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Novel biosensor system model based on fluorescence quenching by a fluorescent streptavidin and carbazole-labeled biotin

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In the present study, a novel molecular biosensor system model was designed by using a couple of the fluorescent unnatural mutant streptavidin and the carbazole-labeled biotin. BODIPY-FL-aminophenylalanine (BFLAF), a fluorescent unnatural amino acid was position-specifically incorporated into Trp120 position of streptavidin by four-base codon method. On the other hand, carbazole-labeled biotin was synthesized as a quencher for the fluorescent Trp120BFLAF mutant streptavidin. The fluorescence of fluorescent Trp120BFLAF mutant streptavidin was decreased as we expected when carbazole-labeled biotin was added into the mutant streptavidin solution. Furthermore, the fluorescence decrease of Trp120BFLAF mutant streptavidin with carbazole-labeled biotin (100 nM) was recovered by the competitive addition of natural biotin. This result demonstrated that by measuring the fluorescence quenching and recovery, a couple of the fluorescent Trp120BFLAF mutant streptavidin and the carbazole-labeled biotin were successfully applicable for quantification of free biotin as a molecular biosensor system. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: molecular biosensor; four-base codon method; fluorescent unnatural mutant streptavidin; carbazole-labeled biotin; fluorescence quenching; fluorescence recovery

INTRODUCTION

Biosensor is a sensor that is based on the use of biological material for its sensing function. Biosensor contains two parts — bioreceptor and transducer. The bioreceptor specifically reacts or interacts with the analyte of interest, resulting in a detectable chemical or physical change by transducer. Molecular biosensor is the part of bioreceptor and transducer integrated in one biological molecular (Hardinge and Crooks, 1960; Hoppner and Lampi, 1992; Zempleni and Mock, 1999).

For the design of bioreceptor molecules, antibodies, enzymes, and binding proteins that chemically modified with one or more functional molecular were usefully applied as a biorecognition component because of their high affinities, highly efficient catalytic activity, and high specificity. Moreover, chemical modification is a general method for providing a new function as fluorescence, oxidation-reduction, and so forth to the biological components. However, the chemical technique suffers the disadvantages in that the chemical modification is not sufficiently position-specific and quantitative. Quantitative modification may not be accomplished even in the case of a highly selective modification of cysteine residue with maleimide derivatives (Pacheco-Alvarez *et al*., 2002; Staggs *et al*., 2004).

In contrast, the unnatural amino acid mutagenesis method using amber and four-base codons is an excellent method which can achieve site-directed introduction of the functional unnatural amino acid that possessed fluorescent or redox-active properties to the designed protein to provide a new function for the molecular biosensor (Cavalcanti *et al*., 2008; Fan *et al*., 2008; Witzens and Hochberg, 2011). In the four-base codon method (Cornish *et al*., 1995; Hohsaka *et al*., 1996; Murakami *et al*., 1998; Hohsaka and Sisido, 2002; Beene *et al*., 2003; Saxl *et al*., 2009; Albani, 2010), one or multiple four-base codons were introduced into an assigned position of the target protein gene. The full-length protein containing the unnatural amino acid will be produced when the four-base codon is successfully decoded by the unnatural aminoacyl-tRNA having the corresponding four-base anticodon, but when the first three bases of the four-base codon are decoded as a three-base codon by a cognate naturally occurring aminoacyl-tRNA, a frame shift occurs to cause the emergence of a stop codon, resulting in the termination of peptide elongation. Therefore, the full-length protein is obtained at only when the four bases are successfully decoded as a single

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codon. For identification and purification of the protein synthesized by four-base method, T7-tag and His-tag were added at the N terminus and C terminus, respectively, which allowed identifying the translated proteins by Western blot analysis and purifying by the full-length of proteins with the His-tag purification system in general.

