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ARTICLE

Preparation of antigen-responsive fluorogenic immunosensor by tyrosine chemical modification of antibody complementarity determining region

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Full-length pharmaceutical antibodies, trastuzumab and rituximab, were chemically modified into Quenchbody, fluorescent immunosensor using a two-step reaction: (1) selective tyrosine residue modification of antibody complementarity determining regions (CDRs), and (2) introduction of fluorescent dye molecules by Cu-free click reaction. Without the need for genetic manipulation and time-consuming examination of protein expression conditions, the antibody-dye combination with good antigen response efficiency was examined in a simple two-hour operation.

Analysis of protein localisation and quantitative concentration is important not only for understanding the biological functions of proteins, but also for the detection of pathogens and viruses and for the diagnosis of diseases. Sensor molecules that change fluorescence brightness based on protein–protein interactions or small molecular ligand-protein interactions provide useful insights.[1,2] For example, recently, Wittrup et al. developed a biosensor that uses a fluorescent dye-conjugated biosensor,[3] which achieved a 100-fold increase in fluorescence by creating an appropriate fluorescence molecule binding sensor; however, the method requires time-consuming genetic engineering of the sensor molecule and cannot be generalised for analysing proteins of interest. On the other hand, antibodies can be made against any protein and are currently being actively applied to diagnostics and disease therapeutics.[4,5] Therefore, antibody modification-based strategies can utilise these previous findings to develop sensor molecules.

A fluorescent immunosensor strategy using Quenchbody, a fluorescence turn-ON system, was developed. The fluorescence of a position-specific fluorolabeled single-chain variable region (scFv) was quenched in its antigen-free state owing to photoinduced electron transfer (PET) from intramolecular tryptophan (Trp) residues to the excited fluorescent dye. However, once the Quenchbody binds to its target antigen, PET does not occur because the dye is expelled from the binding pocket of the antibody and the distance between Trp residues and the dye is increased. The increase in fluorescence is rapid and reflects antigen concentration. Unlike the conventional enzyme-linked immunosorbent assay (ELISA) method, which requires time-consuming washing steps, the proposed method is simple and can measure antigen concentration just by mixing the Quenchbody with the sample. Since Trp residues involved in quenching fluorescence are conserved in antibodies, this method is highly generalizable.[6] Contemporary methods include those based on the modification of Fab,[7,8] nanobody,[9] and full-length Immunoglobulin G (IgG) antibody[10] as well as the scFv. We have also developed various strategies for fluorescent biosensors, such as cell-free translation-mediated position-specific introduction of unnatural amino acid residues,[11] fluorescence-quenched complexes with dye-labelled antibody binding proteins,[7,8] site-specific introduction of Cys-tag,[12] nucleotide-binding site labelling with UV reactive crosslinking,[10] and N-terminal modification.[13]

The properties required for fluorescence turn-ON type immunosensors are as follows: (1) modification that does not inactivate the antigen binding affinity of IgG; (2) fluorescent dye modified near the antigen-binding site; and (3) fluorescent dye whose fluorescence is quenched by PET from Trp residues. On the other hand, we recently developed a tyrosine click that can selectively modify exposed tyrosine residues. In the constant region of full-length IgG structure, tyrosine residues are unexposed, and thus, only tyrosine in the complementarity-determining region (CDR) may become the most exposed tyrosine residue in IgG. Using tyrosine click, we succeeded in CDR-selective modification of trastuzumab and rituximab, anti-human epidermal growth factor receptor 2 (HER2), and anti-cluster of differentiation 20 (CD20).[14] Using CDR modification, the modified antibodies partially maintained

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their antigen recognition ability because tyrosine modification did not result in the loss of phenolic hydroxyl groups and aromaticity, both of which contribute antigen binding affinities.

In this study, we prepared a Quenchbody using CDR-selective tyrosine click. Using purified IgGs, biosensors can be created in approximately 2 h. This procedure uses a two-step reaction comprising IgG tyrosine modification with azide and fluorescent dye introduction with a Cu-free click reaction. Therefore, by using different fluorescent dyes conjugated with distorted alkynes,[15] IgG-specific fluorescent dye screening can be carried out. The dependence of the tyrosine residue structure exposed to CDR on antigen recognition ability differs depending on the antibody type; however, the proposed method can quickly evaluate combinations applicable for fluorescent biosensors. This method can be used to quickly prepare biosensors by converting pharmaceutical antibodies with established industrial mass synthesis without genetic engineering.

