Supporting Information for

A Peptide Inhibitor of Antibody-Dependent Cell-Mediated Cytotoxicity against EGFR/Folate Receptor-α Double Positive Cells

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EXPERIMENTAL

Materials. Rink Amide AM resin (200-400 mesh, amine density of 0.61 mmol g^{-1}), Fmoc-protected amino acids were purchased from Novabiochem (Darmstadt), Fmoc-NH-$(PEG)_5$-COOH was purchased from Merck Millipore, N^{10}-(Trifluoroacetyl)pteroic acid was purchased from Sigma aldrich, (N-[1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethylamino(morpholino)]uronium hexafluorophosphate (COMU), diisopropylethylamine (DIPEA), dichloromethane (DCM), 1-methyl-2-pyrrolidone (NMP), and piperidine were purchased from Watanabe Chemical , and *N, N*-dimethylformamide (DMF) from Kanto Chemical . Dimethylsulfoxide (DMSO), and methanol were purchased from Wako Pure Chemicals . All reagents were used without further purification.

Synthesis of Fc antibody-recruiting molecules (Fc-ARMs). Fc-ARMs were synthesized by standard Fmoc-chemistry using the Rink Amide AM resin, DIPEA as a base, COMU as coupling reagents, and a 20% solution of piperidine in DMF for deprotection of Fmoc group. Presence of free amines was checked by the standard Kaiser (ninhydrin) test. The ivDde protecting group was removed by 2% hydrazine in DMF. TFA protecting group was removed by 1M ammonium hydroxide solution/DMF (1:1, v/v). After completion of the peptide sequence, the resin was washed with DMF, DCM, and methanol (each five times) and dried in vacuo overnight. Cleavage of the peptide from the resin and side chain-deprotection was performed by the treatment of a mixture of TFA/phenol/thioanisole/water/1,2-ethandithiol (80/7.5/5/5/2.5) for 90 min at R.T. The material was filtered and washed with a minimum amount of cleavage reagent described above. The peptide solution was added dropwise into an excess volume of diethyl ether to precipitate peptide, followed by centrifugation (3,500 x g, 4 ºC) to collect precipitate. This cycle was performed three times and dried in vacuo overnight. The crude linear peptide with free cysteine residues was extracted with 2 M AcOH, and the solution was adjusted to pH 7.8 with ammonium hydroxide solution and diluted to the final peptide concentration of 0.1 mM. The solution was stirred slowly at room temperature to form a disulfide bond between two cysteines. The progress of cyclization was monitored by reverse phase HPLC. The cyclized crude peptide was purified by standard reversed phase HPLC using C18 Atlantis column (Waters, 4.6 x 100 mm for analysis and 19 x 100 mm for purification). To modify rhodamine dye to Fc-ARM, 1.2 eq of NHS-rhodamine in dry DMSO (Thermo fisher scientific) and DIPEA was added and reacted with primary amine of Lys side chain. The obtained peptide was further purified by reversed phase HPLC and identified by MALDI-TOF MS.

Preparation of fluorophore-labeled antibody. A fluorescent molecule (NHS-fluorescein (Thermo fisher scientific), SeTau-647 (SETA BioMedicals)) was labelled on anti-CD20 monoclonal antibody (mAb) (ofatumumab) or anti-EGFR mAb (cetuximab) according to the manufacture's protocol. The modification ratio of fluoresenct molecule on the mAb was determined to be as follows; SeTau-647-anti-EGFR mAb (3.5 fluorophores/mAb), fluorescein-anti-EGFR mAb (5.0 fluorophores/mAb), fluorescein-anti-CD20 mAb (3.5 fluorophores/mAb),

