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# Selection of fluorescent biosensors against galectin-3 from an NBD-modified phage library displaying designed α-helical peptides

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ABSTRACT

Fluorescent biosensors are indispensable tools for molecular imaging, detection, and drug screening. Conventionally, fluorescent biosensors were constructed by incorporating fluorophores into ligands. Here, to develop ligand-independent biosensors, we demonstrated biosensor selection from a fluorophore-modified peptide phage library. In this library, the peptides were designed to form α-helical structures, and one cysteine, the probe modification site, was located at the center of four randomized residues on the same face of the helix. By conjugation with 4-nitrobenzoxadiazole (NBD), we constructed an NBD-modified phage library. We conducted selection against galectin-3 (Gal-3), a galactose-specific lectin associated with various biological events such as tumor metastasis and insulin resistance. After biopanning, we obtained NBD-modified peptides that selectively bind to Gal-3 from the library. The fluorescence intensity of the hit biosensors increased with the concentration of Gal-3, and this fluorescent response was visually observed.

Fluorescent biosensors provide powerful biochemical toolboxes for molecule imaging, detection, and analyte quantification. In the design of fluorescent biosensors, a recognition motif that selectively binds to a target analyte is typically coupled to a fluorescent signal transducer.[1] Various semisynthetic biosensors, which consist of a solvatochromic fluorophore and a peptide/protein, have been developed.[2–5] For instance, Hahn's group has developed biosensors by modifying an environment-sensitive dye to a monobody specific for activated Src-family kinases to image the dynamics in living cells.[6] Hamachi's group has demonstrated the in situ construction of fluorescent biosensors by ligand-directed labeling methods to investigate the ligand binding kinetics of receptors[7] and to perform inhibitor discovery.[8] The semisynthetic NADPH sensor developed by Johnsson's group was a powerful tool for metabolic assays.[9]

Although these ligand-based biosensors are indispensable tools, there are two difficulties in rational design and construction.[10] First, in principle, it is inapplicable to target molecules whose ligands or binders are unknown. Second, laborious optimization of the probe conjugation site is required for each target. To obtain a readout of analyte binding as a change in fluorescence properties without perturbing the interaction, a fluorescent probe must be incorporated near but not at the binding interface. Therefore, multiple time-consuming iterations of binder preparation, modification, and testing are required until an ideal sensor is obtained for each target. Considering these drawbacks of conventional construction methods based on peptide/protein engineering with elaborate design, we expect a screening strategy from a fluorophore-modified peptide library to be feasible for the efficient development of biosensors.

Phage display is a representative technique for the selection of peptide ligands. By introducing a randomized DNA sequence into the phage coat protein gene, a phage library displaying a variety of peptides can be constructed.[11] Moreover, the development of an engineered fd phage without any disulfide bonds facilitates selective chemical modifications on Cys residues without losing phage infectivity.[12] Heinis's group first demonstrated the selection of cyclic peptide ligands from chemically modified phage libraries.[13] Our group constructed phage libraries modified with monosaccharides for the selection of modified peptides with high affinity for carbohydrate-binding proteins.[14,15] Moreover, we demonstrated the selection of stapled peptides from helical designed peptide libraries.[16,17]

Some groups have already reported the selection of fluorescent biosensors from fluorophore-modified phage libraries.[18,19] These methods are composed of two steps: (1) the construction of a fluorophore-modified phage library, in which an environment-responsive fluorescent dye is introduced in the vicinity of a random sequence, and (2) the selection of fluorophore-modified phages with

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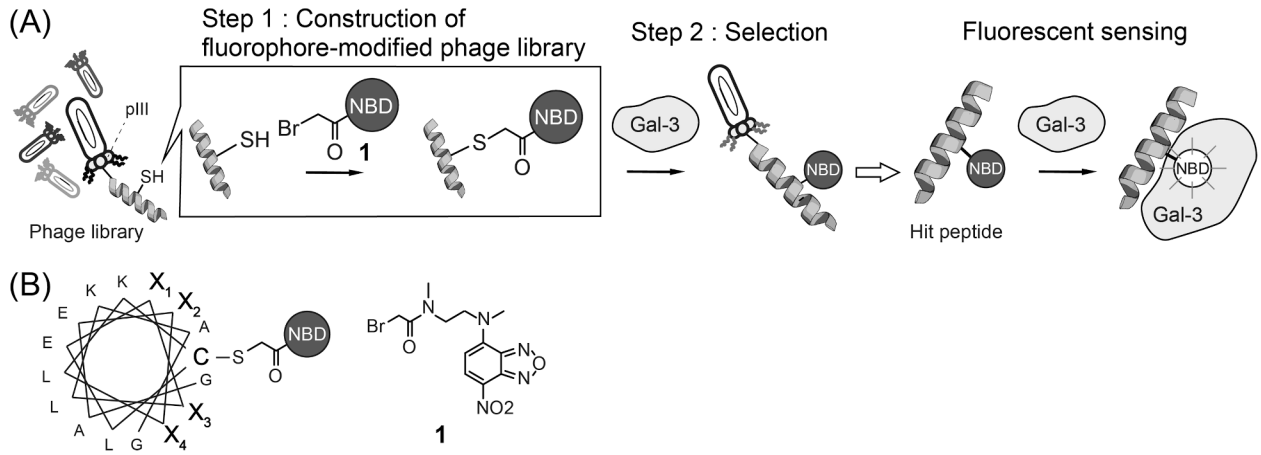


