Supplementary Information for:

Engineering hydrophobic protein–carbohydrate interactions to fine-tune monoclonal antibodies

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Supplementary Materials and Methods

Electrospray ionization (ESI) mass spectrometry

Electrospray mass spectrometry was performed with a Waters Synapt G2 instrument (Waters MS Technologies, Manchester, UK) in negative ion mode. Samples in 1:1 (v:v) methanol:water were infused through Waters thin-wall borosilicate nanospray capillaries. The ion source conditions were: ion source temperature, 120˚C; infusion capillary potential, 1.2 kV; cone voltage 180 V; RF-1 voltage 150 V. Spectra (2 sec scans) were acquired with a digitization rate of 4 GHz and accumulated until a satisfactory signal:noise ratio had been obtained. The ions from neutral glycans were observed as $[M+H_2PO_4]^-$ adducts, the phosphate arising from residual phosphate in the solution whereas acidic (sialylated) glycans gave $[M–H]^-$ ions. For MS/MS data acquisition, the parent ion was selected at low resolution (about 5 *m/z* mass window) to allow transmission of isotope peaks and fragmented with argon at a pressure (recorded on the instrument's pressure gauge) of 0.5 mBar. The voltage on the collision cell was adjusted with mass and charge to give an even distribution of fragment ions across the mass scale (typically 80–120 V). Other voltages were applied as recommended by the manufacturer. Instrument control, data acquisition, and processing were performed with MassLynx software Version 4.1 (Waters). The negative ion collision-induced dissociation (CID) spectra provide highly diagnostic fragmentation spectra that enable structural assignment of the glycans. For a more extensive discussion of these techniques, see Harvey *et al.* (*1, 2*).

Fine structural and compositional analysis of sialylated glycans from the generated Fc mutants was also performed using negative ion ESI CID mass spectrometry (*2*) (Figure 1). This method is illustrated in the spectrum of the $[M–H]^-$ ion of the monosialylated biantennary glycan (Figure 1C). The presence of Neu5Ac is

indicated by the B_1 ion at *m/z* 290 and the absence of an ion at *m/z* 306 indicates that this ion is α2-3-linked (*3*). The C_1 ion at *m/z* 179 shows hexose (galactose) at the non-reducing terminus of the other antenna. The $^{1,3}A_3$ and $^{1,3}A_4/Y_1$ ion at *m/z* 424 contains the Gal-GlcNAc sequence from the antennae and the ion at *m/z* 655 (not labeled) is the B_3 ion Neu5Ac-Gal-GlcNAc. D and D-18 ions arising from the 6-antenna appear at *m/z* 688 and 670 respectively. Ions at *m/z* 1478, 1418 and 1275 are the diagnostic $^{2,4}A_7$, B_6 and $^{2,4}A_6$ ions from the *N*-acetylchitobiose core, with additional loss of the sialic acid and locate the fucose residue to the reducing-terminal GlcNAc.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis

Oligosaccharides were released from target glycoproteins with PNGase F (New England BioLabs) from Coomassie blue-stained NuPAGE gels (*4*). Excised bands were washed five times alternately with acetonitrile and deionized water and rehydrated with 3000 U/mL aqueous PNGase F solution. After 12 h of incubation at 37°C, the enzymatically released N-linked glycans were eluted with water. Samples were analyzed by MALDI-TOF mass spectrometry with a Shimadzu AXIMA MALDI TOF/TOF mass spectrometer (Kratos Analytical, Manchester, U.K.) equipped with delayed extraction and a nitrogen laser (337 nm). Samples were cleaned on a Nafion 117 membrane (Sigma-Aldrich) and then prepared for MALDI-mass spectrometry by adding 0.5 μL aqueous solution of the glycans to the matrix solution (0.3 μL solution of 2,5-dihydroxybenzoic acid in acetonitrile/water; 1:1, v/v) on the stainless steel target plate and allowing it to dry at room temperature. The sample/matrix mixture was then recrystallized from ethanol.

