Development of a fluvoxamine detection system using a Quenchbody, a novel fluorescent biosensor

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Abstract

The misuse of psychotropic drugs intended for medical treatment represents a recent worldwide public health concern. Quenchbody (Q-body) is a novel fluoroimmunosensor that can detect an antigen immediately without additional reagents or washing steps. Here, we describe creating Q-bodies for the detection of the antidepressant fluvoxamine (FLV) and determining optimal conditions to achieve the highest fluorescence intensity (FI). We prepared five Q-bodies with the fluorophore labeled at either the N- or C- terminus and with different linker lengths. Fluorescence was measurable within minutes, indicating the interaction of Q-bodies with FLV. The normalized FI (FI ratio) of the N-terminus labeled Q-body increased approximately 1.5-fold upon FLV addition; Q-bodies labeled at the C-terminus did not significantly increase FI. Among the fluorescence dyes used in this study, Rhodamine 6G labeled Q-body showed the best FI ratio. EC_{50} values of the N-terminus labeled Q-bodies were similar (23.2–224 nM) regardless of linker length or labeling dye. We examined whether the Q-body could be applicable to serum matrix instead of phosphate-buffered saline. The intact serum interfered strongly with the Q-body fluorescence. However, the FI ratios of the Q-body for FLV spiked serum filtrate, for which proteins were removed by filtration, showed a dose-dependency for detecting FLV levels. Deproteinization, which does not interfere with Q-body fluorescence measurements, is likely necessary to detect serum FLV with high sensitivity. This study demonstrates the potential of Q-body probes as a tool towards developing creative immunoassay applications.

Keywords: immunoassay, fluvoxamine, recombinant antibody, fluorescent biosensor, drug screening

Introduction

The misuse of prescription drugs, such as opioids, sedatives, stimulants, and tranquilizers, represent a worldwide public health concern[1]. Psychotropic drugs have been ranked as having one of the greatest rates of exposure increase[2], particularly in the past decade. Because of their high frequency of medical prescription, poisoning cases attributable to psychotropic drugs are increasing[3,4].

In the fields of emergency and forensic medicine, immunoassays are well suited for drug screening because of their simple procedures and near instantaneous results. Although most immunoassay kits used for drug screening can detect well-known illegal drugs and a few psychotropic drugs, they cannot detect newly approved drugs or most psychotropic drugs. Fluvoxamine (FLV, Fig. 1) is a selective serotonin reuptake inhibitor (SSRI) antidepressant and is currently in wide use for treatment of depression and obsessive-compulsive disorders. There are no immunoassay kits able to rapidly detect FLV despite the fact that FLV poisoning cases have been increasing as the number of prescriptions increases[5-7].

In our previous study, we challenged ourselves to develop a drug screening method for psychotropic drugs using a recombinant antibody such as a single chain variable fragment (scFv)[8]. Both the parental and scFv versions of the anti-FLV antibody we developed showed strong FLV binding activities and we demonstrated that anti-FLV scFv could be applied as a tool to screen for FLV. Recombinant antibodies have several advantages, such as 1) they can be produced using in vitro expression systems which is cost-effective and 2) mutations using genetic engineering techniques can be introduced for molecular enhancement of antigen-binding performance. Moreover, recombinant antibodies have the potential to create novel detection methods that differ from existing detection methods using monoclonal antibodies.

Recently, Ueda et al. developed an innovative fluoroimmunosensor named Quenchbody (Q-body)[9]. Q-body is a fluorescent antibody or antibody fragment, such as fragment of antigen binding (Fab)[10] or scFv [11]. Fluorophore quenching by tryptophan (Trp) residues in the Q-body is inhibited when the antigen is bound to Q-body resulting in de-quenching in an antigen concentration-dependent manner. Q-body binding to antigen shows fluorescent change instantly. No additional reagents or washing steps are needed for antigen detection. Q-bodies against variable antigens have been developed as they are highly sensitive and applicable to an extensive range of antigens[9,10,12]. It was also reported that optimum conditions differ depending on the molecular structure of the Q-body, some variable conditions include distance between the antibody molecule and the fluorescent dye, and the type of fluorescent dye used[13].

In this study, we created several anti-FLV Q-bodies, with fluorophore labeling at either the N- or C-terminus of scFv, using different linker lengths consisting of 2 or 5 repeats of a G3S unit, or labeling with different fluorescent dyes. We examined their properties to elucidate the optimal Q-body for FLV detection. We also examined whether the Q-body could be applicable to serums containing three concentration levels of FLV.

Materials and methods

Materials

Fluvoxamine malate and ovalbumin (OVA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluvoxamine conjugated ovalbumin (OVA-FLV) was prepared and used as immobilized antigen for enzyme-linked immunosorbent assays (ELISAs) as described previously[8]. ATTO520-C2-maleimide (ATTO520) was obtained from ATTO-TEC (Siegen, Germany). 5(6)-Tetramethylrhodamine-C5-maleimide (TAMRA-C5) was obtained from Biotium (Hayward, USA). 5(6)-Tetramethylrhodamine-C2-maleimide (TAMRA-C2) and Rhodamine6G-C5-maleimide (R6G) were generous gifts from Prof. Ueda (Tokyo Institute of Technology). All other solvents and chemicals were of analytical grade and were purchased through local suppliers.

Construction of Q-body expression vectors

Five expression vectors, pSQ(FLV)-N0, -N2, -N5, -C2, and -C5, which expressed Q-body precursor anti-FLV scFv, were constructed as described below using PCR, the primers in Table 1, and Prime STAR HS DNA polymerase (Takara Bio, Otsu, Japan) (Fig. 2a). The amino acid sequence of anti-FLV scFv was described previously[14]. Models of the Q-bodies are shown in Fig. 2b.

1) pSQ(FLV)-N0

A V_H-V_L type anti-FLV scFv gene with an internal $(G_4S)_3$ linker sequence was amplified from pETCF-HL[8] using primers 1 and 2 (Table 1). The resulting PCR product was inserted into NdeI/XhoI-digested FLVscFv plasmid[14] using Ligation high Ver.2, resulting in pET-HL.

