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In this study, a novel noncompetitive homogeneous immunoassay for antigen detection was developed. We utilized β-glucuronidase (GUS), a homotetrameric enzyme, the assembly of all of whose subunits is necessary to attain its activity. By using a mutant GUS (GUSm), wherein the dimerization of dimers, which is a rate-limiting step, can be effectively inhibited by a set of interface mutations, we attempted to create a biosensor for detecting various molecules. Usually, the affinity between the two variable region domains (V_H and V_L) of an antibody, especially for a small molecule is relatively low. However, in the presence of an antigen, the affinity increases so that they bind tighter to each other. A pair of fusion proteins, comprising the V_H and V_L regions of antibody as the detector tethered to a GUSm subunit as the reporter, was constructed to detect antigen 4-hydroxy-3-nitrophenylacetyl (NP) and bone Gla protein (BGP) through GUS activity measurement. Colorimetric and fluorescent assays could detect NP, 5-iodo-NP, and BGP within 1 h without separation steps and with a higher signal/background ratio than conventional ELISA. The instantaneous response after simple mixing of the components makes this system convenient and high-throughput. The system could be effective for the analyses of various small molecules in the environmental and clinical settings.

Introduction

Due to rapid industrial development and growth of the world population, the need to detect harmful small molecules in our environment increases rapidly. In addition, the detection of specific molecules related to various diseases is critical for early stage therapies. As a result, the need for a sensitive, effective, and high-throughput analysis method for the detection of a range of molecules has never been higher.

Beta-glucuronidase (GUS) is an enzyme that belongs to the glycosidase family of enzymes that catalyse the breakdown of complex carbohydrates and whose activity can be detected quantitatively and sensitively using both chromogenic and fluorescent substrates. GUS is a homotetramer, the assembly of whose subunits is necessary for its activity [3]. Based on a previous study, a set of interface mutations, M516K and Y517E, is known to effectively inhibit their assembly, resulting in inactive dimers instead of active tetramers [4].

An immunoglobulin G (IgG) consists of heavy chains and light chains, both of which contain constant (C_H and C_L) and variable region domains (V_H and V_L) (Fig. 1a) [5]. Usually, the affinity between the two variable region domains V_H and V_L of an antibody recognizing a small molecule is relatively low. However, in the presence of an antigen, this affinity increases so that they bind to each other more tightly (open sandwich immunoassay principle) (Fig. 1b) [6]. To date, not only small organic molecules but also lipids [7], peptides [8] and their phosphorylation [9], and even larger protein such as influenza hemagglutinin [10] were successfully detected by measuring the interaction strength of V_H and V_L fragments [11]. This leads to the

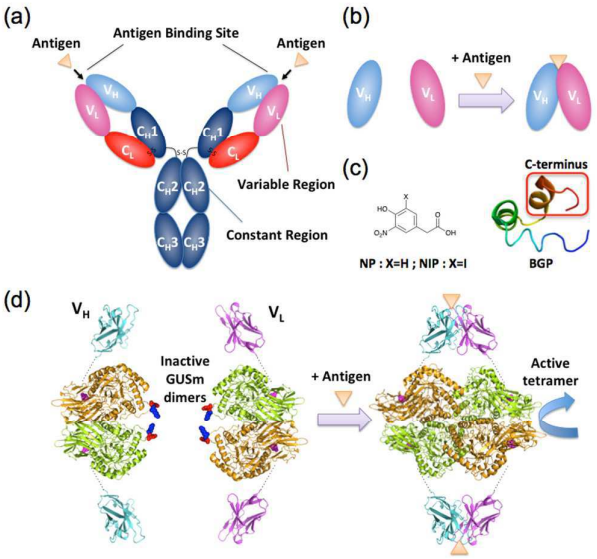

Figure 1. (a) Schematic structure of antibody (IgG). (b) Scheme of open sandwich immunoassay (OS-IA) principle. (c) Molecular structure of the analytes: NP, NIP and human BGP (PDB1Q3M). (d) Scheme of mutant GUS-based immunoassay system, based on the published structures of anti-NP (N1G9) Fab [1] (PDB1NGP) and *E. coli* GUS [2] (PDB3K46). Mutated residues to weaken tetramerization are shown as blue and red balls.