In this study, a new molecular biosensor model that based on the fluorescence quenching of the couple of genetically engineered fluorescent mutant binding protein (antibodies) and quencher-labeled ligand analog (antigens) was designed. The pair of streptavidin and biotin was used as a model in this study because of the extremely high biotin dissociation constant of streptavdin. For this study, a fluorescent unnatural amino acid, BODIPY-FL-aminophenylalanine (BFLAF) (Figure 1(a)) (Hohsaka *et al*., 1996), which was prepared by chemical modification of aminophenylalanine with the BODIPY-FL®, was incorporated at the Tyr54, Tyr83, and Trp120 positions of a streptavidin to synthesize three different monoclonal fluorescent mutant streptavidin by using four-base codon CGGG. On the other hand, carbazole was modified to the carboxyl terminal of biotin as a quencher for BFLAF. The nearest Trp120 of the neighbor subunit in streptavidin tetramer is placed within 15 Å from the carboxyl terminal of biotin (Figure 1(b)) (Hohsaka *et al*., 1999). So, the Trp120BFLAF mutant streptavidin was certainly quenched by carbazole-labeled biotin (Figure 1(c)) when the mutant streptavidin-biotin complex was formed. Whereas in the presence of free biotin existing in the assay solution, the Trp120BFLAF would not be quenched by carbazole-labeled biotin because of the competitive binding reactions of natural biotin and carbazole-labeled biotin to the mutant streptavidin. In this study, the carbazole was chosen not only because of its quenching ability but also from the molecular structure of carbazole. It was slightly larger, so carbazole-labeled biotin was difficult to bind to the streptavidin than nature biotin because of the impairment of carbazole. By this competitive binding assay, free biotin was determined in the concentration range from 20 to 100 nM. Although a molecular biosensor for biotin was designed here, it is more important that the pair of Trp120BFLAF mutant streptavidin and carbazole-labeled biotin should be considered as a molecular biosensor model, which demonstrated the potential of biosensor design based on the couple of genetically engineered fluorescent mutant binding protein and quencher-labeled ligand analog.

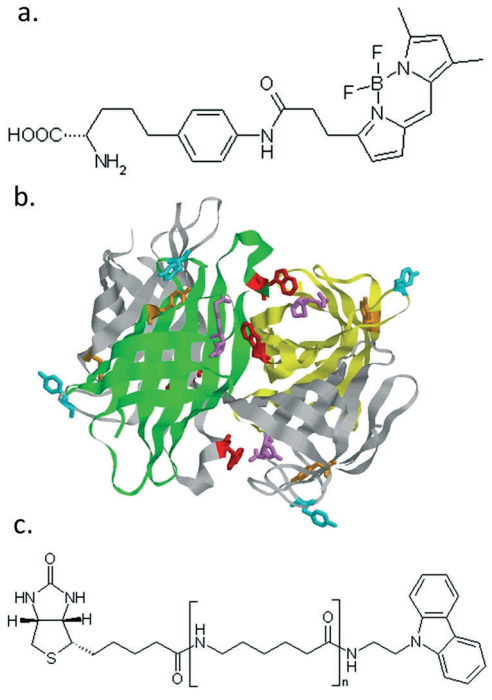

Figure 1. (a) The structure of BODIPY-FL-aminophenylalanine (BFLAF). $\lambda ex^{max} = 505$ nm $\lambda em^{max} = 514$ nm. (b) The homo tetrameric constitution of a natural streptavidin (PDB ID: 1SWE); subunit A was colored with green, and subunit D was colored with yellow; for easy to see, both subunits B and C were colored with white. Tyr54 is colored with orange, Tyr83 is colored with blue, Trp120 is colored with red, and biotin is colored with pink. As shown in Figure 1(a), the biotin is close to the Trp120 of the neighbor subunit in the streptavidin-biotin complex. (c) The structure of carbazole-labeled biotin ($n = 0$: carbazole-labeled biotin, $n = 1$: carbazole-LC-biotin, $n = 2$: carbazole-LC-LC-biotin).

MATERIALS AND METHODS

Materials

The DNAs of wild type and Tyr54, Tyr83, and Trp120 mutant streptavidins (Albani, 2010), DNAs of the tRNA that have a four-base anticodon (Cornish *et al*., 1995) and BFLAF-pdCpA (Hohsaka *et al*., 1996), were prepared as described previously. Alkaline phosphatase-labeled anti-mouse IgG and MagneHis™ Ni-Particles were purchased from Promega. Polymerase chain reaction enzyme (Vent DNA polymerase) and T7 RNA polymerase were purchased from New England Biolabs. T4 RNA ligase was purchased from Takara Bio. Anti-T7-tag antibody was obtained from Novagen.

