A Proximity-Induced Fluorogenic Reaction Triggered by Antibody–Antigen Interactions with Adjacent Epitopes

Kentaro Nishiyama,[a] Hiroki Akiba,[a, b]* Satoshi Nagata,[b] Kouhei Tsumoto,[b, c] Haruhiko Kamada,[a, b] Hiroaki Ohno[a, b]*

[a] K. Nishiyama, H. Akiba, H. Kamada, H, Ohno
Graduate School of Pharmaceutical Sciences, Kyoto University
Kyoto 606-8501, Japan
E-mail: Hiroki Akiba, hakiba@pharm.kyoto-u.ac.jp; Hiroaki Ohno, hohno@pharm.kyoto-u.ac.jp

[b] H. Akiba, S. Nagata, K. Tsumoto, H. Kamada, H. Ohno
National Institute of Biomedical Innovation, Health and Nutrition
Ibaraki City, Osaka 567-0085, Japan

[c] K. Tsumoto
School of Engineering, The University of Tokyo
Tokyo 113-8656, Japan

Supporting information for this article is given via a link at the end of the document.

Abstract: Proximity-induced chemical reactions are site-specific and rapid by taking advantage of their high affinity and highly selective interactions with the template. However, reactions induced solely by antibody–antigen interactions have not been developed. Herein, we propose a biepitopic antigen-templated chemical reaction (BATER) as a novel template reaction. In BATER, reactive functional groups are conjugated to two antibodies that interact with two epitopes of the same antigen to accelerate the reaction. We developed a method for visualizing the progress of BATER using fluorogenic click chemistry for optimal antibody selection and linker design. The reaction is accelerated in the presence of a specific antigen in a linker length-dependent manner. The choice of the antibody epitope is important for a rapid reaction. This design will lead to various applications of BATER in living systems.

Proximity-induced chemical reactions are attractive tools for detection and synthesis of target molecules.[1] In the presence of templates to induce this proximity, local concentration of the substrates is increased by specific and strong interactions, thus promoting the desired reaction even in multimolecular systems. A class of well-known examples is the DNA-templated chemical reaction,[2–9] in which reactive functional groups linked to DNA are brought into proximity by complementary hybridization via duplex formation.[4,5] Various chemical reactions have been demonstrated for nucleic acid detection,[6,7] drug release,[8] and small-molecule drug discovery.[9] In some other approaches, proximity is gained by using interaction of oligonucleotide–peptide nucleic acid (PNA),[10,11] peptide–peptide[12] and protein–peptide.[13] Polyclonal antibody–antigen interactions have also been combined to bring DNA in close proximity to induce hybridization at lower concentrations.[4]

Antibody–antigen interactions are specific, strong, and potentially suitable for proximity-induced chemical reactions. We recently reported that a biparatopic antibody can be generated on an antigen with the assistance of peptide–protein complex formation.[14] However, to the best of our knowledge, reactions solely induced by antibody–antigen interactions have not been developed. This can be partly attributed to the difficulty in the appropriate placement of reactive groups; unlike double-strand formation of DNA to enable proximity in the angstrom range, control of the reacting groups by large protein domains (3–5 nm in diameter) engaging in antibody–antigen interactions has been challenging. We hypothesized that optimization of the structural features of both antibodies and reacting linker groups would enable proximity-induced reactions without the assistance of additional interactions.