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assumption that a fusion protein system comprising V_H and V_L of an antibody, each tethered to a mutant GUS (GUSm) subunit as the reporter, could be used as a biosensor for various molecules. In this study, we aimed to construct this immunosensor and assess its ability to detect 4-hydroxy-3-nitrophenylacetyl (NP) and bone Gla protein (BGP) (Fig. 1c, d). NP (molecular weight, 197.15) is a well-studied model hapten that is generally used for immune response evaluations. Based on previous studies, 5-iodo NP (NIP), a derivative of NP, can be detected more sensitively than NP itself [12]. BGP, a vitamin K-dependent protein synthesized by osteoblasts and measured in blood by radioimmunoassay, has been used as an index of the rate of bone turnover. [13] The association of BGP concentration in serum with bone mineral metabolism [14], and more recently, with endocrine regulation of energy metabolism [15] male fertility [16], and brain development [17] have been suggested, making BGP a very important biomarker for the diagnosis of osteoporotic, metabolic, reproductive and cognitive syndromes.

After successful construction of the system for detecting NP, variable region domains of anti-NP antibody, V_H(NP) and V_L(NP), were simply replaced by V_H(BGP) and V_L(BGP) of an anti-BGP antibody to construct a BGP detection system using the same principle.

Results and Discussion

Preparation of fusion proteins

First, we prepared a pair of antibody V region–mutant GUS fusion proteins with N-terminal thioredoxin, wherein an interdomain linker GESKLAAA or KL(GGGGS)$_3$AAA was placed after V_H or V_L, respectively, followed by GUSm, to detect NP as the antigen. However, the mixture of these proteins showed negligible binding to immobilized NP conjugated with bovine serum albumin (data not shown). We reasoned that the distance between the two N-termini of GUSm (shown as magenta balls in Figure 1d) was too long to gain functional binding activity. Hence, a longer, flexible linker, GESKLAAGGS(GGGGS)$_2$AAA, was placed between V_H and GUSm in the Trx-V_H-GUSm expression vector and used thereafter.

After protein expression using the mutant *E. coli* strain SHuffle T7 Express lysY, whose cytoplasm is oxidative enough to allow disulphide bond formation in each V region, the expressed Trx-V_H(NP)-GUSm and Trx-V_L(NP)-GUSm proteins were tandem purified by immobilized metal affinity chromatography (IMAC) via a His-tag appended to the proteins and by anion exchange chromatography. The purification process was monitored by SDS-PAGE (Fig. 2a, 2b), and, after IMAC purification, a smaller band, whose size did not match the expected size of Trx-V_H(NP)- GUSm, was also observed; this indicated the existence of an undesired degradation product of improperly folded fusion proteins. Therefore, further purification using anion exchange chromatography was performed to increase the purity of Trx-V_H(NP)-GUSm for evaluation, until a single band was observed by SDS-PAGE.

Evaluation of the NP detection system

After mixing the two fusion proteins Trx-V_H(NP)-GUSm and Trx-V_L(NP)-GUSm in the presence or absence of NP, higher enzymatic activity was observed in the reaction mixture with NP when the chromogenic substrate p-nitrophenyl-β-D-glucuronide was added (data not shown). Then, the antigen dependency of the enzymatic activity of each fusion protein and their mixture was evaluated by fluorescence measurement, using the fluorescent substrate 4-methylumbelliferyl-β-D-glucuronide to avoid the effect of NP-derived absorbance (Fig. 3a). The fluorescence intensity increased gradually until plateauing, possibly due to all of the substrate being consumed. While Trx-V_H(NP)-GUSm or Trx-V_L(NP)-GUSm alone in the presence of NP showed indistinguishable signal to that of the fusion protein mixture in the absence of NP, signal derived of the mixture in the presence of NP was significantly higher. This result indicates that the GUS enzymatic activity was regained after association

