Supporting Information

Transglutaminase-mediated proximity labeling of a specific Lys residue in a native IgG antibody

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Materials

Miller lysogeny broth (LB), tryptone, dried yeast extract, tris(hydroxymethyl)aminomethane (Tris), guanidine hydrochloride, L-(+)-arginine monohydrochloride, and hydrochloric acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium hydrogen phosphate 12-water, sodium chloride, thrombin, dithiothreitol, ammonium bicarbonate, trehalose, 4% paraformaldehyde phosphate buffer solution, acetonitrile (ACN), thrombin, and sodium ampicillin were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Glycerol, citric acid, tri-sodium citrate, and potassium chloride were purchased from Kishida Chemical Co., Ltd (Osaka, Japan). Trifluoroacetic acid (TFA) was purchased from Watanabe Chemical Industry (Hiroshima, Japan). Human IgG1 trastuzumab (Herceptin®) and chimeric IgG1 rituximab (MabThera®) were purchased from F. Hoffmann-La Roche, Ltd. (Basel, Switzerland). Human IgG1 ramucirumab was purchased from Eli Lilly Japan K.K. (Kobe, Japan). Imidazole, trypsin, α-cyano-4-hydroxycinnamic acid (CHCA), and TAMRA-YPLQMRG-NH_2 were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). McCoy's 5A medium and Dulbecco's modified Eagle medium (DMEM) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The protein structures were generated by Molecular Operating Environment (Chemical Computing Group Inc., Quebec, Canada).

Construction of pG-fused EzMTG Mutants

The DNA sequence coding the pG sequence was amplified with PCR, and the product was inserted into a pET22b+ vector carrying the gene coding EzMTG using an In-Fusion HD Cloning Kit (Takara Bio Inc., Shiga, Japan). The DNA sequences coding the pG(Fab) and pG(Fc) sequences were amplified with PCR. The products were inserted into the pET22b+ vector carrying the gene encoding EzMTG using the In-Fusion HD Cloning Kit. The amino acid sequences of all the proteins used in this study are summarized in Table S1.

Expression and purification of pG-fused EzMTG mutants

The expression of the pG-fused EzMTG mutants was conducted using *E. coli* BL21 star (DE3). The plasmid vectors encoding MTG mutants were transformed into cells by heat shock and cultured on an LB plate containing 100 μg/mL sodium ampicillin. A single colony was inoculated into 5 mL of LB containing the same amount of sodium ampicillin and shaken at 220 rpm at 37 °C for 6 h. A preculture of cells expressing pG-fused EzMTG was placed into 1 L of Terrific broth medium containing 100 μg/mL ampicillin and cultured with shaking at 220 rpm at 37 °C until the OD_{600} value reached 0.4–0.5. The expression of pG-fused MTG mutants was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) followed by shaking at 18 °C. pG-fused EzMTG mutants were cultured in an auto-induction medium. The cells were harvested by centrifugation at 5,000 ×*g* for 20 min, and the supernatant was discarded. The pellet was resuspended in phosphate-buffered saline (PBS, pH 7.4) and frozen at −80 °C until protein purification.

For the purification of pG-fused EzMTG mutants, the cell pellet was thawed in running water. The cell suspension was sonicated on ice for 12.5 min. The cell debris was removed by centrifugation at 15,000 ×*g* for 60 min at 4 °C. The supernatants were initially applied to a crude column containing 5 mL of HisTrap FF (Cytiva, Tokyo, Japan), which was pre-equilibrated with 93% HisTrap binding buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.4) and 7% HisTrap elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.4). The column was washed with the binding buffer until all the unbound substances had been eluted out; then, the proteins were eluted with an elution buffer with a gradient of 7% to 100%. Each pG-fused MTG variant was purified by size exclusion chromatography using HiLoad 16/600 Superdex 200 pg (Cytiva, Tokyo, Japan) with 1×PBS with 0.1 mM dithiothreitol (pH 7.4) as a running buffer. The purified pG-fused EzMTG mutants were concentrated using 30 kDa molecular weight cutoff (MWCO) Amicon Ultra-15 centrifugal filter units (Millipore, Tokyo, Japan). To determine protein concentrations, the absorbances at 280 nm were measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

Activation of EzMTG-pG(Fab) by thrombin

EzMTG-pG(Fab) (50 μM) was mixed with thrombin (0.14 U/μg of EzMTG-pG(Fab)) in 20 mM phosphate buffer (pH 6.2). The reaction was performed at 25 °C for 60 min.

For the purification of ΔproMTG-pG(Fab), the reaction solution was initially applied to a crude column containing 5 mL of HisTrap FF, which was pre-equilibrated with 93% HisTrap binding buffer and 7% HisTrap elution buffer. HisTrap binding buffer (93%) and HisTrap elution buffer (7%) were applied to the column and the flow-through solution containing ΔproMTG-pG(Fab) was collected. ΔproMTG-pG(Fab) was purified by size exclusion chromatography using HiLoad 16/600 Superdex 200 pg with 1×PBS with 0.1 mM dithiothreitol (pH 7.4) as a running buffer. The purified ΔproMTG-pG(Fab) was concentrated using 30 kDa MWCO Amicon Ultra-15 centrifugal filter units. To determine protein concentrations, the absorbances at 280 nm were measured using a NanoDrop 2000c spectrophotometer.

Evaluation of the antibody-binding ability of pG-fused EzMTG mutants using bio-layer interferometry analysis with BLItz

Bio-layer interferometry was performed with a BLItz (Fortebio, CA, USA) to analyze the binding of each pG-fused EzMTG mutant to trastuzumab. Streptavidin (SA) biosensors (Sartorius AG, Göttingen, Germany) were hydrated in Milli-Q water for at least 10 min before use. To immobilize the trastuzumab on the SA biosensors, trastuzumab was biotinylated using the Biotin Labeling Kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan). After the baseline step with kinetics buffer (Fortebio, CA, USA), 4 μL of biotinylated trastuzumab (30 μg/mL in Milli-Q water) was added to the drop holder to allow the association of biotin-trastuzumab with the SA biosensor surfaces for 120 s. After the baseline step with kinetics buffer for 30 s, the association of samples (0.125, 0.25, 0.50 and 1.0 μM of each pG-fused EzMTG mutant) with the proteins on the tips for 120 s and a dissociation step for 120 s by soaking in kinetics buffer were performed. The binding affinity (K_d) was evaluated as the mean of two experiments ($N = 2$).

Evaluation of the cross-linking activity of pG-fused EzMTG mutants

Trastuzumab (1.3 μM) was mixed with each MTG mutant (2.6 μM) in 40 mM Tris-HCl (pH 8.0). The crosslinking reaction was started by the addition of TAMRA-Q (100 μM, total volume: 50 μL). The reaction was performed at 37 °C for 60 min. A 2× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the reaction solution, and the mixed solution was heated at 98 °C for 2 min. After denaturation treatment, SDS-PAGE was conducted using a 12.5% acrylamide gel with Coomassie Brilliant Blue staining (Quick-CBB, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). The progression of substrate modification was determined using iBright FL1500 (Thermo Fisher Scientific).

