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Analytical Biochemistry 360 (2007) 266–272
ANALYTICAL BIOCHEMISTRY
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Enhanced fluorescence resonance energy transfer immunoassay with improved sensitivity based on the Fab′-based immunoconjugates

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Received 7 August 2006
Available online 7 November 2006

Abstract

Fluorescence resonance energy transfer (FRET) is a powerful technique to monitor protein–protein interaction. Recently, we developed homogeneous and noncompetitive immunoassay based on the enhanced FRET by leucine zipper interaction. Here we improved the assay by establishing a general method for preparation of the Fab′-based immunoconjugate. Anti-human serum albumin Fab′ numbers 11 and 13 were chemically conjugated with recombinant proteins consisting of thioredoxin, flexible linker, and green fluorescent protein color variant tethered with a leucine zipper motif. Compared with single chain antibody variable region-based fusion proteins prepared by the gene fusion method in our previous study, the resultant Fab′-based immunoconjugates accomplished an assay with nearly 10 times greater sensitivity. Furthermore, the conjugation method enabled us to apply the assay generally to measurement of another high-molecular weight antigen for which antibodies prepared for sandwich immunoassay are commercially available. Because of the facility and generality of the preparation method for the immunoconjugate, the assay is expected to be applied to many antigens that require rapid diagnosis and moderate measurement range.

Keywords: Fluorescence resonance energy transfer; Homogeneous immunoassay; Immunoconjugate; Protein–protein interaction; Green fluorescent protein; Antibody

Fluorescence resonance energy transfer (FRET)[1] is a process by which a donor fluorophore in an excited state may nonradiatively transfer its excitation energy to a neighboring acceptor fluorophore [1]. Because of the simple operation under homogeneous conditions, it has been used as a powerful tool for investigating a number of molecular interactions. In particular, the isolation of green fluorescent protein (GFP) [2,3] and the development of its color variants have enabled us to monitor protein–protein interaction [4,5]. Although FRET based on GFP color variants (GFP-based FRET) is a useful technique to monitor the interaction between the proteins of interest fused with GFP variants, it is not always guaranteed to work. For example, in some cases, two fusion proteins might no longer interact with each other due to steric hindrance accompanied by the fusion with GFP variants. In other cases, sufficient energy transfer might not occur because the proximity of two GFP variants within the complex is not ensured by N- or C-terminal fusion of them. Due to these drawbacks, it has been difficult to apply GFP-based FRET to sandwich immunoassay.

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[1] *Abbreviations used:* FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; Fv, antibody variable region; ScFv, single chain Fv; FL4, flexible linker with the amino acid sequence $(GGGGS)_4$; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; HSA, human serum albumin; Trx, thioredoxin; GST, glutathione-*S*-transferase; ERK2, extracellular signal-regulated kinase 2; pERK2, phospho-extracellular signal-regulated kinase 2; MAPK, mitogen-activated protein kinase; LzJun, c-Jun leucine zipper motif; LzFos, FosB leucine zipper motif; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; $BM(PEO)_4$, 1,11-bismaleimidotetraethyleneglycol; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; Ab, antibody.

0003-2697/$ - see front matter © 2006 Published by Elsevier Inc.
doi:10.1016/j.ab.2006.10.025

Recently, we developed a sandwich immunoassay that could address these problems in an optimal way [6]. This assay (enhanced FRET immunoassay) employs two designed molecular probes. One is composed of a single chain antibody variable region fragment (ScFv1) that binds to an epitope of the cognate antigen, a flexible linker (FL4), and a GFP color variant enhanced cyan fluorescent protein (ECFP) with a C-terminally tethered leucine zipper motif, whereas the other is composed of another antibody fragment (ScFv2) that binds to another epitope of the antigen, a flexible linker, and another GFP color variant enhanced yellow fluorescent protein (EYFP) tethered with the same, or a different, leucine zipper motif. In the presence of antigen, these two probes bind to the antigen separately, and then the leucine zipper motifs help these two molecular probes to dimerize with each other due to their increased local concentration. On this leucine zipper interaction, ECFP (donor) and EYFP (acceptor) are located within closer proximity; hence, the FRET can be induced (Fig. 1). Using this assay, we could easily measure human serum albumin (HSA) ranging from 50 to 1000 nM.