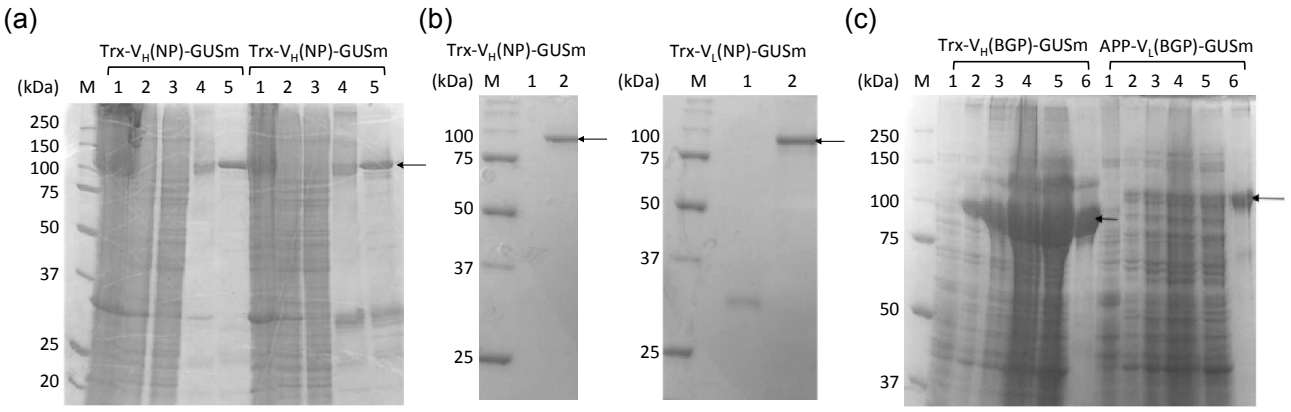

Figure 2. (a) IMAC purification of Trx-V_H(NP)-GUSm (100.9 kDa) and Trx-V_L(NP)-GUSm (98.8 kDa), 1: Insoluble proteins after sonication, 2: soluble proteins after sonication, 3: unbound proteins in IMAC purification, 4 and 5: Elution of IMAC purification. (b) Anion exchange chromatography of Trx-V_H(NP)-GUSm and Trx-V_L(NP)-GUSm, 1: Flow-through (Unbound proteins), 2: Purified proteins. (c) IMAC of Trx-V_H(BGP)-GUSm (99.7 kDa) and APP-V_L(BGP)-GUSm (108.6 kDa) 1: Before induction, 2: After induction, 3: Insoluble proteins after sonication, 4: Soluble proteins after sonication, 5: Unbound proteins in IMAC purification, 6: Elution of IMAC purification.

of the two fusion proteins with the antigen, and it continuously catalysed the breakdown of the substrate, generating the fluorescent product. Comparing the fluorescence signals generated by the binding of the two fusion proteins in the presence and absence of antigen revealed that the highest response, i.e. a 5-fold increase, was observed at around 20 min after reaction start.

Next, the antigen dose-dependency measurements were performed by fixing the reaction time to 20 min and varying the concentration of antigen. When NP or its higher affinity analogue 5-iodo NP (NIP) were added, higher antigen concentrations gave higher fluorescent signals, i.e. higher GUS enzymatic activity (Figure 3c). After curve fitting, the EC_{50} values of this system of NP and NIP detection were 4.8 ± 0.7 μM and 0.44 ± 0.05 μM, respectively, which is in agreement with the 10-fold difference in the K_d of the binding of NP and NIP to the antibody used [18]. Thus, this detection system is capable of detecting model haptens NP and NIP qualitatively and quantitatively in a noncompetitive manner.

In addition, we prepared fusion proteins of the V region and wild-type GUS to evaluate relative specific activity. Compared with the same concentration of wild-type GUS fusion proteins (0.1 μM each), the enzymatic activity of the GUSm fusion proteins in the presence of 1 μM NP was 7.8% (Figure S1). Considering the NP dose-response, the maximal response of this system is about one fifth that of the wild-type GUS.

Evaluation of the BGP detection system

To prove that this system can be applied to noncompetitively detect other small molecule targets by simply swapping the V region of the fusion proteins, a biomarker for bone turnover, BGP, was chosen as a target, and the probes with the V region of the anti-BGP antibody KTM219 were constructed. In this study, a heptameric peptide was used as an epitope peptide at the C-terminus of BGP (BGP-C7).