Immunoblotting. Anti-EGFR mAb-immobilized particle (particle **a**) and Fc-III-immobilized particle (particle **b**) were prepared by using a 3-□m particle (Magnosphere MS300/Low Carboxyl; JSR Life Sciences) following the manufactures protocol. The amounts of immobilized anti-EGFR mAb and Fc-III on the particle were determined to be 1.9 x 10^6 and 3.1 x 10^6 molecules/particle, respectively. KHYG-1/FcγIIIa-158V cells (3 x 10^6 cells) were plated in 25 cm^2 flask and cultured in RPMI 1640 medium supplemented with 10 ng ml^{-1} recombinant human IL-2, 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 □g ml^{-1} streptomycin. Twenty-four to 36 hours after the plating, the cells were left unstimulated or stimulated with particle **a** and/or **b** together with anti-EGFR mAb (200 nM) at 37˚C for 3 min, then the cells were lysed in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 1% Nonidet P-40, 2 mM EDTA, 50 mM NaF, and 0.2 mM Na_3VO_4, supplemented with Complete™ protease inhibitor cocktail (Roche Diagnostics GmbH) on ice for 15 min. The cell lysates were centrifuged to remove insoluble materials and then immunoblotted with anti-phospho-ERK-1/2 rabbit monoclonal antibody (D13.14.4E; Cell Signaling Technology) or anti-ERK1 mouse monoclonal antibody (G-8; Santa Cruz Biotechnology) or anti-actin goat polyclonal antibody (1-19; Santa Cruz Biotechnology) and reacting proteins were visualized by chemiluminescence.

