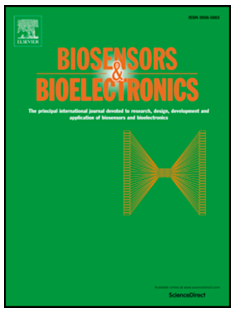

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PM Q-probe: a fluorescent binding protein that converts many antibodies to a fluorescent biosensor

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ABSTRACT

Quenchbody (Q-body) is a fluorescent biosensor in which a fluorescent dye is tagged near the antigen binding site of an antibody. The fluorescence of the dye is quenched by the tryptophan residues present in the variable region of the antibody, and is recovered when the antigen binds. Q-bodies have been prepared using recombinant DNA technology by introducing one or more tag sequence(s) at either the N-terminal of the Fab or the single chain variable region fragment of the antibody, and labeling the tag with a fluorescent dye. However, preparation of recombinant antibody fragments is time-consuming and the performance of the Q-body is unpredictable. Here we report an antibody-binding quenching probe made from protein M from *Mycoplasma genitalium* that can transform the IgG antibody into an immunosensor. By using bacterially expressed and purified protein M and labeling the C-terminal cysteine-containing tag, we prepared a TAMRA-labeled PM Q-probe. When the Q-probe was incubated with Fab or IgG recognizing the bone Gla protein, the fluorescence of the probe was quenched and subsequently recovered by the adding of antigens in a dose-dependent manner. We also succeeded in detecting several small biomarkers with nanomolar sensitivity, including thyroxine extracted from human serum. The clone found to be suitable for the detection of cortisol was confirmed to work as a recombinant Q-body as well, which also worked in 50% human serum. The results suggest that the Q-probe can quickly convert an IgG to a biosensor, which will be useful in rapid diagnosis of small biomarkers.

Keywords: antibody binding protein, immunosensor, protein M, fluorescence quenching, homogeneous immunoassay, sortase

1. Introduction

Quenchbody (Q-body) technology is a fluorescent biosensor-based homogenous immunoassay that does not need washing steps to separate the unbound antibodies (Abe et al. 2011). When a fluorescent dye is used as a label at a site near the antigen-binding pocket of an antibody fragment, it moves inside the antibody fragment. This results in the quenching of dye-fluorescence by amino acid residues, namely tryptophan (Trp), present on the antibody, through a photo-induced electron transfer mechanism. However, when an antigen is added to the quenched Q-body, the fluorescence of the dye is recovered upon antigen-binding. So far, Q-bodies using antibody fragments, such as the single chain variable region (scFv) and antigen binding fragments (Fab) have been reported. The first Q-body was fortuitously discovered when fluorescent dye-labeled aminoacyl transfer RNA was used to label an antibody fragment using a cell-free translation system based on unnatural amino acid incorporation (Abe et al. 2010). Subsequently, Q-bodies with better sensitivity, named Ultra Q-body, were made from Fab fragments, which were either expressed *in vitro* or in bacteria, and labeled with fluorescent dye(s) at the N-terminal cysteine-containing tag(s) (Abe et al. 2014). Q-bodies have been successfully used to detect not only small molecules, such as the bone Gla protein (BGP) peptide, recreational drugs (Abe et al. 2011), protein phosphorylation (Jeong et al. 2013), amyloid beta peptide (Dong et al. 2018), neonicotinoid insecticide (Zhao et al. 2018), and antidepressant fluvoxamine (Sasao et al. 2019), but also larger proteins, such as claudin-4 (Jeong et al. 2017a) and hemagglutinin from influenza viruses (Abe et al. 2014; Jeong et al. 2018). For the preparation of Q-bodies, thiol-maleimide reaction (Abe et al. 2014), transamination reaction (Dong et al. 2016), and photochemical crosslinking to nucleotide-binding sites on the antibody (Jeong et al. 2017b), have been successfully used. However, although the Trp residues in the antibody variable region are well conserved (> 95%), not all antibodies can be converted to a Q-body that shows a good response. This is probably because of structural factors including the accessibility of the dye to Trp residues (Ohashi et al. 2016), but not much is known yet. Hence, a faster and more convenient method to predict the suitability of an antibody to be converted into a Q-body has been long-awaited.

So far, several antibody-binding proteins from bacteria have been reported. Protein A (PA), with a molecular weight of 42 kDa, a component of the cell wall of *Staphylococcus*

aureus, has four antibody-binding domains, each of which binds strongly to the Fc region of IgG (Forsgren and Sjoquist 1966; Langone 1982) and weakly to some Fab fragments at the V_H domain (Roben et al. 1995; Starovasnik et al. 1999). Protein G (PG), a component of the cell wall of groups C and G *Streptococcal* bacteria, also binds strongly to Fc fragments of IgG at its binding domains (Bjorck and Kronvall 1984; Sjobring et al. 1991) but shows weaker binding affinity to the Fab region of IgG. Previously, we reported a fusion protein made of one binding domain each of PA and PG, which showed greater IgG binding activity than either of the domains, presumably due to bivalent binding to the Fab and/or Fc region (Dong et al. 2015). It was also used to convert Fab fragments to Q-bodies (AG Q-probe), which showed a limited but reproducible antigen-dependent fluorescence response of up to ~6% (Jeong et al. 2016). However, it could not convert the whole IgG to a Q-body, because of its stronger binding affinity to the Fc region than the Fab region. Moreover, only 12% of mouse Fab was reported to bind to PA (Graille et al. 2002), indicating its limited utility.

Recently, a novel antibody light chain binding protein Protein M (PM) was discovered in *Mycoplasma genitalium.* It has a larger domain size than PA and PG, and binds tightly to almost all the light chains from many species, with a high affinity at low nanomolar dissociation constants (Grover et al. 2014). Although these better properties are probably due to its wider binding area, the binding of PM to the antibody hinders the binding of protein antigens. However, its effect on small antigen binding is not yet known. Considering the structural proximity of the C-terminus of the PM to the antigen-binding site of the bound antibody, we engineered a PM with an additional cysteine (Cys) residue appended at its C-terminus via a short flexible linker. As a result of the fluorescence labeling of the protein at its C-terminus, a fluorescent probe (Q-probe) capable of converting Fab or IgG antibodies into a fluorescent sensor that acts like a Q-body showing a more than 100% increase in fluorescence was successfully constructed. The Q-probe was successfully used to screen several anti-small biomarker antibodies for their suitability as a Q-body, and an anti-thyroxine IgG was successfully applied to the detection of total thyroxine concentration in serum. Finally, the response of the Q-probe/anti-cortisol IgG complex was compared with the recombinantly made scFv Q-body to evaluate its performance both in a buffer and in serum, to show the potential of this approach in clinical diagnosis.