Identification of modification position

Unmodified trastuzumab and TAMRA-Q-modified trastuzumab were fragmented by reduction-alkylation and trypsin treatment. The resulting peptide fragments were injected into a COSMOSIL 5C18-AR-300 (4.6 ID × 150 mm) column for RP-HPLC analysis. In the RP-HPLC, water and ACN containing 0.1% TFA were used, and the analysis conditions were as follows: flow rate, 1.2 mL min^{-1}; detection wavelengths, 214, 280, and 565 nm; gradient, water/ACN (0.1% TFA) = 100:0 to 60:40 over 40 min. The molecular weight of each peak-derived sample fractionated was determined by MALDI-TOF-MS. Sample solutions were spotted onto a CHCA matrix and mass spectra were obtained using a Bruker Autoflex max MALDI-TOF mass spectrometer (Bruker, Billerica, MA, USA) in positive ion detection mode. The position of modification was identified by calculating the mass of the peptide fragment from the obtained mass using the following formula and comparing it with the theoretical mass ($YADSVK_{65}GR$ = 894.98). Theoretical masses were calculated using Expasy PeptideMass (Swiss Institute of Bioinformatics, Lausanne, Switzerland). N-terminal analysis of the modified antibody fragment was contracted to Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

$$\begin{aligned}\text{Peptide fragment} &= \text{TAMRA-Q modified peptide fragment} - \text{TAMRA-Q} + NH_3 \\ &= 2097.486 - 1218.43 + 17.03 \\ &= 896.086\end{aligned}$$

Evaluation of modification rate

Trastuzumab (1.3 μM) was mixed with each MTG mutant (2.6 μM) in 40 mM Tris-HCl (pH 8.0) with 2 v/v% trehalose. The crosslinking reaction was started by adding TAMRA-Q (100 μM, total volume: 50 μL). The reaction was performed at 37 °C for 120 min. The reaction solution was sampled using 30 μL aliquots, which were mixed with 72 μL of 6 M guanidine hydrochloride containing 100 mM ammonium bicarbonate and 8 μL of 1 M dithiothreitol. The reduction reaction was then carried out at 60 ºC for 30 min. Denatured and reduced samples (100 μL) were injected into a COSMOSIL 5Ph-AR-300 (4.6 ID × 150 mm) column for RP-HPLC analysis. In the RP-HPLC, water and ACN containing 0.1% TFA were used, and the analysis conditions were: flow rate, 1.2 mL min^{-1}; detection wavelength, 214 nm; gradient, water/ACN (0.1% TFA) = 100:0 to 72:28 over 2 min then 72:28 to 62:38 over 16 min. The modification rate was calculated from the following formula using the peak areas of the TAMRA-Q-modified and unmodified heavy chains (Fig. S5a, b). However, 5% of the peak area of the TAMRA-Q-modified heavy chain was corrected for the TAMRA-Q-derived peak area.

$$\text{Modification rate (\%)} = \frac{\text{modified heavy chain} \times 0.95}{\text{unmodified heavy chain} + \text{modified heavy chain} \times 0.95} \times 100$$

Evaluation of aggregation formation

Trastuzumab (1.3 μM) was mixed with each MTG mutant (2.6 μM) in 1×PBS (pH 7.4) (total volume: 50 μL). The mixed solution was incubated at 37°C for 60 min. To measure the OD_{600} value, the absorbances at 600 nm were measured using a NanoDrop 2000c spectrophotometer.

Purification of TAMRA-Q-modified trastuzumab

Trastuzumab (2.6 μM) was mixed with EzMTG-pG(Fab) (5.2 μM) in 40 mM Tris-HCl (pH 8.0). The crosslinking reaction was started by adding TAMRA-Q (200 μM, total volume: 2 mL). The reaction was performed at 37 °C for 120 min. The MTG reaction was stopped and the antibody-binding ability of protein G was inactivated by adding 4 mL of 0.1 M citrate buffer (pH 3.0) to the reaction solution. The sample solution was injected into a Protein Ark HiFliQ S-type column for cation exchange chromatography. In the cation exchange chromatography, 50 mM citrate buffer A (0.2 M arginine hydrochloride, pH 3.0) and 50 mM citrate buffer B (0.5 M arginine hydrochloride, 2 M sodium chloride, pH 3.0) were used, and the analysis conditions were as follows: flow rate, 1.2 mL min^{-1}; detection

wavelength, 280 and 565 nm; gradient, A/B = 95:5 to 45:55 over 20 min. Each fraction was neutralized by adding 100 μL of 1 M Tris-HCl (pH 8.0). The fraction 1 (**F1**) containing TAMRA-Q-modified trastuzumab and unmodified trastuzumab was collected to completely separate the antibody and EzMTG-pG(Fab) (Fig. S8a). The fraction 2 (**F2**) containing primarily EzMTG-pG(Fab) and the residual TAMRA-Q-modified trastuzumab, suggesting the presence of the complex of TAMRA-Q-modified trastuzumab and EzMTG-pG(Fab) that cannot be separated under the experimental conditions. The fractions of **F1** were concentrated, and the buffer was exchanged to 1× PBS (pH 7.4) using 10 kDa MWCO Amicon Ultra-15 centrifugal filter units. The purity of TAMRA-Q-modified trastuzumab in **F1** was determined by RP-HPLC chromatograms (Fig. S8b).

Evaluation of the antigen-binding ability of native or modified trastuzumab with BLItz

Bio-layer interferometry was performed with a BLItz (Fortebio, CA, USA) to analyze the binding of trastuzumab or TAMRA-Q-modified trastuzumab to HER2. Streptavidin (SA) biosensors (Sartorius AG, Göttingen, Germany) were hydrated in Milli-Q water for at least 10 min before use. To immobilize the HER2 on the SA biosensors, the HER2 was biotinylated using the Biotin Labeling Kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan). After the baseline step with kinetics buffer (Fortebio, CA, USA), 4 μL of biotin-HER2 (30 μg/mL in Milli-Q water) was added to the drop holder to allow the association of biotin-HER2 with the SA biosensor surfaces for 120 s. After the baseline step with kinetics buffer for 30 s, the association of samples (50, 100, and 200 nM of TAMRA-Q-modified trastuzumab) with the proteins on the tips for 120 s and a dissociation step for 120 s by soaking in kinetics buffer were performed. The binding affinity (K_d) was evaluated as the mean of two experiments ($N = 2$).

Cell Culture

SK-BR-3 cells and MDA-MB-231 cells were purchased from RIKEN Cell Bank (Ibaraki, Japan). SK-BR-3 cells were cultured in McCoy's 5A medium and MDA-MB-231 cells were cultured in DMEM medium (high glucose). The medium was supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic mixed solution and cultured in a humidified incubator at 37 °C in the presence of 5% CO_2.

CLSM observations

Cells were plated in a multi-well glass-bottom dish (10000 cells per well) and allowed to attach to the glass-bottom dish for 24 h at 37 °C. After washing with Opti-MEM, TAMRA-Q-modified trastuzumab (final conc.: 10 μg/mL) was added and the cells were incubated at 37 °C for 1 h. After washing again with Opti-MEM, 100 μL of 4% paraformaldehyde phosphate buffer solution was added and the cells were allowed to stand for 10 min to fix the cells. The cells were observed using CLSM (Carl Zeiss microscope, Oberkochen, Germany) with diode lasers (567 nm for TAMRA).

Tables

Table S1 The amino acid sequences of pG-fused EzMTG mutants.