In-Gel Tryptic digestion of native and deglycosylated IgG variants

Deglycosylated IgG were prepared by PNGase F digestion (New England Biolabs, UK). In-gel digestion of native and hydrophobic IgG mutants was carried out using the In-Gel Tryptic Digestion Kit (Thermo Scientific, UK) according to manufacturer's protocol. Briefly, gel bands containing IgG were excised and destained extensively, followed by reduction and alkylation. The gel slices were then digested with trypsin for 6 hours at 37˚C and the peptides were extracted with 1% trifluoracetic acid (Sigma-Aldrich, UK). The peptides were analyzed on a MALDI-TOF MS instrument (Kratos Analytical, Manchester, U.K.), using α-Cyano-4-hydroxycinnamic acid as the matrix. The expected tryptic peptide mass of the native and deglycosylated IgG were calculated online using the PeptideMass program at the ExPASy SIB Bioinformatics Resource Portal. The lack of intrinsic N-glycosylation at Fc Asn297 was assessed by the detection of the tryptic peptide EEQYNSTYR, which contains the N-glycosylation site Asn297 and results from non-glycosylated IgG1 Fc. The equivalent tryptic peptide resulting from the normal glycosylated, PNGase F-treated IgG1 Fc, is EEQYDSTYR.

Supplementary References

1. Harvey, D. J., Royle, L., Radcliffe, C. M., Rudd, P. M., and Dwek, R. A. (2008) Structural and quantitative analysis of *N*-linked glycans by matrix-assisted laser desorption ionization and negative ion nanospray mass spectrometry, *Anal. Biochem. 376*, 44-60.
2. Harvey, D. J. (2005) Fragmentation of negative ions from carbohydrates: Part 1. Use of nitrate and other anionic adducts for the production of negative ion

electrospray spectra from *N*-linked carbohydrates, *J. Am. Soc. Mass Spectrom. 16*, 622-630.

3. Wheeler, S. F., and Harvey, D. J. (2000) Negative ion mass spectrometry of sialylated carbohydrates: discrimination of N-acetylneuraminic acid linkages by MALDI-TOF and ESI-TOF mass spectrometry, *Analytical chemistry 72*, 5027-5039.

4. Kuster, B., Wheeler, S. F., Hunter, A. P., Dwek, R. A., and Harvey, D. J. (1997) Sequencing of N-linked oligosaccharides directly from protein gels: in-gel deglycosylation followed by matrix-assisted laser desorption/ionization mass spectrometry and normal-phase high-performance liquid chromatography, *Anal Biochem 250*, 82-101.

5. Harvey, D. J., Merry, A. H., Royle, L., Campbell, M. P., Dwek, R. A., and Rudd, P. M. (2009) Proposal for a standard system for drawing structural diagrams of N- and O-linked carbohydrates and related compounds, *Proteomics 9*, 3796-3801.