2. Materials and methods

2.1 Materials

The materials used for recombinant protein preparation are described in the Supplementary Material. StrepTactin agarose and StrepTactin-Horseradish peroxidase (HRP) conjugate were from IBA (Goettingen, Germany) and Bio-Rad (Hercules, CA, USA), respectively. Fluorescent dye 5-TAMRA-C6-maleimide was purchased from Setareh Biotech, Inc. (OR, USA). Anti-cAMP monoclonal antibody (mAb) SPM486, anti-progesterone mAb (Pg.53), anti-testosterone mAb (4E1G2), anti-thyroxine mAb (ME.125), anti-5-bromodeoxyuridine mAb (MoBu-1), and anti-penicillin mAb (Pen9) were purchased from Abnova (Taoyuan, Taiwan). T3/T4-deficient human serum and pooled human serum were obtained from Sigma (S7144, St. Louis, MO) and MP Biomedicals (Irvine, CA), respectively. Other chemicals, reagents, and antibodies were all obtained from Sigma or Fujifilm Wako Chemicals (Osaka, Japan), unless otherwise indicated.

2.2 Preparation of PM Q-probe

Construction of expression vector pTrx'st2-PM-C to express the fusion protein of Cys-deprived thioredoxin (Trx') and Protein M with C-terminal Cys-tag is described in the Supplementary Material. The expression and purification of the PM probe, fluorescence labeling to yield a PM Q-probe, SDS-PAGE and enzyme-linked immunosorbent assay (ELISA) analyses, and the construction of the scFv (CS4-6)-based cortisol Q-body are described in the Supplementary Materials.

2.3 Fluorescence measurement

The fluorescence of the samples was measured using either a FP-8500 fluorescence spectrophotometer (Jasco, Tokyo, Japan) with a quartz microcuvette (Starna Scientific, Ilford, UK) or a CLARIOstar fluorescence microplate reader (BMG Labtech Japan, Saitama, Japan) with a 96-well half-area black microplate (675076, Greiner Japan, Tokyo). For spectroscopy, the measurement temperature, excitation and emission band width, and scanning speed were

set at 25°C, 5 nm, and 500 nm/min, respectively, and excitation wavelength was set to 546 nm. A dose-response curve was fitted to a four-parameter logistic equation using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). The limit of detection (LOD) was calculated as the concentration corresponding to the mean blank value plus three times the standard deviation, for each assay.

2.4 Detection of T4 and CS in serum

For the detection of total T4 in serum using the Q-probe, 100 µL of T4-spiked T3/T4-deficient human serum at 0 nM or 20 nM was mixed with 210 µL of 95% ethanol by vortex, and centrifuged at 15 kg for 10 min to remove proteins. The supernatant was carefully taken to a fresh 1.5 mL microcentrifuge tube and put into a microcentrifuge-concentrator MV-100 (Tomy, Tokyo, Japan) overnight to evaporate the liquid. The pellet was dissolved in 100 µL of sterilized water and used for the fluorescence assay, wherein 25 µL of sample was added to the same volume of the mixture of 40 nM Q-probe and 20 nM antibody in 10 mM phosphate-buffered saline, 0.05% Tween20, pH 7.4 (PBST), and compared with the T4 standards serially diluted in 50 µL PBST containing the same amounts of Q-probe/IgG.

For the detection of CS, the purified scFv Q-body (2 nM) was mixed with CS dissolved in 2 µL methanol in 100 µL of PBST, or in 50 % human serum in PBST, in a well of microplate. After allowing the reaction to proceed at 25°C for 5 min, the fluorescent intensity was measured with a fluorescence microplate reader with ex/em wavelengths of 530/20 nm and 580/20 nm, respectively.

3. Results and discussion

3.1 Construction of PM probe expression vectors

Protein M (PM) has recently been discovered as an antibody-binding protein that binds tightly to almost all types (κ and λ of many species) of antibody light chains, predominantly at their variable region V_L. The solved crystal structure of the PM-Fab complex shows that the C-terminal region of PM is located just above the antigen-binding pocket and interferes with

Fig. 1

the binding of the protein antigens, which potentially limits its utility as a probe for antibody labeling (Fig. 1A). However, since its inhibitory effect on small antigen binding was not clear, we expressed residues 74 to 468 of *Mycoplasma genitalium* PM (called PM TD) (Grover et al. 2014) as a fusion protein with N-terminal thioredoxin (Trx), which was used as a solubilization tag. In addition, we added a short tag containing C-terminal Cys (SGGGSGGGC) which would be used as the site for fluorescent dye labeling. To avoid the labeling of two Cys residues in the active site (WCGPC) of Trx, both the Cys residues were mutated to Ser, yielding a null-Cys Trx (Trx'). Moreover, to expedite purification after dye-labeling, we added a Twin-Strep-tag in the linker region between the Trx'-His tag and PM to efficiently remove unreacted dye while keeping a high yield (Fig. S1).

3.2 Preparation of PM Q-probe

The fusion protein to be expressed consisted of Trx', His-tag, Twin-Strep-tag, PM, and a C-terminal Cys-tag (Fig. 1B). It was expressed in the cytoplasm of *E. coli*, extracted, and purified by IMAC. As shown in Lane 1 of Fig. 1C, a thick protein band at around 65 kDa was observed, which was in accordance with its theoretical molecular weight of 65.1 kDa, suggesting that it was correctly expressed as designed. We obtained more than 1 mg of the purified protein from 300 mL culture. We then labeled the protein with 5-TAMRA C6 maleimide and purified it using StrepTactin agarose. Fig. 1D shows the fluorescence of labeled PM (TAMRA-PM; PM Q-probe). Lanes 2 and 3 show two fractions eluted during purification, both of which showed strong fluorescence, suggesting that the PM Q-probe was successfully prepared with high yield (>500 μg purified protein from 500 mL culture).

The antibody-binding activity of the PM probe was confirmed by ELISA, using immobilized human IgG and StrepTactin-HRP for detection. As shown in Fig. 1E, the PM probe bound to human IgG and gave a strong signal while no signal was observed for the wells without immobilized IgG. This suggests that the prepared PM probe retained strong antibody-binding activity.

3.3 Expression and purification of KTM219 Fab fragment

KTM219 is a monoclonal antibody against human osteocalcin, which is also known as

bone Gla protein (BGP) (Lim et al. 2007). BGP is a peptide consisting of 49 amino acids and is used as a biomarker for bone metabolism, and known for its unprecedented roles in other body metabolisms such as glucose homeostasis, male fertility and brain development (Lee et al. 2007; Oury et al. 2011; Oury et al. 2013). Previously, we showed that labeling the N-terminal region using a Cys-tag could convert the antibody Fab fragment to a Q-body for detecting BGP at the C-terminal 7-residue epitope (BGP-C7) (Abe et al. 2014). Therefore, we first prepared the KTM219 Fab fragment without a Cys-tag and tested whether the PM Q-probe was capable of converting Fab to a biosensor for BGP. KTM219 Fab was expressed in the oxidative cytoplasm of *E. coli* SHuffle®T7 Express lysY, purified by IMAC, and analyzed by SDS-PAGE. As shown in Fig. 2A, two clear bands were observed at 27 kDa and 25 kDa, for the Fd (V_H1-C_H1) and light chain of the antibody, respectively. The antigen-binding activity was confirmed by ELISA as described (Dong et al. 2016).