EzMTG-pG
MHHHHHHDNGAGEETRSAAETYRLTADDVANINALNESAPAASSGGGSLVPRGSGGGSAPDSDDRVTPPAEPLDRMPD PYRPSYGRAETVVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCVGVTWVNSGQYPTNRLAFASFDEDRFKNELKN GRPRSGETRAEFEGRVAKESFDEEKGFQRAREVASVMNRALENAHDESAYLDNLKKELANGNDALRNEDARSPFYSAL RNTPSFKERNGGNHDPSRMKAVIYSKHFWSGQDRSSSADKRKYGDPDAFRPAPGTGLVDMSRDRNIPRSPTSPGEGFV NFDYGWFGAQTEADADKTVWTHGNHYHAPNGSLGAMHVYESKFRNWSEGYSDFDRGAYVITFIPKSWNTAPDKVKQGW PGGGGSGGGGSGGGGSTYKLVINGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTE

EzMTG-pG(Fc)
MHHHHHHDNGAGEETRSAAETYRLTADDVANINALNESAPAASSGGGSLVPRGSGGGSAPDSDDRVTPPAEPLDRMPD PYRPSYGRAETVVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCVGVTWVNSGQYPTNRLAFASFDEDRFKNELKN GRPRSGETRAEFEGRVAKESFDEEKGFQRAREVASVMNRALENAHDESAYLDNLKKELANGNDALRNEDARSPFYSAL RNTPSFKERNGGNHDPSRMKAVIYSKHFWSGQDRSSSADKRKYGDPDAFRPAPGTGLVDMSRDRNIPRSPTSPGEGFV NFDYGWFGAQTEADADKTVWTHGNHYHAPNGSLGAMHVYESKFRNWSEGYSDFDRGAYVITFIPKSWNTAPDKVKQGW PGGGGSGGGGSGGGGSTYRLVINGRTLRGETTTRAVDAETAEKAFKQYANDYGVDGVWTYDDATRTFTVTE

EzMTG-pG(Fab)
MHHHHHHDNGAGEETRSAAETYRLTADDVANINALNESAPAASSGGGSLVPRGSGGGSAPDSDDRVTPPAEPLDRMPD PYRPSYGRAETVVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCVGVTWVNSGQYPTNRLAFASFDEDRFKNELKN GRPRSGETRAEFEGRVAKESFDEEKGFQRAREVASVMNRALENAHDESAYLDNLKKELANGNDALRNEDARSPFYSAL RNTPSFKERNGGNHDPSRMKAVIYSKHFWSGQDRSSSADKRKYGDPDAFRPAPGTGLVDMSRDRNIPRSPTSPGEGFV NFDYGWFGAQTEADADKTVWTHGNHYHAPNGSLGAMHVYESKFRNWSEGYSDFDRGAYVITFIPKSWNTAPDKVKQGW PGGGGSGGGGSGGGGSTYRLVINGRTLRGETTTRAVDAETAAAAFANYARRNGVDGVWTYDDATRTFTVTE

Pink: Propeptide domain; Orange: MTG domain; Black: pG domain; Red: Mutated amino acid; Light blue: hexahistidine tag; Gray: Linker sequences.

Table S2 Characteristics of the lysine residues in the heavy chain of trastuzumab calculated by MOE, in descending order of the positive residue patch area (PRPA) value.

Position	ASA (Å^2)[1]	Exp (%)[2]	PRPA (Å^2)[3]
65	148.5	63.06	50.4
225	123.4	52.41	49.1
249	138.7	58.91	47.8
363	83.4	35.4	43.2
208	109.7	46.6	43
124	119.2	50.63	42.6
43	143.9	61.1	41.6
395	80.6	34.24	41.6
136	58.4	24.81	40.4
277	145.4	61.74	40.4
213	153.6	65.23	38.4
329	200.3	85.06	37.7
216	55.7	23.66	36
76	152.1	64.58	35
325	58.2	24.74	33.8
291	138.1	58.66	33.4
337	71.2	30.25	33
30	77.9	33.1	31.8
221	97.1	41.22	30
217	94.1	39.95	28.7
343	126.8	53.87	28.1
251	42.4	18.02	27.6
373	21.8	9.26	22
341	16.6	7.07	11.1
293	85.7	36.41	10
150	10.9	4.65	0
320	78.5	33.32	0
323	55.8	23.7	0
412	0.5	0.21	0
417	44.9	19.08	0
442	51.6	21.9	0

[1]ASA: Accessible surface area of the residue.

[2]Exp: Exposure of the residue compared with the ideal surface as determined from Gly_X_Gly triplets.

[3]PRPA: Positive residue patch area. When the surrounding region possesses an appreciable positive charge, and the residue contributes to positive charges exposed on the surface, the value is increased.

Table S3 Characteristics of Lys65 of trastuzumab, rituximab and ramucirumab calculated by MOE.

	trastuzumab	rituximab	ramucirumab
Primary sequence around Lys65	SVK$_{65}$GR	KFK$_{65}$GK	SVK$_{65}$GR
ASA (Å)[1]	148.5	153.6	113.6
Exp (%)[2]	63.06	65.24	48.25
PRPA (Å^2)[3]	50.4	49.5	0.0

[1]ASA: Accessible surface area of the residue.

[2]Exp: Exposure of the residue compared with the ideal surface as determined from Gly_X_Gly triplets.

[3]PRPA: Positive residue patch area.

Figures

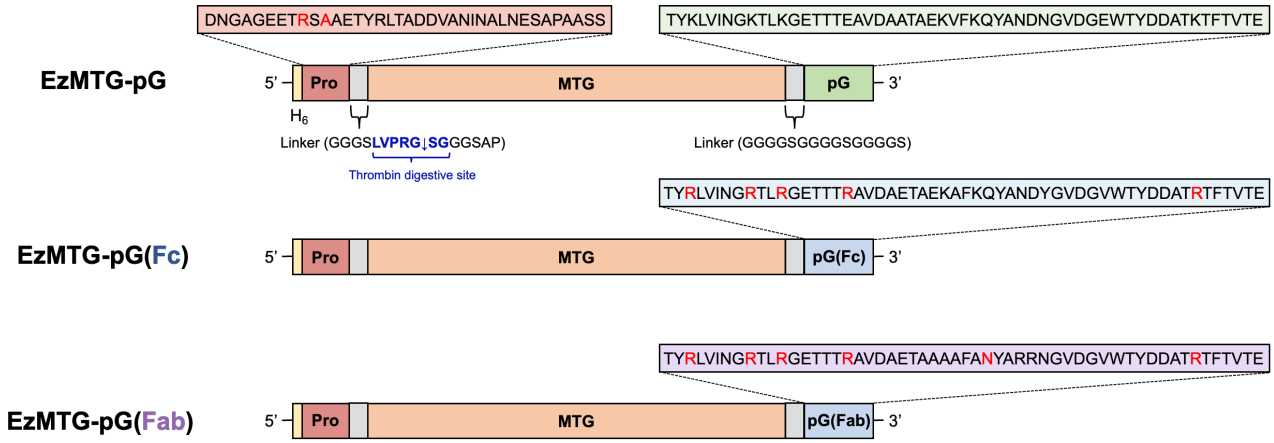


Fig. S1 The pG-fused EzMTG variants designed in this study.