Using pET-HL as a template, two parts of V_H-V_L type anti-FLV scFv genes were amplified using two primer pairs (primers 3 and 4; primers 5 and 6). PCR products were digested with AgeI and SacI or SacI and BamHI, and the two purified PCR products were

inserted into AgeI/BamHI-digested pSQ(KTM219) plasmid[11] (an expression vector for anti-osteocalcin [bone Gla protein] scFv-type Q-body) using Ligation high Ver.2, resulting in pSQ(FLV)-N0.

2) pSQ(FLV)-N2 and pSQ(FLV)-N5

An inverse PCR was performed using primers 7 and 8, with pSQ(FLV)-N0 as template. To remove remaining template plasmid, the PCR product was digested with DpnI and purified (PCR product A). PCR product A was phosphorylated and ligated using T4 Polynucleotide Kinase (Takara Bio, Otsu, Japan) and Ligation high Ver.2 (Toyobo, Osaka, Japan), resulting in pSQ(FLV)-N2.

Linker $(G_3S)_5$ DNA was amplified by overlap PCR using primers 9 and 10, then purified. The PCR product was inserted into PCR product A using an In-Fusion Cloning HD kit (Takara Bio, Otsu, Japan), resulting in pSQ(FLV)-N5.

3) pSQ(FLV)-C2 and pSQ(FLV)-C5

Using pSQ(FLV)-N0 as template, inverse PCR using primers 1 and 11 was performed, amplifying pSQ(FLV) without the N-terminal Cys tag. Then the reaction was digested with DpnI, phosphorylated, and ligated as above, resulting in PCR product B. Next, inverse PCR was performed using primers 12 and 13 with PCR product B as template. The reaction was digested with DpnI and the PCR product was phosphorylated and ligated, resulting in pSQ(FLV)-C2.

Linker $(G_3S)_4$-GGGC DNA was amplified by overlap PCR using primers 14 and 15, then purified. The PCR product was inserted into PCR product B using an In-Fusion Cloning HD kit, resulting in pSQ(FLV)-C5.

The complete sequences of these pSQ(FLV) plasmids were confirmed as previously described[8]. The pSQ(FLV) vectors were transformed into competent *Escherichia coli (E. coli)* BL21(DE3) cells (New England Biolabs, Ipswich, MA, USA).

Preparation of scFv proteins

Expression and purification of the Q-body precursor scFvs was performed as previously described with slight modifications[14]. Briefly, *E. coli* harboring each pSQ(FLV) vector was incubated at 30°C for overnight in Luria-Bertani (LB) broth supplemented with 100 μg/mL ampicillin. Cells expressing each protein were disrupted by sonication and centrifuged to separate the supernatant and insoluble fractions. The insoluble fraction, containing most of the scFv protein, was dissolved in lysis buffer (50 mM Tris-HCl buffer: 6 M guanidine hydrochloride, 500 mM NaCl, pH 8.0) and incubated with rotation at room temperature for 2–4 days. The scFv was purified using Ni-NTA Superflow resin (Qiagen K.K., Tokyo, Japan). Before protein refolding, scFv (≈7.5 μM) was mixed with 375 μM 2-mercaptoethanol at room temperature for 2 h following the method used in Umetsu et al.[15]. Next, it was refolded by gradual removal of guanidine hydrochloride via stepwise dialysis. After dialysis, the solution containing refolded scFv was centrifuged to remove aggregated proteins (12,000 rpm, 10 mins, 4°C), then purified by high-performance liquid chromatography (HPLC) using a TSKgel G3000SW column (300 x 7.5 mm i.d.) (Tosoh, Tokyo, Japan). HPLC conditions were as follows: instrument, D-2000 Elite HPLC (Hitachi High-Tech Science Corp., Tokyo, Japan); mobile phase, 20 mM Tris-HCl (pH 7.0); detector, UV 280 nm; column temperature, room temperature; flow rate, 3.0 mL/min. Purity of scFv was verified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously[14].

Preparation of Q-bodies

To prepare Q-bodies against FLV, scFv fluorescence labeling and purification was performed using the method of Jeong et al.[11] with slight modifications. Briefly, purified scFv (10 μg) and an equal volume of immobilized Tris (2-carboxyethyl) phosphine (TCEP) disulfide reducing gel slurry were mixed and incubated for 1 h at 25°C on a rotating wheel. After centrifugation to remove resin, supernatant was collected and reacted with 20 × mol of fluorescent dye in the dark for 2 h at 25°C. Next, the reaction mixture was incubated with 40 μL of anti-FLAG M2 magnetic beads (Sigma-Aldrich, St. Louis, MO, USA) on a rotating wheel at 4°C overnight. Beads were washed three times with FLAG buffer (20 mM phosphate buffer: 431 mM NaCl, 0.1% polyoxyethylene (23) lauryl ether, pH 7.4) and 0.1 mL FLAG elution buffer (150 ng/mL 3 × FLAG peptide in FLAG buffer) was added. After 30 min incubation at 4°C, eluent was collected, and the elution step was performed again. The total eluent was added to 200 μL Ni Sepharose excel beads (GE Healthcare, Tokyo, Japan) and incubated for 30 min at 25°C on a rotating wheel. Beads were washed three times with 500 μL His wash buffer (20 mM phosphate buffer: 500 mM NaCl, 0.1% polyoxyethylene (23) lauryl ether, pH 7.4). Next, His elution buffer (His wash buffer with 0.5 M imidazole) was added to the beads and the eluent was collected. Buffer was exchanged to PBST (10 mM phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, 0.05% Tween 20, pH 7.4) using centrifugal ultrafiltration and the Q-bodies (N0-, N2-, N5-, C2-, and C5-) were obtained. Q-bodies were stored at 4°C.