REFERENCES

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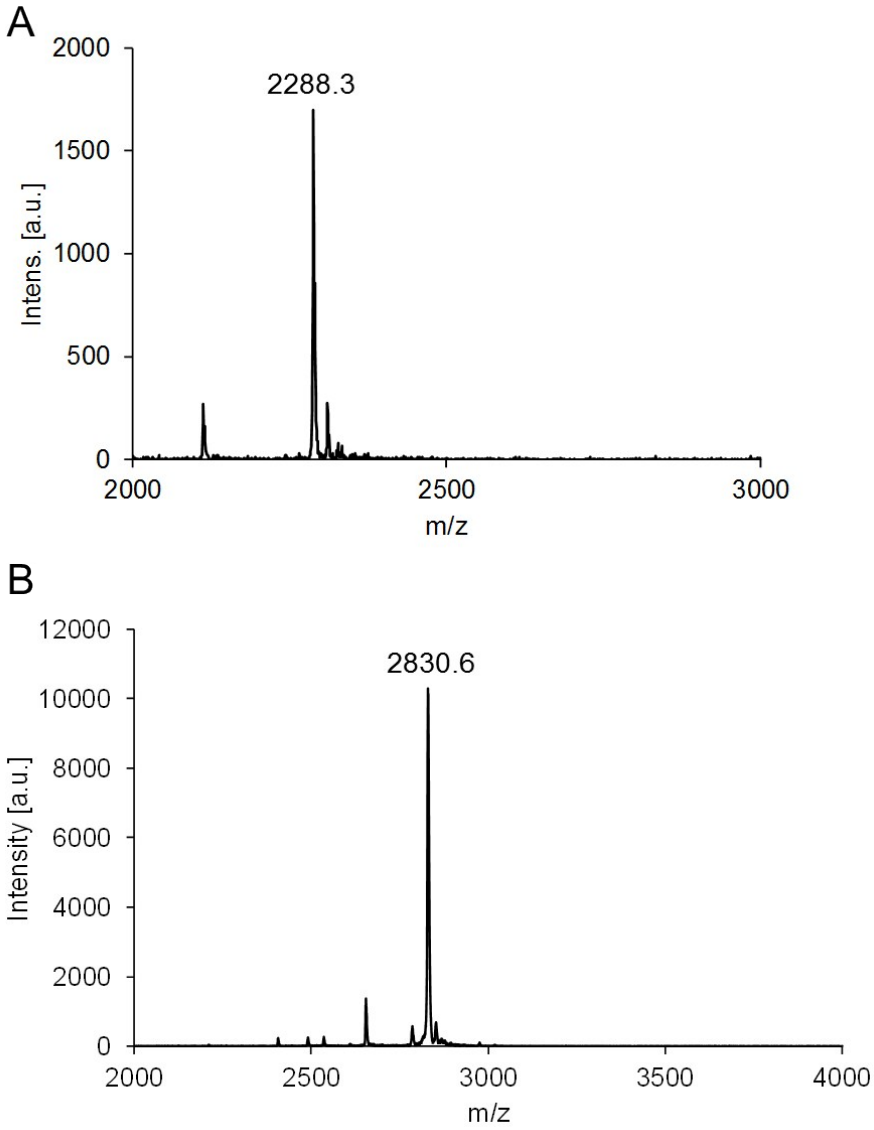
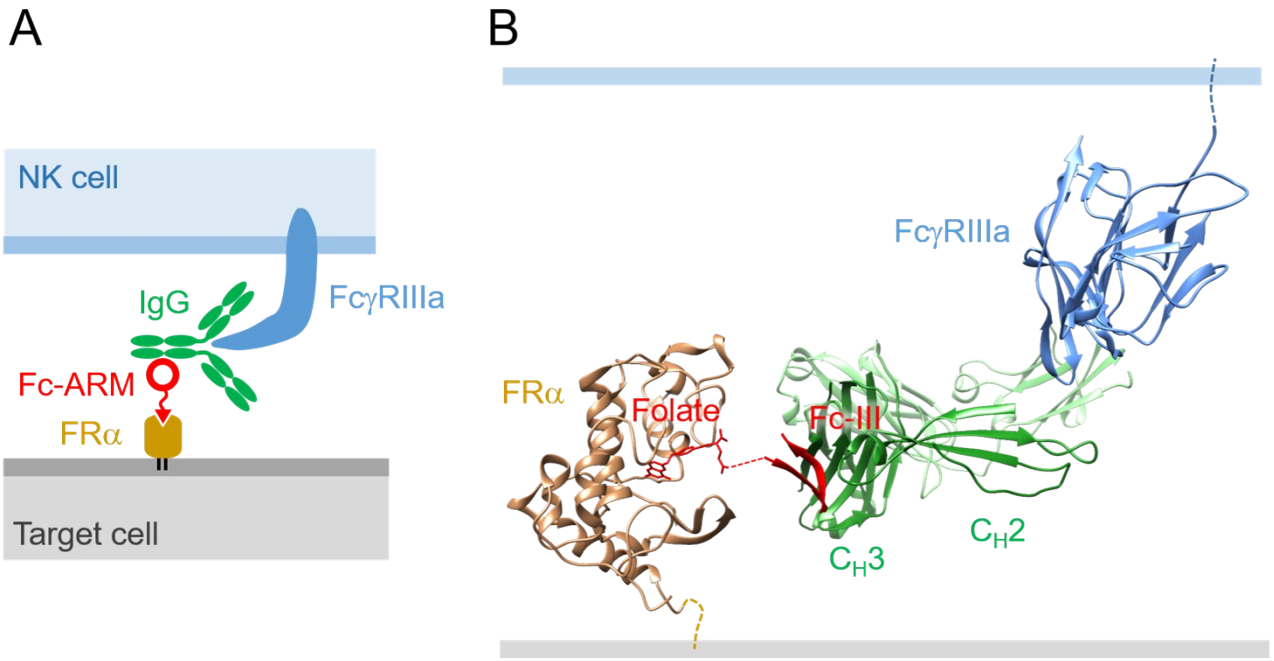
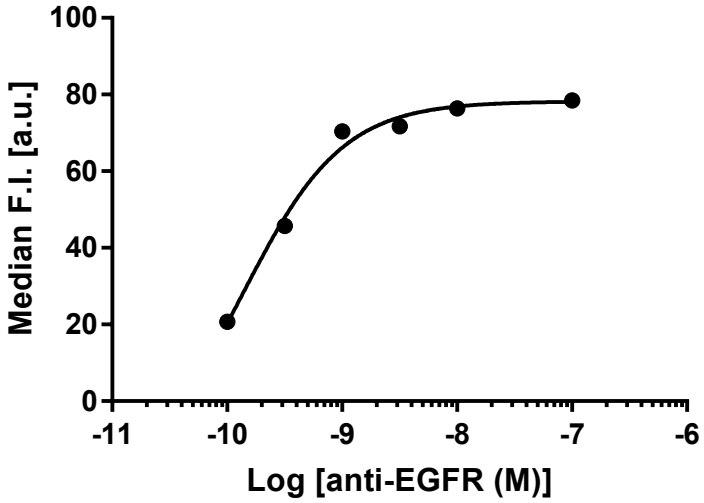
(2) Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004) UCSF Chimera - A Visualization System for Exploratory Research and Analysis. *J. Comput. Chem.* **25**, 1605-1612.



