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Insight into the working mechanism of Quenchbody: Transition of the dye around antibody variable region that fluoresces upon antigen binding

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ABSTRACT: Recently, we reported a novel immunoassay reagent Quenchbody (Q-body): a single chain antibody variable region (scFv) fragment labeled with fluorescent dye, whose fluorescence intensity increases when it binds to the antigen. Here we analyze its working mechanism by immuno- and fluorescence polarization (FP) assays. In an enzyme-linked immunosorbent assay, we found that in the presence of antigen osteocalcin peptide (BGP-C7), more TAMRA-labeled Q-bodies bound to anti-TAMRA antibody than in its absence. Moreover, we found that anti-BGP Q-body with the shortest linker that exhibits the largest antigen-dependency in fluorescence showed the highest binding signal. Similar results were obtained with anti-bisphenol A (BPA) Q-bodies, with inversed correlation with their linker lengths. In the FP assay, when the ATTO 520 labeled Q-body was added with antigen, the Brownian motion of the dye became more active, which resulted in reduced fluorescence anisotropy *r*. In other words, in the presence of antigen, 1/*r* showing the dye mobility is larger than that in the absence of its antigen. In addition, anti-BGP Q-body with the largest antigen-dependency in fluorescence showed the highest mobility. In overall, these results clearly suggest that the antigen-dependent fluorescence quenching and recovery of Q-body is caused by the movement of the dye within and around scFv, which moves out of scFv upon binding with its antigen.

Analytical methods that use antibodies, immunoassays, are becoming increasingly popular in the fields of clinical research and diagnostics to detect various biomarkers in the body fluids with high sensitivity and specificity [1, 2]. However, conventional immunoassay methods such as enzyme-linked immunosorbent assay (ELISA) are generally time and labor consuming before getting final result. Although faster and handier methods such as immunochromatography are being widely utilized, they are generally not very quantitative yet needs more than 15 minutes to complete measurement without compromising sensitivity[3, 4]. As a means to overcome such problems, recently we devised an innovative immunoassay reagent called Quenchbody (Q-body) [5-8]. Q-body is an antibody fragment, generally single chain variable region (scFv) or Fab fragment, whose fluorescence intensity increases upon adding its antigen. This is due to the quenching of the dye labeled at the N-terminus of the variable region Fv, by the intrinsic tryptophan (Trp) residues, and de-quenching (fluorescence recovery) accompanied by antigen binding. By the homogeneous assay using Q-body, a positive fluorescent signal that depends on the antigen concentration in a sample can be readily obtained within few minutes. Unlike ELISA, time-consuming procedures such as washing and enzyme reaction steps are not necessary. The only manipulation required is the addition of antigen to a tube or cuvette containing Q-body. Furthermore, not only proteins and peptides, but also low molecular weight chemical compounds of less than 1000 in molecular weight, like morphine and estradiol can be specifically detected with a similar sensitivity to that obtained in competitive assays [5, 9-11].

Regarding the mechanism of action for Q-bodies, previously we proposed a model that the system relies on the conformational change of antibody Fv upon antigen binding. In the absence of the antigen, the fluorescent dye such as TAMRA and ATTO 520 attached to the N-terminus of single chain Fv or Fab via a short peptide linker interacts transiently with the semi-conserved Trp residues in the variable region. Since the mutagenesis of each one of Trp residues resulted in reduced fluorescence quenching, these Trp residues are more or less responsible for the observed quenching, probably due to photoinduced electron transfer (PeT) [12]. However, in the presence of antigen, since the interaction between the V_H and V_L becomes stronger [13, 14] the dye has to move out of the variable region Fv in a competitive manner, therefore the quenching effect would be removed depending on the antigen concentration, which would result in the increased fluorescence.