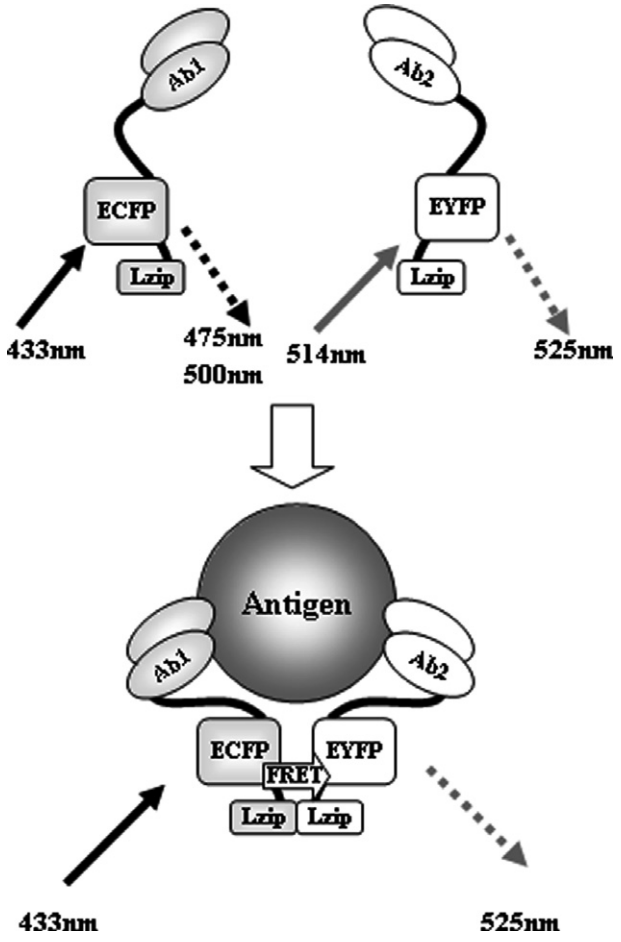

Fig. 1. Principle of the assay. Without antigen, the two probe proteins remain monomeric, and thus FRET between them is negligible. The addition of antigen induces antigen antibody reactions and subsequent leucine zipper dimerization, accompanied by the FRET from ECFP to EYFP.

In spite of the simplicity of the enhanced FRET immunoassay, several obstacles were waiting for the applications of the method to any other antigens. In the previous study, we prepared the molecular probes as two genetically fused chimeric proteins composed of essentially a ScFv and a GFP-based fluorescent probe tethered by a flexible linker simply because the assay required two molecular probes. However, the use of ScFv fusion protein needed to be reconsidered for two reasons. First, sometimes it is difficult to prepare large amounts of ScFv chimeric proteins by using the *Escherichia coli* expression system. In general, the *E. coli* expression system is not optimal to prepare the proteins that require the formation of disulfide bonds for the activity because its cytoplasm is too reductive to form the disulfide bond. Therefore, some strains with more oxidative cytoplasm have been employed to prepare these proteins inside the cell, leading to some successes [7–9]. However, in our case, the yields of chimeric proteins sometimes were very limited even if we employed *E. coli* strains lacking the thioredoxin reductase gene (*trxB*) and the glutathione reductase gene (*gor*) as hosts. Another option of using the secretion expression system also failed to produce large amounts of ScFv-GFP fusion proteins (fluobody) [10,11]. Second, the binding activity of ScFv often is weaker than that of parent antibody because it has no constant domains that contribute to the stability. In fact, Biacore analysis indicated that the affinities of both ScFvs to HSA were approximately 1/100 those of parent IgG. Therefore, we reasoned that further improvement in sensitivity might be possible by using antibody fragment with higher binding activity such as IgG or Fab′.

To improve on these points, in this article we describe a general method to prepare immunoconjugates suitable for enhanced FRET immunoassay that employs antibody fragments Fab′. In addition, further improvement in sensitivity for the assay and an application to another antigen using similar immunoconjugates are demonstrated.

Materials and methods

Apparatus

Fluorescence spectral measurement was performed using the RF-5300PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) with a quartz crystal cuvette.

Antigens

HSA was purchased from Sigma. Glutathione-*S*-transferase (GST)-fused mouse extracellular signal-regulated kinase 2 (ERK2) and GST-fused mouse phospho-ERK2 (pERK2) were purchased from Upstate Biotechnology.

Antibodies

Anti-HSA mouse monoclonal antibodies numbers 11 and 13 were produced previously at our laboratory. Anti-diph-

osphorylated mitogen-activated protein kinase (MAPK) mouse monoclonal antibody, clone MAPK-YT, was purchased from Sigma. Anti-GST goat polyclonal antibody was purchased from Amersham Biosciences.