Fig. 2

3.4 Creation of the BGP biosensor with the PM Q-probe

Having previously established that the KTM219 Fab fragment can be an effective Q-body when the N-terminus of H chain is tethered with the TAMRA dye (Abe et al. 2014), we tested whether the prepared PM Q-probe binds to this Fab fragment to quench the fluorescence of the TAMRA dye attached to the Q-probe (Fig. 2B). To find the best quenching conditions, the PM Q-probe, at a final concentration of 5 nM, was titrated with increasing concentrations of KTM219 Fab and the fluorescence of the mixture was compared with that of the PM Q-probe alone, after incubating for 5 min each at 25°C. As shown in Fig. 2C and D, the fluorescence peak of the PM Q-probe at 500 (a.u.) was quenched to approximately 300 (a.u.) by the addition of the Fab fragment at up to 150 nM, with an EC_{50} of 4.2 nM for the Fab. The fluorescence quenching plateaued at around 15 nM. The EC_{50} observed was in accordance with the reported K_d values of Protein M to antibody (approximately 1.2–5.2 nM) (Grover et al. 2014).

Further, we attempted to detect the dequenching by antigens using a mixture of 5 nM Q-probe and 15 nM Fab (Fig. 2E). When the BGP-C7 antigenic peptide was added to this mixture, the fluorescence of the mixture increased in a dose-dependent manner, up to 2.0-fold compared to the sample with the equivalent volumes of PBST buffer added, with an EC_{50} of

81±8 nM. To further confirm the potential of this system, the dequenching by full-length human BGP was evaluated. A dose-response curve with an EC_{50} value of 315±80 nM was obtained. The observed difference in EC_{50} was also observed for the scFv Q-body, as seen previously, probably reflecting the required unfolding of full-length BGP for the binding (Abe et al. 2011). These results suggest that the PM Q-probe could convert the KTM219 Fab to a complex that behaves like a Q-body, and the complex could detect the target antigens.

Next, we tested this system for whole KTM219 IgG (Fig. 3). In this experiment, the Q-probe was mixed with KTM219 IgG at a concentration of 7.7 nM and 3.8 nM, separately. As in the case of KTM219 Fab, the fluorescence of the PM Q-probe was quenched with IgG and recovered with the addition of BGP peptide, with an EC_{50} of 7.3±0.6 nM. It is worth noting that this value is lower than that obtained with the Fab fragment and also that of scFv Q-body (25 nM) (Abe et al. 2011). This discrepancy might be because the natural IgG has more stable structure and higher activity than the scFv fragment prepared in bacteria. Of note, no increase in fluorescence was observed in the negative control with varied concentrations of Flag peptide, showing the specificity of the Q-probe system.

Fig. 3

3.5 Screening of an anti-hapten antibody panel

Since a similar fluorescence response was obtained with the Q-probe for both Fab and IgG of KTM219, we speculated that the PM Q-probe could be used as a handy tool to find and convert an antibody to a Q-body for detecting small-molecule antigens. To test this possibility, several commercially available anti-hapten monoclonal antibodies (mAbs) including anti-cAMP, anti-progesterone (PG), anti-testosterone (TS), anti-thyroxine (T4) clone ME.125, anti-5-bromodeoxyuridine (BrdU), and anti-penicillin (Pen) were tested. Additionally, another anti-T4 clone 22G8A2 was prepared and tested. The PM Q-probe solution, at a final concentration of 1 nM, was added to each mAb at a final concentration of 25 nM, incubated at 25°C for 5 min, and the fluorescence intensity with and without mAb addition was measured. As shown in Fig. 4A, in this condition, anti-cAMP, anti-PG, anti-TS, and anti-T4 antibodies quenched the PM Q-probe to different extents; 92.2%, 75.2%, 49.9%, and 90.1%, while anti-BrdU and anti-Pen antibodies quenched negligibly at 99.2% and 99.7%, respectively. In another set of experiments, anti-T4 22G8A2 at a lower concentration (5 nM)

Fig. 4

showed stronger quenching to 51.1% (Fig. 4B), suggesting that the degree of quenching of each mAb can be measured easily and that the degree differs depending on the mAb used, not on the target antigen. It also implies that we can expect to obtain suitable mAb clone(s) after Q-probe screening of several mAbs specific to a hapten of choice.

3.6 IgG-type Q-body for haptens

To test whether the quenched Q-probe:IgG complexes could be used as biosensors for the corresponding antigens, Q-probes complexed with anti-PG, anti-TS, and anti-T4 clone 22G8A2 were further investigated. PM Q-probe and anti-PG IgG were mixed at molar ratios ranging from 5:1 to 1:25 and incubated for 10 min. Subsequently, 50 μM of PG was added and fluorescence was measured after a 5 min incubation period. As shown in Fig. S2A, the fluorescence of Q-probe/anti-PG increased up to 1.35-fold after PG addition. Based on this result, the PM Q-probe and anti-PG mAb were mixed at concentrations of 1 nM and 5 nM, respectively, titration with 0 to 1 μM of PG was performed, and fluorescent spectra were obtained from 560 nm to 650 nm, after 5 min of incubation. As shown in Fig. S2B, a single emission peak at around 580 nm was observed, and its peak intensity increased with increased concentrations of PG. Based on these data, a dose-response curve with an EC_{50} of 11±4 nM could be drawn for the detection of progesterone, and the LOD was calculated as 0.96 nM (Fig. 4C).

Similar assays were carried out for anti-TS and anti-T4 antibodies. After the determination of optimal probe:IgG ratios (Fig. S3) and probe concentrations (Fig. S4), the PM Q-probe and corresponding antibody were mixed at 1 nM and 5 nM for TS and at 20 nM and 10 nM for T4, respectively. Titration and subsequent detection with increasing concentrations of antigen were performed (Fig. 4, D and E, Fig. S3B). Dose-response curves with EC_{50} values of 1.37±0.05 nM and 15.8 ± 1.2 nM and LODs of 0.35 nM and 4.05 nM were obtained for TS and T4, respectively. These data clearly show that many mAbs, including commercially available ones, induce the quenching and antigen-dependent dequenching of the Q-probe, both of which are prerequisites of Q-body.