Supplementary Figures

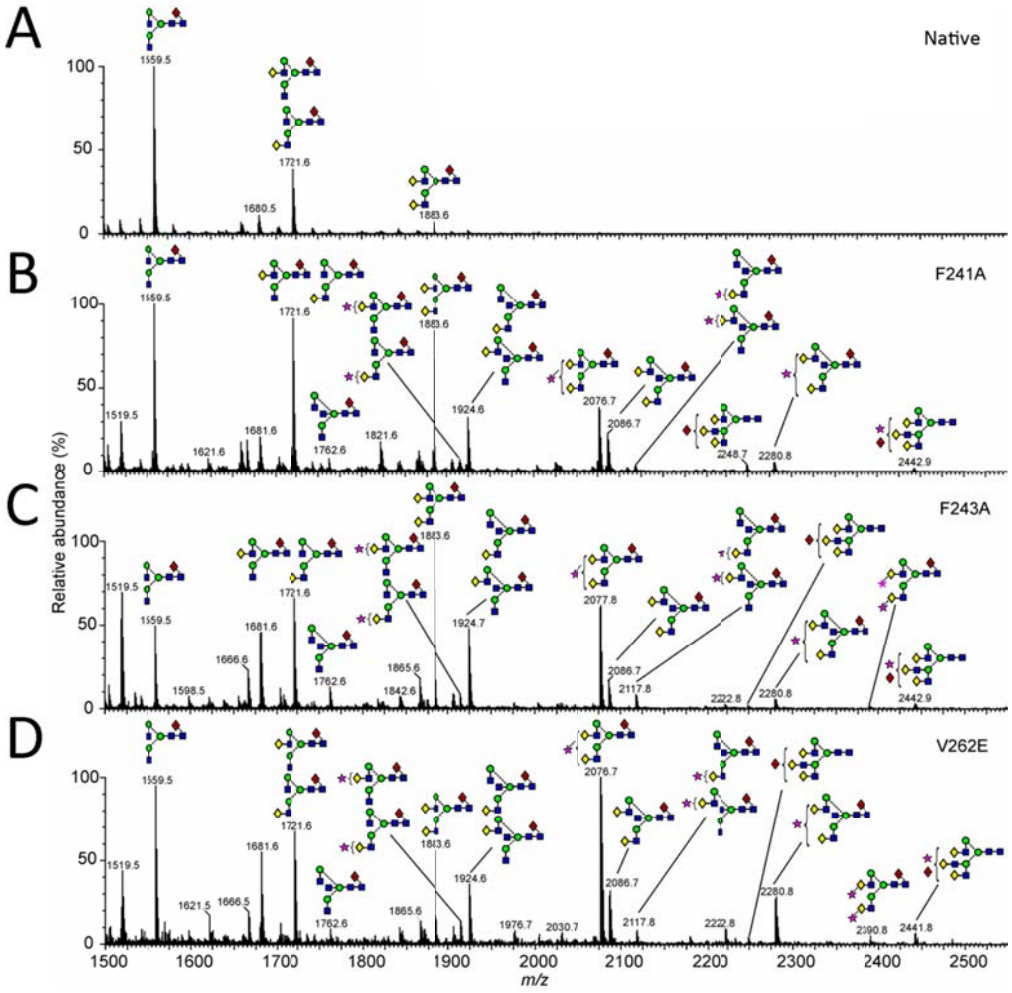


Figure S1. Electrospray ionization mass spectrometric analysis of N-glycans released from IgG1 b12 mutants. Spectra shown represent *singly* charged ions and were extracted using ion mobility from their respective negative ion ESI spectra. Spectra showing doubly charged ions from (A) Native, (B) F241A, (C) F243A, (D) V262E. Masses, compositions and structures of the N-glycans are shown in Table S1.

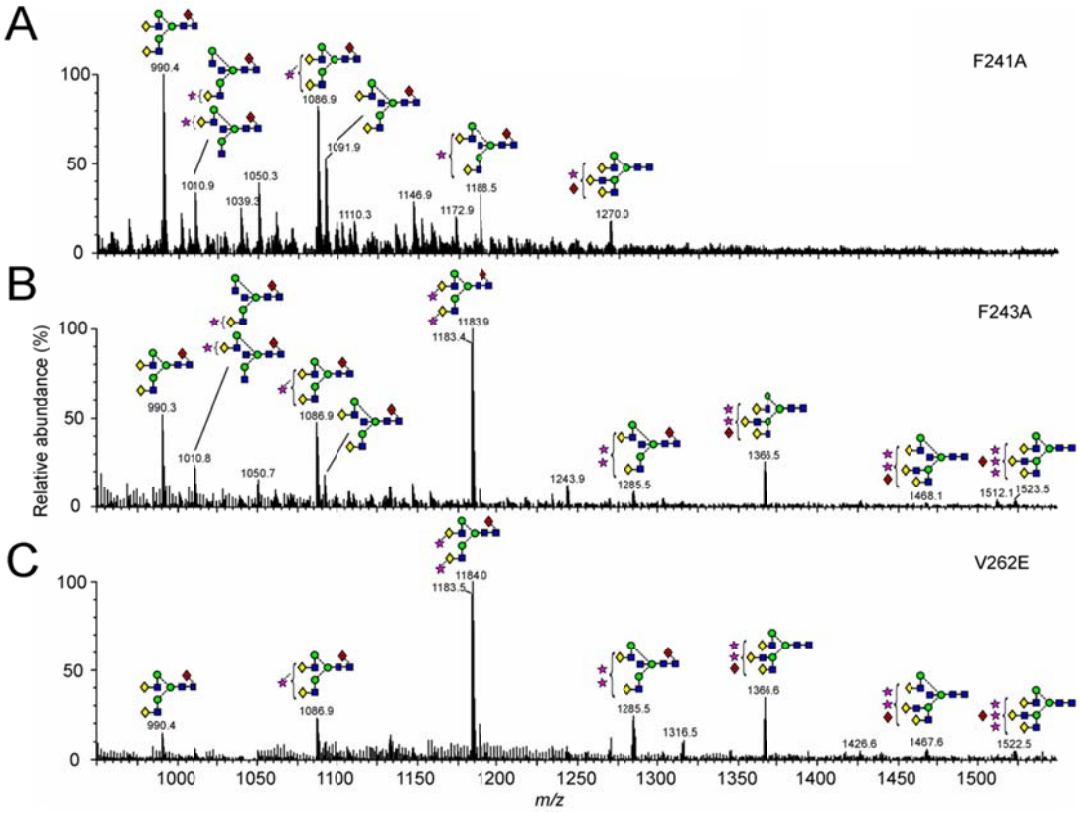


Figure S2. Electrospray ionization mass spectrometric analysis of N-glycans released from IgG1 b12 mutants. Spectra shown represent *doubly* charged ions and were extracted using ion mobility from their respective negative ion ESI spectra. Spectra showing doubly charged ions from (A) F241A, (B) F243A, (C) V262E. Key: The symbolic representation of glycans follows that of Harvey *et al* (*5*) with residues in both the schematic diagrams and molecular graphics following the color scheme of the Consortium for Functional Glycomics. Masses, compositions and structures of the N-glycans are shown in Table S1.