Plasmid construction

Three expression vectors were prepared from the plasmids—pET32/ScFv11-FL4-ECFP-LzJun (p11CJ), pET32/ScFv11-FL4-ECFP-LzFos (p11CF), and pET32/ScFv13-FL4-EYFP-LzJun (p13YJ)—that were constructed in our previous experiment [6]. To delete ScFv fragments, these plasmids were doubly digested with *Eco*RV and *Hin*dIII. After agarose gel electrophoresis, digested plasmid fragment was extracted from agarose gel and purified by using EASYTRAP (version 2, TaKaRa Bio, Shiga, Japan). Purified vector fragment was then ligated via a *Hin*dIII linker (TaKaRa Bio). Consequently, three expression plasmids—pET32/FL4-ECFP-LzJun, pET32/FL4-ECFP-LzFos, and pET32/FL4-EYFP-LzJun—were prepared.

Expression and purification of recombinant protein

For the expression of recombinant proteins, *E. coli* Origami (λDE3) (Novagen, Tokyo, Japan) and pET TRX Fusion System 32 (Novagen), a fusion expression system with *E. coli* thioredoxin (*trxA*), were employed. The cells were transformed with each of three expression plasmids and were selected on LB agar plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar) containing 50 μg/ml ampicillin, 12.5 μg/ml tetracycline, and 15 μg/ml kanamycin. For all of the cultivations thereafter, LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl at pH 7.0) containing 50 μg/ml ampicillin, 12.5 μg/ml tetracycline, and 15 μg/ml kanamycin was used. The medium (1.5 L) was inoculated with 5 ml overnight culture at 30 °C of each strain and was cultured at 30 °C for 7 h. At OD_{600} of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM to induce the expression of the fusion proteins, and the cells were further cultured for 12 h at 16 °C. Harvested cells were resuspended in the lysis buffer (50 mM NaH_2PO_4–NaOH, 300 mM NaCl, and 10 mM imidazole at pH 8.0) and were disrupted by freeze–thaw and sonication. The three cell lysates were obtained by centrifugation at 20 kg for 20 min at 4 °C. Purifications of expressed proteins were performed by His-tag affinity chromatography and anion exchange chromatography. At first, the cell lysates were applied to a 2-ml column of Ni-NTA agarose (Qiagen, Tokyo, Japan). The column was washed with 20 bed volumes of wash buffer (50 mM NaH_2PO_4–NaOH, 300 mM NaCl, and 20 mM imidazole at pH 8.0), and then the bound materials were eluted with 5 volumes of elution buffer (50 mM NaH_2PO_4–NaOH, 300 mM NaCl, and 250 mM imidazole at pH 8.0). For the sequential anion exchange chromatography, the fractions with sufficient fluorescence activity were dialyzed against anion exchanger binding buffer (20 mM Tris–HCl [pH 8.0] for Trx-FL4-ECFP-LzipJun and Trx-FL4-EYFP-LzipJun, 20 mM Tris–HCl [pH 9.0] for Trx-FL4-ECFP-LzipFos). The dialysates were applied to a MonoQ column (Amersham Biosciences, Tokyo, Japan). The column was washed with 20 bed volumes of binding buffer, and then the bound materials were eluted with linear gradient of NaCl. The fractions with sufficient fluorescence activity were collected and dialyzed against phosphate-buffered saline (PBS, 1.48 g/L Na_2HPO_4, 0.43 g/L KH_2PO_4, and 7.2 g/L NaCl at pH 7.2) and stored at −80 °C until use.

Quantification of thiol residues

The number of thiol residues was determined by the method of Hamaguchi and coworkers [12]. For the quantification of thiol residues, 20 μl of the recombinant protein solution was incubated with 480 μl of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.2 mM 4,4′-dithiodipyridine (Sigma, Athens, GA, USA) at room temperature for 20 min. After incubation, the increase in absorbance at 324 nm was measured using the same buffer as a control. The number of thiol residues was calculated using a molar extinction coefficient of 19,800 (cm^{-1} M^{-1}).