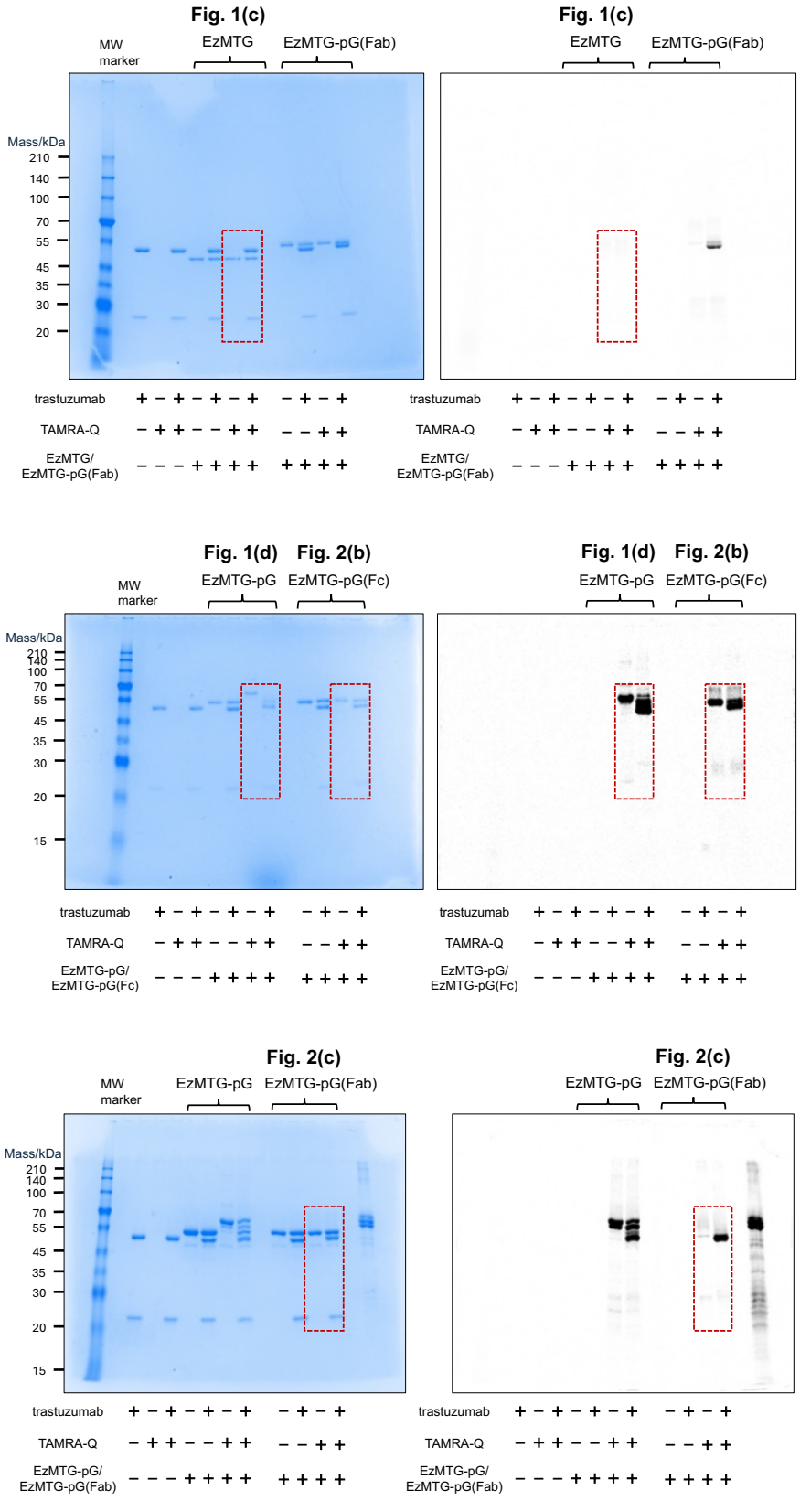


Fig. S2 Raw images of sodium dodecyl sulfate–polyacrylamide gel electrophoretic gels (CBB staining, left) and their fluorescent images (derived from TAMRA, right) for Figs 1c, 1d, 2b, and 2c (Rectangular boxes with red dotted line).

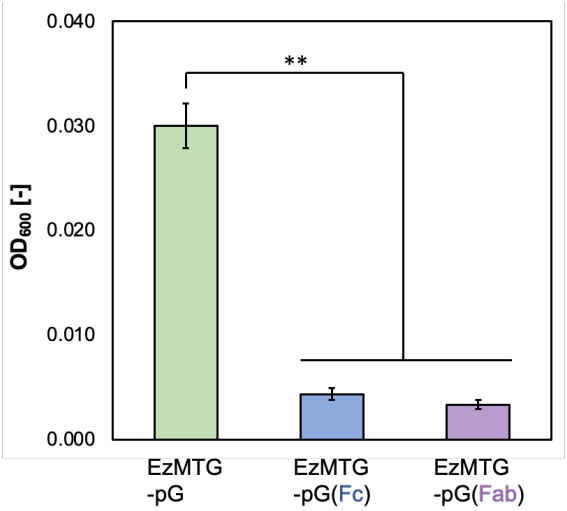


Fig. S3 Evaluation of aggregation formation with IgG using the OD_{600} values. $N = 3$; mean ± SE; $^{**}p < 0.01$. (ANOVA followed by Tukey's post hoc test).

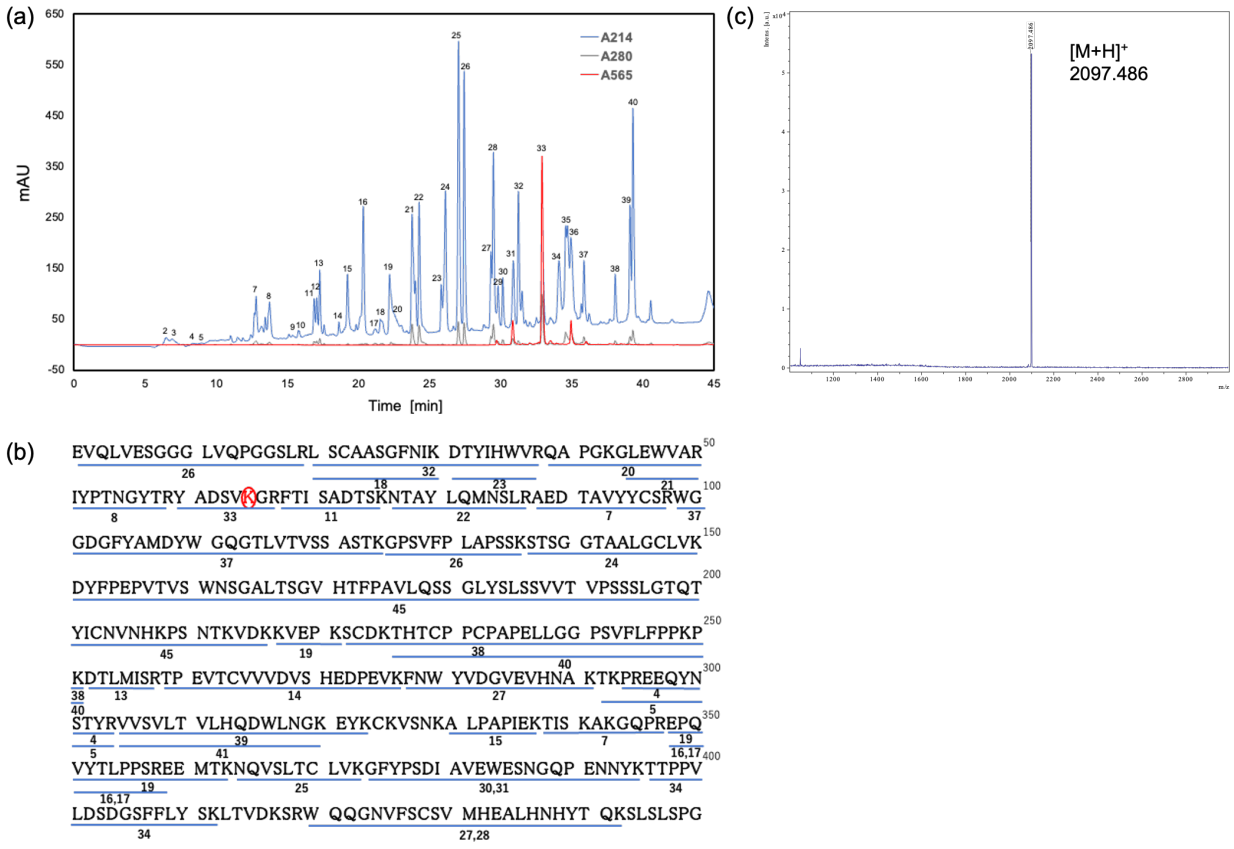


Fig. S4 Identification of modification positions. (a) Chromatogram of TAMRA-Q-modified heavy chain fragmentation peptides and (b) Peptide mapping results on the heavy chain sequence [the numbers at the bottom correspond to the peak numbers on the chromatogram in (a)]. Lys65 is shown in red. (c) MALDI-TOF-MS result for the fragment No. 33 on the chromatogram in (a).