Preparation of quencher-labeled biotin

Synthesis of 9-(2-aminoethyl)-9H-carbazole hydrochloride

The 0.9 M of borane–tetrahydrofuran solution (20 ml) was instilled into the 9-vinylcarbazole (5.8 g, 0.03 mol) tetrahydrofuran solution under a stream of N_2 gas at room temperature. The mixture was stirred at room temperature for 2 h. Thereafter, the trialkylborane solution was diluted with 10 ml of tetrahydrofuran, and hydroxylamine-O-sulfonic acid (2.26 g, 20 mmol) was added; the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was then washed with 1 N of NaOH solution (100 ml) three times after being diluted with diethyl ether (20 ml). The organic phase that was dried by $MgSO_4$ was reacted with ethereal HCl (1 M, 10 ml) to precipitate the amine as 9-(2-aminoethyl)-9*H*-carbazole hydrochloride. The solid was washed with diethyl ether and dried to yield 0.83 g (14%) (Hohsaka *et al*., 2001).

Synthesis of quencher-labeled biotin

The 10 mM of 9-(2-aminoethyl)-9*H*-carbazole hydrochloride in methanol (5 ml) was added into 10 mM of N-Succinimidyl D-Biotin or EZ-Link®. NHS-LC-Biotin or EZ-Link® NHS-LC-LC-Biotin that was dissolved in Tris-HCl buffer pH 8.3 (5 ml) was stirred at 0 °C for 3 h. The produced solid was washed with diethyl ether, water, and evaporated to dry state of carbazole-labeled biotin, carbazole-LC-biotin, and carbazole-LC-LC-biotin. The produces were purified and analyzed by HPLC (GL Sciences, Inertsil ODS-3, and phenomenex ODS, solvent A: 25 mM KH2PO4 pH 2.5), solvent B: acetonitrile, B% = 65%, flow rate: 1.5 ml/min, room

temperature, ultraviolet detection absorption at 240 nm). The yields of the three biotin analogs are more than 95% (Hohsaka *et al*., 2004).

Preparation of fluorescent mutant streptavidin

The mRNAs of streptavidin containing four-base codon (CGGG) at Tyr54, Tyr83, or Trp120 position were synthesized using their DNAs by T7 RNA polymerase. The tRNA derived from yeast phenylalanine that contained the corresponding four-base anticodon (CCCG) and lacked 3′-terminal dinucleotide was also synthesized by using its DNA with T7 RNA polymerase; thereafter, the BFLAF-pdCpA was bound to the tRNA by T4 RNA ligase for obtaining BFLAF-tRNA. A cell-free translation was carried out by adding streptavidin mRNA and BFLAF-tRNA into the reaction mixture containing *Escherichia coli* extract. The reaction mixture (10 μl) contained 7 μl of *E. coli* extract, 0.1 mM each of naturally occurring amino acids, 15 pmol of mRNA, and 0.2 nmol of BFLAF-tRNA. After incubation for 60 min at 37 °C, the product was purified by MagneHis™ Ni-Particles, MicroSpin G25 column, and analyzed by fluorescent imaging analysis and Western blotting using the anti-T7-tag antibody and an alkaline phosphatase-labeled secondary antibody.

Confirmation of the biotin-binding activity of the fluorescent mutant streptavidin

The biotin-binding activity of the wild type and mutant streptavidin was confirmed with dot blot analysis. Two times the amount of the mutant streptavidins as compared with wild-type streptavidin was immobilized on the cellulose nitrate to bind with alkaline phosphatase-labeled biotin and then assayed with western blue for color generation. The coloring image of the dot on the blotting membrane was taken with a scanner and analyzed the spot density by the commercialized image software NIH IMAGEJ.

Fluorescence analysis of fluorescent mutant streptavidin

The carbazole-labeled biotin solution was prepared with dimethyl sulfoxide. The samples contained 10 μl of fluorescent mutant streptavidin, 10 μl of carbazole-labeled biotin, 10 μl of natural biotin, and diluted to 100 μl with HKM buffer (100 mM KCl, 25 mM Hepes, 5 mM $MgCl_2$, pH adjusted to 7.4 with KOH). A fluorescence spectrometer (JASCO FP-6500) was used to measure excitation and emission spectra of the sample solutions with the slit width in 5 nm for both excitation and emission.