Preparation of antibody conjugates

$F(ab')_2$ fragment of anti-HSA antibody, anti-diphosphorylated MAPK antibody, and anti-GST antibody were prepared by the method of Kato and coworkers [13]. Approximately 3 mg of each antibody IgG was incubated with 60 μg of pepsin (Sigma) at 37 °C for 16 h in 1.5 ml of sodium acetate buffer (pH 4.5). After incubation, the pH of the reaction mixture was adjusted to pH 8.0 with 1 N NaOH. The digested $F(ab')_2$ fragment was purified by gel filtration chromatography using Superdex 200 (16/60) column (Amersham Biosciences), equilibrated with PBS (1.48 g/L Na_2HPO_4, 0.43 g/L KH_2PO_4, and 7.2 g/L NaCl at pH 7.2), and concentrated by ultrafiltration. Then 300 μl of 30 μM $F(ab')_2$ solution was incubated with 30 μl of 200 mM β-mercaptoethanol at 37 °C for 1.5 h. Fab′ fragment was separated from β-mercaptoethanol on a Sephadex G25 column, PD-10, equilibrated with sodium phosphate buffer (pH 7.0) containing 10 mM ethylenediaminetetraacetic acid (EDTA). Then 200 μl of 60 μM Fab′ was incubated with 20 μl of 60 mM 1,11-bismaleimidotetraethyleneglycol ($BM(PEO)_4$, Pierce, Rockford, IL, USA) at 30 °C for 1 h. After incubation, Fab′–maleimide was separated from unreacted $BM(PEO)_4$ on a Sephadex G25 column in a manner similar to that described above and was concentrated by ultrafiltration. On the other hand, 300 μl of 100 μM recombinant protein that would be conjugated with antibody fragment was incubated with 30 μl of 200 mM dithiothreitol (DTT) at room temperature for 16 h. After incubation, reduced recombinant protein was separated from DTT on a Sephadex G25 column in a manner similar to that described above and was concentrated by ultrafiltration. To couple antibody fragment to recombi-

nant protein, 200 μl of 50 μM Fab′–maleimide and 200 μl of 50 μM reduced recombinant protein were mixed and incubated at 30 °C for 1 h. To block unreacted free sulfhydryl group, *N*-ethyl maleimide was added at a final concentration of 250 μM. Antibody conjugate was purified by gel filtration chromatography using Superdex 200 (16/60) column (Amersham Biosciences), equilibrated with PBS (1.48 g/L Na_2HPO_4, 0.43 g/L KH_2PO_4, and 7.2 g/L NaCl at pH 7.2). To estimate the molecular weight of the immunoconjugate, we used the gel filtration standard (Bio-Rad, Tokyo, Japan) as the standard. Purified antibody conjugate was concentrated by ultrafiltration to a final concentration of 20 μM.

FRET assay for HSA

The FRET response of each antibody conjugate pair after the addition of antigen was evaluated as follows. The mixtures of two antibody conjugates, anti-HSA number 11 Fab′-Trx-FL4-ECFP-LzJun and anti-HSA number 13 Fab′-Trx-FL4-EYFP-LzJun, were prepared at a concentration of 20 nM in the assay buffer (50 mM Tris–HCl, 50 mM NaCl, and 0.1% gelatin at pH 8.0). Antigen was serially diluted to vary concentrations in the assay buffer, and 20 μl of diluted antigen solution was added to 280 μl of each antibody conjugate mix. After 15 min incubation at 37 °C, the emission spectra ranging from 450 to 600 nm were measured with 433 nm excitation at room temperature. The fluorescence emission ratio I(525 nm)/I(475 nm) was calculated to evaluate FRET efficiency.

FRET assay for pERK2

The mixtures of two antibody conjugates, anti-diphosphorylated MAPK Fab′-Trx-FL4-ECFP-LzFos and anti-GST Fab′-Trx-FL4-EYFP-LzJun, were prepared at a concentration of 40 nM in the assay buffer (50 mM Tris–HCl, 50 mM NaCl, and 0.1% gelatin at pH 8.0). Antigen was serially diluted to vary concentrations in the assay buffer, and 10 μl of diluted antigen solution was added to 290 μl of each antibody conjugate mix. After 15 min incubation at 37 °C, the emission spectra ranging from 450 to 600 nm were measured with 433 nm excitation at room temperature.