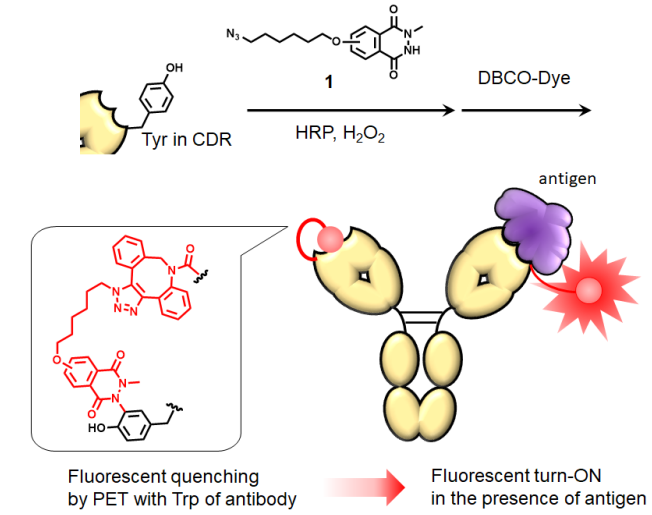

Figure 1. Concept of this work.

We previously reported that trastuzumab and rituximab were labelled CDR-selectively under the reaction conditions using horseradish peroxidase (HRP), H_2O_2, and the labelling reagent, azide-conjugated *N*-methylated luminol derivative (**1**). In the case of trastuzumab, it was shown that **1** is a suitable labelling reagent.[14] To identify suitable labelling reagent for rituximab modification, we evaluated azide-conjugated tyramide (**2**),[16] phenylenediamine derivatives (**3**–**5**),[17] urazole derivatives (**6**, **7**),[14] and luminol derivatives (**1**, **8**)[18] under the reaction conditions using HRP and H_2O_2 (Figure 2A). The azide-modified antibodies were fluorescently modified using dibenzocyclooctyne (DBCO)-Cy3, and the fluorescence intensities on the SDS-PAGE gel were quantified. As with trastuzumab as a substrate, **1** showed the highest efficiency (Figure S1). Cy3-modified rituximab was incubated with CD20-positive SU-DHL-4 cells, and fluorescence on the cells was measured using flow cytometry. It was suggested that the modified rituximab retained antigen recognition abilities (Figure 2B and Figure S2). In addition, the concentrations of HRP and H_2O_2 were optimised for rituximab modification using **1** as a modifier, and when 5 μM rituximab was used, the suitable concentrations of HRP and H_2O_2 were 45 nM (0.9 mol%) and 25 μM (5 equiv), respectively, as observed for trastuzumab (Figure S3). It has been clarified that under these reaction conditions, the exposed heavy chain Tyr57 is modified in trastuzumab, and the heavy chain Tyr32, Tyr52, Tyr101, and Tyr102 are modified in rituximab.[14] Quantitative analysis of the fluorescence intensity of Cy3 revealed that trastuzumab was modified to the 0.80/Fab domain, and rituximab was modified to the 1.76/Fab domain. These modifications were also suggested by intact mass analysis (Figure S4, S5). We previously reported a UV crosslinking method that can also be applied for full-length IgG modification, and this method has a reaction efficiency of 0.379/scFv.[10] Therefore, it is suggested that the proposed method may modify the antibody more efficiently than the conventional photoaffinity labelling technique, although the modification efficiency depends on the CDR structure, exposure level, and number of tyrosine residues in the antibody species.

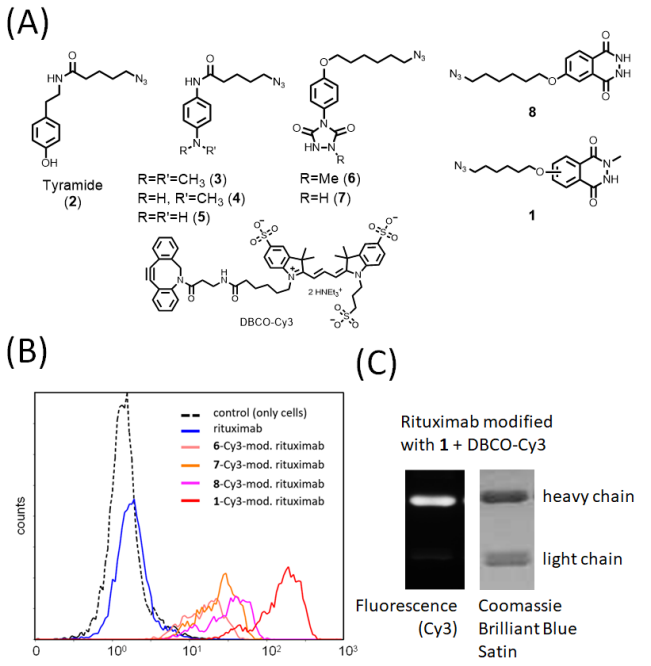

Figure 2. Investigation of modification reagent. (A)Structures of modification reagents. (B) Fluorescence measurement on the SU-DHL-4 cells (CD20+) treated with Cy3-modified rituximabs. (C) Detection of fluorescent modification on rituximab heavy chain.