RESULTS AND DISCUSSION

Confirmation of the synthesis of the fluorescent mutant streptavidin

The wild type, Tyr54BFLAF, Tyr83BFLAF, and Trp120BFLAF mutant streptavidins were synthesized by *in vitro* translation and purified by using the His-tag affinity nickel particles. Three times volume of the fluorescent mutant streptavidin solution as compared with that of the wild-type streptavidin solution was subjected to SDS-PAGE to compare the molecular weight. The bands of three mutant streptavidins were confirmed by Western blotting, in which the T7-tag of the N-terminal of the mutant streptavidin was recognized by anti-T7-tag antibody from mouse, and a mouse secondary antibody labeled with alkaline phosphatase was used to indicate protein band by enzymatic reaction. The band of full-length of streptavidin (20 kDa) was obtained only in the presence of BFLAF-tRNA in the *in vitro* translation system (Figure 2(a)). The bands of the wild type and three mutant streptavidin in Western blotting membrane were seen at the almost same molecular weight of 20 kDa. The relative yield of the full-length mutant streptavidin obtained in the presence of the BFLAF-tRNA was determined by comparing the band density of the mutant streptavidin with those of serially diluted wild-type streptavidin. The bands' densities of Tyr54BFLAF, Tyr83BFLAF, and Trp120BFLAF mutant streptavidin were 26.2%, 26.6%, and 27.4%, respectively, as compared with that of the band density of wild-type streptavidin as 100% with NIH IMAGEJ software. Therefore, the protein yields of Tyr54BFLAF, Tyr83BFLAF, and Trp120BFLAF mutant streptavidin were analyzed to 8.7%, 8.9%, and 9.1%, respectively, as compared with the protein yield of wild-type streptavidin as 100%. The incorporation efficiency of the BFLAF which was estimated by the comparison of density was identical regardless of the incorporated site. The bands of three fluorescent mutant streptavidins were observed with a fluorescence imager to confirm the mutant

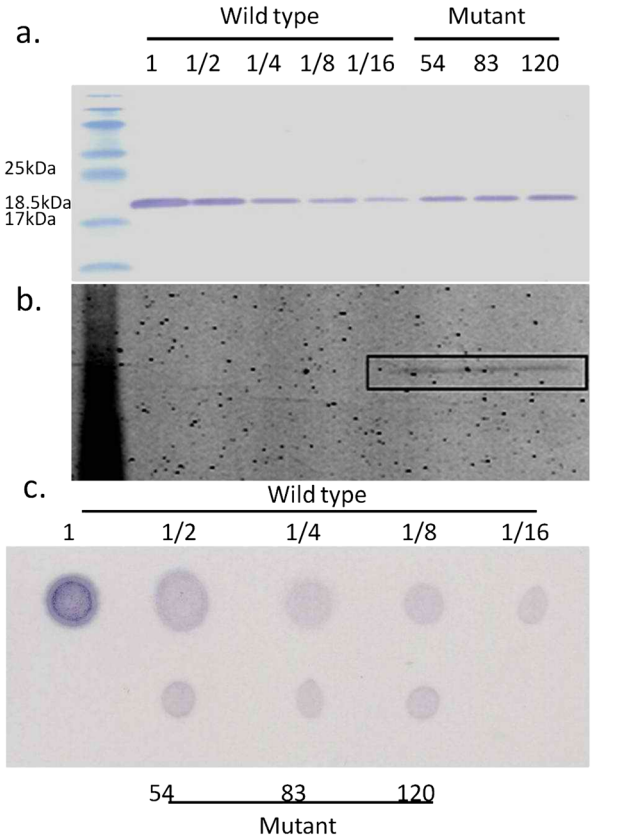

Figure 2. (a) The result of Western blotting. BFLAF was incorporated into streptavidin by using a CGGG four-base codon in the *in vitro* translation system. Applied volumes of the mutant streptavidins were three times larger than that of wild-type streptavidin. The bands of wild type and mutant streptavidins were observed at around 20 kDa. (b) The result of fluorescence imaging analysis. Three fluorescent bands of Tyr54BFLAF, Tyr83BFLAF, and Trp120BFLAF mutant streptavidins were enclosed together with black frame. (c) The result of dot blotting. The biotin binding activities of these fluorescent mutant streptavidins were evaluated by dot blot analysis using an alkaline phosphatase-biotin. The blotting volumes of the mutant streptavidins were three times larger than that of wild-type streptavidin.

streptavidins that were excited at 488 nm, and the fluorescence was measured at 530 nm (Figure 2(b)). The results of Western blotting analysis and fluorescence imaging analysis indicated that the BFLAF were incorporated into streptavidin at each specific position to make the full-length of streptavidin.