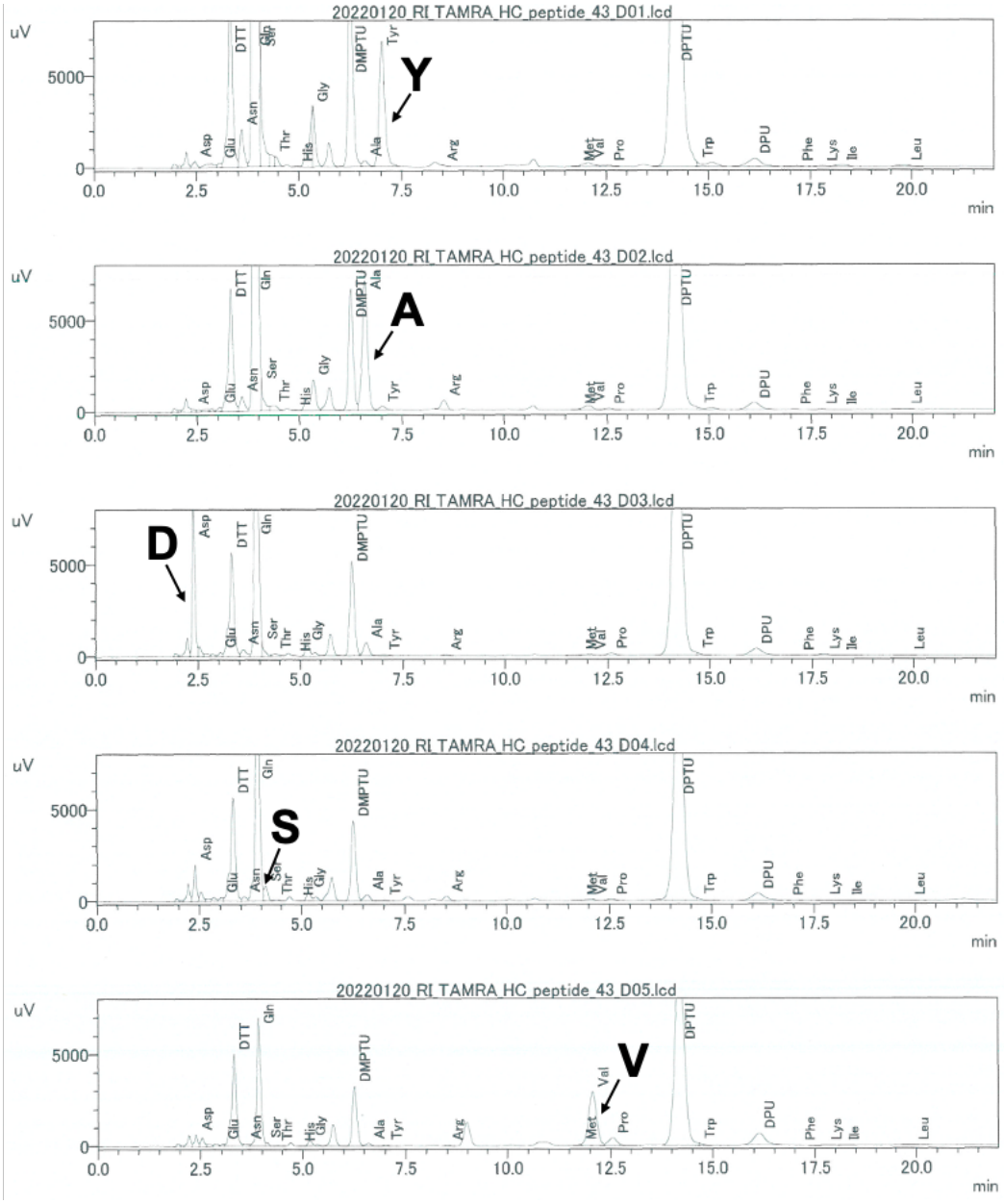


Fig. S5 HPLC chromatograms of each residue obtained from N-terminal sequence analysis of modified antibody fragment.