However, although supported indirectly by the evidences, so far the hypothesis has not been confirmed by direct evidences. Here we analyzed its mechanism using ELISA and fluorescence polarization (FP) assay [15], and obtained results supporting the hypothesis that the fluorescence enhancement is caused by the outbound dye movement within the molecule. FP immunoassays have been successfully used to measure a range of small molecules in a rapid manner [16-18], while also utilized to evaluate internal dynamics of antibody molecule itself [19]. This time, we used this method to evaluate the motion of fluorescent dye in a Q-body.

■ RESULTS

First we analyzed the movement of the dye attached to a Q-body using ELISA (**Figure 1a**). If our prediction is correct, we can detect outbound movement of the dye in a Q-body using an anti-dye (TAMRA) antibody. We prepared two kinds of scFv-type Q-bodies: one for osteocalcin C-terminal peptide (BGP-C7) and one for bisphenol A (BPA). Each Q-body was expressed *in vitro* using an expression vector containing N-terminal amber codon-containing tag (ProX tag: NH_2-MSKQIEVN*SNET-COOH, *=amber codon), and purified using the C-terminally added His_6 tag. To detect the TAMRA dye exposed on the surface of a Q-body, the Q-bodies prepared by an *in vitro* translation reaction were added to the wells of a 96-well microplate that were pre-coated with anti-TAMRA antibody. To this solution, zero to saturating concentration (1000 nM) of cognate antigen, either osteocalcin C-terminal peptide (BGP-C7) or bisphenol A (BPA), were added and incubated [14, 20]. When the wells were washed and probed with peroxidase-labeled anti His-tag antibody to detect well-bound Q-body, we observed a significant antigen-dependent increase in binding signal (**Figure 1b**). From the dose-response curve for BGP-C7 in **Figure 1c**, the EC_{50} was calculated as 21 nM, which was very close to the EC_{50} of Q-body reaction previously reported (25 nM) [5] (**Supplementary Information Figure S1**). This observation clearly indicated antigen-dependent increase in the availability of the dye attached to the Q-body.

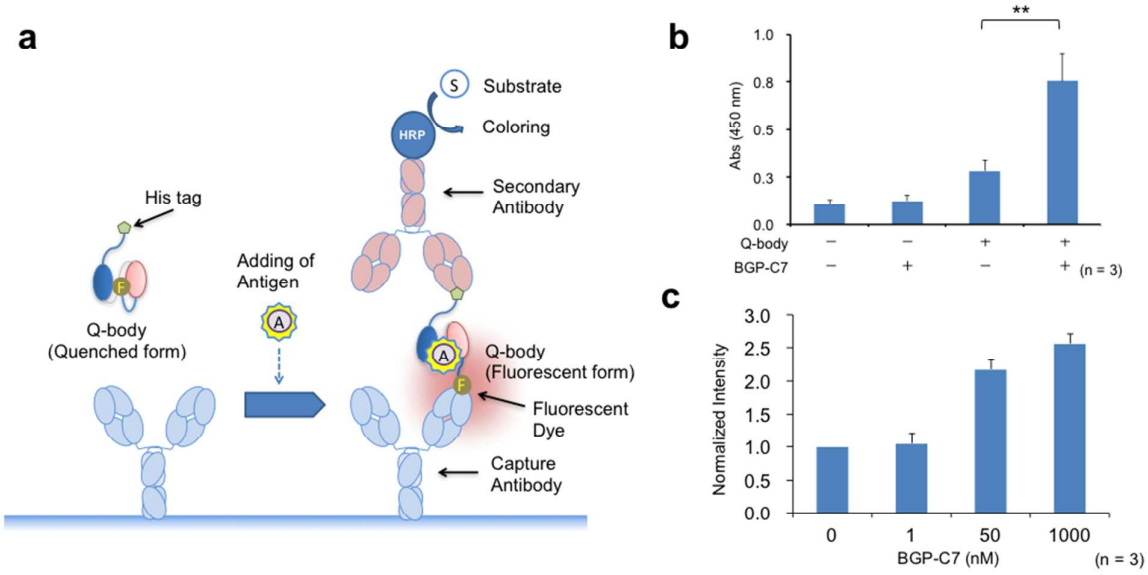

Figure 1. Quenchbody (Q-body) ELISA for the analysis of dye movement upon binding with the antigen. (a) Scheme of the assay. (b) Detection of exposed TAMRA by anti TAMRA antibody. (c) Anti-BGP Q-body ELISA with different concentrations of the antigen. $^{**}P < 0.01$