While the Trx-fused anti-BGP V_H-GUSm protein was expressed in the *E. coli* cytoplasm, most of the expressed Trx-fused V_L-GUSm protein was found in the insoluble fraction, even when *E. coli* was cultured at a lower temperature (<16 °C). To increase the solubility of the V_L(BGP)-GUSm protein, a stronger solubilisation tag including a hyperacidic module derived from amyloid precursor protein (APP)[19] was fused to the N-terminus of the V_L(BGP)-GUSm protein, yielding APP-V_L(BGP)-GUSm. After expression, Trx-V_H(BGP)-GUSm and APP-V_L(BGP)-GUSm protein were both successfully purified by IMAC, and a single band was observed by SDS-PAGE, which indicated a purity of the proteins high enough for the evaluation of their performance (Fig. 2c).

After preparation and mixing the two fusion proteins and the antigen BGP-C7, the antigen dependency of enzymatic activity was monitored by adding a chromogenic substrate. After adding the substrate, the absorbance resulting from the regain of GUS activity gradually increased until plateauing, possibly due to all of the substrate being consumed (Fig. 3b). The results being similar to those of the NP detection system suggests that the enzymatic activity of GUS was regained after

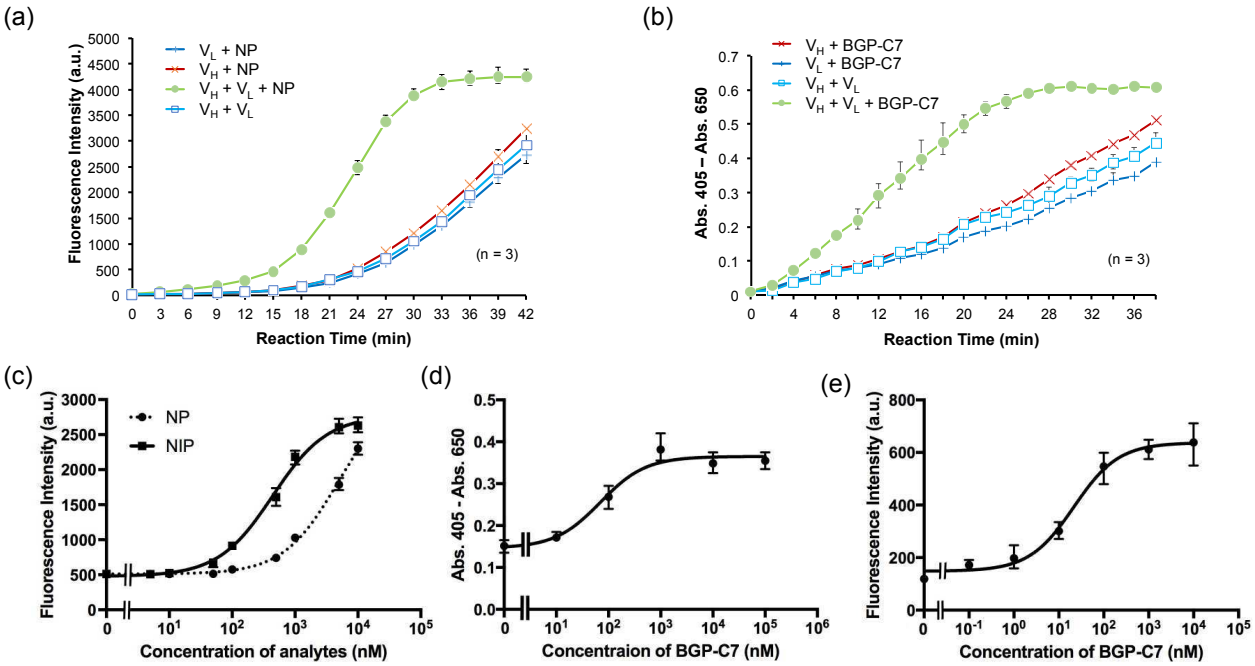

Figure 3. (a) Time course measurement of NP detection system. V_H and V_L stand for Trx-V_H(NP)-GUSm and Trx-V_L(NP)-GUSm proteins, respectively. (b) Time course measurement of BGP detection system. V_H and V_L stand for Trx-V_H(BGP)-GUSm and Trx-V_L(BGP)-GUSm proteins, respectively. (c) Antigen dose-dependency of NP detection system. (d) Antigen dose-dependency of BGP detection system by colorimetric measurement, and (e) the same by fluorescence measurement. Averages of triplicate measurements ± 1 SD are shown.