Enzyme-linked immunosorbent assay (ELISA)

Reactivity of anti-FLV scFv against FLV was assayed using an ELISA described previously, with slight modifications[8]. In brief, 1.0 μg OVA-FLV was coated in each well of a 96-well microtiter plate. The plate was then blocked with 0.5% gelatin. Q-body (dissolved in PBST) was added to the ELISA plate as primary antibodies. Each well was then washed and incubated with horseradish peroxidase-labeled anti-FLAG secondary antibody, followed by addition of 1, 2-phenylenediamine dihydrochloride. The reaction was terminated by addition of 1 M sulfuric acid, and absorbance at 490 nm was read on a micro-ELISA plate reader (Bio Rad, Tokyo, Japan). As a control, wells coated with OVA were used to check the specificity of Q-bodies.

Fluorescence measurements

Reactivity of fluorescence intensity (FI) for each Q-body was measured using a fluorescence spectrometer (F-7100, Hitachi High-Tech Science Corp., Tokyo, Japan). Two hundred ng of Q-body in 200 μL of PBST was added to a micro quartz cell (650-0171, Hitachi High-Tech Science Corporation, Tokyo, Japan), incubated at 25°C for 3 min, and FI of the sample solution was continuously measured 20 × for 20 min (once per min). After the 10th measurement, 1 μL FLV solution (200 μM in PBS [phosphate-buffered saline, pH 7.4]) or PBS was added to the sample solution and mixed well. In the experiment using FLV spiked serum samples, 10 μL of the sample solution was added instead of 1 μL FLV solution.

Fluorescence spectrum of the Q-body with various concentrations of FLV was measured. Two hundred ng of Q-body in 200 μL of PBST was added to a micro quartz cell which was incubated at 25°C for 3 min. Next, 1 μL FLV solution (200 nM to 2 mM in PBS) was added and fluorescent spectra were measured for each FLV concentration.

To examine the maximum FI of the Q-bodies, the Q-body denaturing test was performed as described by Jeong et al.[11] using 200 µL of 7 M guanidium hydrochloride (GdnHCl) and 100 mM of dithiothreitol.

In all of these experiments, conditions of fluorescent measurements were as follows: both the excitation and emission slit widths were set to 10.0 nm; excitation wavelengths were 520, 530, or 546 nm for ATTO520-, R6G-, or TAMRA-labeled Q-body, respectively. Dose-response curves were constructed by fitting the intensities at the maximum emission wavelength of each Q-body using Kaleida Graph 4.1 (Synergy Software, Reading, PA). The 50% effective concentration (EC_{50}) value was calculated using curve fitting to a 4-parameter logistic equation. Normalized FI (FI ratio) was calculated according to following formula:

Normalized FI = [FI(Q-body + FLV) - FI(blank)] / [FI(Q-body) - FI(blank)],

where FI(Q-body + FLV) and FI(Q-body) represent the FI of the samples containing Q-body and FLV, or Q-body, respectively, FI(blank) represents the FI of PBST.

Preparation of FLV spiked serum samples

To investigate whether the Q-body is applicable to practical specimens, we examined the reactivity of the Q-body using FLV spiked serum. Intact serum increased the baseline of FI and strongly interfered with the level of Q-body fluorescence. This may be due to an intrinsic Trp residue in serum albumin (data not shown)[16]. Therefore, we filtered the serum to remove serum proteins before measuring FI. Since FLV binds to serum albumin[17], we examined the FI of the Q-body using both pre-filter FLV-spiked (pre-spiked) and post-filter FLV-spiked (post-spiked) serum filtrates.

For the pre-spiked serum filtrates, 2 µL of FLV solutions (in PBS) were added to 198 µL of unfiltered serum, mixed well, and filtered using centrifugal filters (Amicon® Ultra-0.5mL Centrifugal Filters Ultracel®-3K, Millipore Corporation, Ireland). Post-spiked serum filtrates

were prepared by spiking FLV after the filtration process. FLV spiked PBS was used as a control. The final FLV concentrations in each level were 0.1, 1.0, and 3.0 µg/mL. Finally, the normalized FI of R6G labeled N2-Q-body for FLV pre-spiked serum filtrates, FLV post-spiked serum filtrates, or FLV spiked PBS were measured.

The normal human serum used in this study was obtained from a healthy volunteer.

Ethics

This study was approved by the Ethics Committee for Epidemiology Research and General Research at the Faculty of Life Sciences, Kumamoto University (No. 1269).

Results

Characterization of scFvs and Q-bodies

In an effort to create an optimal Q-body for FLV detection, we constructed five variants of scFv vectors as shown in Figure 2a, which have a cysteine at the N- or C- terminus and different linker lengths from the scFv. Vector sequences were confirmed and *E. coli* transfected with each scFv expression vector produced scFv protein after isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Figure 3a shows that the detected proteins were mainly located in the insoluble precipitate in inactive form, whereas very little scFv protein was present in the soluble fraction. To obtain a sufficient amount of scFv, we tried refolding the proteins contained in the insoluble precipitate using stepwise dialysis. Precursor scFvs were purified successfully as shown in Figure 3b. Q-body molecular weights and protein yields (per 100 mL culture) were as follows: N0-, 30.5 kDa and 530 µg; N2-, 31.1 kDa and 500 µg; N5-, 31.9 kDa and 700 µg; C2-, 29.8 kDa and 670 µg; C5-, 30.6 kDa and 570 µg.

Next, we labeled the purified protein using fluorescent dye and examined its binding activity by ELISA (Fig. 4). Wells immobilized with OVA-FLV showed strong signals, while negligible signals were observed for OVA-coated control wells.

Effect of FLV addition on the fluorescence intensity of Q-bodies

We measured the fluorescence intensity of each Q-body before and after addition of 1 μM FLV to evaluate the Q-bodies reaction rate against FLV in relative FI following FLV addition. As shown in Figure 5, FI of Q-bodies was stable before FLV addition and increased within one minute of FLV addition. The relative FI ratio was increased 1.4-fold after FLV addition, and the increased FI was stable throughout the measurement time period. Adding PBS to Q-bodies as a control did not affect FI.