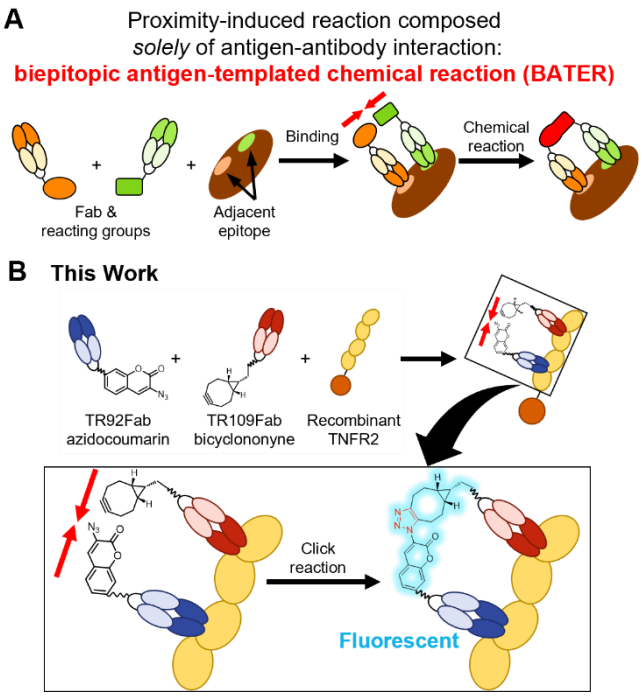


Figure 1. (A) Schematic overview of biepitopic antigen-templated chemical reaction (BATER). (B) Fluorogenic visualization of BATER in this study.

In the present study, we designed a biepitopic antigen-templated chemical reaction (BATER) in which the antigen-binding fragments (Fabs) of monoclonal antibodies interact with different epitopes to increase the local concentration of