Results and discussion

The scheme for the preparation of immunoconjugate is shown in Fig. 2. We prepared a recombinant protein composed of a flexible linker (FL4) and GFP color variant

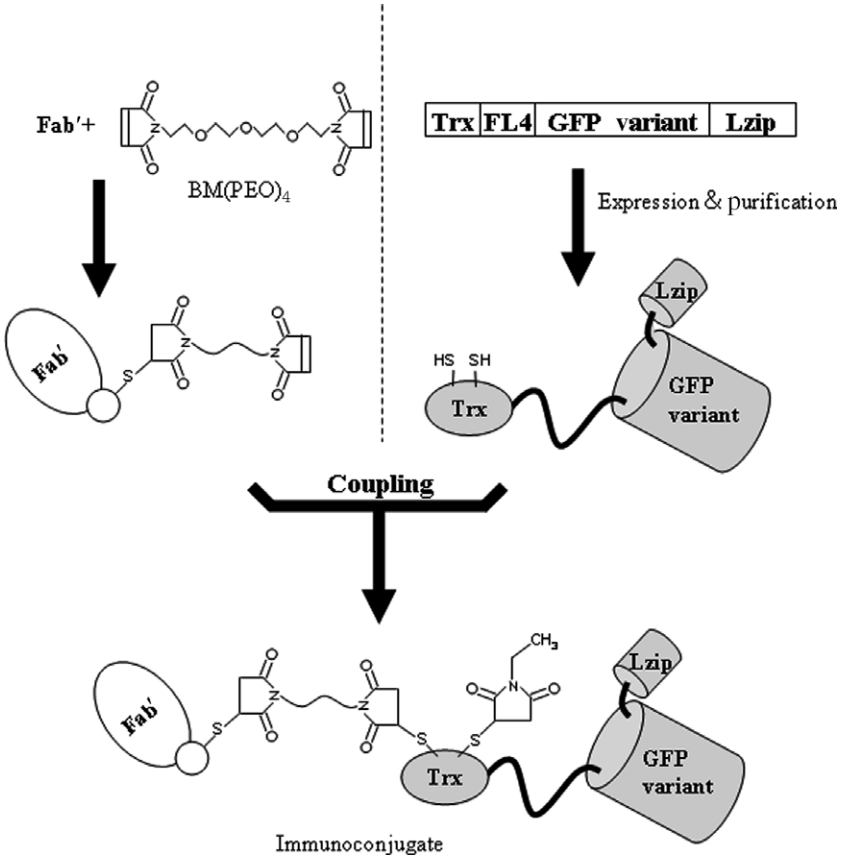

Fig. 2. Scheme of the preparation of immunoconjugates. F(ab′)$_2$ fragment prepared by pepsin digestion of IgG was reduced and treated with homofunctional maleimide reagent. On the other hand, recombinant protein composed of thioredoxin (Trx), flexible linker (FL4), GFP variant (ECFP or EYFP), and leucine zipper (Lzip) was prepared. To conjugate Fab′ to the recombinant protein, Fab′–maleimide and reduced recombinant protein were mixed. Unreacted thiol residues were blocked by *N*-ethylmaleimide.

tethered with a C-terminal leucine zipper motif as a thioredoxin fusion protein by using pET TRX Fusion System 32. The thioredoxin tag was placed at the N-terminal of the fusion protein not only for increasing the solubility of the fusion protein but also for providing thiol residues that would be employed for the coupling to an antibody fragment [14]. These recombinant proteins were expressed successfully in *E. coli* Origami (λDE3) cytoplasm and were purified up to a single band by SDS–PAGE analysis after metal affinity chromatography and subsequent anion exchange chromatography. Finally, we could obtain a 5- to 10-mg amount of each protein from 1 L of *E. coli* culture. Compared with the ScFv fusion proteins in our previous study, the yields of the recombinant fluorescent proteins were increased dramatically, indicating that the ScFv significantly lowered the yields of the fusion proteins in *E. coli* cytoplasm. Using these recombinant proteins, we attempted to prepare immunoconjugates. When the recombinant protein is coupled to Fab′ as shown in Fig. 2, one of two cysteine residues in GFP variant is thought to affect the coupling reaction given that the crystallographic analysis showed that one of them might be available for the thiol coupling reaction, whereas the other was buried inside of the protein [15]. Although another study revealed that neither of them could react with maleimide derivative [16], we initially quantified the number of reactive thiol residues in the recombinant Trx-GFP variant fusion protein before and after reduction. As a result, we confirmed the existence of roughly two thiol residues in the recombinant protein after reduction with DTT, whereas there was no measurable reactive thiols before reduction. These results indicate that one cysteine residue near the molecular surface of GFP variant is not detectable because it is not exposed to the surface to react with thiol reactive reagent. Therefore, we consider that we can employ the two cysteine residues in the active site of thioredoxin for the conjugation after reduction because those in GFP variant will not interfere with the conjugation. Accordingly, we performed the coupling reaction by using anti-HSA Fab′ (no. 13 Fab′) and the recombinant protein (Trx-FL4-EYFP-LzipJun). The gel filtration profile of the coupling mixture is shown in Fig. 3. We could detect three peaks in the gel filtration profile. The estimated molecular weight of peak A to be 200 kDa (Fig. 4). When a designed immunoconjugate, which is composed of Fab′ (46 kDa) and the recombinant protein (51 kDa) in a 1:1 ratio, is dimerized by leucine zipper interaction, the expected molecular weight is approximately 200 kDa, corresponding to the molecular weight of peak A. Therefore, we judged peak A to be the conjugation product. Moreover, from the absorbances at 280 and 514 nm, we could calculate the conjugation ratio of the recombinant protein to Fab′ as approximately 0.8, indicating that the designed immunoconjugate (anti-HSA no. 13 Fab′-Trx-FL4-EYFP-LzJun) was produced. We also prepared the immunoconjugate of number 11 Fab′ and Trx-FL4-ECFP-LzipJun. The resultant immunoconjugate (anti-HSA no. 11 Fab′-Trx-FL4-ECFP-LzJun) also showed similar gel filtration profile and the conjugation ratio.