the association of the two fusion proteins containing the V regions of the anti-BGP antibody by the their antigen BGP-C7. By comparing the absorbance signals generated by the binding of the two fusion proteins and the antigen with the background absorbance signal, it is visible that the highest response was at 15 min after reaction start, and the increase was more than 2.5-fold (data not shown).

In antigen dose-dependency measurements, both colorimetric and fluorescence measurements were performed (Fig. 3d and 3e) at 15 min to study the influence of the measurement methods on the results. The results showed that the antigen concentration is positively related to the colorimetric and fluorescence signals, which are indicators for enzymatic activity. After curve fitting, the EC_{50} value of this system for BGP-C7 detection was estimated to be 69.8 ± 5.6 nM for colorimetric measurement and 21.5 ± 2.3 nM for fluorescence measurement, suggesting that fluorescence measurement was more sensitive than the colorimetric measurement. Thus, this system is capable of detecting small biomarker peptides as well as small haptens with high sensitivity.

Conclusion

A novel homogeneous biosensor system for detecting small molecules based on the regain of enzymatic activity was constructed. Compared with traditional methods, this homogeneous system has a high sensitivity and does not require additional steps such as separation as in heterogeneous assays such as ELISA. [20] The instantaneous response after simply mixing the reagents and analytes makes this system convenient and timesaving and an efficient high-throughput analysis method. In addition, its rapid and high response makes it an effective method for clinical and environmental small molecule detection.

Of note, the IC_{50} and working range obtained from indirect competitive phage ELISA for BGP peptide using the same antibody fragments was 135 ng/mL (88 nM) and 30–1000 ng/mL (20–650 nM), respectively [8]. Considering these values, this assay gives similar or higher sensitivity. Also, the system is expected to work equally well with serum-containing samples, since previously we could detect BGP-C7 in human serum by OS- ELISA using fusion proteins containing the same antibody fragments, after brief pretreatment with albumin adsorption column. [21]

This system is an extension of open sandwich enzyme complementation immunoassay (OS-ECIA), wherein the approximation-induced reconstitution of multimeric β-galactosidase activity derived from $\Delta\alpha/\Delta\omega$ complementation is monitored [22, 23]. Compared with OS-ECIA, which depends on chemiluminescent detection, the efficiency of reconstitution in this system is significantly higher (~20%, compared with 0.6%), which allows the use of both chromogenic and fluorogenic substrates in the assay. Furthermore, the maximum responses of OS-ECIA after extensive optimization was only 1.5-fold for NP.

The remaining problem of background signal propagation observed for this system is probably caused by the insufficient stability and degradation of fusion proteins during the measurement and resultant spontaneous association of mutant GUS dimers. To resolve this, stabilization of the GUSm subunit by introducing stabilization mutations might be effective. However, the current response observed in this study is already useful for detecting many small molecules. In the case of NP, this method gives a more than 5-fold response, which is actually higher than the response of open sandwich ELISA (~3-fold) that measures signal after a washing step [24].

Various commercially available substrates can be used in this system, suiting different needs. In this assay, both fluorometric and colorimetric methods can be used for measurements, the result of the latter can be observed qualitatively, by naked eye, which saves expensive devices in practical usage. In this study, it has been proven that this system can be used for the detection of more than one antigen by just replacing the variable region domains on the fusion protein. Both the easy handling and the versatility of this system make it a very attractive method for environmental and clinical small molecule analysis, as well as that for larger molecules.