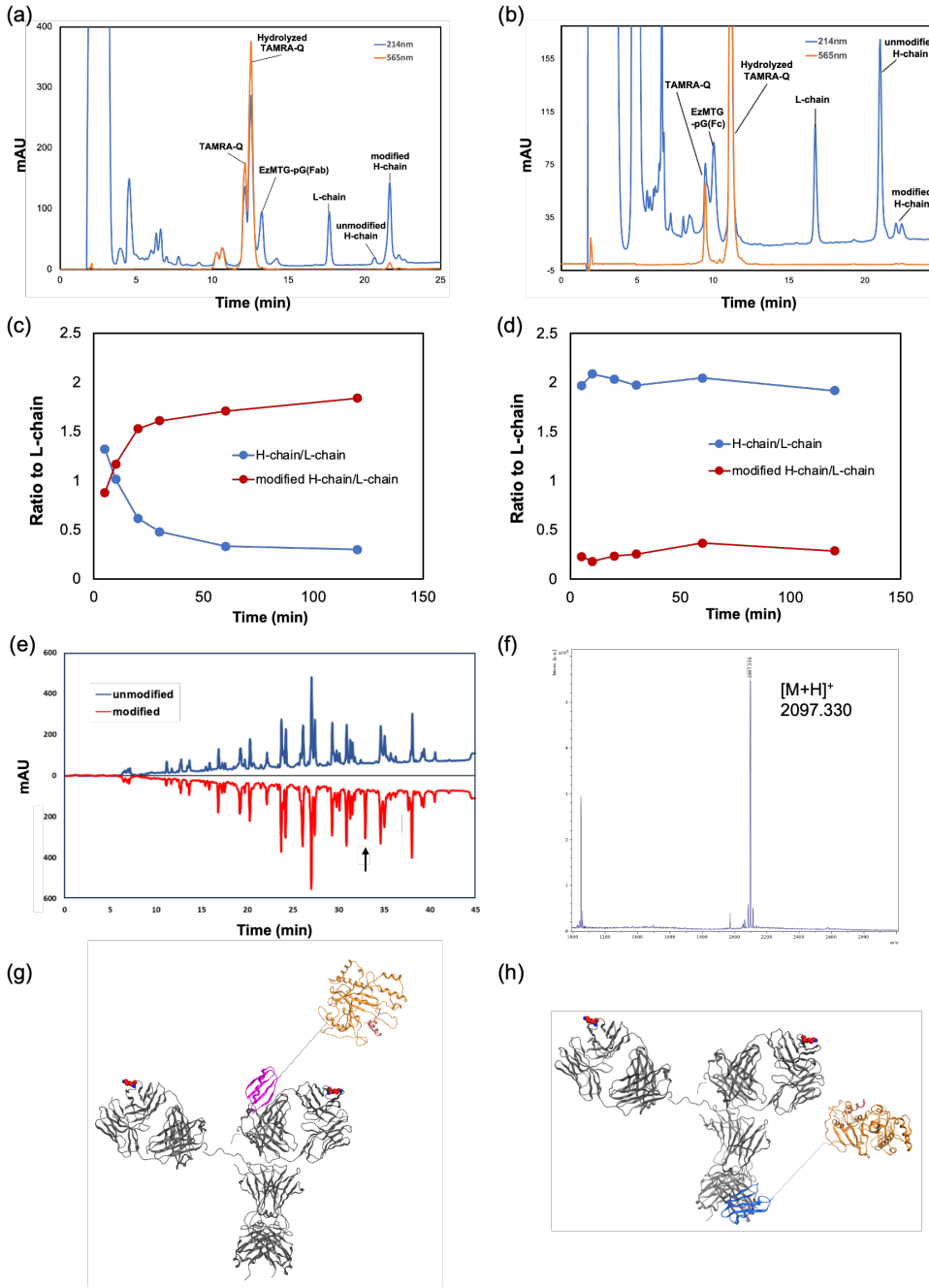


Fig. S6 RP-HPLC chromatograms of reaction solutions with (a) EzMTG-pG(Fab) or (b) EzMTG-pG(Fc). The reaction was performed with trastuzumab (1.3 μM), each pG-fused EzMTG mutant (2.6 μM) and TAMRA-Q (100 μM) in 40 mM Tris-HCl (pH 8.0) with 2 v/v% trehalose at 37 °C for 120 min. Time course of the antibody modification ratio using (c) EzMTG-pG(Fab) or (d) EzMTG-pG(Fc) in 40 mM Tris-HCl (pH 8.0) at 37°C. (e) Identification of Lys residues modified with TAMRA-Q by EzMTG-pG(Fc). RP-HPLC chromatograms of antibodies modified by EzMTG-pG(Fc). (f) MALDI-TOF-MS result for the fragment indicated by the arrow on the chromatogram in (e). The three-dimensional structure of trastuzumab and (g) EzMTG-pG(Fab) or (h) EzMTG-pG(Fc). Trastuzumab, EzMTG, and pG(Fc) are shown in gray, orange, and blue, respectively. The Lys65 of Trastuzumab is shown in red. The PDB entries for Fab, Fc, and Fc-specific pG are 1N8Z.pdb, 1HZH.pdb, and 1FCC.pdb, respectively.

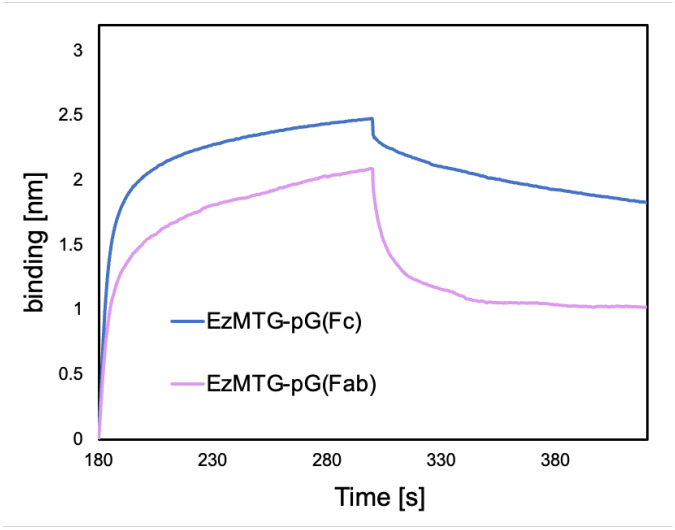


Fig. S7 Representative sensorgrams for evaluation of the binding affinity of each pG-fused EzMTG mutant to trastuzumab by bio-layer interferometry (BLI) analysis.

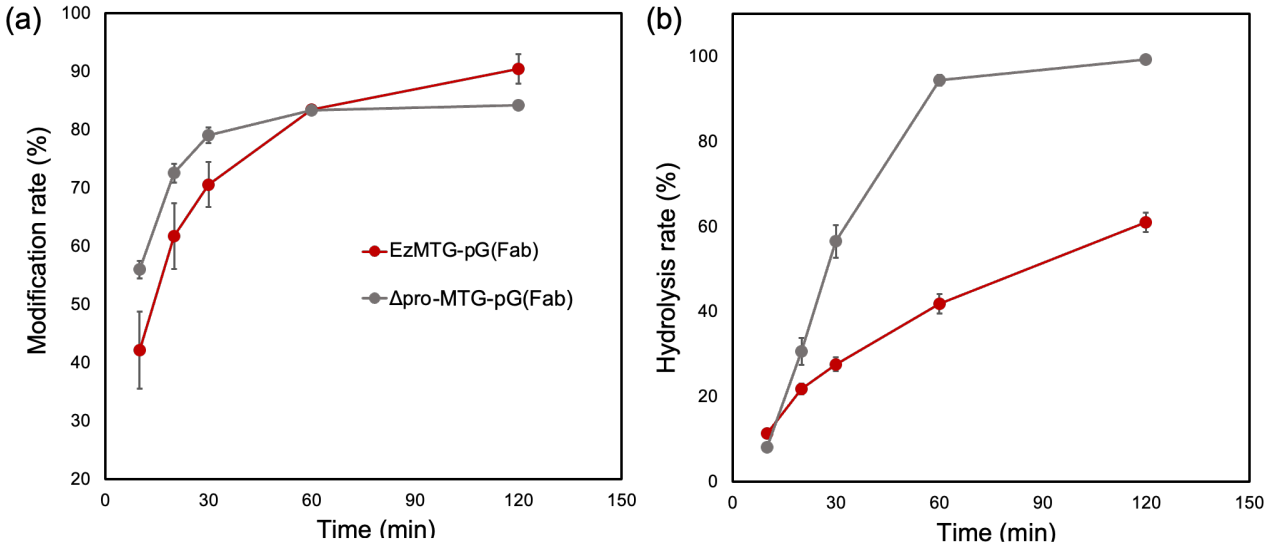


Fig. S8 Time course of (a) the antibody modification ratio and (b) hydrolysis rate of TAMRA-Q substrates using ΔproMTG-pG(Fab) or EzMTG-pG(Fab) in 40 mM Tris-HCl (pH 8.0) at 37°C.

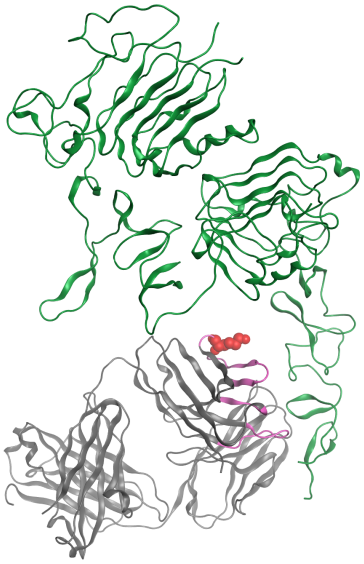


Fig. S9 Three-dimensional structure of trastuzumab Fab domain and its antigen HER2. The Fab domain and HER2 are shown in gray and green, respectively. The Lys65 and CDR of the Fab domain are shown in red and pink, respectively. The PDB entry for the Fab and HER2 complex is 1N8Z.pdb.