Arbitrary number of fluorescent dyes can be introduced into the azide-modified antibody by a Cu-free click reaction using DBCO. Previously, we had succeeded in obtaining an efficient quenching effect using tetramethyl rhodamine (TAMRA).[8] The N-terminal and nucleotide-binding sites were modified, but in this study, the conjugation position of the dye was the tyrosine residue(s) in CDR, and the positional relationship with tryptophan residues involved in PET was different from previous labelling sites. To investigate the linker structure, we conjugated compounds **9** and **10** (Figure 3A) into azide-

modified trastuzumab and rituximab and evaluated their fluorescence quenching efficiencies.

Under denaturing conditions, the modified antibodies unfold the protein structure, and de-quenching occurs when the distance between the Trp residue associated with PET and the dye increases. It is known that electron tunnelling can occur effectively within a 1 nm distance.[19] Therefore, we denatured the modified antibodies by protein denaturation conditions using guanidine and dithiothreitol and estimated the quenching efficiency by comparing the fluorescence intensity before and after denaturation. The results showed that **10**, which has a shorter linker than **9**, was more prone to fluorescence quenching. Modification of trastuzumab and rituximab with **10** resulted in a 1.5-fold and 2.3-fold increase in fluorescence intensity, respectively (Figure 3B and Figure S6). Shortening the linker length favoured the proximity of the dye to the binding pocket of the CDR, which may be the result of efficient PET with Trp residues in the Fab region.

We used cell lysates of SU-DHL-4 cells and CD20 positive B lymphocytes to test whether the antigen could be detected using rituximab modified with **1** and **10**. As a negative control, SK-BR-3 cells were used as CD20 negative cell line. By observing the response of the fluorescence increase, a selective fluorescence increase was observed in the lysate of SU-DHL-4, indicating that modified rituximab functions as a Quenchbody in response to the antigen (Figure 3C).

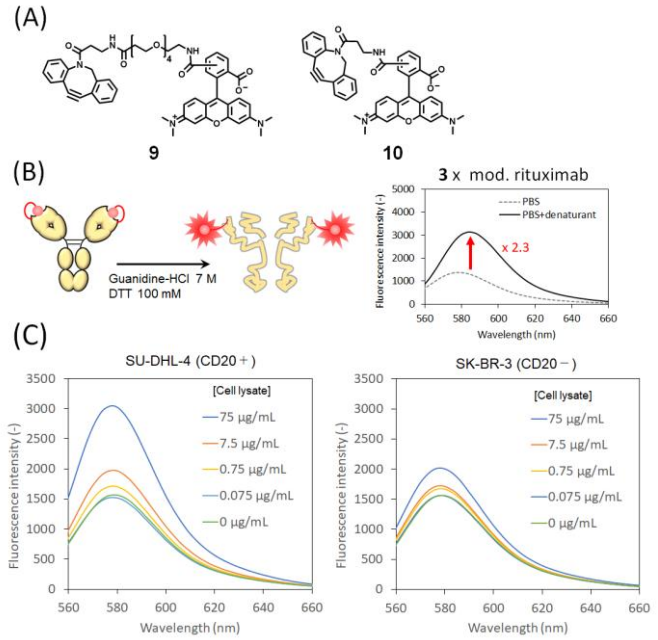

Figure 3. Modified rituximab exhibiting Quenchbody properties. (A) Chemical structure or **9** and **10**. (B) Dequenching of fluorescence by antibody denaturation. (C) Selective fluorescent de-quenching in antigen-expressing cell lysate using rituximab modified with **1** and **10**.