Experimental

Materials

Oligonucleotides were synthesized by Eurofins Japan (Tokyo, Japan). KOD-Plus-Neo DNA polymerase was obtained from Toyobo (Osaka, Japan). PCR was performed using a Thermo Cycler T3000 (Biometra, Göttingen, Germany). The *E. coli* strain used for DNA preparation was XL10-Gold (Agilent Technologies, Carlsbad, CA, USA). The strain for protein expression SHuffle T7 Express lysY and the restriction enzymes were obtained from NEB Japan (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemicals (Tokyo, Japan) unless otherwise indicated.

Fluorescence intensity was measured by a fluorescence microplate reader (Genios Pro; Tecan, Männerdorf, Switzerland) using a black 96-well half-area microplate (675077, Greiner Bio-one, Frickenhausen, Germany), whereas optical absorbance was measured by an SH-1100 microplate reader (Corona Electronics, Ibaraki, Japan), using a transparent 96-well microplate (Costar 3590, Corning, NY, USA).

Oligonucleotides

The following nucleotides were used for the construction of plasmids: GUS_NotBack, 5′-ATAAGAATGCGGCCGCTATGTTAC-GTCCTGTAGAAA-3′; GUS_XhoFor, 5′-CCGCTCGAGTAGTCATT-GTTTGCCTCCCTG-3′; MY2KE_Back, 5′-CTGCACTCAAAGG-AGACCGACATGTGGAGTGAAG-3′; MY2KE_For, 5′-CTCCTTT-GAGTGCAGCCCGGCTAACGTATCC-3′, G4Sbottom, 5′-CGTAACA-TAGCGGCCGCGCTACCGCCACCGCCGG-3′; G4S_Top2, 5′-TCCAAGCTTGCGGCCGGTGGATCCGGT-3′; VH(KTM)NcoBack, 5′-ATATGCCATGGATCAAGTAAAGCTGCAGCAGTC-3′; VH_HindFor, 5′-CCCAAGCTTGCTCGAGAGACGGTGACCGT-3′;

Vk(KTM)NcoSalBack, 5'-CATGCCATGGGGTCGACGGACATTG-AGCTCACCCAG-3'; and Vk_HindFor, 5'-CCCAAGCTT-CCGTTTTATTTCCAGCTT-3'.

Plasmid Constructions

The wild-type *E. coli GUS* gene was obtained by PCR using GUS_NotBack and GUS_XhoFor as primers and pCA24N-GUS (National Institute of Genetics, Shizuoka, Japan) as a template. PCR was carried out with 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C), extension (1 min at 68 °C), with 10 ng of template in a 50 μL reaction mixture containing 50 pmol of each primer and 1 U of KOD-Plus-Neo DNA polymerase. All PCRs in this study were carried out under the same conditions. The PCR products were mutated by overlap extension PCR, using the primers MY2KE_Back and MY2KE_For to introduce the M516K and Y517E mutations. They were digested by *Not*I and *Xho*I and inserted into the linearized plasmids pET32-VH(NP)-ECFP and pET32-VL(NP)-GS3-EYFP (HU, unpublished), which carried the genes of the variable region domains of anti-NP antibody and were digested with same restriction enzymes, to yield the pET32-VH(NP)-GUSm and pET32-VL(NP)-GS3-GUSm vectors, respectively. To insert a linker between V_H and GUSm, a 15-mer flexible linker (AAGGSGGGGSGGGGS) gene was obtained by PCR using G4S_Top2 and G4Sbottom as primers and pET32-VL(NP)-GS3-EYFP as a template, digested with *Hin*dIII and *Not*I, and inserted into pET32-VH(NP)-GUSm digested with the same enzymes using Ligation High (Toyobo, Osaka, Japan) to construct pET32-VH(NP)-GS3-GUSm. The same constructs using the wild-type GUS instead of GUSm (pET32-VL(NP)-GS3-GUS and pET32-VH(NP)-GS3-GUS) were also made for the comparison.