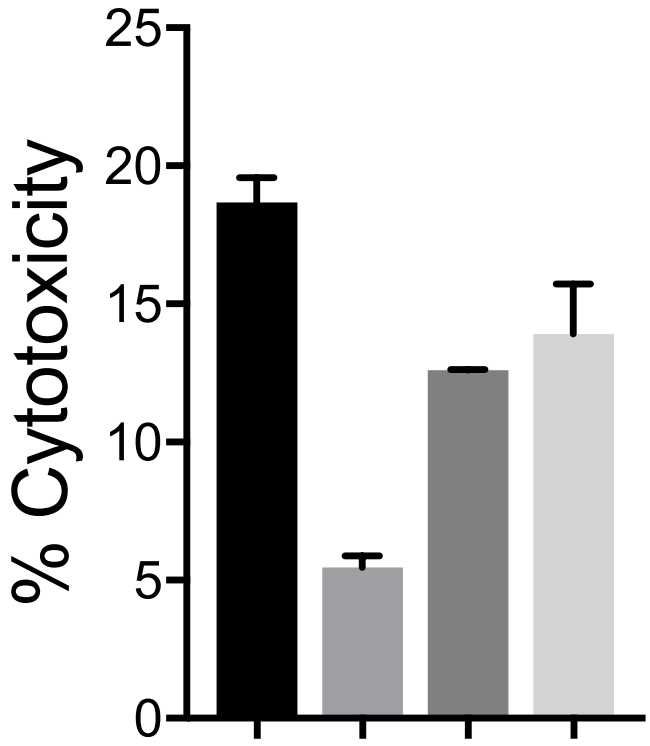
Supplementary Figure 1: Identification of Fc-ARM **1** (A) and **2** (B). Calculated exact mass of Fc-ARM **1** and **2** were 2286.9 and 2829.2, respectively.



Supplementary Figure 2: Schematic drawing (A) and molecular model (B) of ternary complex (FRα/Fc-ARM/IgG) bound with FcγRIIIa. Fc-ARM is shown without oligoethyleneglycol spacer. IgG is only shown as Fc region (C_H2 and C_H3 domains). FRα and FcγRIIIa are shown as extracellular region. This model is generated by UCSF chimera from three crystal structures (PDB ID: 1DN2, 3RJD, 4LRH).[2] A closest distance between *N*-terminus of Fc-III and γ-carboxyl group of folate is ca. 1.1 nm.