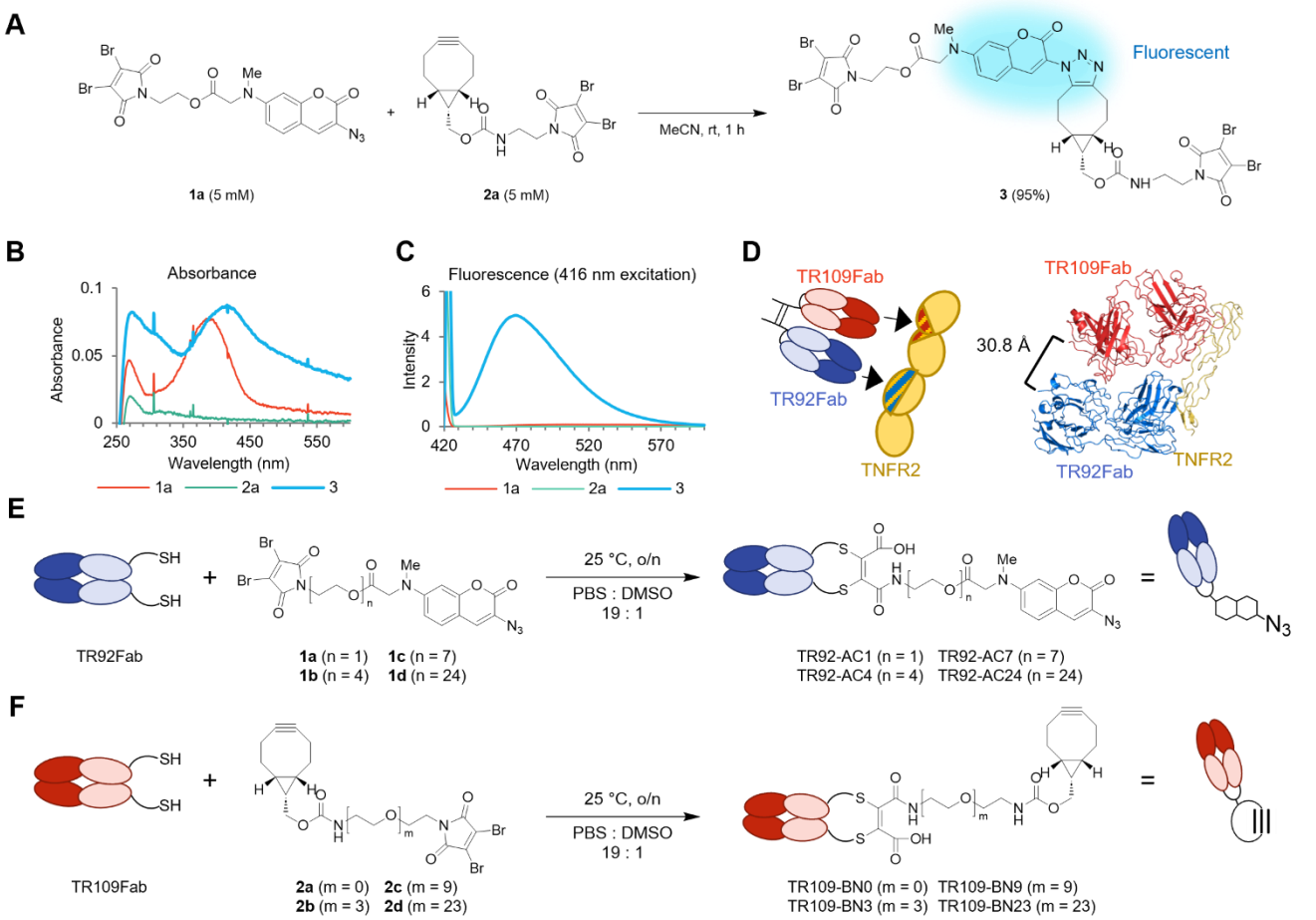


Figure 2. Spectroscopic characterization of fluorogenic groups and preparation of Fab conjugates. (A) Click reaction between **1a** and **2a**. (B,C) Absorption (B) and emission (C) spectra of **1a**, **2a,** and **3**. (D) Schematic (left) and cryo-EM resolved structure (right, PDB ID: 8HLB) of Bp109-92 in complex with TNFR2. (E,F) Schematic representation of conjugate reaction between Fab and linker **1a-d** (E), **2a-d** (F).

conjugated reactive functional groups (Figure 1A). Among the templated reactions, this strategy offers an advantage in that the biological functions of the antibody–antigen interaction itself can lead to biotherapeutics. For example, the alteration of monovalent Fab to bivalent F(ab′)$_2$-like molecules on the target cell surface may extend the retention of small fragment antibodies. Another potential application is the conjugation of a prodrug and a reacting group to different antibodies, leading to the generation of active drug molecules in an antigen-specific manner. These applications would overcome the problems in current antibody therapeutics.[15] Herein we describe a fluorescence-based method to track the progression of BATER based on the interaction between Fab antibodies bearing two reacting groups with different epitopes of tumor necrosis factor receptor 2 (TNFR2) as a model antigen. Notably, the fluorogenic reaction only occurred when the two reacting groups were in close proximity (Figure 1B). We also demonstrated that BATER is feasible under the strict control of epitope arrangement.

To visualize the progress of BATER, a copper-free click chemistry between 3-azidocoumarin[16] (AC) and bicyclononyne[17] (BN) was used (Figure 2A). AC fluoresces when an azide group reacts with an alkyne group. We designed **1a** and **2a** (Figure 2A) as reactive AC and BN derivatives, respectively, using dibromomaleimide[18] (DBM) for conjugation to the C-terminal disulfide bond between the light and heavy chains of Fab. Before proceeding to bioconjugation, the absorbance and fluorescence properties of the chemically synthesized compound **3** were analyzed (Figure 2B and C). Compound **3** exhibits an absorption maximum at 416 nm (Figure 2B). When excited at this wavelength, the fluorescence of **3** was observed with a maximum wavelength of 469 nm, whereas the AC and BN derivatives (**1a** and **2a**) were silent (Figure 2C), suggesting that only the chromophore generated by the copper-free click reaction exhibited fluorescence. Fluorogenic reaction between **1a** and **2a** (1 mM each, 37 °C) was complete in 3 hours (Figure S1). To optimize the linker length, the AC and BN derivatives **1b–d** and **2b–d**, respectively, were additionally synthesized using various PEG linkers (n = 4, 7, or 24; m = 3, 9, or 23).

As Fabs for conjugation with **1** and **2**, the Fab proteins from three monoclonal antibodies binding different epitopes of TNFR2 (TR92, TR96, and TR109) were used. We recently developed a biparatopic antibody against TNFR2, Bp109-92,[19] composed of two different Fabs, TR92 and TR109. Bp109-92 is characterized by two Fabs binding to a single TNFR2 molecule simultaneously (Figure 2D, left).[19] Cryo-electron microscopy (cryo-EM) structure of the Bp109-92–TNFR2 complex revealed that the epitopes of TR92 and TR109 were adjacent, and the C-termini of the two Fab proteins were close to each other (Figure 2D, right). Thus, we selected TR92 and TR109 for our initial investigations, expecting these antibodies to be suitable for inducing proximity in BATER. SDS-PAGE analysis of the prepared Fab proteins, C-terminally conjugated with reacting groups (Figure 2E and F), indicated an

approximately 50% yield of the conjugated Fab proteins as the product of crosslinking between two cysteine residues from the light and heavy chains by a DBM group (Figure S2). The subsequent analyses were conducted without further purification.