Genes for the V region domains of the anti-BGP antibody, V_H(BGP) and V_L(BGP), were obtained by PCR from the pUQ1H(KTM219) [25] and pMAL-VL(BGP)ΔT [26] vectors, using the primers VH(KTM)NcoBack, VH_HindFor, Vk(KTM)NcoSalBack, and Vk_HindFor. The PCR products were digested by *Nco*I and *Hin*dIII and inserted into a linearized pET32-GS3-GUSm plasmid digested with *Nco*I and *Hin*dIII to yield the vectors pET32-VH(BGP)-GUSm and pET32-VL(BGP)-GS3-GUSm, respectively. Afterwards, the open reading frame for VL(BGP)-GS3-GUSm was cloned by PCR, with Vk(KTM)NcoSalBack and GUS_XhoFor as primers, digested by *Nco*I and *Xho*I, and inserted into a linearized pRsetA plasmid, carrying the murine gene of hyperacidic module derived from the amyloid precursor protein (APP) [19, 27], to yield pRsetA-VL(BGP)-GS3-GUSm to improve the solubility of the VL(BGP)-GUSm fusion protein.

Protein Expression and Purification

For the preparation of proteins, SHuffle T7 Express lysY cells transformed with the expression vector and grown at 30 °C in LB medium containing 100 μg/mL ampicillin were used. The protein expression was induced by adding isopropyl-thio-β-galactopyranoside to a final concentration of 0.5 mM at a cell density of A_{600} = 0.5-0.6. The cells were cultured for 16 h at 16 °C. Talon IMAC resin (Clontech, Takara-Bio) was added to the cellular extracts, which were then washed by Talon washing buffer (Talon buffer supplemented with 0.34 g/L imidazole) and eluted by Talon elution buffer (Talon buffer supplemented with 3.4 g/L imidazole). To improve purity, anion exchange chromatography (Enrich™ Q; Bio-Rad, Hercules, CA, USA) was performed to purify the Trx-V_L(NP)-GUSm protein. The expression and purification processes were confirmed by SDS-PAGE.

Time Course Measurement

Because the inherent colour of NP and NIP (5-iodo-NP) solutions interferes with colorimetric measurement, fluorescence measurement was performed for the evaluation of the NP system. After mixing 0.1 μM each of Trx-V_H(NP)-GUSm and Trx-V_L(NP)-GUSm proteins with PBST (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween20; pH 7.4), 1 μM antigen and 0.3 mg/mL 4-methylumbelliferyl-β-D-glucuronide in PBST were added, and the solution was incubated for 5 min at room temperature. Afterwards, fluorescence intensity was measured every 2 min. Three samples for each group were taken and 100 μL of each sample was added into plate wells. The fluorescent intensity was obtained from triplicate samples from three independent experiments at the excitation wavelength of 340 nm and emission wavelength of 480 nm. Same measurement was carried out for the BGP detection system, i.e. Trx-V_H(BGP)-GUSm and APP-V_L(BGP)-GUSm, 0.1 μM each in PBST buffer. As the antigen solution is colourless and thus does not interfere with colorimetric measurement, a colorimetric substrate (p-nitrophenyl-β-D-glucuronide, 0.3 mg/mL in PBST; same conditions as for other measurements) was used for the evaluation of BGP detection system. The BGP-C7 peptide (NH_2-RRFYGPV-COOH; molecular weight, 893) was synthesized by Lifetein (Somerset, NJ, USA) and diluted to 10 μM in PBST as an analyte. Optical absorbance was measured at 405 nm, with 650 nm measurement as a control.

Antigen Dose-dependency Measurement

Antigens (NP and NIP) were prepared in gradient concentrations (0 to 100 μM in PBST) and mixed with the Trx-V_H(NP)-GUSm and Trx-V_L(NP)-GUSm proteins (0.1 μM in PBST) and the fluorescent substrate, and the fluorescence intensity was measured. For the BGP detection system, BGP-C7 was prepared in gradient concentrations (0 to 100 μM in PBST) and mixed with the Trx-V_H(NP)-GUSm and Trx-V_L(NP)-GUSm proteins (0.1 μM in PBST) and the fluorescent and colorimetric substrates. Both fluorescence intensity and optical absorbance were measured.

Conflicts of interest

There are no conflicts to declare.

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