3.7 Detection of total T4 in serum

T4 is a major thyroid hormone and an important biomarker for the diagnosis of hyperthyroidism and hypothyroidism. To verify whether the system can be applied to the determination of biomarker concentration in the blood samples, we attempted to measure total T4 in human serum. To this end, we spiked T4 to T3/T4-deficient human serum at concentrations of 0 and 20 nM. After a brief ethanol extraction of the samples to denature and remove interfering proteins, including T4-binding proteins and intrinsic antibodies that could affect the Q-probe response, the same volume of Q-probe/antibody mixture that made up the spiked concentration of T4 to 0 and 10 nM was added to the samples, and the signals were compared with the standard curve (Fig. 4E). After subtracting the background fluorescence of the non-spiked (0 nM) sample, the T4 concentration was calculated as 8.28 (±1.31) nM, giving a recovery rate of 82.8%. The result showed the utility of the Q-probe/antibody system to detect T4 in serum at less than the physiological concentration (normal range: 5-12 μg/dL ~ 64-154 nM) (Burtis 1996).

3.8 Screening and conversion of anti-cortisol IgG to a Q-body

To evaluate the utility of the PM Q-probe for the screening of suitable antibody clones as Q-bodies, a panel of murine mAbs established for cortisol, namely CS3-3, CS4-6, CS10-2, and CS13-11 (Oyama et al. 2015), in which the antibodies were denoted as Ab#3, #4, #10, and #13, respectively, was tested. The hybridoma clone producing each antibody was cultured and the antibody secreted into the culture medium was purified using Protein G magnetic beads. Thereafter, it was used for the Q-probe assay (Fig. S5A). Upon mixing the PM Q-probe with each of the above-mentioned antibodies, clones CS4-6 and CS13-11 quenched Q-probe fluorescence significantly, while the others did not (Fig. S5B). When cortisol was added at 25 μM to the Q-probe/IgG complex, we found an increase in fluorescence of the Q-probe/CS4-6, while no significant increase was observed for Q-probe/CS13-11 (Fig. S5C). Therefore, CS4-6 was considered suitable as a biosensor for detecting cortisol. As before, after the optimization of the Q-probe:IgG ratio at a final Q-probe concentration of 1 nM (Fig. S6A), the ratio of 2:1 was adopted. To measure dequenching, cortisol was added to the mixture, and time-dependent fluorescence recovery was measured. As shown in Fig. S6B, dequenching reached a maximum of ~2.2-fold after 5 min, when the ratios were larger than

2:1. Therefore, when the ratio of Q-probe to IgG was 2:1, 10 min incubation for quenching and 5 min for dequenching were long enough to perform the assay.

Based on the above results, a mixture of 1 nM of PM Q-probe and 500 pM of CS4-6 IgG was prepared in PBST, incubated for 10 min, and used to detect cortisol. When cortisol was added for titration at concentrations ranging from 0 to 100 μM, the fluorescence intensity increased with the increased concentration of cortisol. A dose-response curve is shown in Fig. 5A. The maximum response and EC_{50} of the assay for cortisol were 2.17-fold and 7.3±1.4 μM, respectively, and the LOD was calculated as 0.56 μM.

Fig. 5

3.9 Comparison with recombinant cortisol Q-body

The Q-probe can be used as a tool to find a good backbone mAb to be used to make an effective Q-body. To compare the performance of the PM Q-probe with the recombinant Q-body, the previously cloned single-chain Fv gene for CS4-6 was used to prepare a Q-body. This time, we used *E. coli* secretory expression system combined with a sortase-mediated fluorescence labeling method, to avoid unintended promiscuous labeling of Cys inside antibody domains (Fig. 5B). Although the SHuffle®T7 Express lysY strain can form disulfide bonds of the expressed Fab and scFv fragments, we experienced different folding and soluble expression yields depending on the clone. In this case, we successfully expressed, purified, and labeled two types of scFv fragment with an N-terminal Sortase substrate sequence (GGGTG), with and without a short (GGGS) linker. After reacting with TAMRA-LPETGG peptide, sufficient amounts of labeled scFv fragments were obtained. The normalized dose-responses are shown as Fig. 5C. The maximum response and EC_{50} of scFv Q-bodies were, 2.23-fold and 26.7 μM for no linker-type and 2.46-fold and 45.2 μM for the G_3S linker-type. The maximum response of recombinant Q-bodies was somewhat higher than that of the Q-bodies made with the IgG/Q-probe, and their EC_{50} values were also higher, probably due to their decreased affinity.

The G_3S linker-type Q-body was also used to measure CS in human serum. As shown in Fig. 5D, an equivalent or even higher response was observed in 50% human serum with similar sensitivity. Possibly, this was due to higher quenching/antigen-dependent de-quenching of TAMRA dye in the presence of serum.

4. Conclusions

As a fluorescence-labeled antibody-based biosensor, Q-body can detect many targets—from small molecules to large proteins—with high sensitivity and selectivity. Moreover, because of its very simple detection principle, the assay requires only seconds to minutes to obtain a signal, which is suitable for real-time monitoring of antigens *in situ*. Q-body enables homogeneous immunoassay and has many merits. It requires no washing steps, nor a large sample volume. Hence, these unique properties enable Q-body to be used in a range of applications in many fields such as the detection of illegal drugs, food inspection, and point-of-care testing. However, widespread application has been hindered by the difficulty in obtaining suitable Q-bodies for the desired targets in a timely manner.

To overcome this problem, we developed a method using Protein M to make and/or select Q-bodies with practical utility. The response received from the PM Q-probe with KTM219 Fab was superior to that of the AG Q-probe, probably owing to its higher affinity. More importantly, it also works in combination with whole IgG, showing better sensitivity in terms of EC_{50} for BGP-C7 and cortisol. This is probably because IgG has a more stable structure and higher affinity than recombinant antibodies. Although the maximum response of the KTM219 IgG/Q-probe complex was lower than that of the corresponding conventional Q-bodies, this may be due to the difference in dye location, which is attached near the L chain rather than the N-terminus of the H chain, in conventional Q-bodies. However, higher sensitivity and low reduction in response, compared with recombinant Q-body, was observed for the anti-CS IgG/Q-probe, which suggests the potential widespread utility of the method.

There is another antibody light-chain-binding protein Protein L (PL) from *Peptostreptococcus magnus,* which binds to certain classes of kappa light chain from some human, mouse, and rat antibodies at the V_L region with high (K_d~ nM) affinity. However, not all kappa chains and no lambda chains can bind to PL (Bjorck 1988). Specifically, only one subtype (VkI) of the mouse kappa chain can bind to it. In theory, PL-based Q-probe, if any, can also convert some IgGs posessing kappa light chains. However, considering its limited binding repertoire and two kappa binding sites in the domain (Graille et al. 2001), PL is not expected to have applications similar to the PM Q-probe.