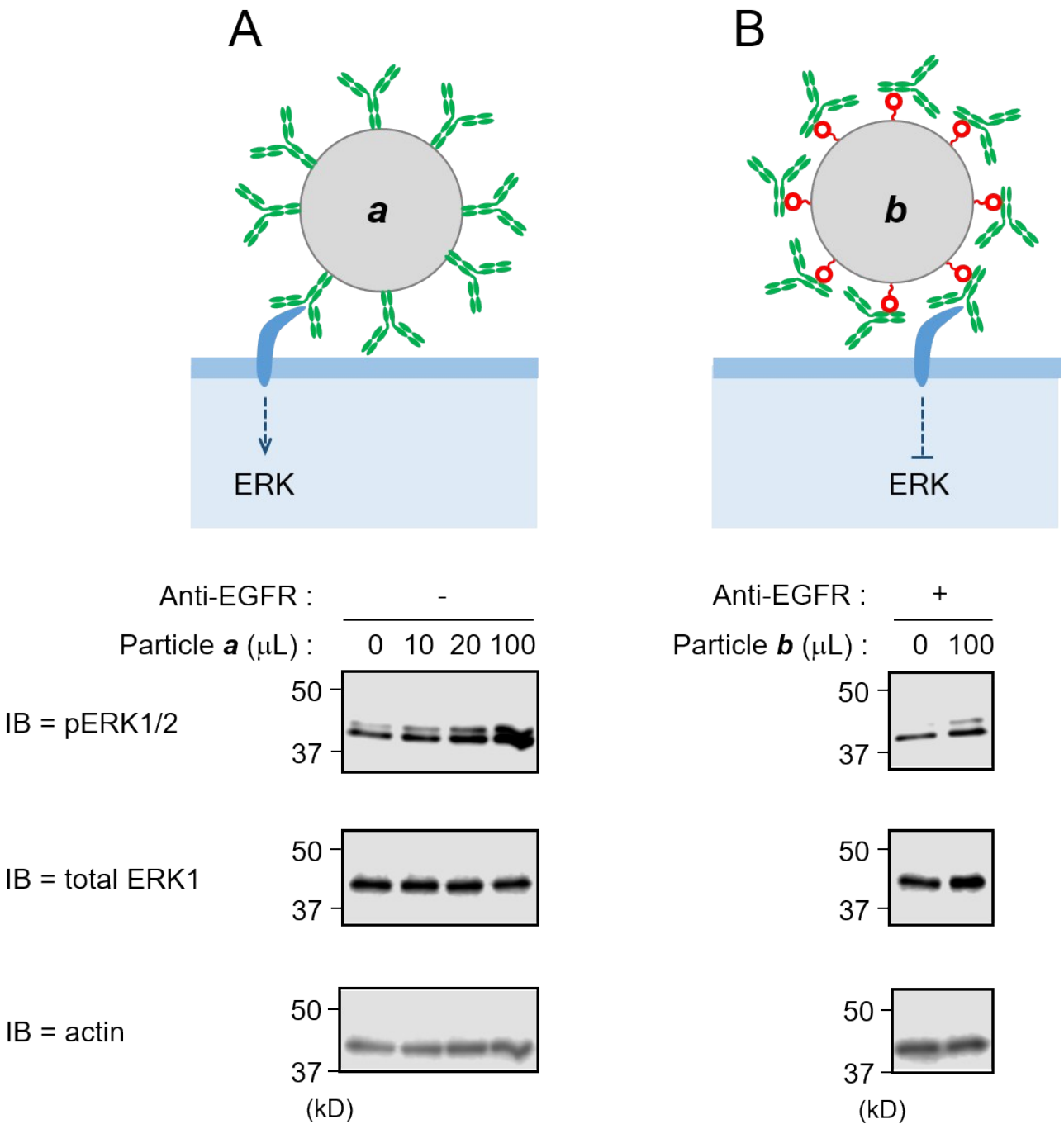


Supplementary Figure 3: Binding capacity of anti-EGFR mAb (rhodamine-labelled) to IGROV-1 cells determined by flow cytometry. Experimental conditions are same with Figure 3D-F. Binding of anti-EGFR mAb was saturated at around 1 nM.