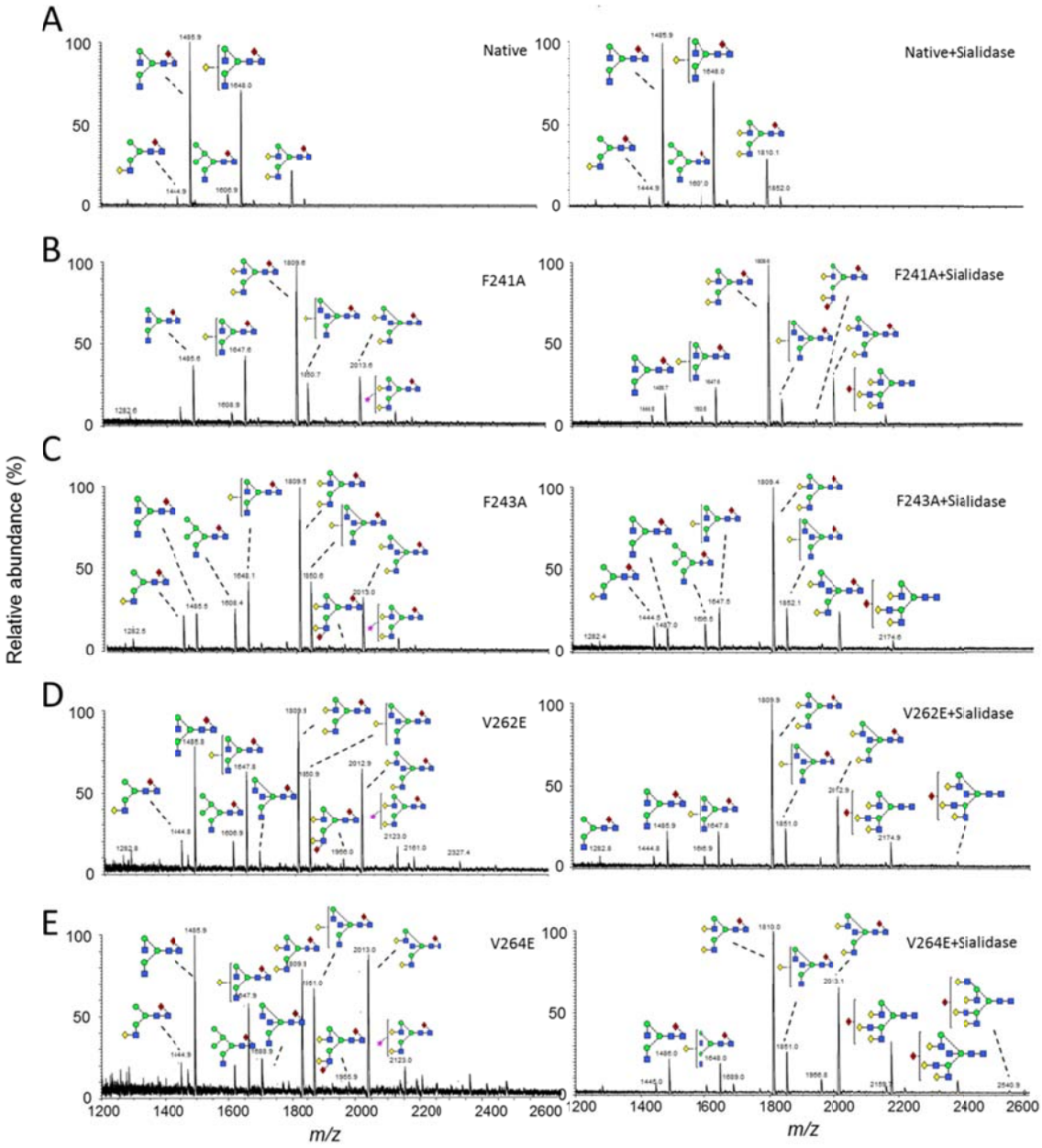


Figure S3. MALDI-TOF mass spectrometric analysis of N-linked glycans released from IgG b12 variants and their desialylated counterparts. N-linked glycans were released from IgG by PNGase F digestion. For desialylation, released sugars were treated with neuraminidase. N-linked glycans were analyzed by MALDI-TOF mass spectrometry, DHB was used as the matrix. (A) Wild type. (B) F241A. (C) F243A. (D) V262E. (E) V264E.