When we tested six commercially available mAbs and four anti-CS mAbs, two each of them did not show detectable fluorescence quenching. As reported previously, the fluorescence quenching is mostly attributable to the photoinduced electron transfer from tryptophan (Trp) residues of the antibody V region to the fluorescent dye (Abe et al. 2011). However, the quenching efficiency depends on the antibody and the dye used, not the antigen, probably due to the interaction strength of the dye with the antigen-binding pocket. Possibly, the antibodies with weak V_H/V_L interaction (Ueda et al. 1996) can accept the intrusion of the dye into the pocket, while those with strong interaction cannot. It is difficult to gauge the structural plasticity of the V region based on the exterior structure of full-sized antibodies. Our PM Q-probe is the first to measure the "quenchability" of many antibody-dye pairs just by mixing them.

In this study, we successfully prepared a PM Q-probe that can quickly find an antibody suitable for use as a Q-body, and also quickly convert the antibody to a Q-body. For the first time, the testing and conversion of a small amount (less than microgram) of many full-sized IgGs to a sensitive fluorescent immunosensor is possible, which has never been achieved by the AG Q-probe. It is also worth mentioning that the PM Q-probe/IgG complexes show similar or higher sensitivity compared with the recombinant Q-body, while PM was reported to hinder antigen binding. It seems that the PM Q-probe does not affect antigen binding in the cases of small haptens and peptides, which allows easier signal-on detection of these antigens that usually need competitive (signal-off) immunoassays to quantify. The upper limit of detectable antigen size for the PM Q-probe is now under investigation, but full-length BGP (5.8 kD) has already been successfully detected. In future, detection of larger antigens might be possible by introducing appropriate mutations or deletions to PM.

In conclusion, we believe that this work will contribute to further development of Q-body technology and its wider application in the analysis of biological, environmental, industrial, and clinical samples.

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Figure legends

Fig. 1. Preparation of PM Q-probe. A. The 3D structure of Protein M-Fab complex based on PDB entry - 4NZR. The C-terminal Arg of PM TD (orange) and Trp residues in the variable region of the antibody (blue for H chain and magenta for L chain) are shown as a space-filling model. **B**: Scheme of the expression vector for the PM probe. TrxAΔC: Thioredoxin A devoid of Cys residues; His: His_6 tag; ST: Twin-Strep-tag; Cys: a flexible tag containing C-terminal Cys. **C**: Coomassie Brilliant Blue staining of the gel after SDS-PAGE of the purified PM probe before (lane 1) and after (lanes 2 and 3) dye labeling. M: Precision Plus protein unstained standards (Bio-Rad) to which one fiftieth of Dual Color marker was mixed. **D**: The red fluorescence image of the same gel. **E**: IgG-binding activity of the PM probe measured by ELISA. hIgG: human IgG immobilized in the wells. Error bars represent a standard deviation (SD) of triplicate samples.

Fig. 2. Quenching of the PM Q-probe upon Fab binding. **A**: SDS-PAGE analysis of purified anti-osteocalcin KTM219 Fab. M: Precision plus protein unstained standards (Bio-Rad); **B**: Scheme of PM Q-probe assay. BGP-C7: C-terminal 7-mer peptide of osteocalcin; **C**: Fluorescence spectra showing the Fab dose-dependent quenching of PM Q-probe. **D**: Dose-response curve for KTM219 Fab. Emission at 580 nm was plotted. **E**: Dose-response curves for BGP-C7 and full-length human BGP obtained with PM Q-probe/Fab complex. Mean values with error bars of 1 SD are shown.

Fig. 3. Conversion of IgG to an osteocalcin sensor by PM Q-probe. **A**: Schematic image for PM Q-probes converting IgG to a biosensor; **B**: Dose-response curves for BGP-C7 antigen and Flag negative control peptides. Mean values with error bars of 1 SD are shown.

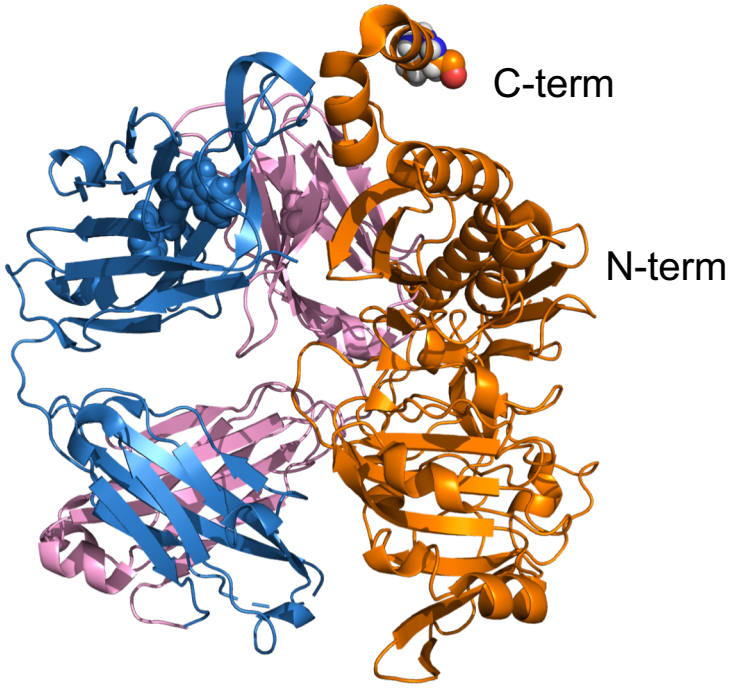
Fig. 4. Testing of a panel of monoclonal antibodies. A: Quenching of a panel of commercially available anti-hapten mAbs. **B:** Quenching of an anti-thyroxine (T4) IgG clone 22G8A2. **C–E**: Normalized dequenching dose-response curve for progesterone **(C)**, testosterone **(D)**, and T4 using the clone 22G8A2 **(E)**. Mean values with error bars of 1 SD

are shown.

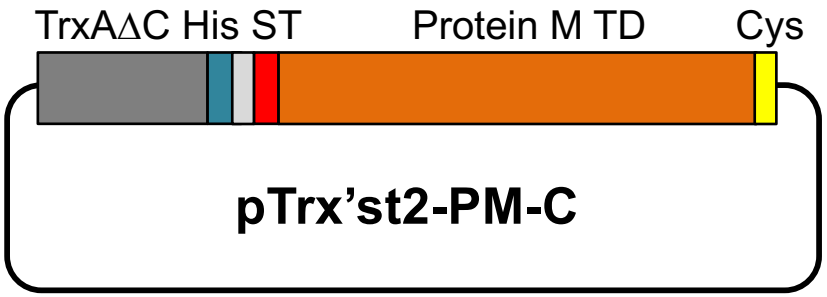
Fig. 5. Comparison of the responses obtained with Q-probe/IgG complex and recombinant Q-bodies. **A**: Dose-response curve for cortisol obtained with the mixture of 1 nM Q-probe/500 pM CS4-6 IgG. **B**: Scheme of sortagging-based Q-body assay. **C:** Dose-response curves for cortisol obtained with recombinant CS4-6 scFv Q-bodies (0.5 nM) with and without GGGS linker between the sortag and the V_H N-terminus. **D**: Dose-response curve for cortisol in the presence of 50% human serum obtained with recombinant scFv Q-body with GGGS linker (2 nM). Mean values with error bars of 1 SD are shown.