The biotin binding activities of the wild type and the mutant streptavidins were evaluated by dot blotting using alkaline phosphatase-labeled biotin. For confirmation of the biotin binding activities of the mutant streptavidins, two times volume of each mutant streptavidin solution as compared with wild-type streptavidin solution was blotted on the membrane. The protein amounts of Tyr54BFLAF, Tyr83BFLAF, and Trp120BFLAF mutant streptavidins blotted on the membrane were 17.4%, 17.8%, and 18.2% of that of wild-type streptavidin from Western blotting analysis. Furthermore, the blot densities of Tyr54BFLAF, Tyr83BFLAF, and Trp120BFLAF mutant streptavidins were 9.6%, 9.6%, and 9.7%, respectively, as compared with the wild-type streptavidin with the NIH IMAGEJ software. The results indicated that the mutant streptavidin containing BFLAF at positions of Tyr54, Tyr83, and Trp120 retained enough biotin binding activity as wild-type streptavidin (Figure 2(c)). These positions are located at the surface regions of the beta-barrel structure or at an interface region of a biotin binding pocket that may be little influential in biotin binding activities.

Fluorescence quenching of fluorescent mutant streptavidin upon addition with carbazole-labeled biotin

Before the experiment of fluorescence quenching of fluorescent mutant streptavidin by addition with carbazole-biotin, 100 nM of natural biotin was added into the solution of the each fluorescent mutant streptavidin for confirming the effect of the natural biotin. Actually, the effect of fluorescence intensity from natural biotin was little (Figure 3(a–c)).

The fluorescence spectrum change of each fluorescent mutant streptavidin of Tyr54BFLAF, Tyr83BFLAF, or Trp120BFLAF by the addition of carbazole-labeled biotin was measured. There was almost no change in the each fluorescence spectrum of Tyr54BFLAF and Tyr83BFLAF in the presence (100 nM) and absence of carbazole-labeled biotin. Tyr54 and Tyr83 are far from

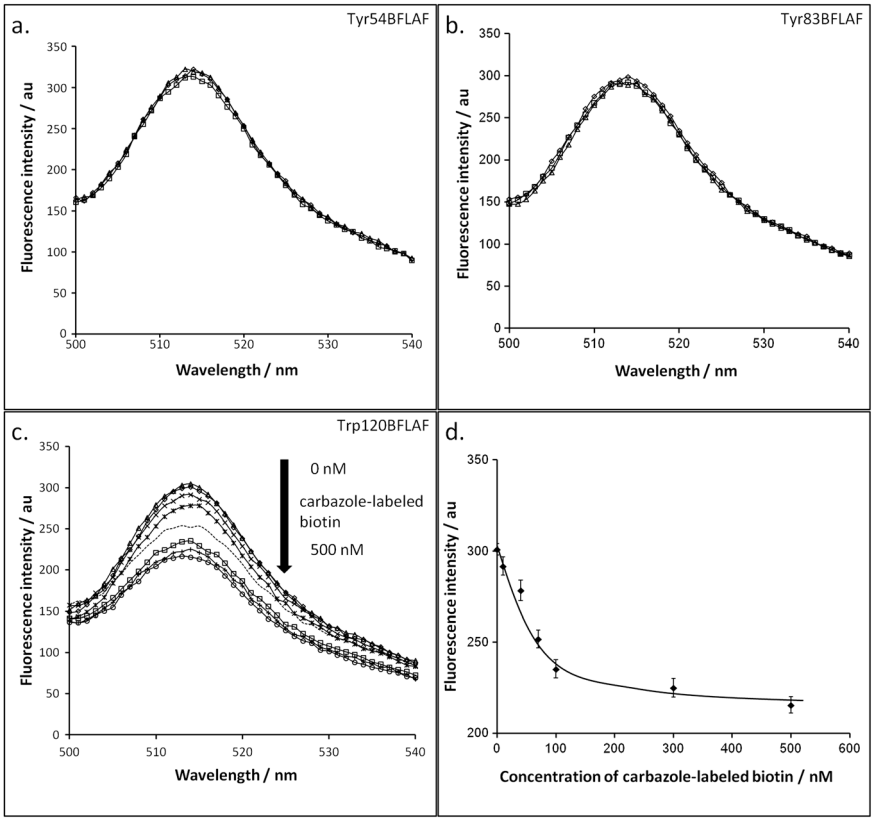

Figure 3. (a) Fluorescence spectra of the Tyr54BFLAF mutant streptavidin only (—◇—); in the presence of 100 nM of natural biotin (—△—) or 100 nM of carbazole-labeled biotin (—□—), there was no change in the fluorescence intensity upon addition of natural biotin or carbazole-labeled biotin for the Tyr54BFLAF mutant streptavidin. (b) Fluorescence spectra of the Tyr83BFLAF mutant streptavidin only (—◇—); in the presence of 100 nM of natural biotin (—△—) or 100 nM of carbazole-labeled biotin (—□—), there was no change in the fluorescence intensity upon addition of natural biotin or carbazole-labeled biotin for the Tyr83BFLAF mutant streptavidin. (c) Fluorescence spectra of the Trp120BFLAF mutant streptavidin only (—◇—); in the presence of 100 nM of natural biotin (—△—) or 10 nM (—×—), or 50 nM (—■—), or 80 nM (—▪▪▪—), or 100 nM (—□—), or 300 nM (—|—), or 500 nM (—○—) of carbazole-labeled biotin. Marked fluorescence quenching was observed for the Trp120BFLAF mutant streptavidin by addition of carbazole-labeled biotin. The fluorescence intensities of the Trp120BFLAF mutant streptavidin decreased as the concentration of carbazole-labeled biotin increased, and the fluorescence intensity was 1.4 times in the absence of carbazole-labeled biotin as compared with that in the presence of carbazole-labeled biotin (100 nM). (d) The fluorescence intensities at 510 nm of the Trp120BFLAF mutant streptavidins were plotted against the added carbazole-labeled biotin concentration. Fluorescence intensity of Trp120BFLAF mutant streptavidin decreased linearly against the concentration of carbazole-labeled biotin in the range of 10 to 100 nM, and the fluorescence quenching was saturated over 100 nM. All of the fluorescence spectra of mutant streptavidins were measured at the excitation wavelength of 490 nm.

the carbazole group that was modified to the biotin bound into the streptavidin pocket. The quenching of BFLAF incorporated into the Tyr54 or Tyr83 position of streptavidin by carbazole-labeled biotin might be very difficult to occur (Figure 3(a and b)).

By contrast, in the case of Trp120BFLAF mutant streptavidin, a marked quenching was observed upon the addition of carbazole-labeled biotin (100 nM). The fluorescence intensity of the Trp120BFLAF mutant streptavidin in the absence of carbazole-labeled biotin was 1.4 times larger than that of Trp120BFLAF mutant streptavidin in the presence of carbazole-labeled biotin (100 nM) (Figure 3(c)). Furthermore, the concentration dependence of the fluorescence quenching of Trp120BFLAF mutant streptavidin by the binding of carbazole-labeled biotin was shown in Figure 3(d).

For optimization of the spacer length between biotin and carbazole, the carbazole-LC-biotin and carbazole-LC-LC-biotin were also synthesized, and the fluorescence quenching of Trp120BFLAF mutant streptavidin was examined by addition of these biotin analogs with spacer chain. Here, LC shows the spacer chain of aminocaproic acid. The spacer arm length of carbazole-LC-biotin and carbazole-LC-LC-biotin were, respectively, 22.4 and 30.5 Å. The fluorescence spectra of Trp120BFLAF mutant streptavidin upon titration with carbazole-LC-biotin indicated almost no change (Figure 4(a and b)). However, the fluorescence spectra of Trp120BFLAF mutant streptavidin showed a significant enhancement by addition of carbazole-LC-LC-biotin (Figure 4(c and d)). In our previous research, a similar phenomenon that showed fluorescence enhancement of the Trp120BFLA mutant streptavidin by binding of the biotin analog with a LC-LC-hydrazide was demonstrated. We speculated that this enhancement may be a result of the disturbing of the fluorescence quenching between Trp120BFLAF and Trp79 or Trp108 of the neighbor subunit by the long spacer tail of the biotin analog. Therefore, in the case of the pair of Trp120BFLAF mutant streptavidin and carbazole-LC-biotin, the fluorescence quenching of BFLAF by carbazole moiety and fluorescence enhancement of BFLAF by LC chain effect were occurring simultaneously, and the fluorescence quenching counteracted the fluorescence enhancement to induce no fluorescence change when the carbazole-LC-biotin was added into the Trp120BFLAF mutant streptavidin. But in the case of the pair of Trp120BFLAF mutant streptavidin and carbazole-LC-LC-biotin, the fluorescence quenching effect of BFLAF by carbazole moiety which has a long flexible spacer chain might be less than the effect of fluorescence enhancement by LC-LC block. Therefore, the fluorescence enhancement might be observed totally when the carbazole-LC-LC-biotin was added into the Trp120BFLAF mutant streptavidin solution.

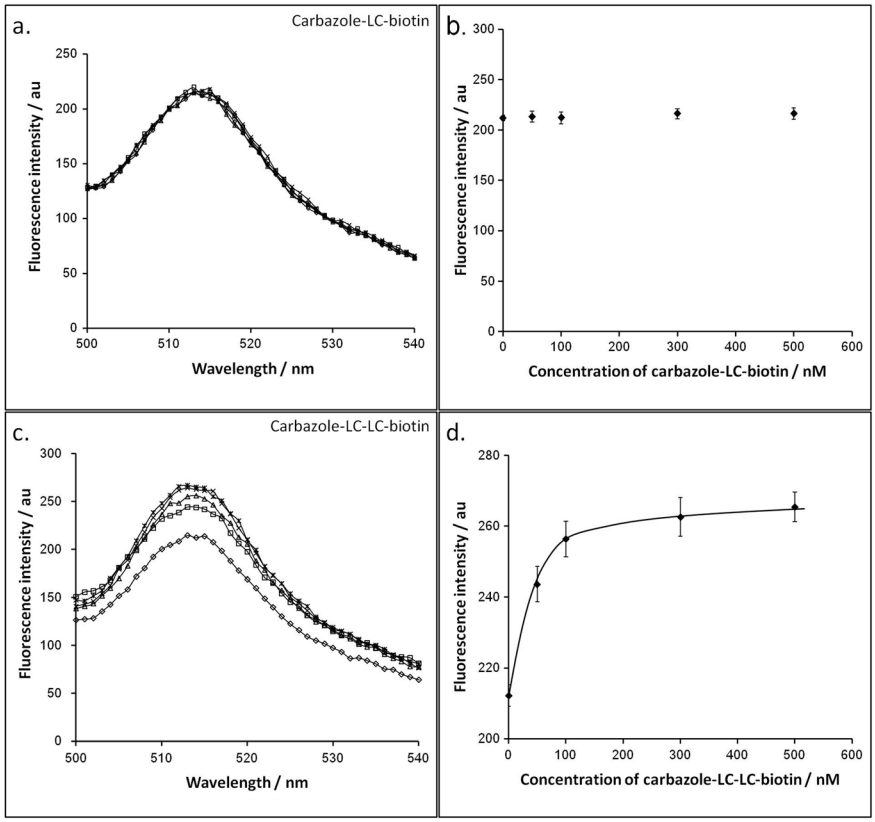

Figure 4. (a) Fluorescence spectra of the Trp120BFLAF mutant streptavidin in the presence of 0 nM (—◇—), or 50 nM (—□—), or 100 nM (—△—), or 300 nM (—×—), or 500 nM (—■—) of carbazole-LC-biotin. (b) The dependence of fluorescence intensity of the Trp120BFLAF mutant streptavidin on the concentration of carbazole-LC-biotin at 510 nm. Excitation wavelength was 490 nm. (c) Fluorescence spectra of the Trp120BFLAF mutant streptavidin in the presence of 0 nM (—◇—), or 50 nM (—□—), or 100 nM (—△—), or 300 nM (—×—), or 500 nM (—■—) of carbazole-LC-LC-biotin. (d) The dependence of fluorescence intensity at 510 nm of the Trp120BFLAF mutant streptavidin on the concentration of carbazole-LC-LC-biotin at 510 nm. Excitation wavelength was 490 nm.