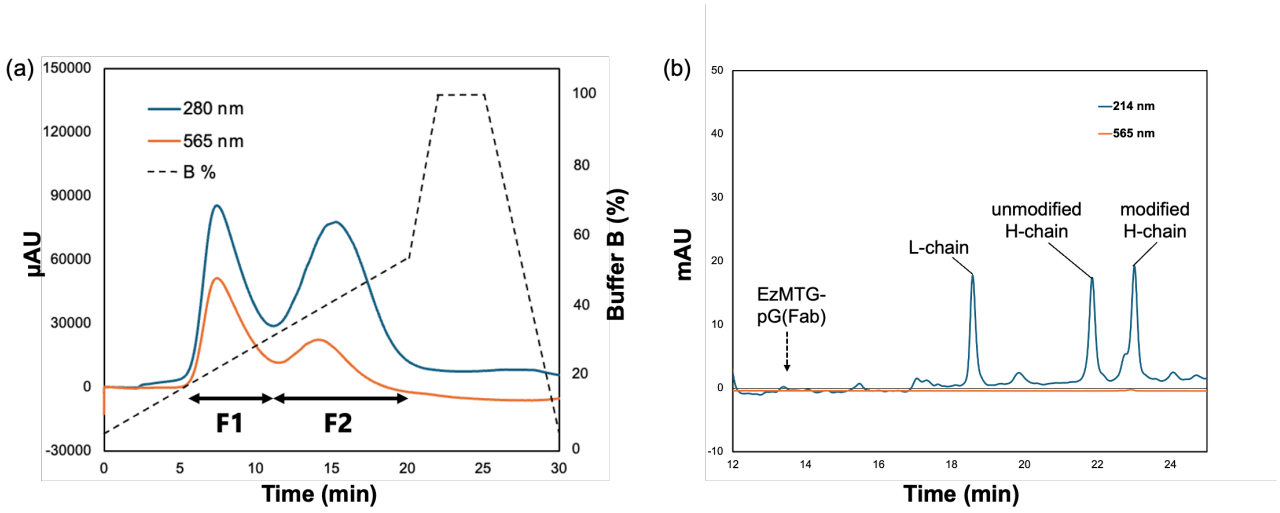


Fig. S10 (a) Cation exchange chromatogram for the purification of TAMRA-Q-modified trastuzumab. Fraction 1 (**F1**) contains TAMRA-Q-modified and unmodified trastuzumab, whereas fraction 2 (**F2**) contains mainly EzMTG-pG(Fab) and the remaining TAMRA-Q-modified trastuzumab. (b) RP-HPLC chromatogram of the **F1** fraction, used as purified TAMRA-Q-modified trastuzumab for evaluation of antigen binding properties.

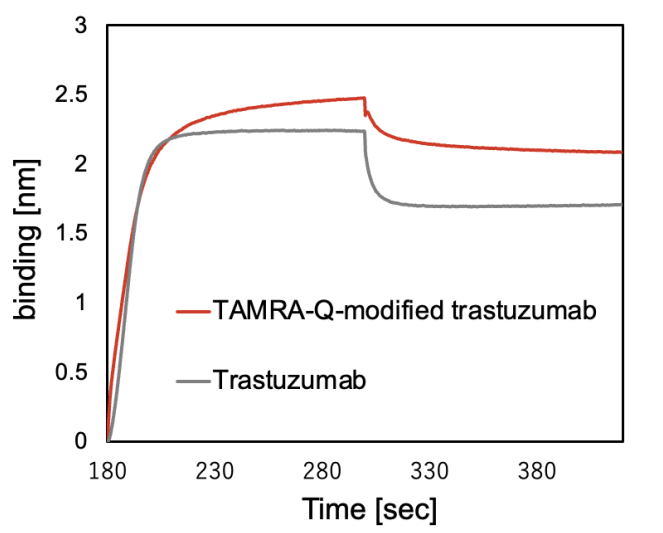


Fig. S11 Representative sensorgrams for evaluation of the binding affinity of trastuzumab and TAMRA-Q-modified trastuzumab to HER2 by bio-layer interferometry (BLI) analysis.

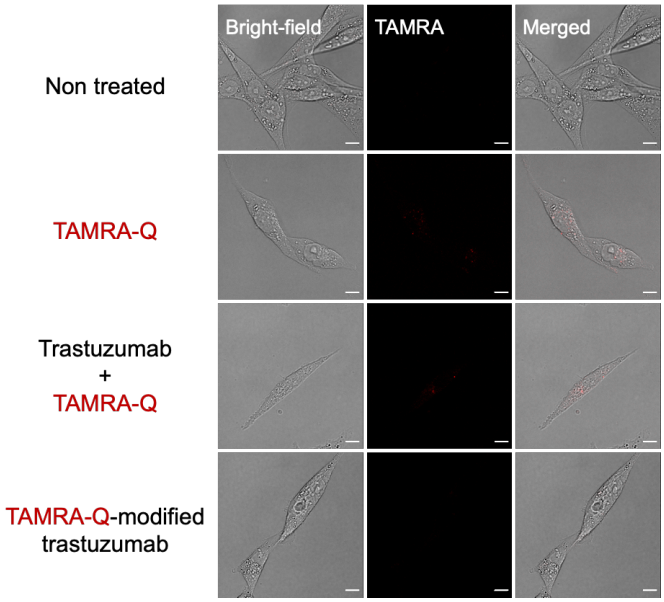
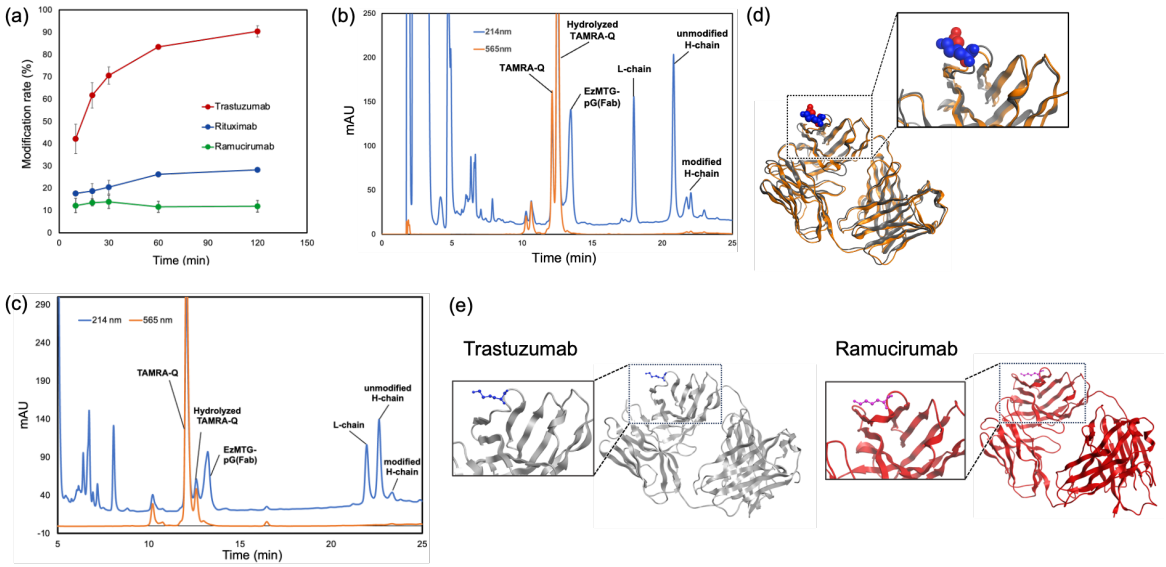


Fig. S12 Binding of TAMRA-Q-modified trastuzumab to the SK-BR-3 cell line. Representative fluorescence images obtained by CLSM. MDA-MB-231 cells were incubated with each sample at 37 °C for 1 h. Bars: 10 μm.