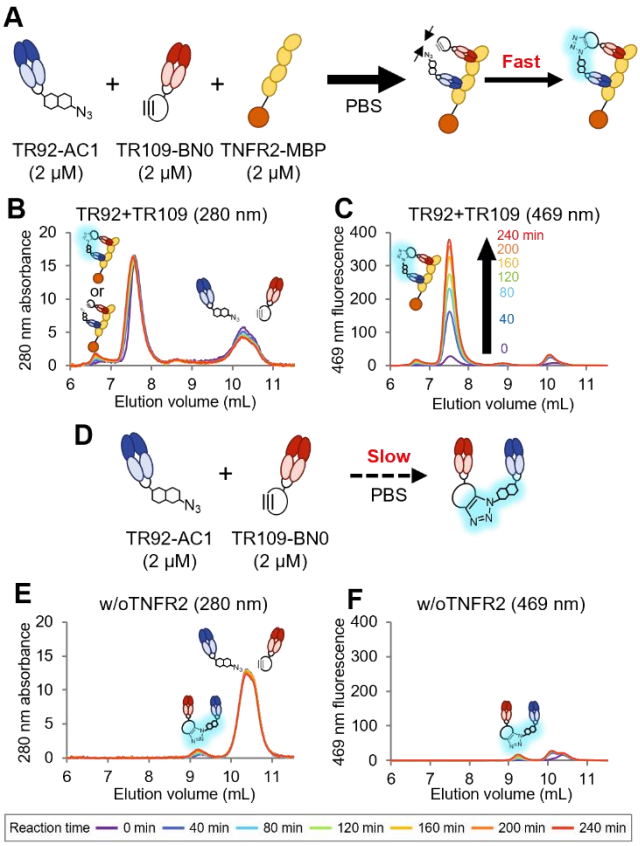
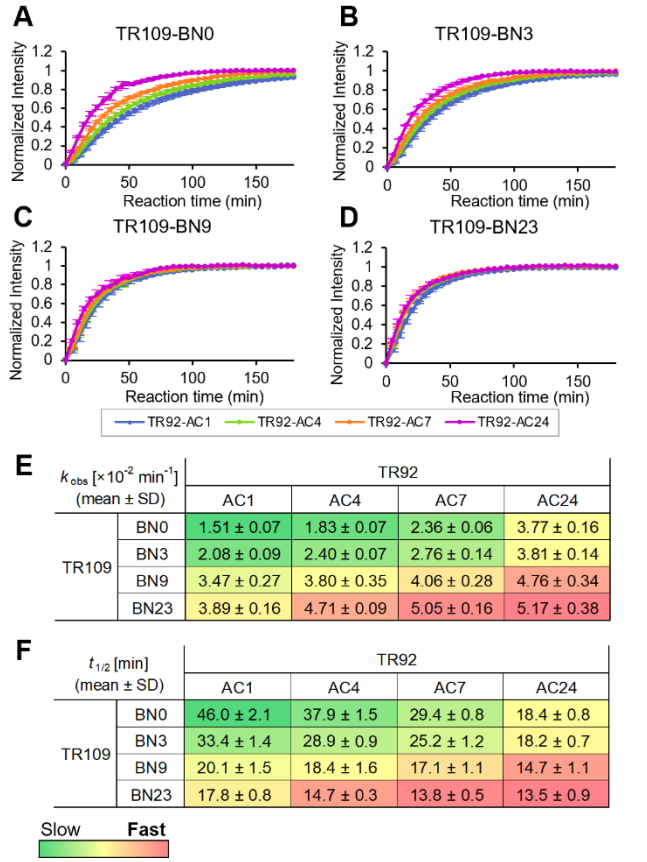


Figure 3. Analysis of fluorogenic reactions using size-exclusion chromatography. (A–C) Biepitopic antigen-templated chemical reaction in the combination of TR92-AC1 and TR109-BN0 with TNFR2-MBP. (D–F) Reaction without TNFR2. (A,D) Schematic representation of the reaction. (B,C,E,F) Size-exclusion chromatograms detected by absorbance at 280 nm (B,E) or excitation at 416 nm / emission at 469 nm (C,F).