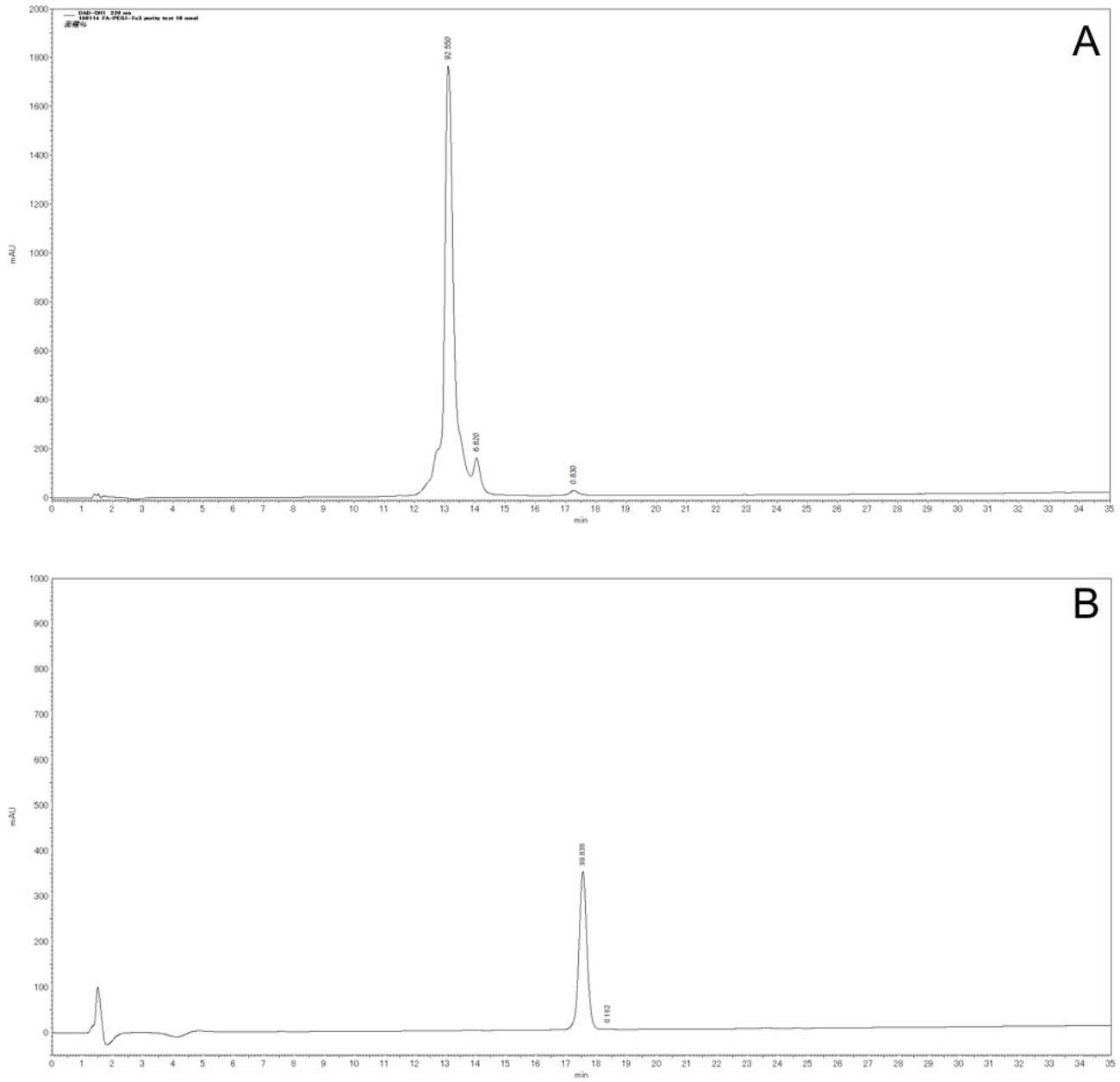


anti-EGFR (nM) :	100	100	100	100
Fc-ARM **1** (M^{-1}) :	10^{-12}	10^{-8}	10^{-8}	10^{-8}
folate (∝M) :	-	-	1	-
Fc-III (∝M) :	-	-	-	20

Supplementary Figure 4: Reversal of inhibition of ADCC by Fc-ARM **1** by adding excess amount of competing ligand, folate or Fc-III (n = 3, mean ± SD).



Supplementary Figure 5: Stimulation of NK cells with anti-EGFR mAb-immobilized particle **a** (A) and Fc-III-immobilized particle **b** together with anti-EGFR mAb (B) for 3 min at 37 ˚C. Amount of activated ERK (pERK) increased with loaded amount of particle **a**, while activation of ERK was negligible in the case of particle **b**/anti-EGFR mAb complex.



Supplementary Figure 6: Purity test of Fc-ARM **1** (A) and **2** (B) by HPLC. The system ran at 1 mL/min at room temperature. 0.1% TFA/water (mobile phase A) and 0.1% TFA/CH_3CN (mobile phase B) were used. The gradient was started with 25% of mobile phase B, and increased to 60% (1%/min).