(f)

* **Trastuzumab**	LEWVARIYPTNG--YTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTA	Secukinumab	LEWVAAINQDGS--EKYYVGSVKGRFTISRDNAKNSLYLQMNSLRVEDTA
* **Rituximab**	LEWIGAIYPGNG--DTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSA	Durvalumab	LEWVANIKQDGS--EKYYVDSVKGRFTISRDNAKNSLYLQMNSLRAEDTA
* **Ramucirumab**	LEWVSSISSSSS--YIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTA	Elotuzumab	LEWIGEINPDSS--TINYAPSLKDKFIISRDNAKNSLYLQMNSLRAEDTA
Tocilizumab	LEWIGYISYSG---ITTYNPSLKSRVTMLRDTSKNQFSLRLSSVTAADTA	Daratumumab	LEWVSAISGSGG--GTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA
Necitumumab	LEWIGYIYYSG---STDYNPSLKSRVTMSVDTSKNQFSLKVNSVTAADTA	Alirocumab	LDWVSTISGSGG--TTNYADSVKGRFIISRDSSKHTLYLQMNSLRAEDTA
Alemtuzumab	LEWIGFIRDKAKGYTTEYNPSVKGRVTMLVDTSKNQFSLRLSSVTAADTA	Avelumab	LEWVSSIYPSGG--ITFYADTVKGRFTISRDNSKNTLYLQMNSLRAEDTA
Basiliximab	LEWIGAIYPGNS--DTSYNQKFEGKAKLTAVTSASTAYMELSSLTHEDSA	Omalizumab	LEWVASITYDG---STNYNPSVKGRITISRDDSKNTFYLQMNSLRAEDTA
Isatuximab	LEWIGTIYPGDG--DTGYAQKFQGKATLTADKSSKTVYMHLSSLASEDSA	Satralizumab	LEWIGFISYSG---ITNYNPSLQGRVTISRDNSKNTLYLQMNSLRAEDTA
Obinutuzumab	LEWMGRIFPGDG--DTDYNGKFKGRVTITADKSTSTAYMELSSLRSEDTA	Panitumumab	LEWIGHIYYSG---NTNYNPSLKSRLTISIDTSKTQFSLKLSSVTAADTA
Tildrakizumab	LEWMGQIFPASG--SADYNEKFEGRVTMTTDTSTSTAYMELRSLRSDDTA	Denosumab	LEWVSGITGSGG--STYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA
Vedolizumab	LEWIGEIDPSES--NTNYNQKFKGRVTLTVDISASTAYMELSSLRSEDTA	Eculizumab	LEWMGEILPGSG--STEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTA
Benralizumab	LAWMGYINPYND--GTKYNERFKGKVTITSDRSTSTVYMELSSLRSEDTA	Ravulizumab	LEWMGEILPGSG--HTEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTA
Risankizumab	LEWIGYIYPRDD--SPKYNENFKGKVTITADKSTSTAYMELSSLRSEDTA	Evolocumab	LEWMGWVSFYNG--NTNYAQKLQGRGTMTTDPSTSTAYMELRSLRSDDTA
Ustekinumab	LDWIGIMSPVDS--DIRYSPSFQGQVTMSVDKSITTAYLQWNSLKASDTA	Brodalumab	LEWMGWISTYSG--NTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTA
Bezlotoxumab	LEWMGIFYPGDS--STRYSPSFQGQVTISADKSVNTAYLQWSSLKASDTA	Inotuzumab	LEWIGGINPGNN--YATYRRKFQGRVTMTADTSTSTVYMELSSLRSEDTA
Guselkumab	LEWMGIIDPSNS--YTRYSPSFQGQVTISADKSISTAYLQWSSLKASDTA	Natalizumab	LEWMGRIDPANG--YTKYDPKFQGRVTITADTSASTAYMELSSLRSEDTA
Atezolizumab	LEWVAWISPYGG--STYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTA	Ixekizumab	LEWMGVINPMYG--TTDYNQRFKGRVTITADESTSTAYMELSSLRSEDTA
Certolizumab	LEWMGWINTYIG--EPIYADSVKGRFTFSLDTSKSTAYLQMNSLRAEDTA	Gemtuzumab	LEWIGYIYPYNG--GTDYNQKFKNRATLTVDNPTNTAYMELSSLRSEDTA
Ranibizumab	LEWVGWINTYTG--EPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTA	Pembrolizumab	LEWMGGINPSNG--GTNFNEKFKNRVTLTTDSSTTTAYMELKSLQFDDTA
Bevacizumab	LEWVGWINTYTG--EPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTA	Galcanezumab	LEWMGAIYEGTG--KTVYIQKFADRVTITADKSTSTAYMELSSLRSEDTA
Pertuzumab	LEWVADVNPNSG--GSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTA	Nivolumab	LEWVAVIWYDGS--KRYYADSVKGRFTISRDNSKNTLFLQMNSLRAEDTA
Polatuzumab	LEWIGEILPGGG--DTNYNEIFKGRATFSADTSKNTAYLQMNSLRAEDTA	Dupilumab	LEWVSSISGSGG--NTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA
Inebilizumab	LEWVGRIYPGDG--DTNYNVKFKGRFTISRDDSKNSLYLQMNSLKTEDTA	Emicizumab	LEWVSSISPSGQ--STYYRREVKGRFTISRDNSKNTLYLQMNSLRAEDTA
Ipilimumab	LEWVTFISYDGN--NKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA	Cetuximab	LEWLG-VIWSGG--NTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTA
Canakinumab	LEWVAIIWYDGD--NQYYADSVKGRFTISRDNSKNTLYLQMNGLRAEDTA	Ibritumomab	LEWIGAIYPGNG--DTSYNQKFKGKATLTVDKSSSTAYMQLSSLTSEDSA
Golimumab	LEWVAFMSYDGS--NKKYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA	Idarucizumab	LEWIGVIWAGGS---TGYNSALRSRVSITKDTSKNQFSLKLSSVTAADTA
Sarilumab	LEWVSGISWNSG--RIGYADSVKGRFTISRDNAENSLFLQMNGLRAEDTA	Brolucizumab	PKLLIYLASTLAS---------GVPSRFSGSGSGAEFTLTISSLQPDDFA
Adalimumab	LEWVSAITWNSG--HIDYADSVEGRFTISRDNAKNSLYLQMNSLRAEDTA	Blinatumomab	PKLLIYDASNLVS---------GIPPRFSGSGSGTDFTLNIHPVEKVDAA
Ofatumumab	LEWVSTISWNSG--SIGYADSVKGRFTISRDNAKKSLYLQMNSLRAEDTA	Palivizumab	LEWLADIWWDDK---KDYNPSLKSRLTISKDTSKNQVVLKVTNMDPADTA
Mogamulizumab	LEWVATISSAST--YSYYPDSVKGRFTISRDNAKNSLYLQMNSLRVEDTA		

Fig. S13 (a) Time course of the modification rate of rituximab using EzMTG-pG(Fab) in 40 mM Tris-HCl (pH 8.0) at 37 °C. RP-HPLC chromatograms of reaction solutions with EzMTG-pG(Fab). The reaction was conducted with (b) rituximab or (c) ramucirumab (1.3 μM), each pG-fused EzMTG mutant (2.6 μM) and TAMRA-Q (100 μM) in 40 mM Tris-HCl (pH 8.0) with 2 v/v% trehalose at 37 °C for 120 min. (d) Model of the Fab three-dimensional structures of trastuzumab and rituximab shown in gray and orange, respectively. Lys65 of trastuzumab and Lys65 of rituximab represented as the space-filling model are shown in blue and red, respectively. The PDB entries used for Fab of trastuzumab and Fab of rituximab are 1N8Z.pdb and 4KAQ.pdb, respectively. (e) Model of the Fab three-dimensional structures of trastuzumab and ramucirumab shown in gray and red, respectively. Lys65s of trastuzumab and ramucirumab are represented as a ball-and-stick in blue and pink, respectively. The PDB entries used for Fab of ramucirumab and Fab of trastuzumab are 3S36.pdb and 1N8Z.pdb, respectively. (f) Amino acid sequence around Lys65 of commercial monoclonal antibodies. The red box highlights the sequence surrounding Lys65 (SVKGR), which is identical to that of trastuzumab.