To demonstrate BATER with the aid of fluorescence, the Fab conjugates with the shortest linker (TR92-AC1 and TR109-BN0) were first used with TNFR2-MBP (Figure S3) as the template. These three components (2 μM each) were incubated in phosphate buffered saline (PBS) at 25 °C (Figure 3A). The mixture was analyzed every 40 min by size-exclusion chromatography (SEC). When the proteins were detected by absorbance at 280 nm, ternary complex consisting of two Fabs and TNFR2 was found at the elution volume of 7.5 mL (Figure 3B). Residual Fab conjugates eluted at 10–11 mL (see Figure S4 for each component). No significant change in the time course was observed at 280 nm. In contrast, when fluorescence at 469 nm (excitation at 416 nm) was detected, the intensity increased over time at the elution volume of the ternary complex (Figure 3C), reflecting generation of a fluorescent group. Different time-course observations of 280 nm absorbance and 469 nm fluorescence indicated that the interaction between TNFR2-MBP and two antibody Fabs occurred in a short period of time, and the fluorogenic click reaction was slower. In contrast, in the absence of TNFR2-MBP, the increase in fluorescence of only 4.5% relative to its presence was observed at 9.2 mL (Figure 3D–F). These observations revealed successful BATER, in that the interaction between Fab and TNFR2 promoted the fluorogenic click reaction.

Note that the use of mixture of modified and non-modified Fab proteins limited the maximum yield to approximately 25%, which may be overcome by higher degree of Fab modification.



E

k_{obs} [$\times 10^{-2}$ min^{-1}] (mean ± SD)		TR92			
		AC1	AC4	AC7	AC24
TR109	BN0	1.51 ± 0.07	1.83 ± 0.07	2.36 ± 0.06	3.77 ± 0.16
	BN3	2.08 ± 0.09	2.40 ± 0.07	2.76 ± 0.14	3.81 ± 0.14
	BN9	3.47 ± 0.27	3.80 ± 0.35	4.06 ± 0.28	4.76 ± 0.34
	BN23	3.89 ± 0.16	4.71 ± 0.09	5.05 ± 0.16	5.17 ± 0.38

F

$t_{1/2}$ [min] (mean ± SD)		TR92			
		AC1	AC4	AC7	AC24
TR109	BN0	46.0 ± 2.1	37.9 ± 1.5	29.4 ± 0.8	18.4 ± 0.8
	BN3	33.4 ± 1.4	28.9 ± 0.9	25.2 ± 1.2	18.2 ± 0.7
	BN9	20.1 ± 1.5	18.4 ± 1.6	17.1 ± 1.1	14.7 ± 1.1
	BN23	17.8 ± 0.8	14.7 ± 0.3	13.8 ± 0.5	13.5 ± 0.9

Slow Fast

Figure 4. Kinetic analysis. (A–D) Time-course measurement of fluorescence intensity for TR92-AC of variable linkers reacted with TR109-BN0 (A), TR109-BN3 (B), TR109-BN9, (C) and TR109-BN23 (D) in the presence of TNFR2-MBP. (E, F) Calculated observed first-order rate constants k_{obs} (E) and $t_{1/2}$ (F) for all linker combinations. The charts and values show mean ± SD of triplicate experiments.