Next, we examined the reactivity of TAMRA-C5 labeled Q-bodies to clarify the differences resulting from the fluorophore labeling position or linker length (Fig. 6). FI of Q-bodies was increased following an increase in FLV concentration; the maximum emission wavelength was 580 nm (Fig. 6a). Therefore, the FI ratio of each Q-body at each FLV concentration was measured at 580 nm (Fig. 6b). Maximum FI ratios of the Q-bodies with fluorophore labeling at the N-terminus were 1.5-, 1.4-, and 1.4-fold for N0-, N2-, and N5-Q-body, respectively. However, Q-bodies with fluorophore labeling at the C-terminus showed a slight increase of FI, which was 1.0- and 1.0-fold for C2- and C5-Q-body, respectively. The N0-Q-body showed the best reactivity against FLV, however, the precursor scFv had severe aggregation compared with N2- and N5-Q-bodies. EC_{50} values were 31.4, 43.9, and 33.2 nM for N0-, N2-, and N5-Q-body, respectively.

Characterization of Q-bodies labeled with different dyes

To determine the most suitable fluorescent dye for FLV detection, we created Q-bodies labeled with four different fluorescent dyes, TAMRA-C5, TAMRA-C2, R6G, or ATTO520. FI of each Q-body was monitored at its maximum emission wavelength (TAMRA-C5 and TAMRA-C2: 580 nm; R6G: 557 nm; ATTO520: 546 nm). Figure 7 shows the FLV dose dependency of FI ratio for each Q-body. The maximum FI ratio of TAMRA-C5, TAMRA-C2, and R6G labeled Q-bodies were 1.3-, 1.2-, and 1.5-fold, respectively. The FI ratio of ATTO520 labeled Q-body did not change even when 10 μM FLV was added. EC_{50} values were 55.2, 23.2, and 224 nM for TAMRA-C5, TAMRA-C2, and R6G labeled Q-body, respectively.

Reactivity of the Q-body for FLV spiked serum filtrates

Figure 8 shows the normalized FIs of the Q-body in FLV spiked PBS, FLV pre-spiked serum filtrate, and FLV post-spiked serum filtrate. The FI ratios for 0.1, 1.0, and 3.0 μg/mL of FLV, respectively, were as follows: PBS (1.1±0.04, 1.3±0.07, 1.3±0.11), FLV pre-spiked serum filtrate (1.0±0.03, 1.1±0.08, 1.1±0.10), and FLV post-spiked serum filtrate (1.2±0.03, 1.3±0.06, 1.2±0.14). The FI ratios in each group showed near dose-dependent increases. The FI ratios of FLV pre-spiked serum filtrate were lower than the other groups. The FI ratios of FLV post-spiked serum filtrates were nearly the same as the levels for the control at each FLV concentration level.

Discussion

We created a Q-body for detecting SSRI antidepressant FLV and determined the optimum conditions for the best fluorescent response. Conditions tested included distance between the antibody molecule and fluorescent dye, and the type of fluorescent labeling dye

used. Q-bodies were primarily created using a cell-free transcription system[9,12,18,19]. Recently, to improve production yield, Jeong et al. developed a method for large-scale production of Q-bodies, which combined *E. coli* expression of recombinant scFv and post-fluorescence chemical labeling[11]. They prepared a precursor scFv from the soluble fraction of *E. coli*, and the yield was 400-600 μg/100 mL culture. We tried to create scFv using this method, however, we did not obtain sufficient amounts of scFv for experimentation. In this study, we prepared scFv from inclusion bodies using a refolding technique with stepwise dialysis. Precursor scFvs were prepared successfully with yields of 500–700 μg/100 mL culture using the procedure described by Umetsu et al.[15] and the yields were sufficient for creating Q-bodies. This is the first report describing successful preparation of Q-body precursor scFv from inclusion bodies using an *E. coli* expression system.

Antigen detection systems using Q-body are based on the photoinduced electron transfer (PET) mechanism through interactions between fluorophores and intrinsic Trp residues of the antibody protein (Trp36H, Trp47H, Trp103H, and Trp35L; using the Kabat numbering scheme[20]), which are highly conserved among immunoglobulins of various origins[9] as well as anti-FLV scFv[14]. Q-body works using the following mechanism: in the absence of antigen, a fluorophore penetrates between the V_H/V_L interface and interacts with Trp residues (Trp47H and Trp103H) by hydrophobic and/or π–π stacking interactions; these interactions lead to quenching of electron transfers from Trp residues to the dye through transient contact between each Trp residue and the fluorophore; binding of an antigen to antibody (scFv) induces a tighter complex of V_H and V_L, thus releasing the dye from interactions with Trp residues near the V_H/V_L interface[9].

In our study, Q-bodies labeled at the N-terminus of scFv showed a better fluorescence response than Q-bodies labeled at the C-terminus. Fig. 2b shows the models of the N- or C-terminal labeled Q-bodies we created. Since all of the Q-bodies bound FLV sufficiently

(Fig. 4), this result reflected that Q-body labeling at the C-terminus showed less PET mediated quenching effect. PET mediated quenching is expected to occur more efficiently in Q-bodies labeled at the N-terminus of the V_H domain because it contains two important Trp residues for PET mediated quenching effects[9,12,18,21].

The fluorescent responsiveness of Q-bodies with different linker lengths depends on each scFv used for creating Q-bodies[13]. For example, the fluorescent response of anti-bone gla protein Q-body decreased 5.6- to 3.8-fold using the longer linker, whereas, anti-bisphenol A Q-body increased 1.1- to 2.0-fold using the longer linker[9]. The fluorescent response for the anti-FLV Q-body was slightly reduced using the longer linker, and maximum response was observed in the Q-body using the shorter linker (Fig. 6). Our results indicate that the PET mediated quenching effect could occur more effectively using a shorter linker length that provides the fluorophore with optimal positioning or orientation against Trp residues in the scFv.

Q-body labeled with R6G showed the maximum fluorescent response in our experiments (Fig. 7). Jeong et al. noted that R6G is easily quenched around the hydrophobic V_H/V_L interface because it is more hydrophobic than TAMRA[11], this observation supports our results. However, Q-bodies labeled with ATTO520 showed less fluorescent response in our experiments. In the pilot study, ATTO520 labeled Q-body that was denatured using guanidine hydrochloride and dithiothreitol, showed a fluorescent response of 1.5-fold compared with the control (data not shown). This result showed that PET mediated quenching would occur sufficiently in ATTO520 labeled Q-body. Hence, we speculated that ATTO520 could not be released by the interaction between Q-body and FLV. In other words, FLV addition would not lead to de-quenching and therefore FI would not change. Considering these results, one noteworthy finding is that benzoic acid residues and/or methyl groups of xanthene, which are identical parts of the chemical structures of TAMRA and R6G,

might be crucial for de-quenching in our Q-body system. Incidentally, the EC_{50} values of Q-bodies were almost same (23.2 – 224 nM) regardless of linker length or labeling dye; these results indicate that Q-bodies have high sensitivity for FLV detection. EC_{50} values were below the therapeutic serum concentration for FLV (190 – 720 nM)[20].

We examined the FI of the Q-body for FLV pre-spiked serum filtrates in which proteins were removed by centrifugal filtration because proteins of intact serum interfered strongly with the fluorescence of the Q-body (Fig. 8). The FI ratios of the Q-body for FLV pre-spiked serum filtrates were lower than the FI ratios of the Q-body for FLV post-spiked serum filtrates. FLV post-spiked serum filtrates were samples in which the FLV recovery rate by filtration was 100%. Our results show that most of the FLV in the serum was not recovered by centrifugal filtration since FLV binds to serum proteins (plasma protein binding of FLV: 77%)[17]. The FI ratio of the Q-body showed dose-dependency even when it was being tested in FLV pre-spiked serum filtrates. For practical use of the Q-body system, it is necessary to perform deproteinization which does not interfere with Q-body fluorescence measurements to successfully detect serum FLV with high sensitivity. Improvement of the Q-body which results in a greater FI ratio could lead to detection of more distinct differences in each FLV concentration level.

Q-body assays, which can detect an antigen instantaneously without any other reagents, would be valuable in clinical medicine as an on-site drug test for emergency rooms or testing for medication management. These assays would also be useful in criminal investigation for detecting drugs at a crime scene[23]. Our study showed that Q-body could detect FLV within minutes and with high-sensitivity, however, the fluorescent response should be improved for practical purposes. Furthermore, if optimal Q-body structure could be predicted in advance, based on the amino acid sequence of the antibody, it would be convenient for rapid Q-body development and production.

In conclusion, we prepared a Q-body precursor scFv from inclusion bodies using an *E. coli* expression system and successfully created Q-bodies for FLV detection. Our results showed that the Q-body labeled at the N-terminus with a shorter linker length yielded the best FI increase for FLV detection and that R6G was the optimal fluorescence dye in our system. Furthermore, we demonstrated the Q-body could be applicable for serum samples if they are deproteinized to prevent interference with FI. Considering that the misuse of pharmaceutical drugs is a great concern for worldwide public health, rapid development of a simple drug screening method would be helpful in the fields of clinical and forensic toxicology.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1 Nucleotide sequences of primers used in this study

Primer	Nucleotide sequence (5′–3′)
1	GATATACATATGGAGGTGAAACTGCAGGAG
2	GTGGTGCTCGAGTTTGATTTCCAGCTTGG
3	AATGAGACCGGTGAGGTGAAACTGCAGGAG
4	ATTTCCAGCTTGGTCCCCCCTCCG
5	CGCTATCACATGTCTGACATTGAGCTCACCCAG
6	GTAGTCGGATCCGCCGTGGTGGTGGTGGTGGTG
7	GGAGGCGGTAGTACCGGTGAGGTGAAACTG
8	CGAACCTCCGCCGGTCTCATTAGAGCAG
9	ACCGGCGGAGGTTCGGGAGGTGGCAGCGGCGGCGGGTCCGG
10	GGTACTACCGCCTCCAGATCCGCCCCCGGACCCGCCGCCG
11	TCCTTCTTAAAGTTAAACAAAATTATTTC
12	GGAGGCGGTTGTCACCACCACCACCACCACGGCGG
13	CGAACCTCCGCCCTCGAGTTTGATTTCCAG
14	GAGGGCGGAGGTTCGGGAGGTGGCAGCGGCGGCGGGTCCGG
15	GTGACAACCGCCTCCAGATCCGCCCCCGGACCCGCCGCCG

Figure 1

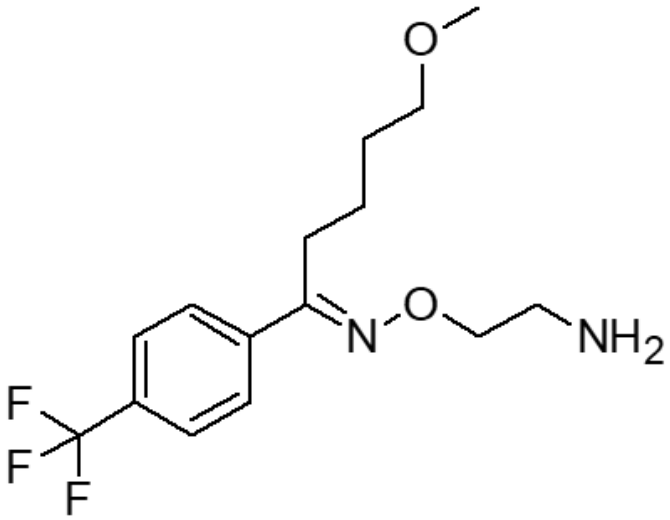

Figure 1. Chemical structure of fluvoxamine (FLV).

Figure 2

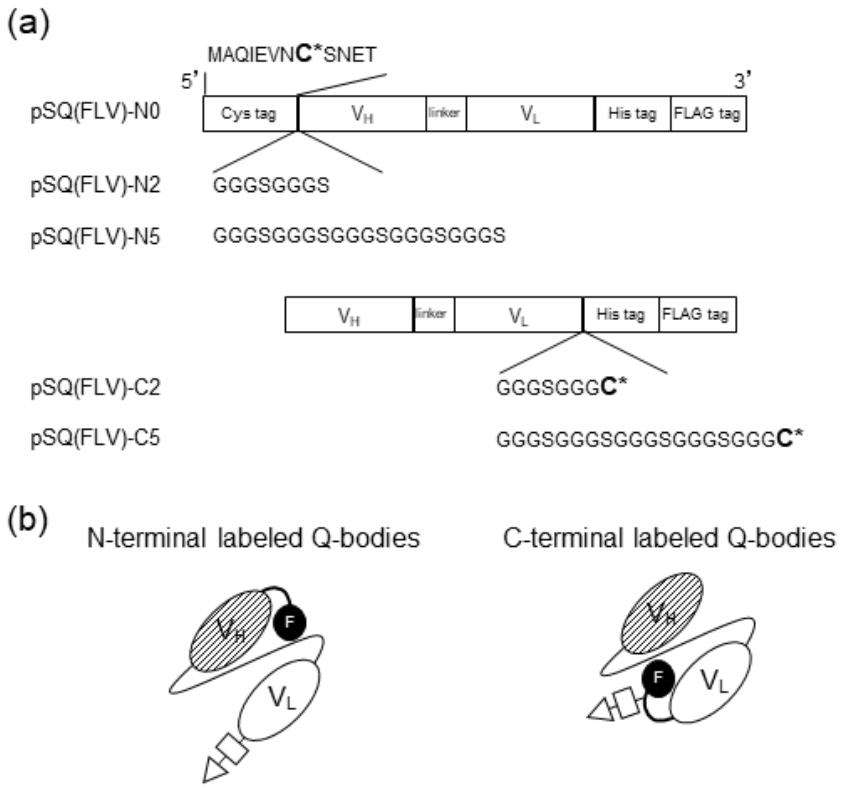

Figure 2. Q-body expression vectors and diagrams with fluorophore labeling. (a) Vectors were constructed for expression of Q-body precursor single-chain variable fragments (scFvs) against fluvoxamine (FLV) (pSQ(FLV)-N0, -N2, -N5, -C2, and -C5). Each gene was ligated into AgeI/BamHI-digested pSQ (KTM219) then transformed into BL21 (DE3) competent *E. coli*. * indicates the cysteine that was used for fluorophore labeling. (b) Diagram of Q-bodies with the fluorophore labeled at either the N- or C-terminal and with different linker lengths. Shaded oval, variable domain of heavy chain (V_H); white oval, variable domain of light chain (V_L); black circle, fluorophore; white rectangle, His tag: white triangle, FLAG tag; bold black line, linker consisting of 0, 2, or 5 repeats of a G3S unit.

Figure 3

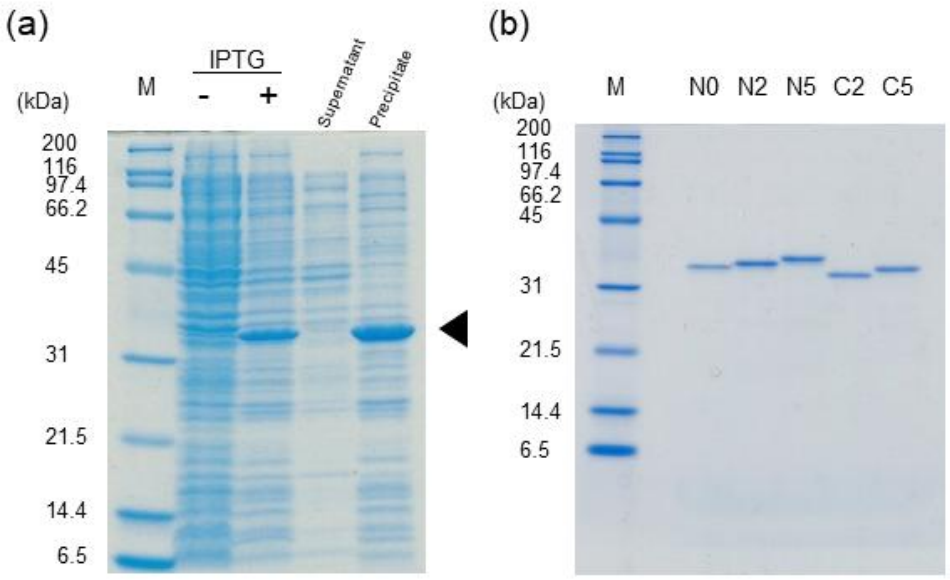

Figure 3. (a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of *E. coli* crude samples and (b) Q-body precursor single-chain variable fragments (scFvs) stained with Coomassie brilliant blue R‐250. (a) *E. coli* transfected with pSQ(FLV)-N(0) without or with 1 mM IPTG, respectively; supernatant and precipitate fraction of sonicated *E. coli*. The scFv proteins are indicated by black arrows, (b) purified precursor scFvs (N0, N2, N5, C2, and C5). M: molecular protein marker (Bio Rad Laboratories, Inc., Tokyo, Japan).

Figure 4

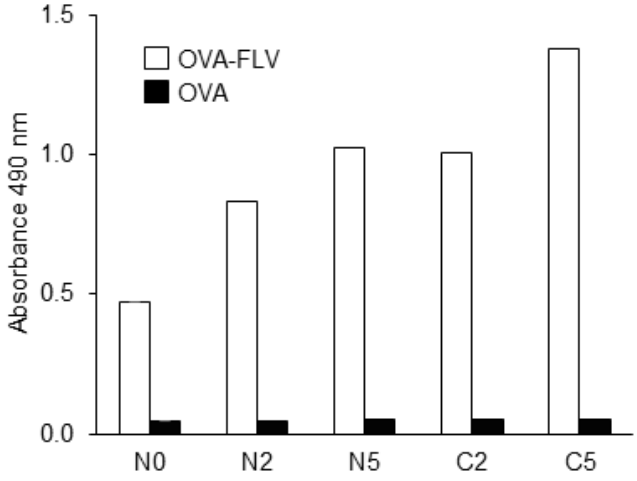

Figure 4. Specific binding of TAMRA-C5 labeled Q-bodies to immobilized OVA-FLV by ELISA. Q-bodies were used for ELISA as follows: N0-, 16.7 ng/well; N2-, N5-, C2-, and C5-, 50 ng/well. White bars represent the signal of OVA-FLV with Q-bodies, black bars represent the signal of OVA with Q-bodies. Data are shown as mean ± standard deviation (n = 3).

Figure 5

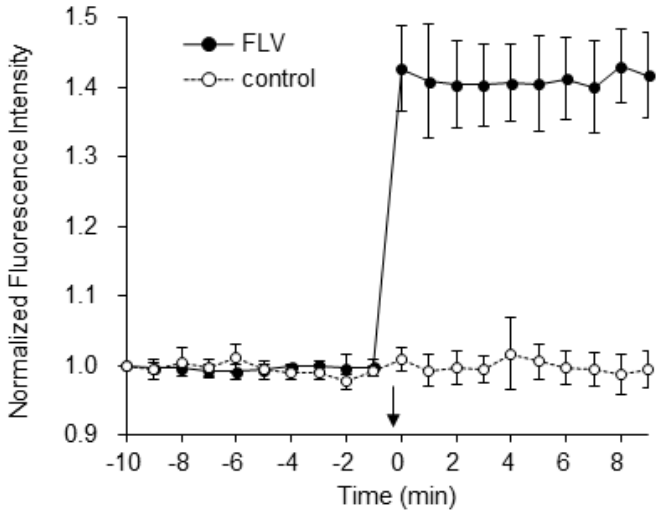

Figure 5. Changes in normalized fluorescence intensity (FI) through interactions of Q-body and FLV. Two-hundred ng N2-Q-body in 200 µL of PBST was added to a micro quartz cell then incubated at 25°C for 3 min. FI of the sample solution was continuously measured 20 × for 20 min. After the 10th measurement (arrow), 1 µL FLV solution (final concentration: 1 µM) or PBS (control) was added to the sample solution and mixed well. Normalized FIs are relative to FI values in the absence of FLV. Data are shown as mean ± standard deviation (n = 3).

Figure 6

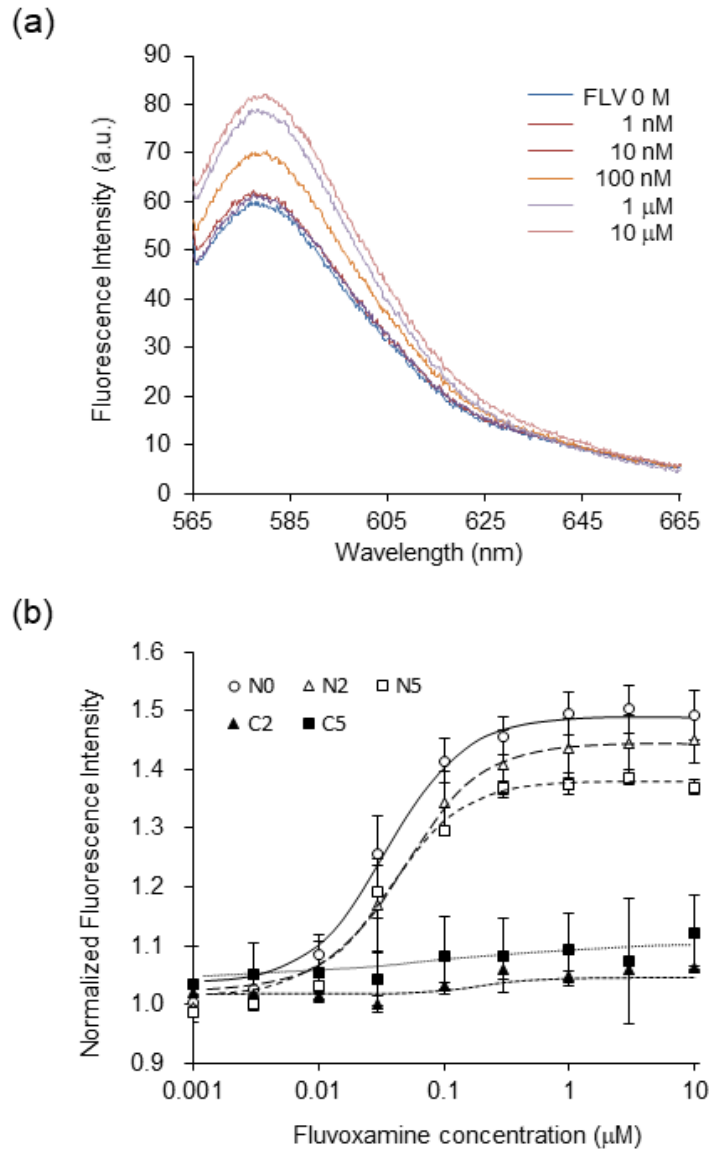

Figure 6. Antigen-dependent fluorescence enhancement of TAMRA-C5 labeled Q-bodies. (a) Fluorescence spectra of TAMRA-C5 labeled N2-Q-body with excitation at 546 nm in the absence and presence of FLV. Fluorescence intensity is expressed as arbitrary units. (b) Titration curves of the fluorescence intensity (FI) at 580 nm using each Q-body. Normalized FIs are relative to FI values in the absence of FLV. Data are shown as mean ± standard deviation (n = 3).