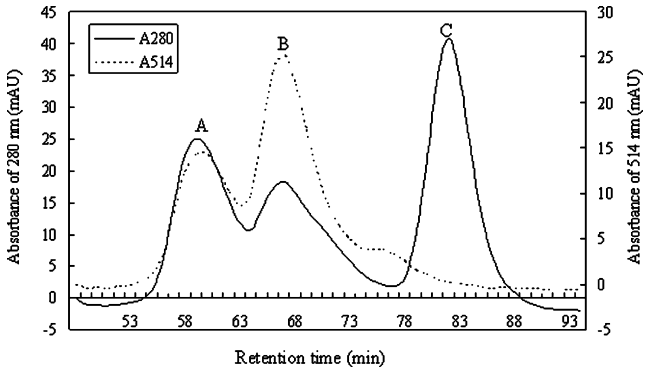

Fig. 3. Elution profile of the immunoconjugate (Fab′ no. 13 EYFP-LzipJun) from a column of Superdex 200. Peak A, immunoconjugate; peak B, unreacted recombinant protein; peak C, unreacted Fab′ fragment. Solid line indicates absorbance at 280 nm. Broken line indicates absorbance at 514 nm.

Using these immunoconjugates, we performed FRET immunoassay for HSA. Fig. 5 shows the HSA concentration-dependent change in the emission spectra of the mixtures. As the HSA concentration increased, a decrease in emission at 475 nm (ECFP emission maximum) and an increase in emission at 525 nm (EYFP emission maximum) were observed. Moreover, on the addition of excess HSA solution, the change in fluorescence emission spectrum decreased (data not shown). These phenomena were quite similar to our previous observation using ScFv chimeric proteins, indicating that the coexisting antigen induced successful FRET between the immunoconjugates. Fig. 6 shows the relationship between

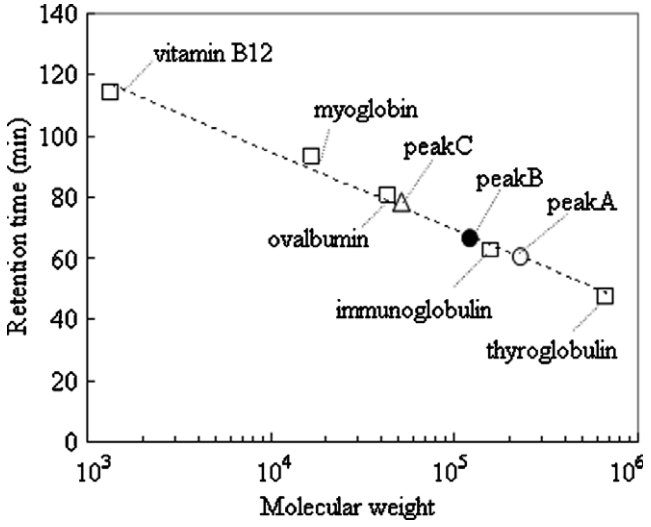

Fig. 4. Size estimation of the immunoconjugate. Peak A was eluted at a position corresponding to 200 kDa, reflecting dimerization of the immunoconjugate. Peak B was eluted at a position corresponding to 100 kDa, reflecting dimerization of the recombinant protein. Peak C was eluted at a position corresponding to 50 kDa, reflecting the Fab′ fragment. Size markers used were as follows: thyroglobulin, 670 kDa; immunoglobulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; and vitamin B12, 1.4 kDa.

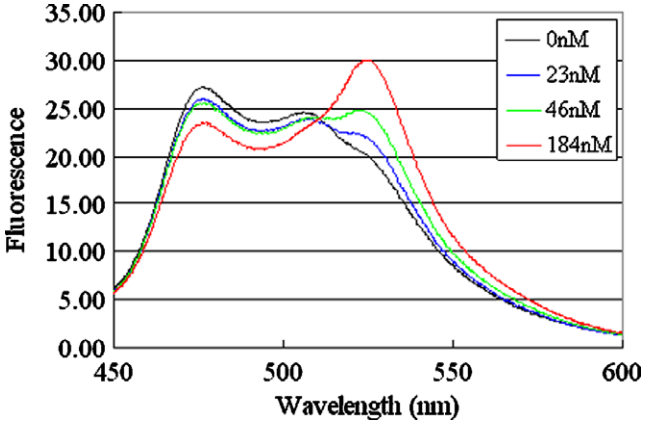

Fig. 5. Change in fluorescence spectra by FRET due to the addition of HSA. Shown are fluorescence spectra 15 min after the addition of 0-, 23-, 46-, and 184-nM HSA solutions to the immunoconjugate mixtures. (For interpretation of the references to color in the legend at the top right of the figure, the reader is referred to the Web version of this article.)

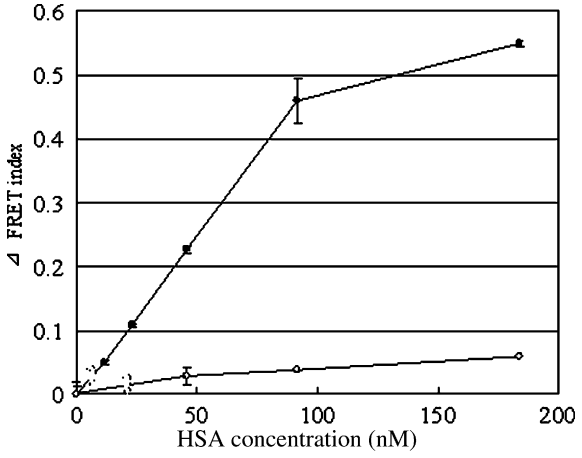

Fig. 6. Dose–response curves for HSA using Fab′ immunoconjugates (●) and ScFv fusion proteins (○) [6]. Data are averages ±1 standard deviation ($n = 3$).

the antigen concentration and the increase in fluorescence ratio I(525 nm)/I(475 nm) as a FRET index. The increase in fluorescence ratio due to FRET was detected on addition of the antigen solution in a range from 11.5 to 183.8 nM. Although the dynamic range of the assay was narrower than that of the ScFv-based FRET assay, the slope in the dose–response curve was shifted leftward to nearly one tenth HSA concentration range compared with that of our previous study. Because the equilibrium dissociation constants of these anti-HSA antibodies, numbers 11 and 13, were measured to be approximately 10^{-10} M (data not shown), the measurable range from 0.8 to 12.3 nM as the final concentration was reasonable.

To prove the generality of the assay as well as the conjugation method, we prepared another pair of immunoconjugates employing commercially available antibodies for diphosphorylated MAPK and GST, and then applied them to the FRET immunoassay. Similar to the case of HSA, we could successfully prepare the two kinds of immunoconjugates: anti-diphosphorylated MAPK Fab′-Trx-FL4-ECFP-LzFos and anti-GST Fab′-Trx-FL4-EYFP-LzJun. Then we performed the FRET immunoassay for GST-fused phosphorylated MAPK (GST-pERK2) using these immunoconjugates. Fig. 7 shows antigen concentration-dependent change in the normalized emission spectra of the immunoconjugate mixtures. As the GST-pERK2 concentration increased, a decrease in emission at 475 nm and an increase in emission at 525 nm were observed. When we also added the same amounts of nonphosphorylated GST-MAPK (GST-ERK2) instead of GST-pERK2 to the mixture of the immunoconjugates, no change in fluorescence emission spectra was detected. This result also indicates that the coexisting antigen induced successful FRET between the immunoconjugates.

By establishing a method for preparation of the Fab′-based immunoconjugate, we showed two improvements in enhanced FRET immunoassay. First, the conjugation method described here enabled a facile preparation of immunoconjugate for many antigens. When we develop a practical immunoassay method, we should establish not only assay principle but also a feasible method for the preparation of immunoconjugate. Due to general difficulty in preparing a large amount of soluble active ScFv by using the *E. coli* expression system, the refolding method from the inclusion body has been reported to be very useful [17]. In our previous study, we attempted to refold fully active ScFv fusion proteins because of the low yield from the soluble fractions. However, the examination of the refolding condition was rather complicated because of the different character of each protein domain of fusion protein. Compared with these difficulties, the conjugation method described here is much simpler because investigating the conjugation condition for each antibody is not necessary. Although the conjugation method is easy, currently it has a drawback in that the yield of immunoconjugate is approximately 30% that of the source materials (Fig. 3). We

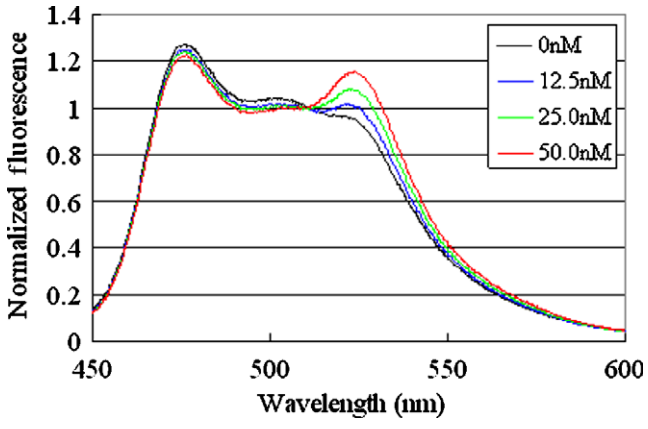

Fig. 7. Change in fluorescence spectra by FRET due to the addition of GST-pERK2. Shown are normalized fluorescence spectra 15 min after the addition of GST-pERK2 solutions to the immunoconjugate mixtures. Antigen concentrations indicated are final concentrations. These data of fluorescence intensity are normalized at 510 nm. (For interpretation of the references to color in the legend at the top right of the figure, the reader is referred to the Web version of this article.)

consider that one reason for the low yield of the immunoconjugate is the number of reactive thiol residues at the thioredoxin moiety of the recombinant protein. In the recombinant protein, the reduced disulfide bond at the thioredoxin domain is considered to have a tendency to be reoxidized. Therefore, we suspect that the yield of the immunoconjugate can be increased by solving this problem.

Second, we accomplished leftward shift in the slope of the dose–response curve with the use of Fab′. In the HSA assay, the slope of the dose–response curve of the Fab′-based immunoconjugate was shifted to nearly a 10 times lower concentration range than that of the ScFv-based one. The improvement in the reactivity is considered to be attributable to the higher affinity/stability of the Fab′. In general, the binding activities of whole IgG or Fab′ are superior to those of ScFv [18]. Therefore, establishment of the method for preparation of the Fab′-based immunoconjugate means that we can perform enhanced FRET immunoassay with higher sensitivity than ever.

As described here, we could improve enhanced FRET immunoassay by using Fab′-based immunoconjugates. However, there might be room for improvement in the FRET efficiency, albeit aided by the leucine zipper interaction. The maximum FRET efficiency calculated from the donor quenching at saturation was approximately 15% (Fig. 5). In antigen–antibody complex, the donor and acceptor GFP variants could be placed as close as the Förster distance R_0 between ECFP and EYFP (~50 Å) [3] because two GFP variants were tethered with leucine zipper motif via short polypeptide linker composed of Gly-Gly. Taking these molecular designs into consideration, the actual FRET efficiency attained was lower than expected. We suspect that the insufficient FRET efficiency can be attributed to the orientation of the FRET pair juxtaposed by leucine zipper interaction because FRET efficiency depends not only on the distance between FRET pairs but also on the orientation of them. Taking these into consideration, further enhancement of the FRET efficiency might be possible by optimizing the polypeptide linker tethering the leucine zipper motif to GFP variant.

Although the improved sensitivity is lower than that of conventional heterogeneous immunoassay, including enzyme-linked immunosorbent assay (ELISA), the enhanced FRET immunoassay has great potential because it is a homogeneous assay requiring merely fluorescence measurement after mixing of the antigen and two immunoconjugates. Because of the generality of the assay principle and the preparation method for the immunoconjugate, the assay is expected to be applied to many antigens that require rapid diagnosis and moderate but wide measurement range.

Acknowledgment

We are thankful for the financial support from the New Energy and Industrial Technology Development Organization (NEDO) of Japan for this work.

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