Next, we investigated the dependence of BATER on PEG linker length. For all 16 linker combinations ([4 for AC] × [4 for BN]), the reactions occurred in a similar manner to that of the TR92-AC1–TR109-BN0 combination (Figure S5). To determine the reaction rates, kinetics were observed by the incubation of TR92-ACn, TR109-BNm (n, m: PEG length; 1.2 μM each), and TNFR2-MBP (1 μM) at 37 °C and fluorescence intensity was measured every 5 min (Figure 4A–D). The fluorogenic reaction was assumed to occur via first-order kinetics based on SEC observations. The observed rate constant k_{obs} and $t_{1/2}$ was calculated from the time-dependent change in the normalized fluorescence intensity (Figure 4E, F). k_{obs} for all linker combinations ranged from 0.0151 to 0.0517 min^{-1}, and $t_{1/2}$ ranged from 13.5 to 46.0 min. Considering that $t_{1/2}$ of the fluorogenic reaction using 1 mM of the linker **1a** and **2a** (without Fab and TNFR2) was approximately 50 min (Figure S1), BATER apparently brought over 1000-fold enrichment effect. Interestingly, the reaction rate was faster with longer PEG for both the AC and BN reactive groups. Compared with the original combination with the shortest PEG, the combination of TR92-AC24 and TR109-BN23 with the longest PEG was 3.4 times faster (k_{obs} = 0.0151 vs. 0.0517 min^{-1}). The reaction rate of BATER was increased by using a linker with a longer PEG, reaching a plateau with a combination of AC24 and BN23 bearing the longest linkers. When the longest

linker was used on one side, the effect of length of the other linker was relatively small.

In the complex structure of Bp109-92 and TNFR2 (Figure 2D), the distance between C-termini of heavy chains of TR92Fab (Ser221) and TR109Fab (Pro217) was 30.5 Å. [19] Assuming that separately-binding two Fab antibodies forms similar complex, the distance between the two DBM groups of the reactive linkers conjugated each to TR92Fab and TR109Fab would be approximately 30 Å. It was previously estimated that the average end-to-end dis-tances of PEG3 and PEG27 were 8.3 ± 0.1 Å and 26.5 ± 0.9 Å[20], respectively, by molecular dynamics simulation. Thus, it is not unnatural to observe a slow reaction rate with the shortest linkers, owing to the low collision frequency of the reacting groups. In contrast, long PEG linkers are more flexible, resulting in more frequent collisions between the reactive functional groups suitable for cycloaddition. This naturally describes why the effect of shorter linker is small when one of two linkers was sufficiently long.

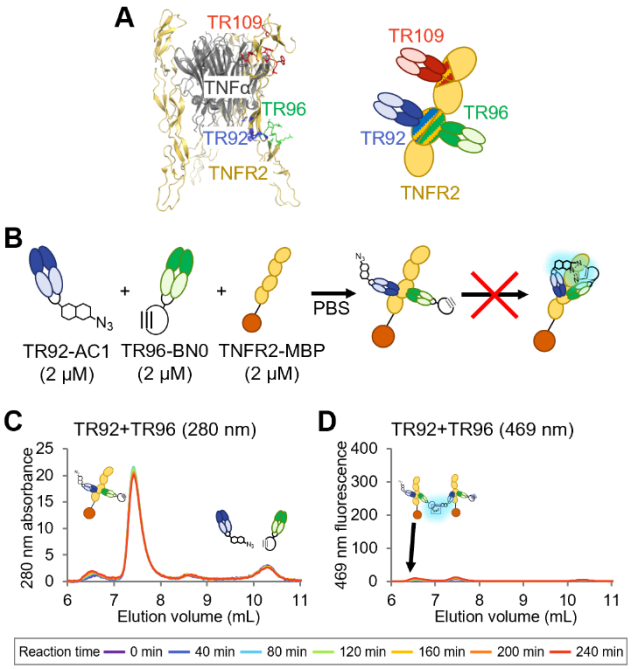


Figure 5. Fluorogenic reaction in combination of TR92-AC1 and TR96-BN0. (A) Topographical (left) and schematic (right) epitope mapping of anti-TNFR2 Fabs used in this study. Gray: TNFα, yellow: TNFR2, blue: TR92 epitope, green: TR96 epitope, red: TR109 epitope. (B) Schematic representation of BpAb formation. (C,D) Size-exclusion chromatograms detected by absorbance at 280 nm (C) or excitation at 416 nm / emission at 469 nm (D).