In addition, we conducted Q-body ELISA by using Q-bodies with different dye-protein linker peptide lengths. To this end, Q-bodies that have a flexible peptide linker $(Gly_3Ser)_n$ between ProX tag and scFv for BGP (n = 0, 2, 3) or BPA (n = 0, 2, 3, 5), were prepared. Previously, depending on the

scFv used, different linker length dependency in the maximum fluorescent response was observed. For anti-BGP Q-body, the fluorescent response was slightly reduced from 5.6- to 3.8-fold for the longer linker (n = 3), while the response of anti-BPA Q-body increased from 1.1- to 2.0-fold for the longer linker (n = 5) [5]. Therefore, this time we applied these Q-bodies with different lengths of a linker to the Q-body ELISA. As a result, the anti-BGP Q-bodies showed a generally increasing binding signal for longer linkers, while the ratio with and without antigen decreased for the longer linkers (**Figure 2, a and b**). On the other hand, the anti-BPA Q-bodies showed an increasing signal for longer linkers, as well as the significantly increased absorbance ratio (**Figure 2, c and d**). These results are in good agreement with the results previously obtained by the Q-body assay.

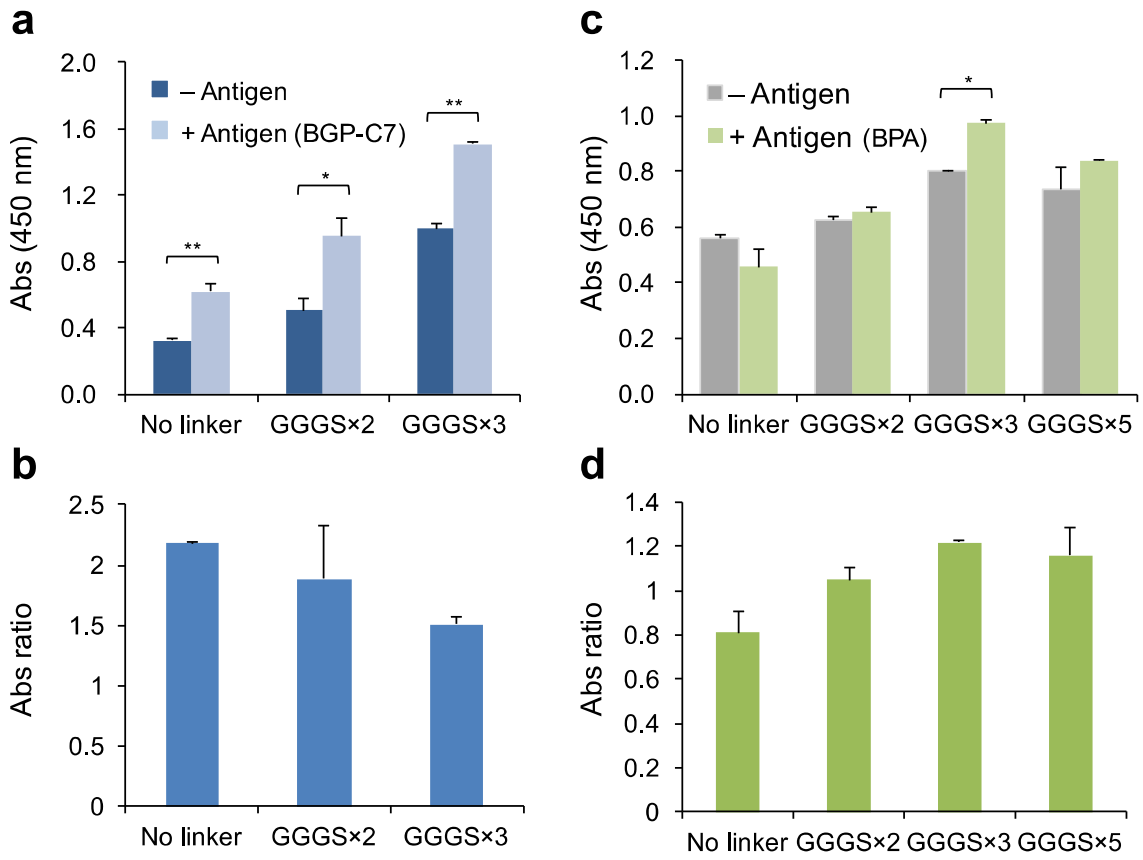

Figure 2. Effect of linker length between TAMRA and scFv in the cases of (a, b) BGP Q-body and (c, d) BPA Q-body. The absorbance with and without antigen (a, c), and their ratios (b, d) are shown. $^{*}P < 0.05$, $^{**}P < 0.01$

