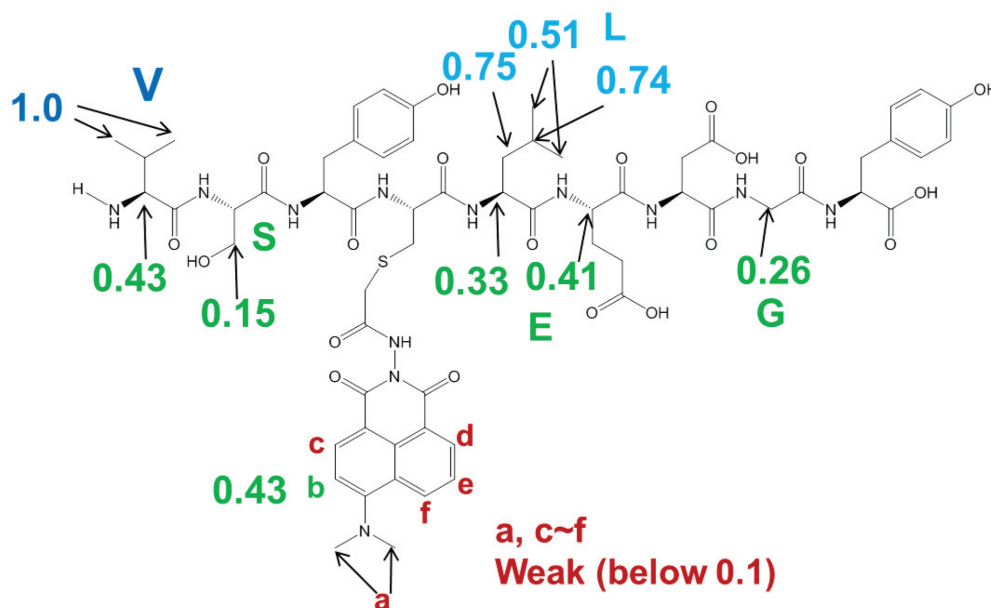

FIGURE 4. Quantitative epitope mapping of the 4-DMN-evolver VSYC*LEDGY upon GST binding on the basis of STD-NMR measurement. Relative saturation rate constant of each proton (k; shown here) was obtained from relative STD signal intensity (STD) at each saturation time (t) and maximum STD signal intensity (STD_{max}) by using an equation of $STD = STD_{max} (1-e^{kt})$. Tyrosine protons could not be quantified because their peak resolutions were too poor.

CONCLUSION

We successfully have found turning-on GST-binders which are excitable by widely applicable visible light. Very recently, Ito and his coworkers independently evolve 7-nitro-2,1,3-benzoxadiazole (NBD) derivatives using ribosome display system [15] to obtain turning-on NBD-peptide hybrid which exclusively binds to calmodulin. They further replace NBD to DMN derivative on the same peptide motif, and confirm that the dissociation constant seldom changes upon the fluorophore replacement [16]. This fluorophore-replacement method might be useful when the peptide moiety shows the major contribution for the target recognition. If the fluorogenic core is pre-optimized such as using virtual screening, independent selection using the optimized fluorogenic core would be rapid and straightforward to obtain strong binders.

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EXPERIMENTAL SECTION

In general, all experiments were performed according to our previously reported method / condition [9] with commercially available reagents and kits.

2-bromo-N-(6-(dimethylamino)-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)acetamide (DMN-BA; **1**) was prepared as previously reported [17].

2-bromo-N-(2-(7-(N,N-dimethylsulfamoyl)benzo[c][1,2,5]oxadiazol-4-ylamino)ethyl)acetamide (DBD-BA; **2**) was prepared as follows. 7-(2-aminoethylamino)-*N,N*-dimethylbenzo[c][1,2,5]oxadiazole-4-sulfonamide (also known as DBD-ED; 0.12 mmol; cat. No. A5574, TCI), and *N, N*-diisopropylethylamine (0.25 mmol) were mixed in 2 mL of CH_2Cl_2 at – 10 °C. Then, bromoacetyl bromide (0.19 mmol; cat. No. B56412, Sigma-Aldrich) in 2 mL of CH_2Cl_2 was added dropwise over 10 minutes, and the mixture was stirred for 3 hours at room temperature. The bright yellow precipitate was collected, and rinsed with 4 mL of CH_2Cl_2, and the remained solvent was completely evaporated to obtain pure DBD-BA (30 mg: 60% yield). It was analyzed both using NMR spectrometer and an Shiseido Nanospace SI-2 semi-micro HPLC system using a 0-100% gradient of acetonitrile containing 0.1% formic acid during 20 min at a flow rate of 300 μL per minute, equipped with a C18 reverse-phase column (Hypersil GOLD, 2.1 × 100 mm, Thermo Fisher Scientific) connected to a LCQ-Fleet ion trap mass spectrometer.
^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.89 (d, 1H, J = 8 Hz), 6.91 (s(br), 1H, NH), 6.41 (s(br), 1H, NH), 6.17 (d, 1H, J = 8 Hz), 3.94 (s, 2H), 3.72-3.58 (m, 4H), 2.87 (s, 6H); LC-MS (*m/z*) 405.84 and 407.86 ($[M + H]^+$; retention time = 9.1 min).

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