Fig. 1

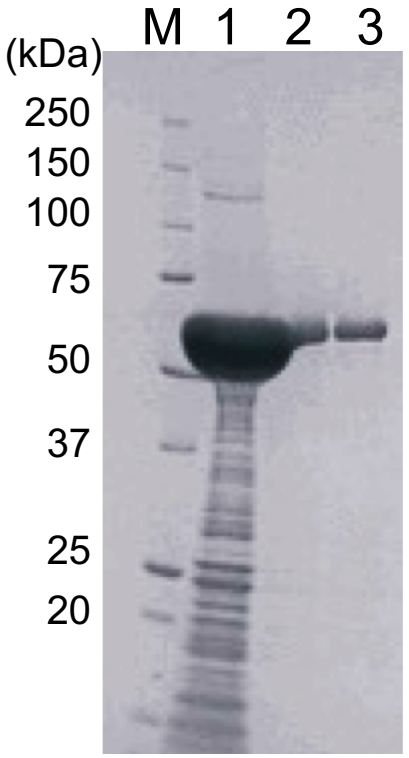
A


C-term
N-term

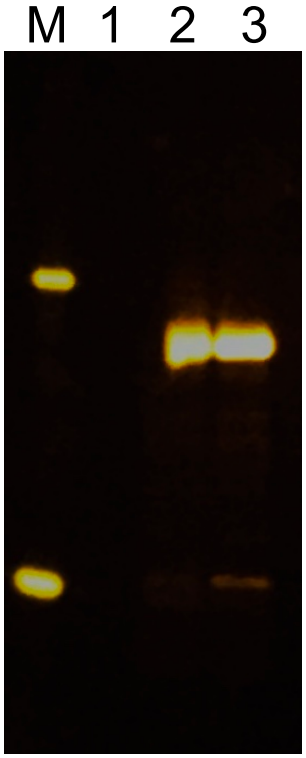
B


TrxAΔC His ST
Protein M TD
Cys
pTrx'st2-PM-C

C


M 1 2 3
(kDa)
250
150
100
75
50
37
25
20

D


M 1 2 3

E

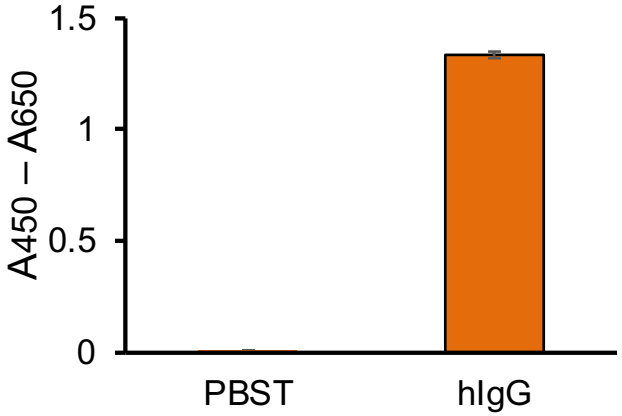

A450 – A650
1.5
1
0.5
0
PBST
hIgG

Fig. 2

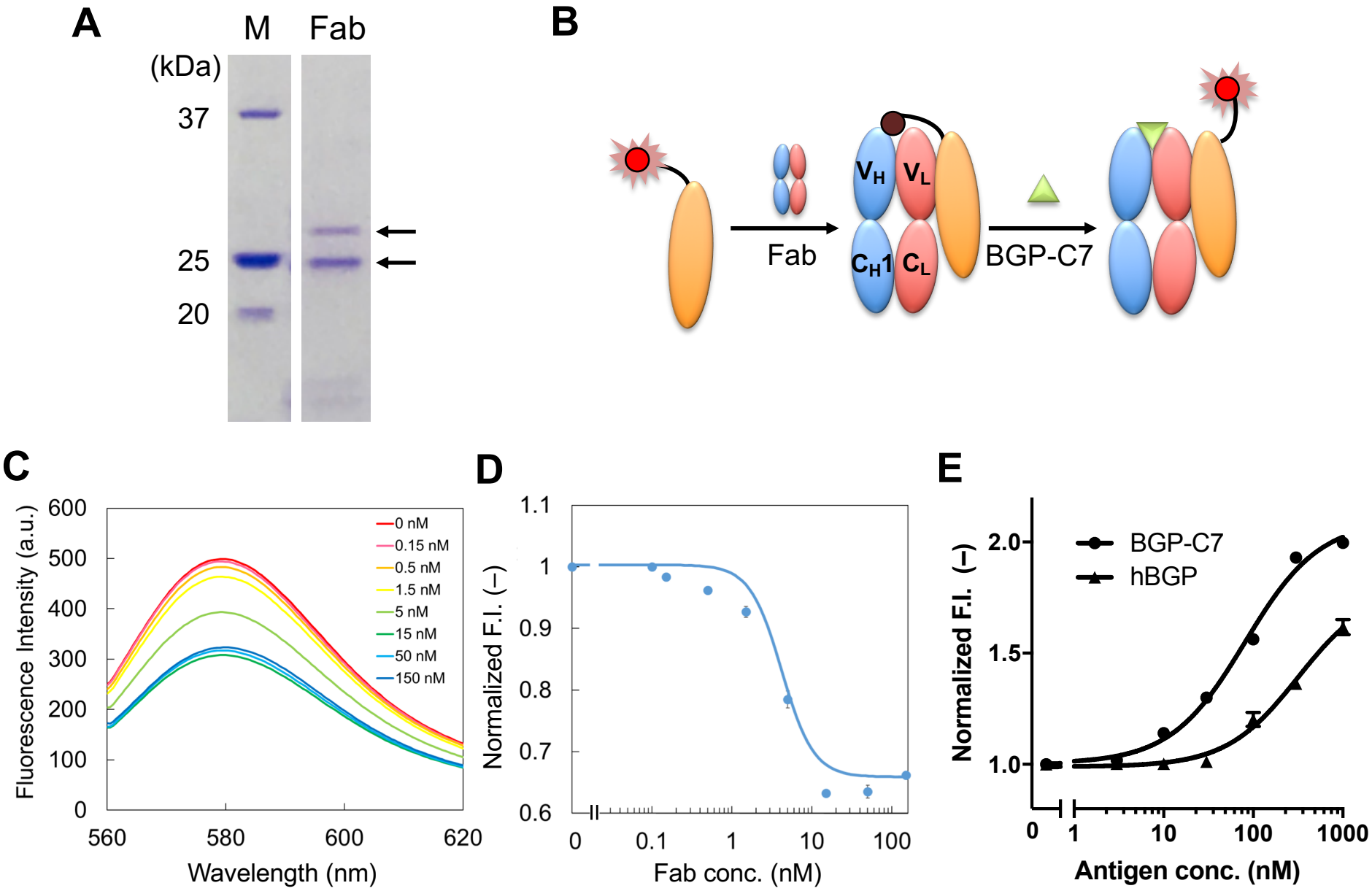

A
M
Fab
(kDa)
37
25
20
B
Fab
V_H
V_L
C_H1
C_L
BGP-C7
C
Fluorescence Intensity (a.u.)