Finally, we investigated the influence of the antibody epitopes used for BATER. The epitopes of TR92 and TR109 are on the same side with TNFα-interacting face, while that of TR96 is located on the opposite face (Figure 5A).[21] TR92-AC1, TR96-BN0, and TNFR2-MBP (2 μM each) were incubated and analyzed by SEC (Figure 5B). A ternary complex of two Fabs and TNFR2 was observed at a 7.5 mL elution volume (Figure 5C), similar to the combination of the TR92-AC1–TR109-BN0 pair (Figure 3B). However, no increase in the fluorescence intensity was observed at 7.5 mL (Figure 5D). This result suggests that a proximity-induced reaction did not occur for the TR92-AC1–TR96-BN0 pair. A slight increase in the fluorescence intensity observed at an elution volume of 6.5 mL, earlier than that of the ternary complex, was derived from a cross-linking reaction between two molecules of TNFR2. The slow rate of this reaction, as observed in the absence of TNFR2 (Figure 3F), also supports a cross-linking intermolecular reaction.

The difference in the reaction rate by different combinations of Fab antibodies can be explained as follows. For the combination of TR92 and TR109, the two Fabs interacting with TNFR2 are adjacent, and the reacting groups are proximal for the click reaction. However, the distance between the C-termini of TR92 and TR96 bound to TNFR2 was too large for the reacting groups to undergo intramolecular collisions, thus leading to a slow intermolecular crosslinking reaction. The use of the longest linkers (TR92-AC24 and TR96-BN23) only slightly facilitated the intermolecular reactions (Figure S6). These results suggested that the selection of Fabs that interact with adjacent epitopes is important for BATER.

In conclusion, we successfully observed that chemical reactions proceeded in an antigen-specific manner using reactive functional groups in proximity to each other, using only antibody–antigen interactions. Conjugation of bioorthogonal functional groups to antibodies has been conducted using only fast reactions between tetrazine and BN or *trans*-cyclooctyne (second-order reaction rate constant is 10^2–10^5 $M^{-1}s^{-1}$),[22,23] and has been utilized for cross-linking of antigens at the cell surface[22] and delivery of radiopharmaceuticals (pretargeting).[24] Compared to these reactions, the reaction between azide and BN is far slower (second-order reaction rate is approximately 0.10 $M^{-1}s^{-1}$),[25] potentially useful for controllable reactions on the target antigen molecules. The BATER strategy is applicable to other antigens and bioorthogonal reactions, providing a basis for synthesizing compounds on antigens *in vivo* for diagnosis and therapy.

Supporting Information

The authors have cited additional references within the Supporting Information.[26–28]

Acknowledgements

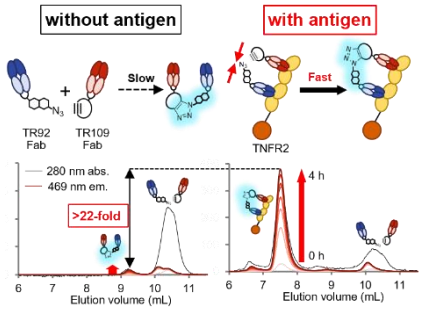
This work was supported by JSPS grant number JP21K06453 and AMED grant numbers JP22ak0101099 and JP23ama121042.

Keywords: Template synthesis • Antibodies • Click chemistry • Epitope • Bioconjugation

[1] O. Seitz, *J. Pept. Sci.* **2019**, *25*, e3198.
[2] X. Li, D. R. Liu, *Angew. Chem. Int. Ed.* **2004**, *43*, 4848–4870.
[3] M. Di Pisa, O. Seitz, *ChemMedChem* **2017**, *12*, 872–882.
[4] L. B. Pellejero, M. A. D. Nijenhuis, F. Ricci, K. V. Gothelf, *Small* **2023**, *19*, 2200971.
[5] L. B. Pellejero, M. Mahdifar, G. Ercolani, J. Watson, T. Brown, F. Ricci, *Nat. Commun.* **2020**, *11*, 6242.
[6] K. K. Sadhu, N. Winssinger, *Chem. Eur. J.* **2013**, *19*, 8182–8189.
[7] H. Wu, B. T. Cisneros, C. M. Cole, N. K. Devaraj, *J. Am. Chem. Soc.* **2014**, *136*, 17942–17945.
[8] Z. Ma, J.-S. Taylor, *Proc. Natl. Acad. Sci.* **2000**, *97*, 11159–11163.
[9] R. K. O'Reilly, A. J. Turberfield, T. R. Wilks, *Acc. Chem. Res.* **2017**, *50*, 2496–2509.
[10] Y. Ura, J. M. Beierle, L. J. Leman, L. E. Orgel, M. R. Ghadiri, *Science* **2009**, *325*, 73–77.