In this method, Quenchbody is prepared using a two-step reaction: (1) modification of the antibody by tyrosine modification with azide-conjugated compound **1,** and (2) click reaction between the azide group and the DBCO-dye. This operation makes it easy to change the fluorescent molecules to be bound simply by changing the type of DBCO-dye. We tested rhodamine dyes **10**–**16**, 6-JOE (**17**), ATTO610 (**18**), coumarin dyes **19**–**21**, NBD-X (**22**), ATTO495 (**23**), and BODIPY dyes **24**–**28** (Figure 3A, See Figure S7 for chemical structures of dyes).[20–22] DBCO-dyes **11**–**28** were prepared by the reaction of DBCO-amine with NHS dye and used for the second step reaction with the **1**-modified antibody without purification. We confirmed that the quenching efficiency using the protocol without purification was comparable to that using the purified DBCO-dye (Figure S8). Each dye-conjugated antibody was then prepared by removing the unreacted DBCO-dyes by gel filtration. The dye-modified antibodies were then examined for fluorescence recovery under denaturing conditions and by the addition of antigen-expressing cell lysate. In the case of rituximab modification, ATTO520, ATTO610, and ATTO495 showed high dequenching activities under denaturing conditions, but the fluorescence recovery effects by antigen addition were not significant (Figure 4B). These dyes have cationic structures, and it is possible that the charge-changing modification of CDRs impairs antigen recognition ability. Among the tested dyes, BODIPY dyes were particularly notable for their good quenching effect (Figure 4B). BODIPY is known to cause PET in conditions where it is in close proximity to tryptophan residues.[23] In addition, and the BODIPY dyes have hydrophobic properties,[24] which means that they tend to bind the internal structure in CDR, resulting in efficient fluorescence quenching by PET from tryptophan residues in the Fab region.

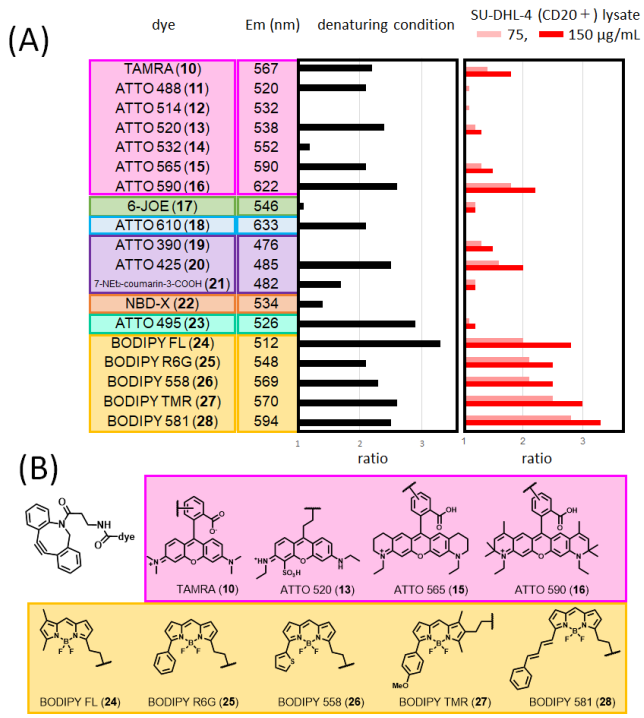

Figure 4 Fluorescent response of modified rituximab (A) and structures of representative dyes (B). See Figure S7, and S9-12 for structures of all tested dyes, and detailed spectral changes.

Not only in the case of modified rituximab but also in the case of modified trastuzumab, BODIPY showed high fluorescence

response under both antibody denaturation and antigen addition conditions (Figure S7). Turn-ON properties were observed in several BODIPY derivatives with different fluorescence wavelengths, indicating that multi-colour multi-antigen detection will be possible using this concept in the future. BODIPY is known to cause PET not only by its proximity to Trp but also to Tyr,[23] and changes in the proximity of BODIPY molecules to Tyr on antibodies may contribute to quenching.

In conclusion, we proposed an antibody chemical modification method to quickly and efficiently create Quenchbodies suitable for each antibody by tyrosine selective modification in CDRs. This procedure is based on a two-step reaction consisting of an IgG tyrosine click with an azide-conjugated compound and fluorescent dye conjugation with a Cu-free click reaction. We succeeded in obtaining Quenchbody properties by targeting trastuzumab and rituximab. This method is advantageous because it can be used to modify available antibody species, such as pharmaceutical antibodies, without the need for genetic manipulation and examination of antibody expression conditions. However, this method is not applicable to all antibodies; if the tyrosine residue in the CDR is essential for antigen recognition, this tyrosine residue modification may result in the inactivation of antigen recognition. In fact, when the Fab of KTM219 was modified, we confirmed that the reaction proceeded selectively in the CDR region and that the fluorescence of the modified dye was efficiently quenched, but no response to the antigen was observed because the antigen recognition ability of the antibody was impaired (see Figure S13–18). Despite these limitations and the fact that there is a certain percentage of inapplicable antibody species, this method still has wide potential for converting natural antibodies into sensor molecules in a screening manner that is quick and readily applicable to various antibodies.[25] Although it did not work in the case of KTM219, the first step tyrosine modification reaction in the presence of antigen may allow the reaction to be selective at tyrosine residues in the CDR that contribute less to antigen recognition while protecting tyrosine that is essential for antibody recognition.