We inferred that the movement of a small fluorescent dye from the inside to the outside of the antibody Fv (MW ~30 kDa) will result in the increased Brownian motion (mobility) of the dye. Based on this assumption, next we tried to measure the change of fluorescence polarization (FP) according to the movement of fluorescent dye attached by a flexible linker to the Q-body. We predicted that the

mobility of the dye would increase in a linker length-dependent way when Q-body binds to its antigen (**Figure 3a**). FP is based on the principle that when a fluorescent molecule (the tracer) is excited with plane-polarized light, the emitted light is largely depolarized. The molecule rotates rapidly in solution during its fluorescence lifetime. If the fluorescent molecule's rotation is slowed, the emitted light remains in the same plane as it was excited [15]. Generally, anisotropy: r stands for the degree of the polarization of the light, which is calculated by the two fluorescence intensities $F_{\parallel}$ and$F_{\perp}$, where $F_{\parallel}$ is the emission intensity parallel to the excitation plane and $F_{\perp}$ is the intensity perpendicular to the excitation plane. In this study, we used $1/r$, which is considered as the degree of mobility of a fluorescent dye in a Q-body. First, we prepared three BGP Q-bodies with different lengths of flexible peptide linkers $(Gly_3Ser)_n$ (n = 0, 2, 3) between ProX tag and scFv. As a dye, we used ATTO 520, a Rhodamine 6G-like dye that is also suitable for making a Q-body, [6] since the maximum excitation/emission wavelengths of ATTO 520 was more suitable than TAMRA to the measurable wavelengths of the FP microplate reader.