[11] J. Sayers, R. J. Payne, N. Winssinger, *Chem. Sci.* **2018**, *9*, 896–903.
[12] U. Reinhardt, J. Lotze, S. Zernia, K. Mörl, A. G. Beck-Sickinger, O. Seitz, *Angew. Chem. Int. Ed.* **2014**, *53*, 10237–10241.
[13] N. Brauckhoff, L. Fang, A. Haim, T. N. Grossmann, *Chem. Commun.* **2023**, *59*, 5241–5244.
[14] H. Akiba, K. Takayanagi, O. Kusano-Arai, H. Iwanari, T. Hamakubo, K. Tsumoto, *Biotechnol. Rep.* **2020**, *25*, e00418.
[15] P. K. Mahalingaiah, R. Ciurlionis, K. R. Durbin, R. L. Yeager, B. K. Philip, B. Bawa, S. R. Mantena, B. P. Enright, M. J. Liguori, T. R. Van Vleet, *Pharmacol. Ther.* **2019**, *200*, 110–125.
[16] K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill, Q. Wang, *Org. Lett.* **2004**, *6*, 4603–4606.
[17] J. Dommerholt, S. Schmidt, R. Temming, L. J. A. Hendriks, F. P. J. T. Rutjes, J. C. M. van Hest, D. J. Lefeber, P. Friedl, F. L. van Delft, *Angew. Chem. Int. Ed.* **2010**, *49*, 9422–9425.
[18] E. A. Hull, M. Livanos, E. Miranda, M. E. B. Smith, K. A. Chester, J. R. Baker, *Bioconjug. Chem.* **2014**, *25*, 1395–1401.
[19] H. Akiba, J. Fujita, T. Ise, K. Nishiyama, T. Miyata, T. Kato, K. Namba, H. Ohno, H. Kamada, S. Nagata, K. Tsumoto, **2022**, *bioRxiv* preprint DOI: 10.1101/2022.12.15.520217.
[20] S. Hezaveh, S. Samanta, G. Milano, D. Roccatano, *J. Chem. Phys.* **2012**, *136*, 124901.
[21] Y. Mukai, T. Nakamura, M. Yoshikawa, Y. Yoshioka, S. Tsunoda, S. Nakagawa, Y. Yamagata, Y. Tsutsumi, *Sci. Signal.* **2010**, *3*, ra83.
[22] T. Komatsu, E. Kyo, H. Ishii, K. Tsuchikama, A. Yamaguchi, T. Ueno, K. Hanaoka, Y. Urano, *J. Am. Chem. Soc.* **2020**, *142*, 15644–15648.
[23] R. García-Vázquez, U. M. Battisti, M. M. Herth, *Pharmaceuticals* **2022**, *15*, 685.
[24] M. Altai, R. Membreno, B. Cook, V. Tolmachev, B. M. Zeglis, *J. Nucl. Med.* **2017**, *58*, 1553–1559.
[25] J. Dommerholt, O. van Rooijen, A. Borrmann, C. F. Guerra, F. M. Bickelhaupt, F. L. van Delft, *Nat. Commun.* **2014**, *5*, 5378.
[26] L. Castañeda, Z. V. F. Wright, C. Marculescu, T. M. Tran, V. Chudasama, A. Maruani, E. A. Hull, J. P. M. Nunes, R. J. Fitzmaurice, M. E. B. Smith, L. H. Jones, S. Caddick, J. R. Baker, *Tetrahedron Letters* **2013**, *54*, 3493–3495.
[27] C.-S. Tsai, P.-Y. Liu, H.-Y. Yen, T.-L. Hsu, C.-H. Wong, *Chem. Commun.* **2010**, *46*, 5575.
[28] H. Akiba, K. Tsumoto, S. Nagata, WO 2021 200840, **2021**.

Entry for the Table of Contents



We designed a biepitopic antigen-templated chemical reaction by using two monoclonal antibodies to interact with different epitopes of an antigen. Accelerated reaction was observed using a fluorescence-based method and the importance of epitope selection was demonstrated.