Conflicts of interest

The authors have no conflicts of interest to declare. A patent application relating to the use of this method has been filed by the Tokyo Institute of Technology.

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Notes and references

1 M. A. Dwyer and H. W. Hellinga, *Curr. Opin. Struct. Biol.*, 2004, **14**, 495–504.
2 S. Sakamoto, S. Kiyonaka and I. Hamachi, *Curr. Opin. Chem. Biol.*, 2019, **50**, 10–18.
3 S. de Picciotto, P. M. Dickson, M. W. Traxlmayr, B. S. Marques, E. Socher, S. Zhao, S. Cheung, J. D. Kiefer, A. J. Wand, L. G. Griffith, B. Imperiali and K. D. Wittrup, *J. Mol. Biol.*, 2016, **428**, 4228–4241.
4 S. A. Ejazi, S. Ghosh and N. Ali, *Immunol. Cell Biol.*, 2021, **99**, 21–33.
5 A. Beck, L. Goetsch, C. Dumontet and N. Corvaïa, *Nat. Publ. Gr.*, 2017, **16**, 315–337.
6 R. Abe, H. Ohashi, I. Iijima, M. Ihara, H. Takagi, T. Hohsaka and H. Ueda, *J. Am. Chem. Soc.*, 2011, **133**, 17386–17394.
7 H. Jeong, T. Kojima, J. Dong, H. Ohashi and H. Ueda, *Anal. Methods*, 2016, **8**, 7774–7779.
8 J. Dong, C. Miyake, T. Yasuda, H. Oyama and I. Morita, *Biosens. Bioelectron.*, 2020, **165**, 112425.
9 A. Inoue, Y. Ohmuro-matsuyama, T. Kitaguchi and H. Ueda, *ACS Sensors*, 2020, **5**, 3457–3464.
10 H. Jeong, K. Matsumoto, S. Itayama, K. Kodama, R. Abe, J. Dong, M. Shindo and H. Ueda, *Chem. Commun.*, 2017, **53**, 10200–10203.
11 R. Abe, H. Jeong, D. Arakawa, J. Dong, H. Ohashi, R. Kaigome, F. Saiki, K. Yamane, H. Takagi and H. Ueda, *Sci. Rep.*, 2014, **4**, 4640.
12 H. Jeong, T. Kawamura, J. Dong and H. Ueda, *ACS Sensors*, 2016, **1**, 88–94.
13 J. Dong, H. Jeong, H. Ueda and J. B. I. B. Ioeng, *J. Biosci. Bioeng.*, 2016, **122**, 125–130.
14 S. Sato, M. Matsumura, T. Kadonosono, S. Abe, T. Ueno, H. Ueda and H. Nakamura, *Bioconjug. Chem.*, 2020, **31**, 1417–1424.
15 P. V Chang, J. A. Prescher, E. M. Sletten, J. M. Baskin, I. A. Miller, N. J. Agard, A. Lo and C. R. Bertozzi, *Proc. Natl. Acad. Sci.*, 2010, **107**, 1821–1826.
16 J. S. Rees, X. W. Li, S. Perrett, K. S. Lilley and A. P. Jackson, *Curr. Protoc. Protein Sci.*, 2015, 19.27.1-19.27.18.
17 S. Sato, M. Yoshida, K. Hatano, M. Matsumura and H. Nakamura, *Bioorganic Med. Chem.*, 2019, **27**, 1110–1118.
18 S. Sato, K. Nakamura and H. Nakamura, *ChemBioChem*, 2017, **18**, 475–478.
19 C. C. Page, C. C. Moser, X. Chen and P. L. Dutton, *Nature*, 1999, **402**, 47–52.
20 L. Jespers, T. P. Bonnert and G. Winter, *Protein Eng. Des. Sel.*, 2004, **17**, 709–713.
21 B. Zhang, X. Li, B. Li, C. Gao and Y. Jiang, *Expert Opin Ther Pat*, 2014, **24**, 647–664.
22 A. Loudet and K. Burgess, *Chem. Rev.*, 2007, **107**, 4891–4932.
23 N. Marmé, J. P. Knemeyer, M. Sauer and J. Wolfrum, *Bioconjug. Chem.*, 2003, **14**, 1133–1139.
24 N. Dorh, S. Zhu, K. B. Dhungana, R. Pati, F. T. Luo, H. Liu and A. Tiwari, *Sci. Rep.*, 2015, **5**, 1–10.
25 Sato, S; Nakamura, H; Ueda, H. Fluorescent-labeled antibody or antibody fragment. 2020. JP Patent WO/2020/026733.