600
500
400
300
200
100
0
560
580
600
620
Wavelength (nm)
0 nM
0.15 nM
0.5 nM
1.5 nM
5 nM
15 nM
50 nM
150 nM
D
Normalized F.I. (–)
1.1
1
0.9
0.8
0.7
0.6
0
0.1
1
10
100
Fab conc. (nM)
E
Normalized F.I. (–)
2.0
1.5
1.0
0
1
10
100
1000
Antigen conc. (nM)
BGP-C7
hBGP

Fig. 3

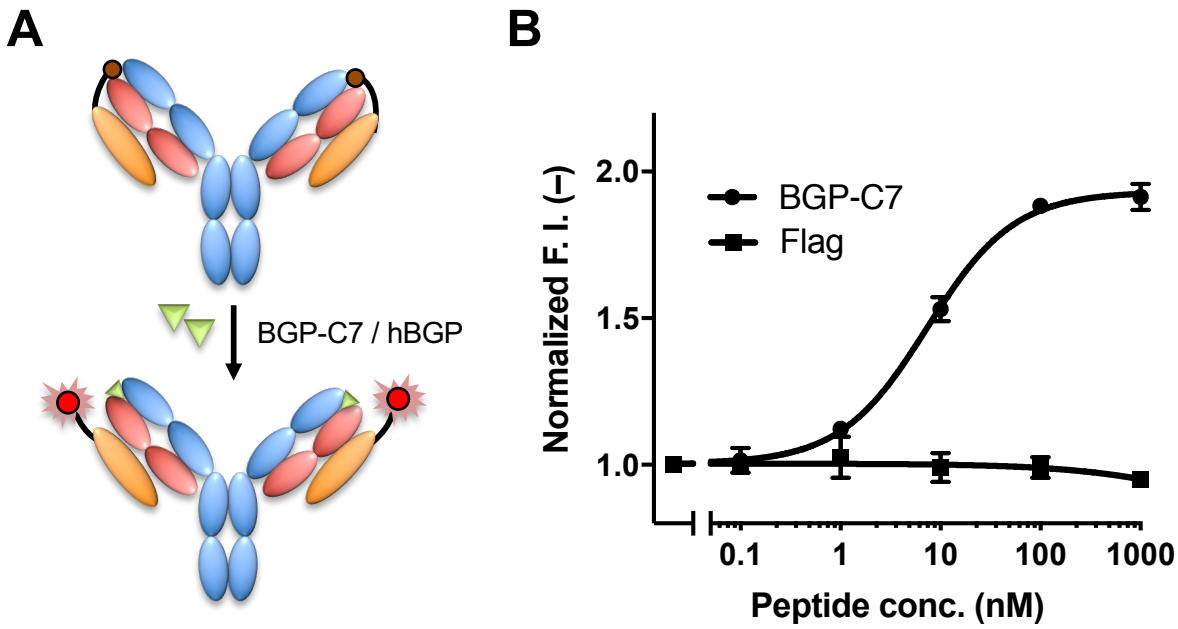

A
BGP-C7 / hBGP
B
Normalized F. I. (–)
2.0
1.5
1.0
BGP-C7
Flag
0.1
1
10
100
1000
Peptide conc. (nM)