**Fig. 1.** (A) A schematic illustration of the construction of an NBD-modified phage library and selection of NBD-modified peptide biosensors against Gal-3. (B) A helical wheel diagram of an NBD-modified α-helical peptide library (GAE**X$_1$X$_2$**LKCLE**X$_3$X$_4$**LKAG) and a chemical structure of compound **1**.

high affinity to a target molecule from the library. In particular, Winter's group constructed fluorophore-modified single chain Fv (scFv) libraries and developed a labeled scFv that selectively responds to 2-phenyloxazolone-conjugated BSA.[18] Although folded proteins have the advantage of properly defining random residues and modification sites in the structure, peptide-based biosensors are preferred since small peptides have high stability and can be organically synthesized, which reduces the cost and facilitates chemical modification.[20] Additionally, peptides have the potential for intracellular applications.[21] Taki's group has constructed fluorophore-modified T7 phage libraries ($X_3$-C-$X_{5\text{-}7}$-C-$X_3$, Cys are modified with a fluorescent dye, X = any amino acids) and selected a peptide biosensor against glutathione S-transferase.[19] However, the affinity-based selection of unstructured peptide libraries carries the risk of selecting inappropriate peptides where the fluorophore is not a core part of the interaction.

In this research, for the rational development of peptide biosensors integrating a dye into the binding interface, we devised a strategy to stabilize the secondary structure that defines the positions of fluorescent dye and randomized residues. Here, we report the construction of a phage library displaying fluorophore-modified α-helical peptides and the selection of peptide biosensors from the library (Fig. 1A).

We chose a designed helical peptide as the biosensor backbone. Helical structures play a vital role in protein–protein interactions (PPIs), and approximately 62% of PPIs occur on one face of helical structures.[22] For this reason, four residues located on one face of the helical peptide were randomized. To stabilize the helical structure, a hydrophobic Leu residue was introduced on one side to obtain an amphiphilic nature, and hydrophilic Glu and Lys ion pairs were introduced on the other side for salt bridges.[23] Thus, we designed an amphipathic 16-mer helical peptide library (GAE**X$_1$X$_2$**LKCLE**X$_3$X$_4$**LKAG, Xn = randomized positions), as reported previously.[15] Furthermore, we have shown that a model peptide in which all random residues are replaced with Ala (GAE**AA**LKCLE**AA**LKAG) forms a helical structure (86% helicity).[15] The microenvironments around the conjugated fluorochromes need to be changed between unbound and bound states to read out the analyte binding as a fluorescent response. Therefore, a Cys residue was placed at the center of four randomized residues and modified with a dye by nucleophilic substitution reaction (Fig. 1B). In this study, 4-nitrobenzoxadiazole (NBD) was selected as a fluorescent dye since NBD is frequently used for biomolecule imaging due to its high sensitivity to solvent polarity.[24–26] For chemical modification of the Cys residue with NBD, we synthesized compound **1**, which has an electrophilic bromoacetyl group (Fig. 1B).

First, to check the NBD modification of the synthetic model peptide, the reaction was traced by HPLC. The model peptide disappeared, and the conjugate was detected after a one-hour incubation at 42 °C (Fig. S1). Next, to examine the NBD modification of expressed peptides on pIII, model peptide phage was mixed with 1.0 μM compound **1** after 1.0 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride) reduction. The NBD-labeled phages were subjected to SDS-PAGE and analyzed by fluorescence gel imaging. A fluorescent band was observed at a molecular weight corresponding to pIII of the model peptide phage (Fig. S2), suggesting successful conjugation with NBD. In contrast, for the negative control phage, in which Cys was substituted with Ala (C8A), no fluorescent band was detected, indicating that the Cys residue employed in the model helical peptide was modified with NBD. The reaction yield of the NBD modification was almost saturated at 1.0 μM compound **1** (Fig. S3). The NBD modification did not significantly decrease the infectivity of the phage at 1.0 μM, while a higher concentration of **1** attenuated the phage activity (Fig. S4). From these results, we determined that a one-hour incubation with 1.0 μM of **1** at 42 °C was the optimum condition.