Biotin sensing by measurement of fluorescence intensity of the Trp120BFLAF fluorescent mutant streptavidin and carbazole-labeled biotin system

For the application of the fluorescence quenching of the Trp120BFLAF mutant streptavidin by the binding of carbazole-labeled biotin to the molecular biosensing system for free biotin detection, we performed the next experiments with the following two steps: (i) natural free biotin was first added into the Trp120BFLAF mutant streptavidin solution; and (ii) 100 nM of carbazole-labeled biotin solution was next added for binding to the mutant streptavidin solution. The fluorescence intensity of Trp120BFLAF mutant streptavidin with carbazole-biotin (100 nM) was increased by increasing added free biotin concentration. Furthermore, the fluorescence intensity of Trp120BFLAF mutant streptavidin with carbazole-labeled biotin (100 nM) depended on the added free biotin concentration. (Figure5(a and b)). This result demonstrated that the molecular biosensor for biotin based on the fluorescence quenching of Trp120BFLAF fluorescent unnatural streptavidin and carbazole-biotin was successfully designed, and the free biotin could be determined in the concentration from 20 to 100 nM in this method.

In this study, the fluorescence of Trp120BFLAF mutant streptavidin was quenched by an addition of carbazole-labeled biotin. A new molecular biosensor for biotin was developed by using the fluorescence quenching of Trp120BFLAF unnatural streptavidin with carbazole-labeled biotin. Moreover, our model experiment suggested that the potential of biosensor design based on the couple of genetically engineered fluorescent mutant binding protein (antibodies) and quencher-labeled ligand analog (antigens).

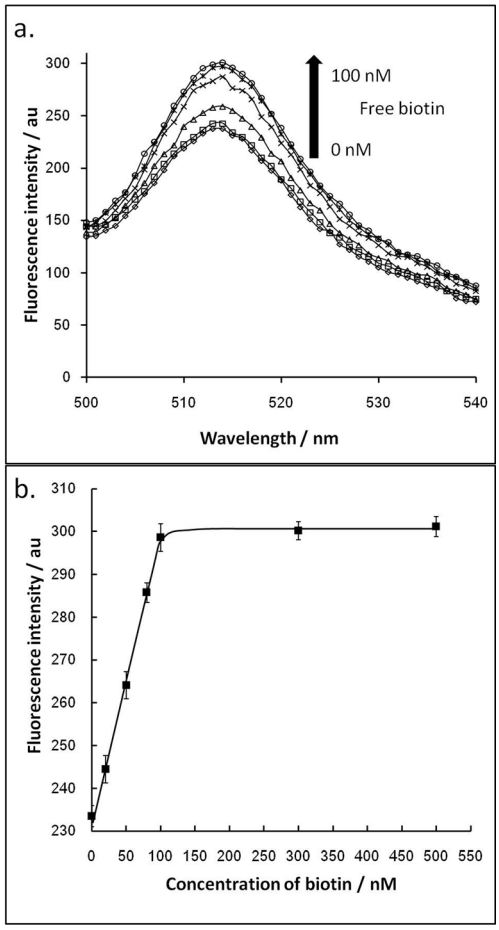

Figure 5. (a) Fluorescence spectra of the Trp120BFLAF mutant streptavidin only (—○—) and in the presence of the pairs of 0 nM (—◇—), or 20 nM (—□—), or 50 nM (—△—), or 80 nM (—×—), or 100 nM (—✳—), of natural biotin and 100 nM of carbazole-labeled biotin. Fluorescence recovery of Trp120BFLAF mutant streptavidin quenched by carbazole-labeled biotin (100 nM) was observed upon biotin addition. (b) The dependence of fluorescence intensity of the Trp120BFLAF mutant streptavidins on the concentration of added free biotin in the copresence of carbazole-labeled biotin (510 nm). Fluorescence quenching recovery at 510 nm by biotin addition depended on the concentration of added free biotin. This result demonstrated that fluorescence quenching recovery-based biosensor for biotin was successfully fabricated by a combination of the Trp120BFLAF mutant streptavidin and the carbazole-labeled biotin. All of the fluorescence spectra of mutant streptavidins were measured at the excitation wavelength of 490 nm.

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COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of Interest

All authors in this research declare that they have no conflicts of interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent

Not applicable.

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