Fig. 4

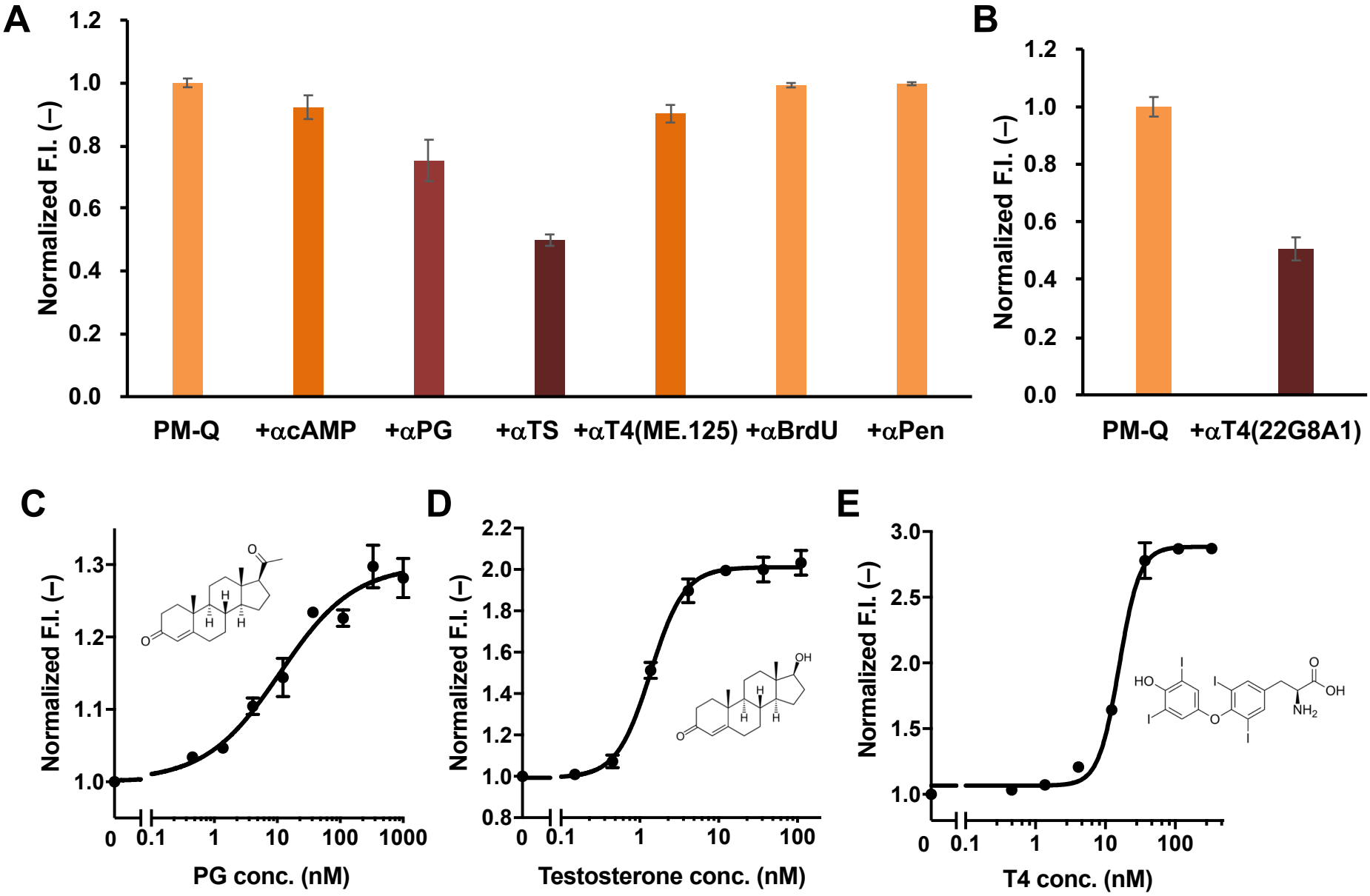
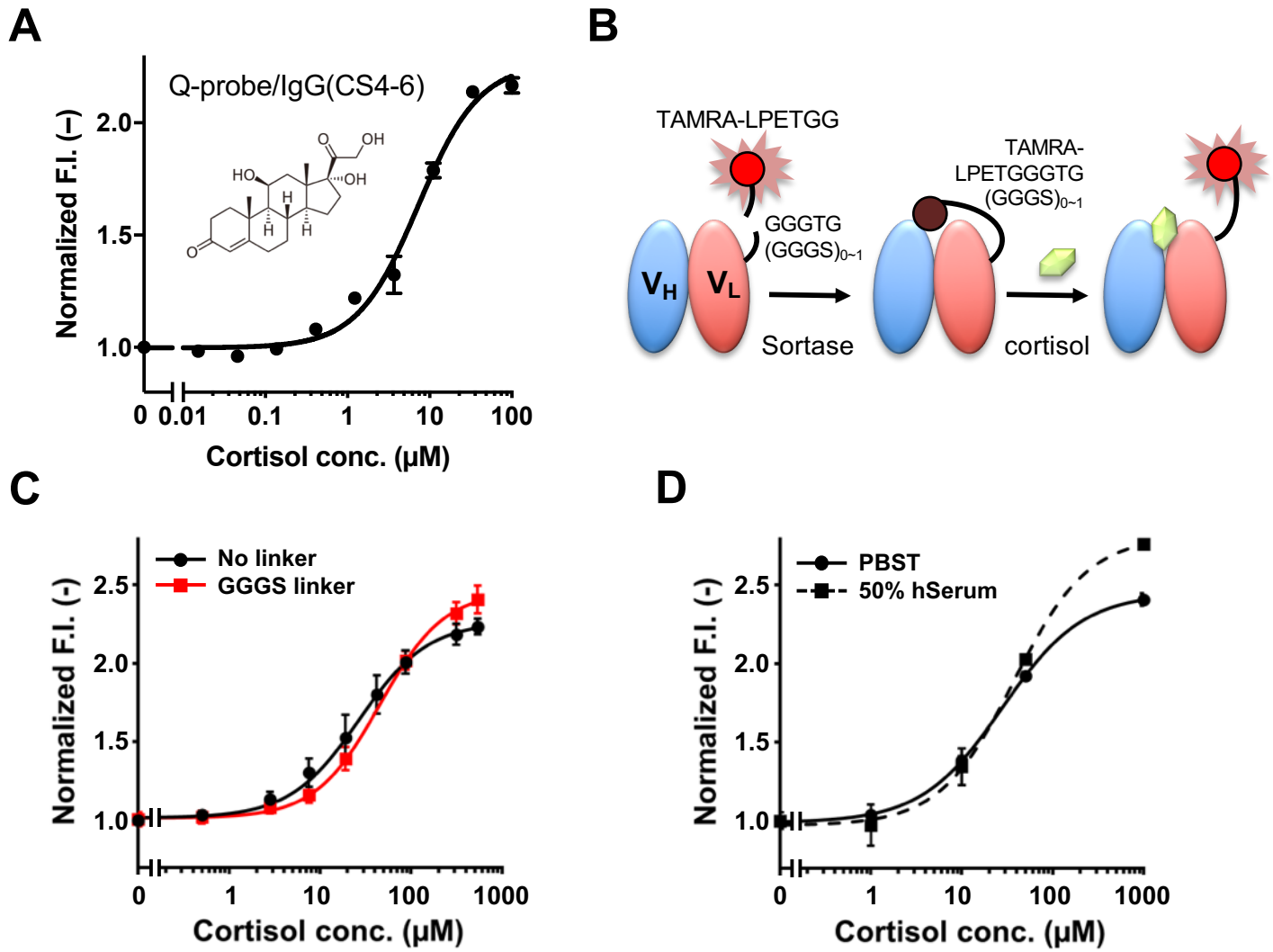

A
Normalized F.I. (–)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PM-Q
+αcAMP
+αPG
+αTS
+αT4(ME.125)
+αBrdU
+αPen
B
Normalized F.I. (–)
PM-Q
+αT4(22G8A1)
C
Normalized F.I. (–)
PG conc. (nM)
0
0.1
1
10
100
1000
D
Normalized F.I. (–)
Testosterone conc. (nM)
E
Normalized F.I. (–)
T4 conc. (nM)

Fig. 5


A
Q-probe/IgG(CS4-6)
Normalized F.I. (–)
2.0
1.5
1.0
0
0.01
0.1
1
10
100
Cortisol conc. (μM)
B
TAMRA-LPETGG
GGGTG
(GGGS)0~1
VH
VL
Sortase
TAMRA-
LPETGGGTG
(GGGS)0~1
cortisol
C
No linker
GGGS linker
Normalized F.I. (-)
2.5
2.0
1.5
1.0
0
1
10
100
1000
Cortisol conc. (μM)
D
PBST
50% hSerum
Normalized F.I. (-)
2.5
2.0
1.5
1.0
0
1
10
100
1000
Cortisol conc. (μM)

- A fluorescent probe that transforms antibodies to an immunosensor is made
- Protein M from mycoplasma was C-terminally labeled with TAMRA
- The resultant Q-probe showed quenching upon binding with many antibodies
- The quenched complex showed small antigen-dependent fluorescence recovery
- Biomarkers including thyroxine extracted from human serum was readily detected

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: