ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

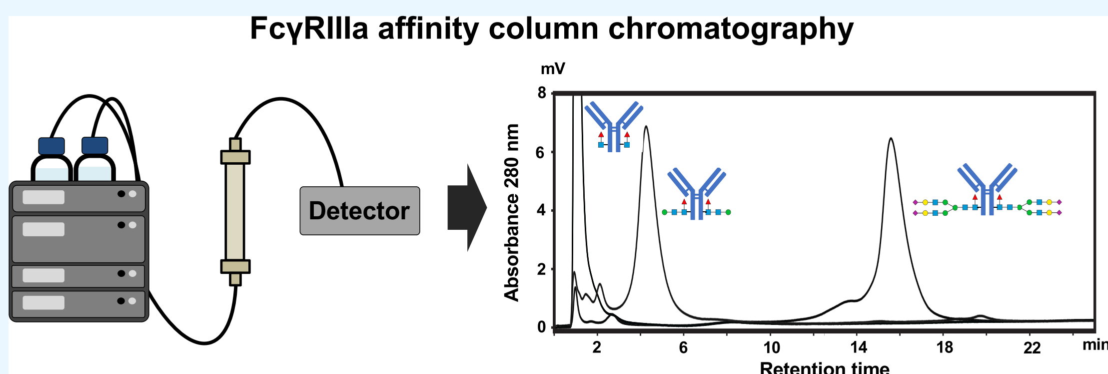
Chromatographic Analysis of the *N*-Glycan Profile on Therapeutic Antibodies Using FcγRIIIa Affinity Column Chromatography

Methanee Hiranyakorn, Shogo Iwamoto, Asako Hoshinoo, Ryo Tsumura, Hiroki Takashima, Masahiro Yasunaga, and Shino Manabe*

Cite This: *ACS Omega* 2023, 8, 16513–16518

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information



ABSTRACT: *N*-Linked glycosylation on IgG has a profound impact on antibody functions. The relationship between the *N*-glycan structure and the binding affinity of FcγRIIIa, relating to antibody-dependent cell-mediated cytotoxicity (ADCC) activity, is important for the efficient development of a therapeutic antibody. Here, we report an influence of the *N*-glycan structure of IgGs, Fc fragments, and antibody-drug conjugates (ADCs) on FcγRIIIa affinity column chromatography. We compared the retention time of several IgGs with heterogeneous and homogeneous *N*-glycans. IgGs with a heterogeneous *N*-glycan structure provided several peaks in column chromatography. On the other hand, homogeneous IgGs and ADCs gave a single peak in column chromatography. The length of glycan on IgG also affected the retention time of the FcγRIIIa column, suggesting that the length of glycan is also impacted by binding affinity to FcγRIIIa, resulting in ADCC activity. This analytic methodology provides evaluation of the binding affinity of FcγRIIIa and ADCC activity, not only full-length IgG but also Fc fragments, which are difficult to measure in a cell-based assay. Furthermore, we showed that the glycan-remodeling strategy controls the ADCC activity of IgGs, Fc fragment, and ADCs.

■ INTRODUCTION

Immunoglobulin G (IgG) is a major antibody used in therapeutics. *N*-Glycosylation is conserved at asparagine position 297 (Asn-297) in IgGs (Figure 1a). *N*-Glycan is composed of core hepta-saccharide involving *N*-acetylglucosamine and mannose. Moreover, the additional heterogeneous part included galactose and *N*-acetyl neuraminic acid (Figure 1b). *N*-Linked glycosylation is crucial for the structural and functional properties of IgGs in pharmaceutics and affects their stability, pharmacokinetics, safety, and clinical efficacy. For instance, the absence of core fucosylation significantly increases the affinity of IgG for FcγRIIIa receptors, resulting in increased antibody-dependent cellular cytotoxicity (ADCC). Moreover, non-reducing terminal galactosylation affects the ADCC activity and thermal stability.[1−3] Thus, glycan composition monitoring is important for the development of effective therapeutic antibodies.

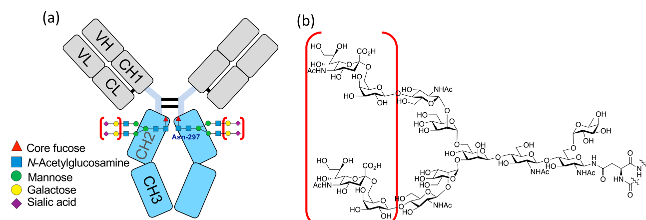
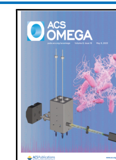


Figure 1. (a) Structure of the antibody *N*-linked glycan depicted by symbol nomenclature for glycans and (b) *N*-glycan structure depicted by chemical drawing. The brackets indicate the heterogeneous part of *N*-glycan.

Received: April 8, 2023
Accepted: April 18, 2023
Published: April 28, 2023



© 2023 The Authors. Published by American Chemical Society

Therapeutic IgGs recognize specific cell surface-expressed antigens and elicit immune effector functions representative of ADCC activity via the interaction between the Fc region and Fcγ receptors (FcγRs). The *N*-glycan structure can also affect the dynamic behavior of the CH2 domain on Fc.[4,5] Since there is a relationship between the binding ability of IgG to FcγRIIIa and ADCC activity, FcγRIIIa-immobilized column chromatography has been used to evaluate ADCC activity without the cleavage of a glycan from IgGs.[6,7]

In general, therapeutic antibodies show three peaks in FcγRIIIa-immobilized affinity column chromatography because of the microheterogeneity of the Fc-glycan, and a longer retention time exhibits higher ADCC activity. The increase in retention time correlates with an increased number of terminal galactose. In this paper, we describe the retention time difference of FcγRIIIa affinity column chromatography with several glycan-modified IgGs, Fc fragments, and antibody-drug conjugates (ADCs).

■ RESULTS AND DISCUSSION

We commenced our research with the HPLC analysis of several therapeutic antibodies using FcγRIIIa affinity column chromatography. Rituximab **1**, trastuzumab **2**, and cetuximab **3** exhibited three peaks (Figure 2a−c), suggesting the microheterogeneity of the Fc-glycan profile on IgG effects on binding on FcγRIIIa.[6,7] In addition, an apparent shoulder peak was observed before the first main peaks in cetuximab **3** analysis. Mogamulizumab **4**, which is an anti-C-C chemokine receptor type 4 antibody without core fucose, showed a longer retention time than that of therapeutic antibodies **1**, **2**, and **3** (Figure 2d). Since core fucose interferes with the interaction between the Fc region and FcγRIIIa, core fucose-deficient IgGs have been developed for ADCC-enhanced therapeutic antibodies. Thus, the FcγRIIIa affinity column reflects the high ADCC activity of mogamulizumab **4**. The Fc fragment of rituximab **5** also showed a slightly longer retention time (Figure 2e). Recently, the Fc fragment of an anti-FcRn antibody, efgartigimod, was approved for generalized myasthenia gravis.[8,9] Since the Fc fragment does not possess Fab, it is difficult to measure the ADCC activity using cell-based assay. ADCC measurement by FcγRIIIa would be helpful for Fc-based medicines.

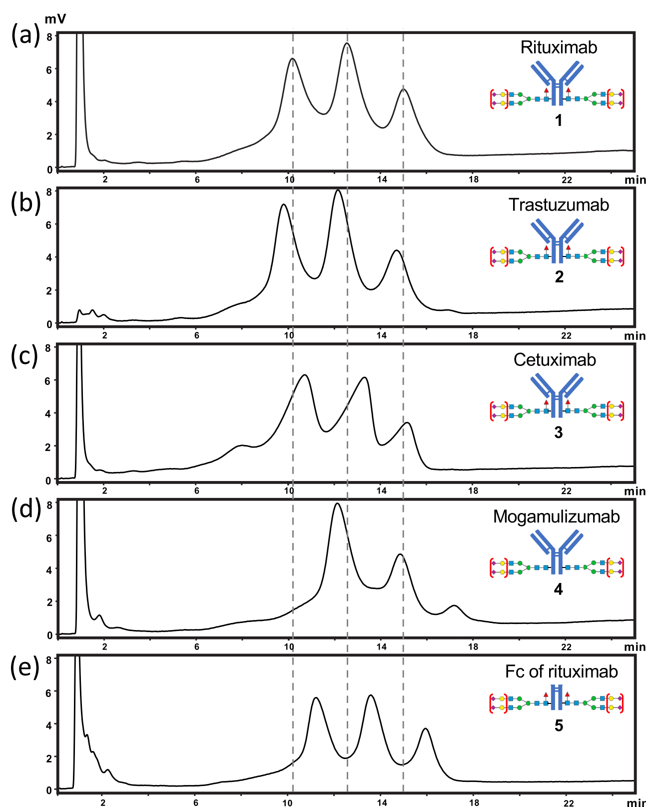


Figure 2. FcγRIIIa affinity chromatographic profile of (a) full-length rituximab **1** (retention times: 10.2, 12.6, and 15 min), (b) full-length trastuzumab **2** (retention times: 9.8, 12.2, and 14.7 min), (c) full-length cetuximab **3** (retention times: 10.7, 13.3, and 15.1 min), (d) full-length mogamulizumab **4** (retention times: 12.1, 14.8, and 17.2 min), and (e) Fc fragment of rituximab after papain digestion **5** (retention times: 11.2, 13.6, and 16 min).

We subsequently tested the HPLC profile of glycan-modified rituximab. IgG with homogeneous glycan was prepared to clarify the relationship between the glycan structure and the retention time in FcγRIIIa column chromatography.[10] IgGs with homogeneous glycan have been prepared using a combination of endo-*β*-*N*-acetylglucosaminidase (ENGase) mutants and glycan oxazolines as donors.[11] The prepared IgGs carrying homogeneous glycan showed a single peak in FcγRIIIa affinity column chromatography. After cleaving the glycan using endoS, an ENGase, glycan-truncated rituximab **6** was not retained, indicating that the glycan structure is crucial for retention time (Figure 3a). The trisaccharide-carrying rituximab **7** showed a medium retention time (Figure 3b). A homogeneous full-length glycan-carrying rituximab **8** was prepared using endoS D233Q and glycan oxazoline, and the retention time was significantly longer (Figure 3c). The same phenomenon was observed in the glycan-remodeled Fc fragments **9** and **10**. Namely, the GlcNAc-Fc fragment **9** was not retained in the column (Figure 3d). However, the Fc fragment with full-length glycan **10** showed a longer retention time (Figure 3e).

Glycopeptide (Glu-Glu-Gln-Tyr-Asn(glycan)-Ser-Thr-Tyr-Arg)[12] **11**, a digestion fragment by trypsin of an antibody containing an *N*-glycan attachment position at Asn297, did not bind to FcγRIIIa (Figure 3f). This result showed that the peptide sequence near Asn297 was insufficient and that a higher-order structure of the Fc moiety was required for FcγRIIIa binding.

Finally, we evaluated several ADCs and the precursor of radioimmunotherapy (RIT). ADCs and RIT focus on efficient antibody-based medicine,[13,14] and several ADC and RIT have been approved in recent years. Potent cytotoxic compounds are conjugated to the antibody via a linker in ADCs. The potent cytotoxicity of ADC is mainly caused by the toxic agents cleaved after internalization. ADCC activity has not been focused on due to the high potency of ADC cytotoxicity.

However, trastuzumab deruxtecan, an efficient ADC, was reported to show ADCC activity similar to that of the native antibody trastuzumab.[15] Furthermore, with various types of ADCs being approved, including photoimmunotherapy,[16] the potential need for ADCC activity measurement will be increased. The traditional methodology of conjugation at the Lys or Cys residue results in various species of ADCs.

Cys residue conjugation is the first choice for ADC preparation at present. The HPLC profile of a Cys-conjugated ADC, rituximab-Mal-Val-Cit-PAC-MMAE (monomethyl auristatin E) **12**, presented six main peaks (Figure 4c) as Matsuda et al. reported.[17] The reason of split peaks is not clear yet.

https://doi.org/10.1021/acsomega.3c02374
ACS Omega 2023, 8, 16513−16518

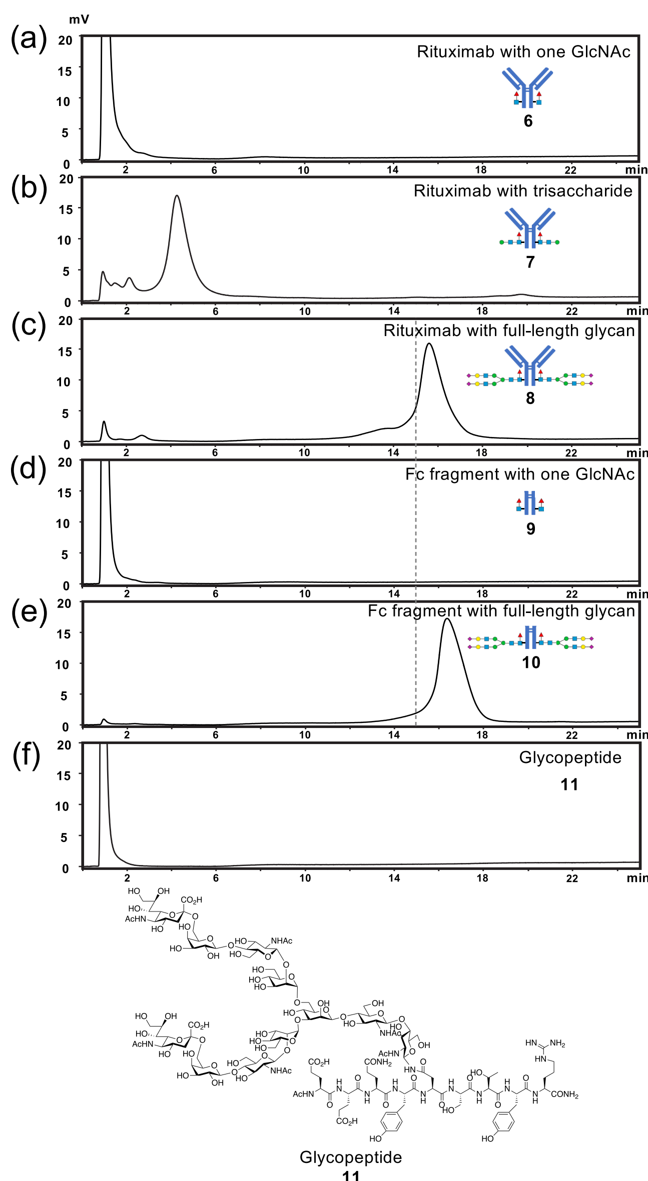


Figure 3. FcγRIIIa affinity chromatographic profile of glycan-modified rituximab: (a) full-length rituximab with one GlcNAc **6** (retention time: 1 min), (b) full-length rituximab with trisaccharide **7** (retention time: 4.3 min), (c) full-length rituximab with full-length glycan **8** (retention time: 15.6 min), (d) Fc fragment of rituximab with one GlcNAc **9** (retention time: 1 min), and (e) Fc fragment of rituximab with full-length glycan **10** (retention time: 16.4 min). (f) Glycopeptide **11**, Glu-Glu-Gln-Tyr-Asn(glycan)-Ser-Thr-Tyr-Arg (retention time: 1 min).

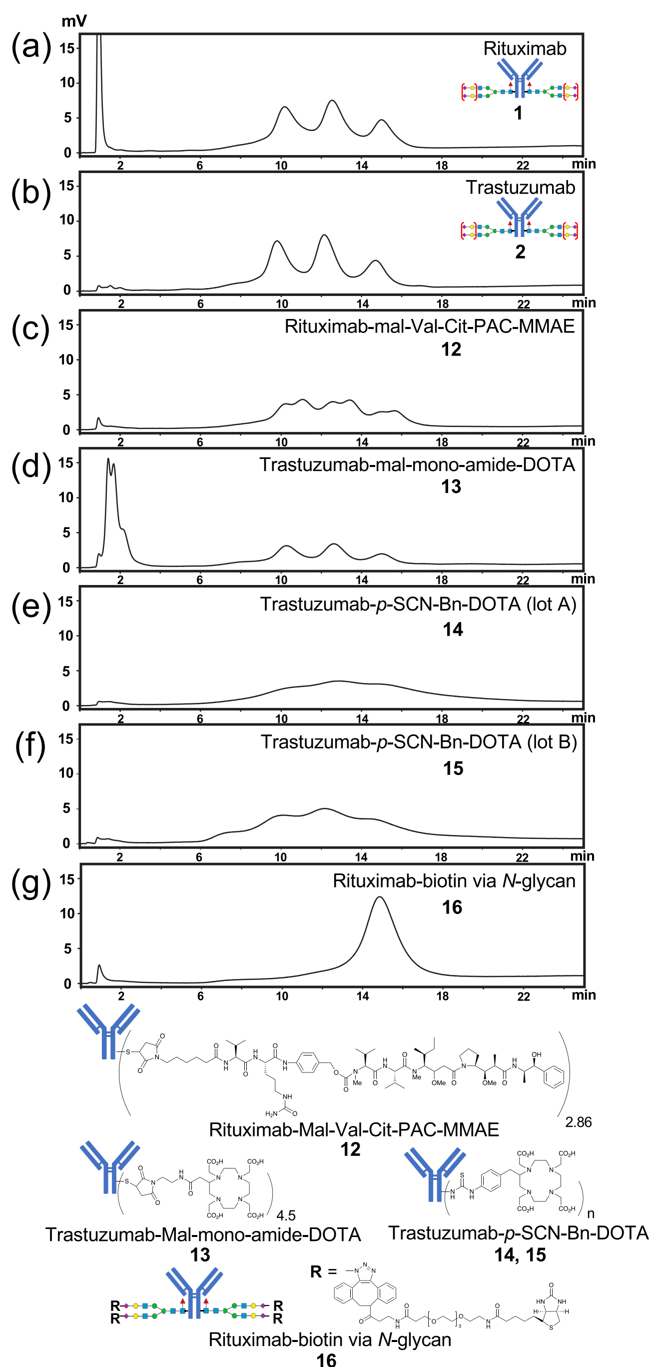


Figure 4. FcγRIIIa affinity chromatographic profile of ADCs: (a) rituximab **1**, (b) trastuzumab **2**, (c) rituximab-mal-Val-Cit-PAC-MMAE via Cys-conjugate **12** (drug-to-antibody ratio (DAR), 2.86), (d) trastuzumab-mal-mono-amide-DOTA via Cys-conjugate **13** (DAR, 4.5), (e) trastuzumab-*p*-SCN-Bn-DOTA via Lys-conjugate **14** (DAR, not determined (n)), (f) trastuzumab-*p*-SCN-Bn-DOTA via Lys-conjugate **15** (DAR, not determined (n); different batch from (e)), and (g) rituximab-conjugated biotin via *N*-glycan (as a mimic of glycan-conjugated ADC) **16** (DAR, 4).

Similarly, trastuzumab with chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) at the Cys residue **13** showed three peaks (Figure 4d). These results suggest that ADC and RIT precursors from random conjugation may also show heterogeneous ADCC activity. When DOTA was conjugated to trastuzumab at the Lys residue **14**, an inseparable broad peak was observed as >70 Lys residues on the IgG surface can result in numerous species of functionalized trastuzumab. This result indicates that the ADCC activity of Lys-conjugated IgG would have a broad spectrum (Figure 4e). Because conjugation on the Lys residue cannot control the drug-to-antibody ratio and conjugation sites, different batches of Lys-conjugated RIT **15** gave different HPLC profiles by FcγRIIIa affinity column chromatography (Figure 4f). On the other hand, the preparation of homogeneous ADCs expands their therapeutic window.[18,19] Many methodologies for the preparation of homogeneous ADCs have been developed based on the advantage of homogeneous ADCs. We also reported the preparation of homogeneous ADCs at the glycan site through glycan

https://doi.org/10.1021/acsomega.3c02374
ACS Omega 2023, 8, 16513−16518

remodeling of IgG, with the prepared ADCs exhibiting high cytotoxicity.[20] To mimic homogeneous glycan-conjugated ADC, we conjugated biotin at the *N*-glycan site of rituximab in our procedure. The homogeneity of the biotin-conjugated IgG was confirmed using MS. The prepared biotin-conjugated IgG **16** showed a single peak in FcγRIIIa column chromatography (Figure 4g). Based on the relationship between FcγRIIIa and ADCC activity, homogeneous ADC can be expected with consistent ADCC activity.