Figure 7

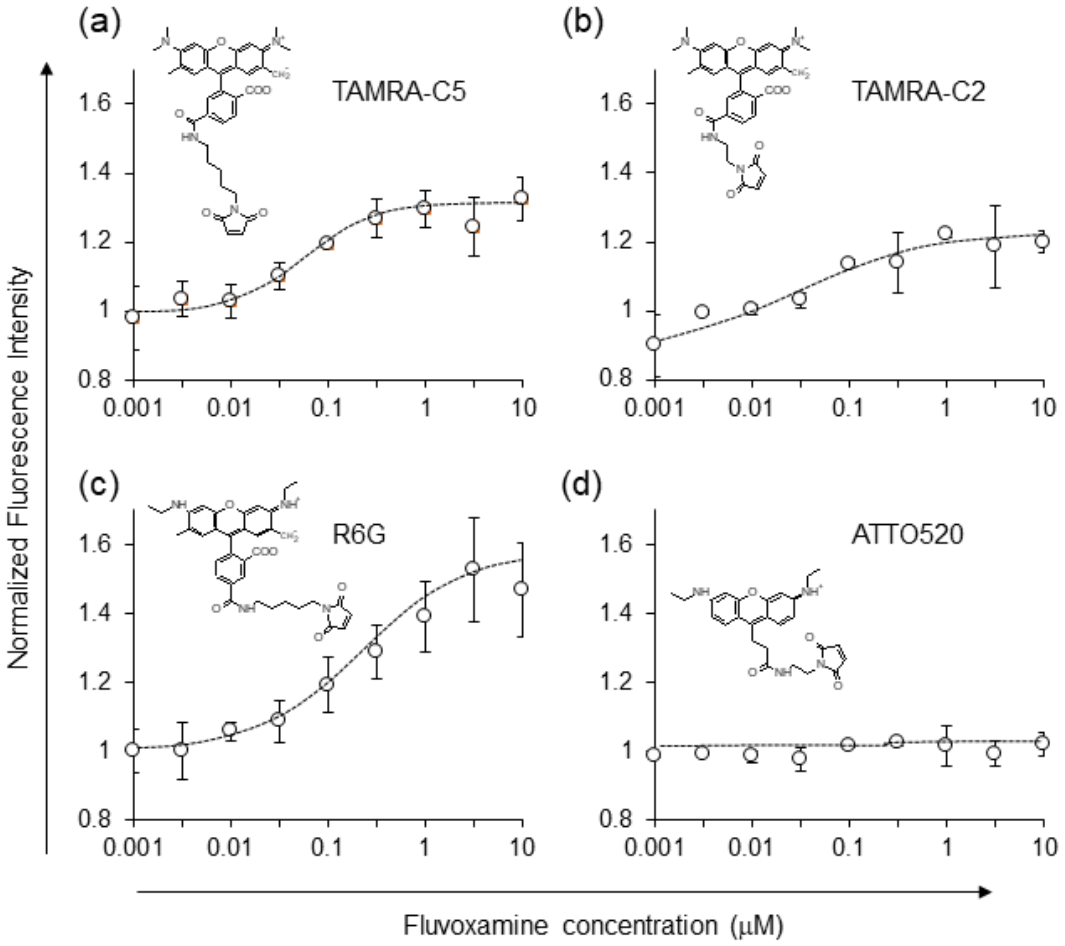

Figure 7. Titration curves of fluorescence intensity using N5-Q-body labeled with each of the dyes. The chemical structure of each dye is also shown. (a) TAMRA-C5, (b) TAMRA-C2, (c) R6G, (d) ATTO520. The excitation wavelength was 546 nm for TAMRA-C5- and TAMRA-C2-, 530 nm for R6G-, and 520nm for ATTO520-labeled Q-body, respectively. The normalized fluorescence intensities are relative to fluorescent intensity values in the absence of FLV. Data are shown as mean ± standard deviation (n = 3).

Figure 8

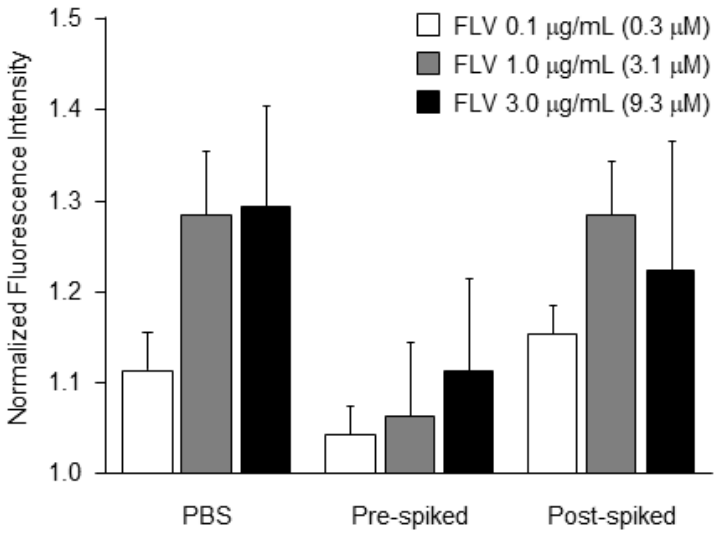

Figure 8. Normalized fluorescence intensities (FIs) of R6G labeled N2-Q-body for FLV spiked PBS (PBS), FLV pre-spiked serum filtrate (Pre-spiked), and FLV post-spiked serum filtrate (Post-spiked). The FIs are normalized to FI values in the absence of FLV. White, grey, and black bars represent the normalized FIs of the Q-bodies for the samples containing 0.1, 1.0, and 3.0 μg/mL FLV, respectively. These concentrations are estimated as therapeutic, toxic, and fatal FLV levels[22]. Data are shown as mean ± standard deviation (n = 3).