As a proof-of-concept, we performed a selection of fluorescent biosensors from an NBD-modified peptide phage library against Gal-3. Gal-3 is a galactose-specific lectin involved in tumor metastasis and angiogenesis.[27] In addition, secreted Gal-3 is known to induce cellular insulin resistance, and the blood Gal-3 levels in subjects with obesity are high.[28] Therefore, we selected Gal-3 as a physiologically significant target. In the selection process, the carbohydrate recognition domain of Gal-3 was chemically biotinylated and captured on streptavidin-magnetic beads. The NBD-modified phage library was incubated with the beads for 30 min at 4 °C. Then, unbound phages were washed out, and the bound phages were eluted by acidic glycine buffer (pH 2.2). The eluted phages were infected with *E. coli* TG-1 and amplified for subsequent rounds of selection. Selections were repeated 5 times in total. The recovery yields in each round were determined by phage titration (Fig. S5). The yields were dramatically increased in the 5th round. In addition, we conducted enzyme-linked immunosorbent assays (ELISAs) to evaluate the binding activity of phage pools to Gal-3. The results indicated that the amounts of phage binding to Gal-3 dramatically increased in the 5th round (Fig. S6), which is consistent with the recovery yields in Fig. S5.

We identified 33 kinds of peptides by DNA sequencing of individual clones in the 5th round phage pool (Table S1). In general, Cys and Gln residues were found in randomized positions at high frequency (4 times higher than the theoretical frequency). Most of the peptides (17/33) had an additional Cys at random residues in addition to the designed Cys. In addition, peptides (10/33) without any Cys were also identified due to mutation of the designed Cys to Arg, which resulted from PCR byproducts during the library construction. We attribute the enrichment of clones lacking Cys or clones with two Cys residues to the low propagation efficiency of phages with unpaired Cys residues (odd numbers of Cys residues).[29] We excluded them from the candidates because they did

**Table 1**
Sequences of selected NBD-modified peptides against Gal-3.

| Library | Peptide sequence | | | | | Charge |
|---|---|---|---|---|---|---|
| | GAE | $X_1X_2$ | LKC(NBD)LE | $X_3X_4$ | LKAG | |
| Clone 1 | GSE | **AR** | LKC(NBD)LE | **QH** | LKAG | +1 |
| Clone 2 | GAE | **FR** | LKC(NBD)LE | **SV** | LKAG | +1 |
| Clone 3 | GAE | **AR** | LKC(NBD)LE | **RR** | LKAG | +3 |
| Clone 4 | GAE | **SQ** | LKC(NBD)LE | **KQ** | LKAG | +1 |
| Clone 5 | GAE | **SQ** | LKC(NBD)LE | **RQ** | LKAG | +1 |
| Clone 6 | GAE | **FC(NBD)** | LKRLE | **DW** | LKAG | 0 |

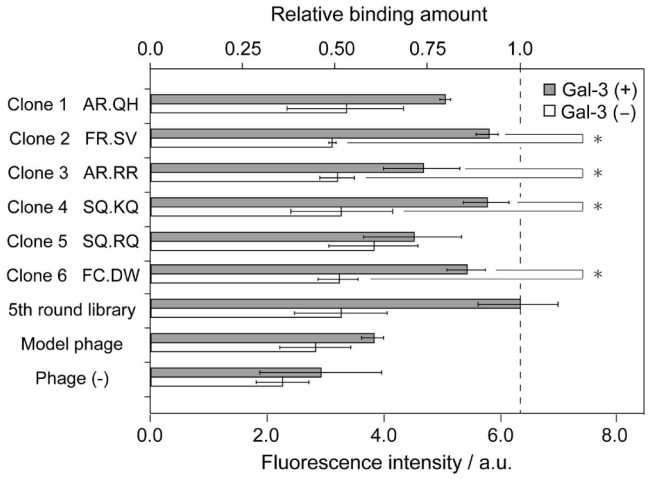


**Fig. 2.** ELISA for the binding activities of phages identified after 5 rounds of selection to Gal-3. The four letters that follow the clone number indicate the sequence of random residues ($X_1X_2.X_3X_4$). The relative binding amounts were estimated based on the fluorescence intensities of the horseradish peroxidase substrates after the enzyme reaction. Gal-3 (1 μg) was immobilized on each well, and phage concentrations were 10 nM. For all samples, n = 3. Error bars represent the standard deviation. (*: $p < 0.05$ , $p = 5.0 \times 10^{-4}$, 0.047, 0.025, and $1.3 \times 10^{-3}$ for Clone 2, 3, 4, and 6, respectively.)

not match our original design and focused on six sequences containing a single Cys, which are summarized in Table 1. Clones 1, 2 and 3 had Arg at the $X_2$ position in common, and Clones 1 and 3 contained Ala at the $X_1$ position. Clones 4 and 5 have similar sequences, in which the $X_1$, $X_2$, $X_4$ positions were the same residues (Ser, Gln, and Gln, respectively), and $X_3$ was cationic Lys or Arg. All five sequences were cationic. In Clone 6, one Cys was included in the $X_2$ position, although the designed Cys was mutated to Arg. Interestingly, Clone 6 showed a similar sequence to Clone 7, which was found at the highest frequency (Table S1). Both peptides had Arg instead of the designed Cys and had Asp and Trp at the $X_3$ and $X_4$ positions. Therefore, Clone 6 was included as a candidate for the next evaluation, although it was different from our original design. The relative binding activities of the six individual phage clones to Gal-3 were evaluated by ELISA. In the case of Clones 2, 3, 4, and 6, the binding amounts of phage were significantly increased in the presence of Gal-3 compared to the absence of Gal-3 (Fig. 2). On the other hand, Clones 1 ($p = 0.098$) and 5 ($p = 0.36$) did not show significant differences with and without Gal-3, as was the case for the model phage ($p = 0.10$, negative control).

To assess the fluorescent responses of the six peptides (P1-P6) to Gal-3 binding, we synthesized these peptides by the Fmoc solid-phase method and modified them with compound **1**. The changes in fluorescence intensities upon Gal-3 binding were measured by adding 10 μM Gal-3 to 2.0 μM NBD-modified peptide solutions. Among the six NBD-modified peptides, only P2 (FR.SV) and P4 (SQ.KQ) peptides showed significant increases in fluorescence intensity upon the addition of Gal-3 (Fig. S7). Notably, these two hit peptide clones showed the highest binding activities to Gal-3 in ELISA studies (Fig. 2). On the other hand, P1, P5, and the model peptide showed lower binding activities to Gal-3 and no fluorescent responses, suggesting that these peptides had a low affinity to Gal-3. In addition, P6, whose sequence is different from the original design, did not show any fluorescent response, although ELISA studies showed high binding to Gal-3.

We further investigated the affinity and selectivity of P2 and P4 peptides for Gal-3. The fluorescence intensity increased 1.4-fold and 1.5-fold in a concentration-dependent manner for P2 and P4, respectively (Fig. 3A). No shift in the fluorescence wavelength was observed. The fluorescence intensities were curve-fitted by a fractional saturation (*f*)

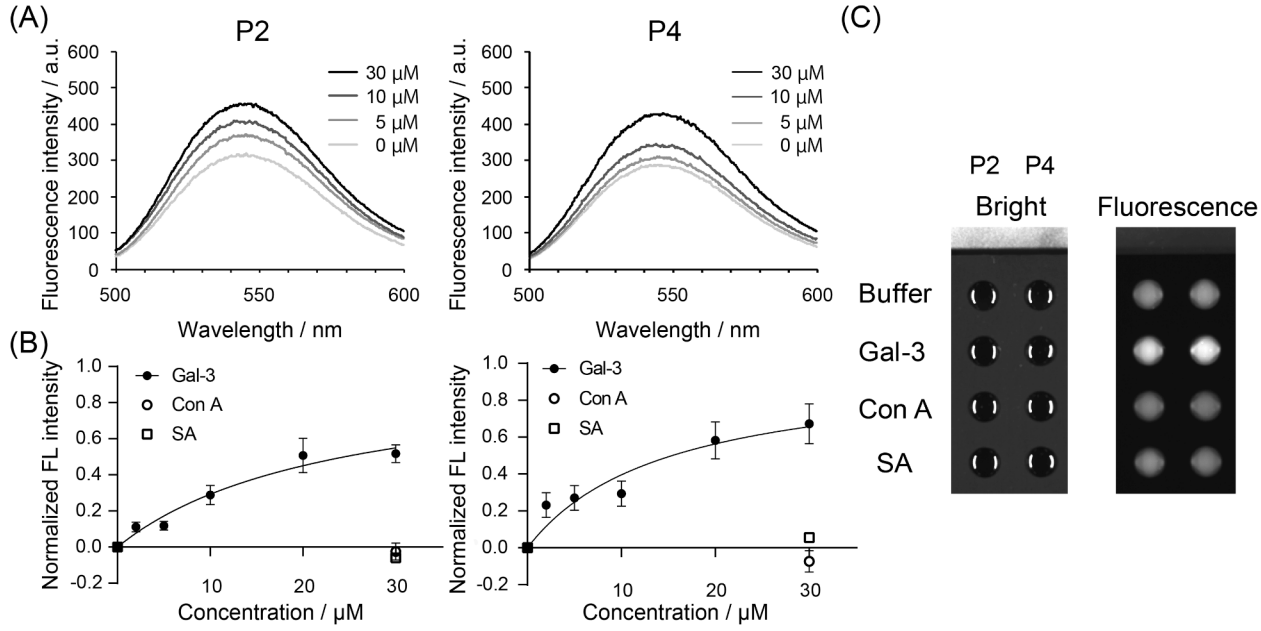


**Fig. 3.** Fluorescence properties of hit NBD-modified peptides (P2 (FR.SV) and P4 (SQ.KQ)). (A) Fluorescence spectra at various concentration of Gal-3. Peptide concentrations were 2.0 μM. Excitation wavelength was 460 nm. (B) Normalized fluorescence intensities and titration curves. The data were fitted to one-site specific binding model. For all samples, n = 3. Error bars represent the standard deviation. ConA; concanavalin A, SA; streptavidin. (C) Fluorescence detection of Gal-3 on a glass slide.

equation, and the dissociation constants ($K_d$) values were calculated to be 23.5 μM for P2 and 14.8 μM for P4 (Fig. 3B). This fluorescent response was driven by the selective binding to Gal-3, since no change was observed against streptavidin and concanavalin A, a glucose/mannose-specific lectin, at 30 μM. The fluorescent responses to Gal-3 could be visually observed on a glass slide (Fig. 3C). Besides, P2 and P4 also worked in complex environments, cell lysates, and the fluorescence intensities were enhanced in the presence of Gal-3 (Fig. S8). This result indicated that the hit NBD-modified peptides enable easy and rapid detection of Gal-3 without special equipment.

Finally, we evaluated whether these NBD-modified peptides formed α-helical structures (Fig. S9). The CD measurements of two peptides exhibited typical α-helical spectra with negative Cotton effects at 208 and 222 nm in 30% trifluoroethanol/20 mM phosphate buffer (these conditions mimicked the protein binding environments[30]). The α-helical contents of P2 and P4 were estimated to be 55% and 58%, respectively, suggesting that the modified NBD was located at the interface of binding sites.

In conclusion, we demonstrated the selection of biosensors from a fluorophore-modified phage library displaying designed α-helical peptides. After biopanning against Gal-3, we found six NBD-modified peptide candidates containing one Cys among the sequences. In ELISA studies using phage clones and fluorescence assays of synthetic peptides, two NBD-modified peptides (P2 and P4) were identified as hit biosensors. These biosensors selectively bound to Gal-3 and visually detected the target as an increase in fluorescence. Thus, our screening approach has the potential to develop fluorescent biosensors without using known ligands, unlike conventional construction depending on ligands or binders. Moreover, our helical design forces a fluorescent probe to be located at the center of the binding interface and facilitates reading out the binding to the target molecule as a change in fluorescence.

In addition, we noticed some issues in our strategies. First, the affinity of hit peptides (P2 and P4) was not high, presumably because the diversity of our library is not large. We expect that the elongation of our helical peptide and increase of randomized residues improve the diversity and enhance the chance to obtain peptides with high affinity to a target protein. Second, the majority of isolated peptides did not match our design, affording the peptides with an additional Cys residue. We suspect that phages tethering a single Cys were eliminated during repeats of amplification due to amplification bias in *E. coli*. This should be the main reason why we could not find a strong consensus sequence among isolated peptides containing a single Cys. To prevent the enrichment of undesired peptides, we expect it to be effective to use 19 trinucleotide cassettes without Cys codons instead of NNK codons. We envision that these improvements based on our strategy lead to more robust and efficient tools for developing peptide-based fluorescent biosensors.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.127835.

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