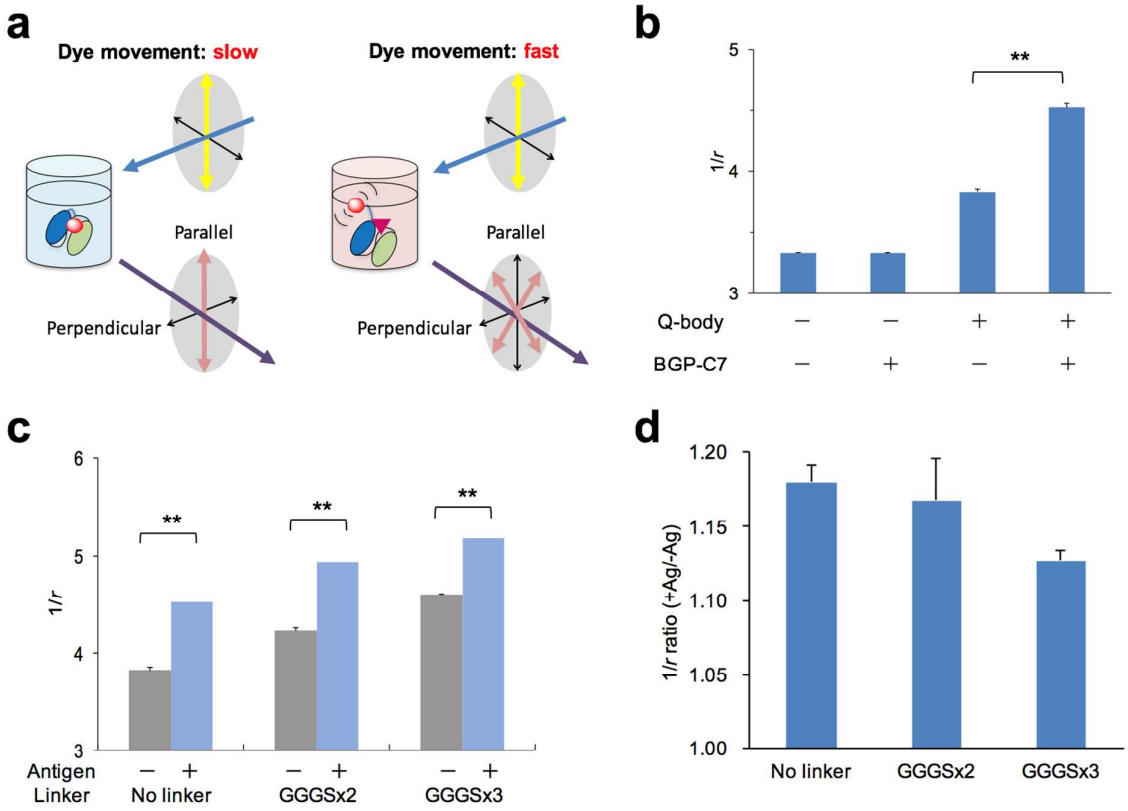

Figure 3. Fluorescence Polarization (FP) assay of anti-BGP Q-body (a) Schematic illustration of the assay. ATTO 520-labeled Q-body was subjected to FP assay in the presence and absence of antigen. (b) $1/r$ showing the dye mobility in the presence or absence of Q-body with zero linker and/or antigen. (c) Comparison of $1/r$ using the Q-bodies with different N-terminal tag lengths. (d) Ratios of $1/r$. The average of three measurements with 1 SD is shown. $^{**}P < 0.01$

The FP experiment showed that with the use of ATTO 520-labeled anti-BGP Q-body with no linker, a significant increase in 1/*r* was observed when the antigen BGP-C7 was added (**Figure 3b**). Since the molecular weight of antigen (MW: 894.03) was considerably smaller than that of scFv, its effect on 1/*r* seemed relatively small. In the FP experiment of Q-bodies with different lengths of linker, the Q-bodies showed somewhat different responses according to linker length. Irrespective of the presence or absence of antigen, increased 1/*r* was observed for the Q-body with longer linker (**Figure 3c**). On the other hand, when the ratio of 1/*r* in the presence and absence of antigen was calculated, it showed decreased value for longer linkers (**Figure 3d**). These trends were in good agreement with that of Q-body ELISA (**Figure 2, a and b**). Hence, in general, the longer the linker length is, the higher the degree of dye mobility becomes. However, their antigen dependency gets higher for the anti-BGP Q-body with a shorter linker. Similar results were obtained for anti-BPA Q-body with a different length of linker (**SI Figure S1**). In this case, a positive correlation between the antigen concentration and the linker length (**SI Figure S1a**) and a positive correlation between the 1/*r* ratio and the linker length was observed (**SI Figure S1b**).

To further validate the antigen dose-dependency of dye availability and 1/*r*, we employed an anti-methamphetamine (MP) [7] and an anti-deoxynivalenol (DON) [10] Fab-type Q-bodies (UQ-bodies). These UQ-bodies are incorporated with a TAMRA dye at the Pro-X tag with $(GlySer)_2$ linker added to the N-terminus of either heavy (MP) or light (DON) chain, and show antigen-dependent fluorescence increase up to 6-(MP) or 4-(DON) fold. When these UQ-bodies were subjected to Q-body ELISA, modest but significant antigen-dependent increase in signal was observed (**Figure 4, a and c**). The higher background signals (availability of the dye) in the absence of antigen might reflect lower quenching of the dye compared with anti-BGP Q-body. However, when the FP assay for these UQ-bodies was performed, and an MP analog- and DON-dose dependencies of 1/*r* plotted, a significantly higher increase than previously observed for anti-BGP and anti-BPA scFvs was observed (**Figure 4, b and d**). Moreover, for anti-DON UQ-body, even lower EC_{50} (44.1±2.9 nM) was observed than previously described for its fluorescent response (30 μg/mL= 101 nM). The apparently higher EC_{50} of anti-MP UQ-body (23.0±3.4 μM) compared with previously described (~5 μM) might reflect the use of MP analog and/or the difference in dye conjugation method (Cys-maleimide linkage). These results clearly show the higher rotational freedom of the dye in the presence of antigen.

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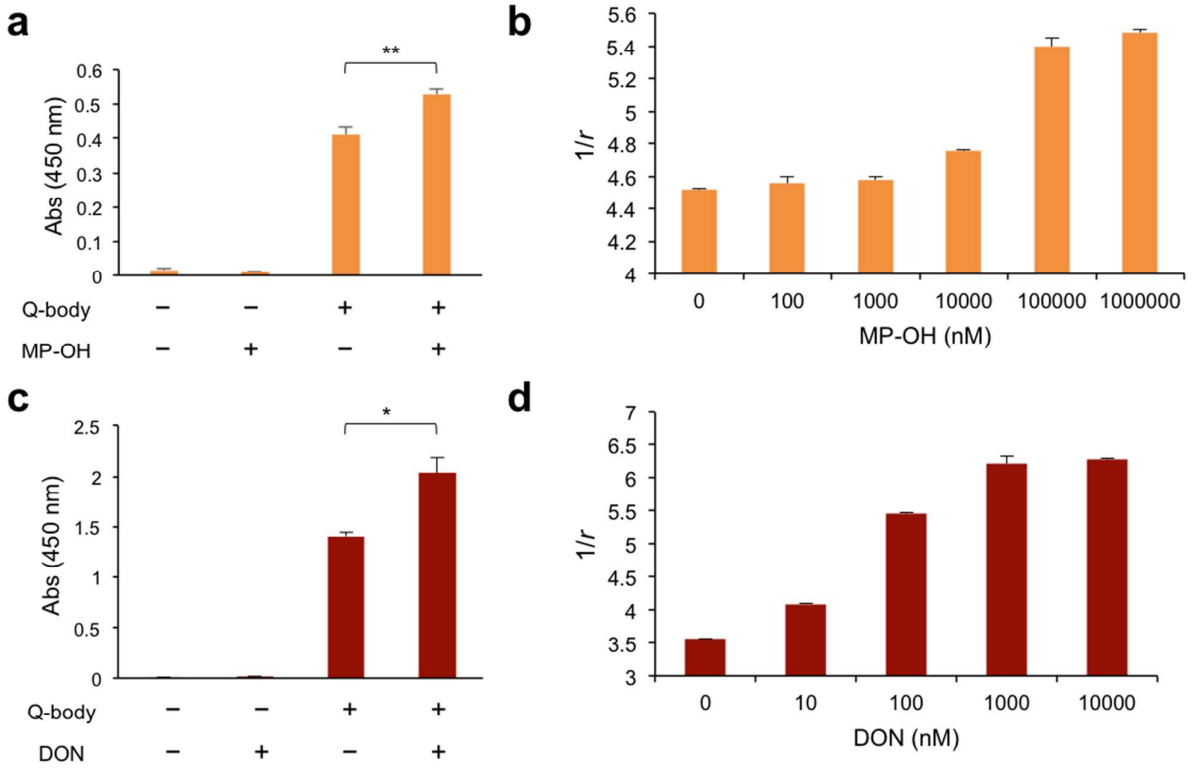

Figure 4. Dye-availability ELISA (a,c) and antigen dose-dependency evaluation by the FP assay (b,d) of TAMRA-labeled Fab-type Q-bodies. Anti-MP UQ-body (a,b) and anti-DON UQ-body (c,d) were used. MP-OH is an MP analog that reacts with this antibody. The average of three measurements with 1 SD is shown. $^{*}P < 0.05$, $^{**}P < 0.01$

DISCUSSION

In the ELISA of anti-BGP and anti-BPA Q-bodies with different lengths of a linker, the order of absorbance ratios was in good agreement with the order of fluorescent responses upon antigen addition. Before adding the antigen, probably being located at the certain position(s) inside the Fv, the dye in Q-body is most quenched by the nearby Trp residues. After adding the antigen, the dye is released from the position according to the antigen-antibody reaction. We assume that the optimal dye position and orientation vary depending on the Q-body used, and the relationship between the dye position and the linker length affects the degree of quenching. We consider that the shorter linker of anti-BGP Q-body, or the longer linker of anti-BPA Q-body, results in the placement of the dye to the position enabling the higher incidence of quenching that leads to the higher fluorescent response. In the presence of both Q-body and antigen (+Q-body/+antigen), when the linker becomes longer, higher absolute signal was obtained for both Q-bodies except anti-BPA with the longest linker. Probably, due to longer linker, the fluorescent dye becomes generally more exposed to anti-dye antibody, which resulted in the higher absolute signals.

In the FP assay of anti-BGP Q-bodies, similar trends of linker length dependency were

observed for the Q-body antigen response and the antigen response in terms of dye mobility ($1/r$). Probably, as well as in the ELISA, this was also due to the relationship between the optimal position of the dye and the linker length before antigen-antibody reaction. Also, $1/r$ shows a similar tendency to that of ELISA signal strength because the shorter the linker of anti-BGP Q-body or the longer the linker of anti-BPA Q-body allows the fluorescent dye to locate at the most suitable position before the antigen-antibody reaction. Previously our model was supported by several indirect observations including i) the observed maximum fluorescence intensity of Q-body roughly matches that obtained by the denaturation by 7 M guanidium hydrochloride and 100 mM dithiothreitol, and ii) the fluorescence species with shorter life time, which implied PeT-derived quenching, decreased after adding antigen. Here we showed an evidence that the response of Q-body in antigen-antibody reaction is strongly associated with fluorophore motion. Our result supports the following motion of the fluorescent dye: The fluorescent dye quenched by the Trp residues staying inside Q-body before antigen-antibody reaction exposes itself outside the Q-body due to the conformational change of scFv caused by the antigen-antibody reaction, increasing its degree of freedom. As a result, the fluorescent intensity increases, it becomes easier for the fluorescent dye to bind to the anti-dye antibody, and the mobility of the dye also increases. However, a mystery remains on the reason why the linker length dependency and also the absolute degrees of quenching differ from antibody to antibody. Further analysis based on the defined antibody structures will clarify this phenomenon.

In general, the FP assay of Q-bodies showed lower signal fluctuation due to ratiometric measurement. Especially, Fab-type Q-bodies showed higher signal change probably reflecting the higher molecular weight of Fab than scFv. Due to its simplicity and no need of fluorolabeled hapten for the competition, it might be a method of choice for small molecule detection.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:
Experimental procedures, a figure for the FP analysis of anti-BPA Q-body.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BGP, bone Gla protein or osteocalcin; BGP-C7, C-terminal 7 residue peptide of BGP; BPA, bisphenol A; DON, deoxynivalenol; ELISA, enzyme-linked immunosorbent assay; FP, fluorescence polarization; Fv, antibody variable region; MP, methamphetamine; PBS, phosphate buffered saline; Q-body, Quenchbody; scFv, single chain Fv; V_H, antibody heavy chain variable region domain; V_L, antibody light chain variable region domain;

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