■ CONCLUSIONS

We determined the relationship between the glycan structure of IgG and the retention time in FcγRIIIa affinity column chromatography. The glycan length of IgG and the presence of the Fab fragment influence the retention time in FcγRIIIa column chromatography. In addition, we showed that glycan remodeling to IgGs and functional IgGs would be useful for consistent ADCC activity. Although surface plasmon resonance (SPR)[21] and biolayer interferometry (BLI)[22] are reported as conventional technologies for the measurement of the binding ability of IgG to FcγRIIIa, HPLC-based measurement is operationally convenient and economically advantageous. We also clearly demonstrated that the glycan-remodeling strategy can control ADCC activity. Since eight amino acid-mutated FcγRIIIa without glycosylation is used for this affinity column chromatography,[6] the relationship between ADCC activity and the retention time in the column will be reported in a more detailed manner in due course. Investigation on the FcγRIIIa column chromatography-based analysis and *N*-glycan structure would be helpful for the development of efficient antibody-based therapeutics.

■ EXPERIMENTAL PROCEDURES

General Procedure. Rituximab (Rituxan; lot: R1102AB) and trastuzumab (Herceptin; lot: 19B020E) were available from Chugai Pharmaceutical Co. Ltd. (Chuo-ku, Tokyo, Japan). Cetuximab (Erbitux; lot: ERBA012) and mogamulizumab (Poteligeo; lot: 19303P) were purchased from Merck Biopharma (Meguro-ku, Tokyo, Japan) and Kyowa Hakko Kirin Co. Ltd. (Chiyoda-ku, Tokyo, Japan), respectively. Payload (dibenzocyclooctyne-PEG$_4$-biotin) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bifunctional chelating agents, *p*-SCN-Bn-DOTA and maleimido-mono-amide-DOTA, were purchased from Macrocyclics (Dallas, TX, USA). Sialylglycan and 2-chloro-1,3-dimethyl-1*H*-benzimidazol-3-ium chloride for preparing oxazoline were from Fushimi Pharmaceutical Co., Ltd. (Marugame, Kagawa).

A TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm) column from TOSOH (Minato-ku, Tokyo, Japan) was used for the analysis of all samples. Protein A column chromatography was available from TOSOH (Minato-ku, Tokyo, Japan). HPLC (Prominence, SHIMADZU, Kyoto, Japan) was used for analyses and purification. The antibody concentration was measured by a NanoDrop Lite (Thermo Fisher Scientific, Waltham, MA, USA). The ^{1}H-spectra of oxazolines were measured on a JEOL JMN-ECZL spectrometer (400 MHz) at ambient temperature in D_2O. Sephadex G-25 was purchased from GE Healthcare (Chicago, IL, USA) for purification of glycan oxazoline.

FcγRIIIa Affinity Column Chromatography Analysis and Purification. Mobile phase A was 50 mM citrate at pH 6.5, and mobile phase B was 50 mM citrate at pH 4.5. A total of protein was 50 μg was dissolved with mobile phase A. A linear gradient of buffer B (0 to 100%) at a flow rate of 1 mL/min was applied to the column for 18 min to elute IgG employing a Shimadzu purifier system with fluorescence absorbance at 280 nm detection. The gradient was started at 2 min (Figure S1). Samples were injected into the 50 μL sample loop at a column temperature of 25 °C. Homogeneous glycan carrying IgGs and compound **15** were prepared according to a previously reported procedure.[20]

Removal of *N*-Glycan on IgG by EndoS. Rituximab (10 mg/mL in 50 mM sodium citrate buffer, pH 6.5) was incubated with 30 μg of endoS for 16 h. The reaction was monitored by TSKgel FcR-IIIA-NPR column chromatography. The product was purified using a protein A column by HPLC. The product was loaded into the column with a buffer of 50 mM sodium phosphate at pH 6.5 and eluted with a buffer of 50 mM citrate at pH 3.0 at 25 °C at a flow rate of 1 mL/min.

General Procedure for Addition of *N*-Glycan on IgG by EndoS D233Q. Glycan-modified rituximabs were prepared by *in vitro* enzymatic reaction using endoS D233Q according to a previous report.[23] Rituximab with GlcNAc (3 mg/mL in 50 mM citrate buffer, pH 6.5) was incubated with endoS D233Q in the presence of glycan oxazoline (Figures S2−S4), and a TSKgel FcR-IIIA-NPR column (TOSOH) was used for monitoring the reaction. TSKgel FcR-IIIA-5PW (7.8 mm I.D. × 7.5 cm) was used for purification of the product. The mobile phases were buffer A (50 mM citrate at pH 6.5) and buffer B (50 mM citrate at pH 4.5). Separations were carried out at 25 °C at a flow rate of 1 mL/min.

Preparation of the Fc Fragment by Papain Digestion. Full-length rituximab (10 mg/mL in 75 mM sodium phosphate buffer (pH 7.0) containing 75 mM NaCl and 2 mM EDTA) was digested with papain at a ratio of papain/IgG was 1:50. The reaction was incubated at 37 °C for 14 h, and the product was purified by protein A column chromatography. (Figure S5).

MS Measurement of IgGs with Homogeneous Glycans. The LC−MS/MS system was comprised of a Waters Acquity UPLC H-Class Bio System with an MS/MS detector. A Waters Vion IMS Qtof instrument was operated in positive ion/sensitivity mode at an *m*/*z* range of 400−4000. The capillary voltage was set at 3 kV and the cone voltage was 150 V with a source temperature of 120 °C and a deconvolution temperature of 300 °C. Instrument control, data processing, and deconvolution were performed using Waters UNIFI software v. 1.9.4.053 with an advanced maximum entropy (MaxEnt)-based procedure. The samples were analyzed on a Waters MassPREP Micro Desalting Column (1000 Å, 20 μm, 2.1 × 5 mm) at 80 °C with a gradient of 0.1% formic acid in water and acetonitrile (Figures S6−S11).

Preparation of Antibody-Drug Conjugates (ADCs) and RIT Precursors. According to the previously described procedure with minor modification, mc-vc-PAB-MMAE was attached to rituximab.[24] In short, rituximab was reduced at 37 °C for 30 min with 20 mM cysteamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA). Then, the buffer solutions were exchanged for phosphate-buffered saline (PBS) containing 5 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich). Reactive sulfhydryl groups were determined using 5,5′-dithiobis (2-nitrobenzonic acid) (Sigma-Aldrich). Subsequently, the antibodies were incubated at 4 °C overnight with 4.5-fold molar excess of mc-vc-PAB-MMAE over the sulfhydryl groups. To remove unconjugated mc-vc-PAB-

https://doi.org/10.1021/acsomega.3c02374
ACS Omega 2023, 8, 16513−16518

MMAE, the buffers solutions were exchanged for PBS. Then, the residual sulfhydryl groups were determined as described above. The drug−antibody ratio (DAR) was calculated using the formula DAR = $SH_{initial} - SH_{ADC}$, where $SH_{initial}$ is the number of reactive sulfhydryl groups per antibody before the conjugation and SH_{ADC} is that after the conjugation. The ADC was stored at −80 °C until the analysis.

Preparation of Antibodies Conjugated with 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic Acid (DOTA). To attach *p*-SCN-Bn-DOTA to trastuzumab, the antibodies were dissolved in 0.1 M $NaHCO_3$ (pH 8.5) by buffer exchange and the antibody concentration was adjusted to 5 mg/mL. Then, the antibodies were incubated for 1 h at 37 °C with a 25-fold excess of *p*-SCN-Bn-DOTA (20 mg/mL in DMSO). Subsequently, the buffer solutions were exchanged for PBS and unconjugated *p*-SCN-Bn-DOTA was removed.

According to the ADC preparation protocol described above, maleimido-mono-amide-DOTA was attached to trastuzumab. For DOTA conjugation, trastuzumab was reduced at 37 °C for 30 min with 20 mM cysteamine hydrochloride (Sigma-Aldrich) and incubated at 4 °C overnight with 5-fold molar excess of maleimido-mono-amide-DOTA over the sulfhydryl groups. Similarly, the DOTA−antibody ratio was determined. The DOTA-conjugated antibodies were stored at −80 °C until the analysis.

Preparation of Rituximab-Conjugated Biotin via *N*-Glycan (Mimic of Glycan-Conjugated ADC). The purified rituximab with glycan-azide (1.4 mg/mL) was dissolved in 50 mM citrate buffer at pH 6.5, and 30 equiv of payload (dibenzocyclooctyne-PEG_4-biotin) was added to the final concentration of 10% (v/v) dimethyl sulfoxide (DMSO). The solution was incubated at 25 °C for 16 h, and the reaction mixture was purified by a TSKgel FcR-IIIA-5PW column (TOSOH).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02374.

MS and NMR spectra of glycan oxazoline donors and MS spectra of chemically modified IgGs (PDF)

AUTHOR INFORMATION

Corresponding Author

Shino Manabe − *Laboratory of Functional Molecule Chemistry, Pharmaceutical Department and Institute of Medicinal Chemistry, Hoshi University, Tokyo 142-8501, Japan; Research Center for Pharmaceutical Development Graduate School of Pharmaceutical Sciences & Faculty of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan;* orcid.org/0000-0002-2763-1414; Phone: + 81-3-5498-5770; Email: s-manabe@hoshi.ac.jp, shino.manabe.e1@tohoku.ac.jp; Fax: +81-3-5498-5837

Authors

Methanee Hiranyakorn − *Laboratory of Functional Molecule Chemistry, Pharmaceutical Department, Hoshi University, Tokyo 142-8501, Japan*

Shogo Iwamoto − *Fushimi Pharmaceutical Co. Ltd., Marugame, Kagawa 763-8605, Japan*

Asako Hoshinoo − *Fushimi Pharmaceutical Co. Ltd., Marugame, Kagawa 763-8605, Japan*

Ryo Tsumura − *Division of Developmental Therapeutics, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa City 277-8577, Japan*

Hiroki Takashima − *Division of Developmental Therapeutics, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa City 277-8577, Japan;* orcid.org/0000-0001-6487-7344

Masahiro Yasunaga − *Division of Developmental Therapeutics, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa City 277-8577, Japan;* orcid.org/0000-0003-3356-0197

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c02374

Author Contributions

M.H. and S.M. conceived and designed the study. M.H. prepared the glycan-remodeled antibodies and performed the HPLC analysis of FcγRIIIa affinity column chromatography. S.I. and A.H. prepared the azide-carrying glycan and performed the MS measurements. R.T., H.T., and M. Y. prepared the ADCs and RIT precursors. M.H. and S.M. drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare the following competing financial interest(s): M.H., R.T., H.T., M. Y., and S. M. declare no conflicts of interest. S.I. and A. H. are employees of Fushimi Pharmaceutical Co. Ltd.

ACKNOWLEDGMENTS

S.M. received support through a Grant-in-Aid for Scientific Research (B) (grant no. 19H03357) from the Japan Society for Promotion of Science and A-STEP from Japan Science and Technology Agency (grant no. JPMJTR20U9). We also thank Dr. Mayumi Ikegami-Kawai, Faculty of Pharmaceutical Sciences, Hoshi University, for NMR maintenance.

ABBREVIATIONS

ADC, antibody-drug conjugate; IgG, immunoglobulin G; ADCC, antibody-dependent cellular cytotoxicity; FcγR, Fcγ receptors; ENGase, endo-*β*-*N*-acetylglucosaminidase; RIT, radioimmunotherapy; MMAE, monomethyl auristatin; DOTA, 1,4,7,10-tetraazacyclododecan-1,4,7,10-tetraacetic acid; SPR, surface plasmon resonance

REFERENCES

(1) Arnold, J. N.; Wormald, M. R.; Sim, R. B.; Rudd, P. M.; Dwek, R. A. The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu. Rev. Immunol.* **2007**, *25*, 21−50.

(2) Nimmerjahn, F.; Anthony, R. M.; Ravetch, J. V. Agalactosylated IgG antibodies depend on cellular Fc receptors for *in vivo* activity. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 8433−8437.

(3) Shinkawa, T.; Nakamura, K.; Yamane, N.; Shoji-Hosaka, E.; Kanda, Y.; Sakurada, M.; Uchida, K.; Anazawa, H.; Satoh, M.; Yamasaki, M.; Hanai, N.; Shitara, K. The absence of fucose but not the presence of galactose or bisecting *N*-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J. Biol. Chem.* **2003**, *278*, 3466−3473.

(4) Lee, H. S.; Im, W. Effects of *N*-glycan composition on structure and dynamics of IgG1 Fc and their implications for antibody engineering. *Sci. Rep.* **2017**, *7*, 12659.

https://doi.org/10.1021/acsomega.3c02374
ACS Omega 2023, 8, 16513−16518

(5) Yanaka, S.; Yogo, R.; Inoue, R.; Sugiyama, M.; Itoh, S. G.; Okumura, H.; Miyanoiri, Y.; Yagi, H.; Satoh, T.; Yamaguchi, T.; Kato, K. Dynamic views of the Fc region of immunoglobulin G provided by experimental and computational observations. *Antibodies* **2019**, *8*, 39.
(6) Kiyoshi, M.; Caaveiro, J. M. M.; Tada, M.; Tamura, H.; Tanaka, T.; Terao, Y.; Morante, K.; Harazono, A.; Hashii, N.; Shibata, H.; Kuroda, D.; Nagatoishi, S.; Oe, S.; Ide, T.; Tsumoto, K.; Ishii-Watabe, A. A. Assessing the heterogeneity of the Fc-glycan of a therapeutic antibody using an engineered FcγReceptor IIIa-immobilized column. *Sci. Rep.* **2018**, *8*, 3955.
(7) Chakrabarti, A.; Kervinen, J.; Müller, E.; Tanaka, T.; Muranaka, K. *Analytical characterization of mAbs with a novel Fc receptor-based chromatography technique.* Monoclon. Antibodies, IntechOpen, 2021, 1−25.
(8) Ulrichts, P.; Guglietta, A.; Dreier, T.; van Bragt, T.; Hanssens, V.; Hofman, E.; Vankerckhoven, B.; Verheesen, P.; Ongenae, N.; Lykhopiy, V.; Enriquez, F. J.; Cho, J.; Ober, R. J.; Ward, E. S.; de Haard, H.; Leupin, N. Neonatal Fc receptor antagonist efgartigimod safely and sustainably reduces IgGs in humans. *J. Clin. Invest.* **2018**, *128*, 4372−4386.
(9) Yang, C.; Guo, X.; Gong, R. Engineering of Fc fragments with optimized physicochemical properties implying improvement of clinical potentials for Fc-based therapeutics. *Front. Immunol.* **1860**, *2018*, 8.
(10) Wada, R.; Matsui, M.; Kawasaki, N. Influence of *N*-glycosylation on effector functions and thermal stability of glycoengineered IgG1 monoclonal antibody with homogeneous glycoforms. *mAbs* **2019**, *11*, 350−372.
(11) Wang, L.-X.; Tong, X.; Li, C.; Giddens, J. P.; Li, T. Glycoengineering of antibodies for modulating functions. *Annu. Rev. Biochem.* **2019**, *88*, 433−459.
(12) Manabe, S.; Yamaguchi, Y.; Abe, J.; Matsumoto, K.; Ito, Y. Acceptor range of endo-*β*-*N*-acetylglucosaminidase mutant endo-CC N180H: from monosaccharide to antibody. *R. Soc. Open Sci.* **2018**, *5*, 171521.
(13) Drago, J. Z.; Modi, S.; Chandarlapaty, S. Unlocking the potential of antibody-drug conjugates for cancer therapy. *Nat. Rev. Clin. Oncol.* **2021**, *18*, 327−344.
(14) Wang, D.-R.; Wu, X.-L.; Sun, Y.-L. Therapeutic targets and biomarkers of tumor immunotherapy: response versus non-response. *Sig. Trans. Target. Ther.* **2022**, *7*, 93.
(15) Ogitani, Y.; Aida, T.; Hagihara, K.; Yamaguchi, J.; Ishii, C.; Harada, N.; Soma, M.; Okamoto, H.; Oitate, M.; Arakawa, S.; Hirai, T.; Atsumi, R.; Nakada, T.; Hayakawa, I.; Abe, Y.; Agatsuma, T. DS-8201a, A novel HER2-targeting ADC with a novel DNA topoisomerase I inhibitor, demonstrates a promising antitumor efficacy with differentiation from T-DM1. *Clin. Cancer Res.* **2016**, *22*, 5097−5108.
(16) Kobayashi, H.; Ogawa, M.; Alford, R.; Choyke, P. L.; Urano, Y. New strategies for fluorescent probe design in medical diagnostic imaging. *Chem. Rev.* **2010**, *110*, 2620−2640.
(17) Matsuda, Y.; Chakrabarti, A.; Takahashi, K.; Yamada, K.; Nakata, K.; Okuzumi, T.; Mendelsohn, B. A. Chromatographic analysis of site-specific antibody-drug conjugates produced by AJICAP first-generation technology using a recombinant FcγIIIa receptor-ligand affinity column. *J. Chromatography B.* **2021**, *1177*, 122753.
(18) Junutula, J. R.; Raab, H.; Clark, S.; Bhakta, S.; Leipold, D. D.; Weir, S.; Chen, Y.; Simpson, M.; Tsai, S. P.; Dennis, M. S.; Lu, Y.; Meng, Y. G.; Ng, C.; Yang, J.; Lee, C. C.; Duenas, E.; Gorrell, J.; Katta, V.; Kim, A.; McDorman, K.; Flagella, K.; Venook, R.; Ross, S.; Spencer, S. D.; Wong, W. L.; Lowman, H. B.; Vandlen, R.; Sliwkowski, M. X.; Scheller, R. H.; Polakis, P.; Mallet, W. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat. Biotech.* **2008**, *26*, 925−932.
(19) Tsuchikama, K.; An, Z. Antibody-drug conjugates: recent advances in conjugation and linker chemistries. *Protein Cell* **2018**, *9*, 33−46.
(20) Manabe, S.; Yamaguchi, Y.; Matsumoto, K.; Fuchigami, H.; Kawase, T.; Hirose, K.; Mitani, A.; Sumiyoshi, W.; Kinoshita, T.; Abe, J.; Yasunaga, M.; Matsumura, Y.; Ito, Y. Characterization of antibody products obtained through enzymatic and nonenzymatic glycosylation reactions with a glycan oxazoline and preparation of a homogeneous antibody-drug conjugate via Fc *N*-Glycan. *Bioconjugate Chem.* **2019**, *30*, 1343−1355.
(21) Forest-Nault, C.; Gaudreault, J.; Henry, O.; Durocher, Y.; De Crescenzo, G. On the use of surface plasmon resonance biosensing to understand IgG-FcγR interactions. *Int. J. Mol. Sci.* **2021**, *22*, 6616.
(22) Geuijen, K. P. M.; Oppers-Tiemissen, C.; Egging, D. F.; Simons, P. J.; Boon, L.; Schasfoort, R. B. M.; Eppink, M. H. M. Rapid screening of IgG quality attributes − effects on Fc receptor binding. *FEBS Open Bio* **2017**, *7*, 1557−1574.
(23) Tang, F.; Wang, L. X.; Huang, W. Chemoenzymatic synthesis of glycoengineered IgG antibodies and glycosite-specific antibody−drug conjugates. *Nat. Protoc.* **2017**, *12*, 1702−1721.
(24) Tsumura, R.; Manabe, S.; Takashima, H.; Koga, Y.; Yasunaga, M.; Matsumura, Y. Influence of the dissociation rate constant on the intra-tumor distribution of antibody-drug conjugate against tissue factor. *J. Controlled Release* **2018**, *284*, 49−56.

https://doi.org/10.1021/acsomega.3c02374
ACS Omega 2023, 8, 16513−16518