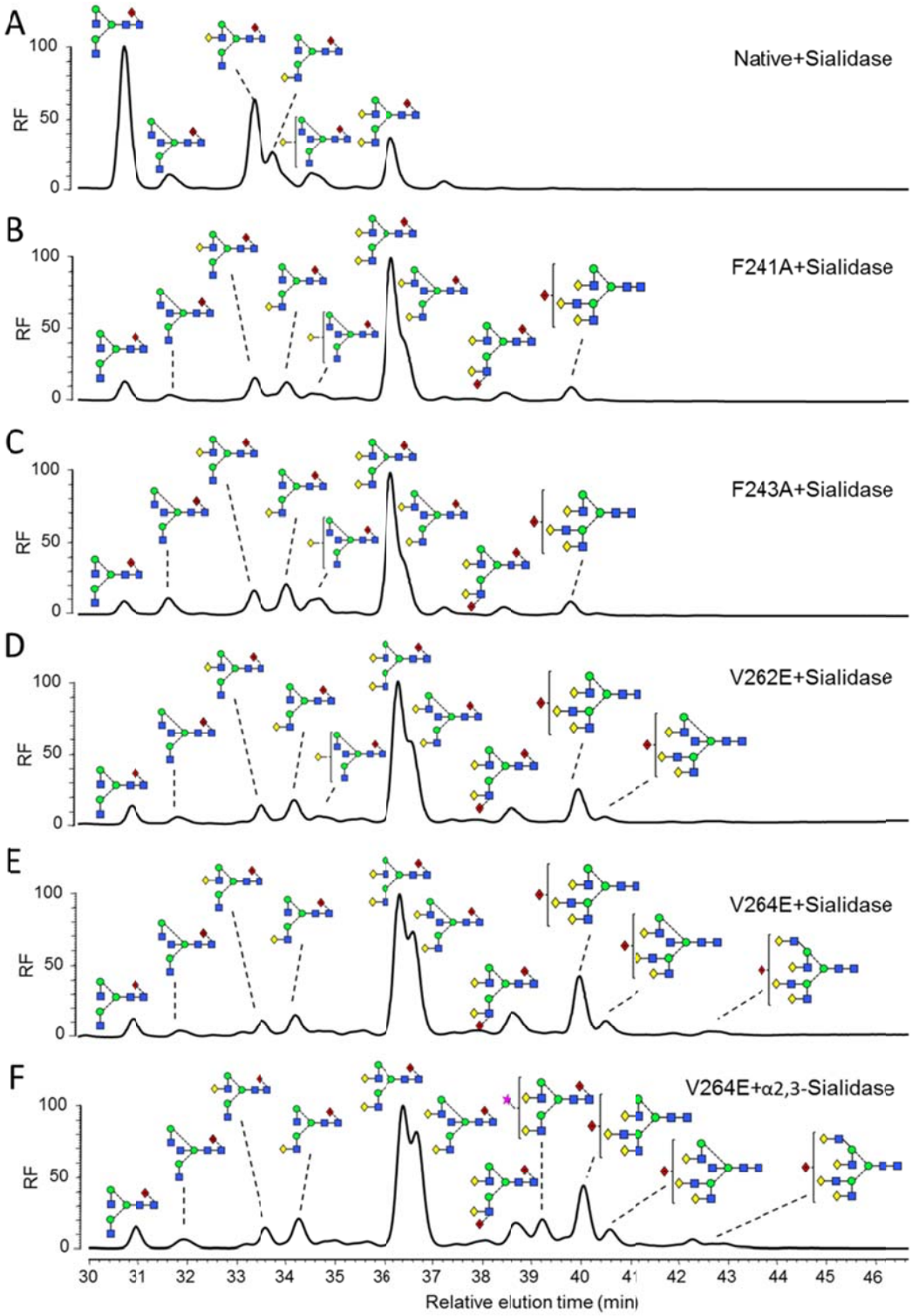


Figure S4. HPLC analysis of 2AA-labelled, desialylated N-linked glycans from monoclonal IgG1 b12 mutants expressed in HEK 293T. Normal-phase HPLC analysis of 2AA-labelled N-linked glycans, released from target antibody glycoforms by in-gel PNGase F digestion, followed by neuraminidase digestion. Glycan profile of neuraminidase-treated IgG1 b12 variants expressed in HEK 293T: (A) Wild type. (B) F241A. (C) F243A. (D) V262E. (E) V264E. (F) V264E treated with only α2,3-specific neuraminidase.

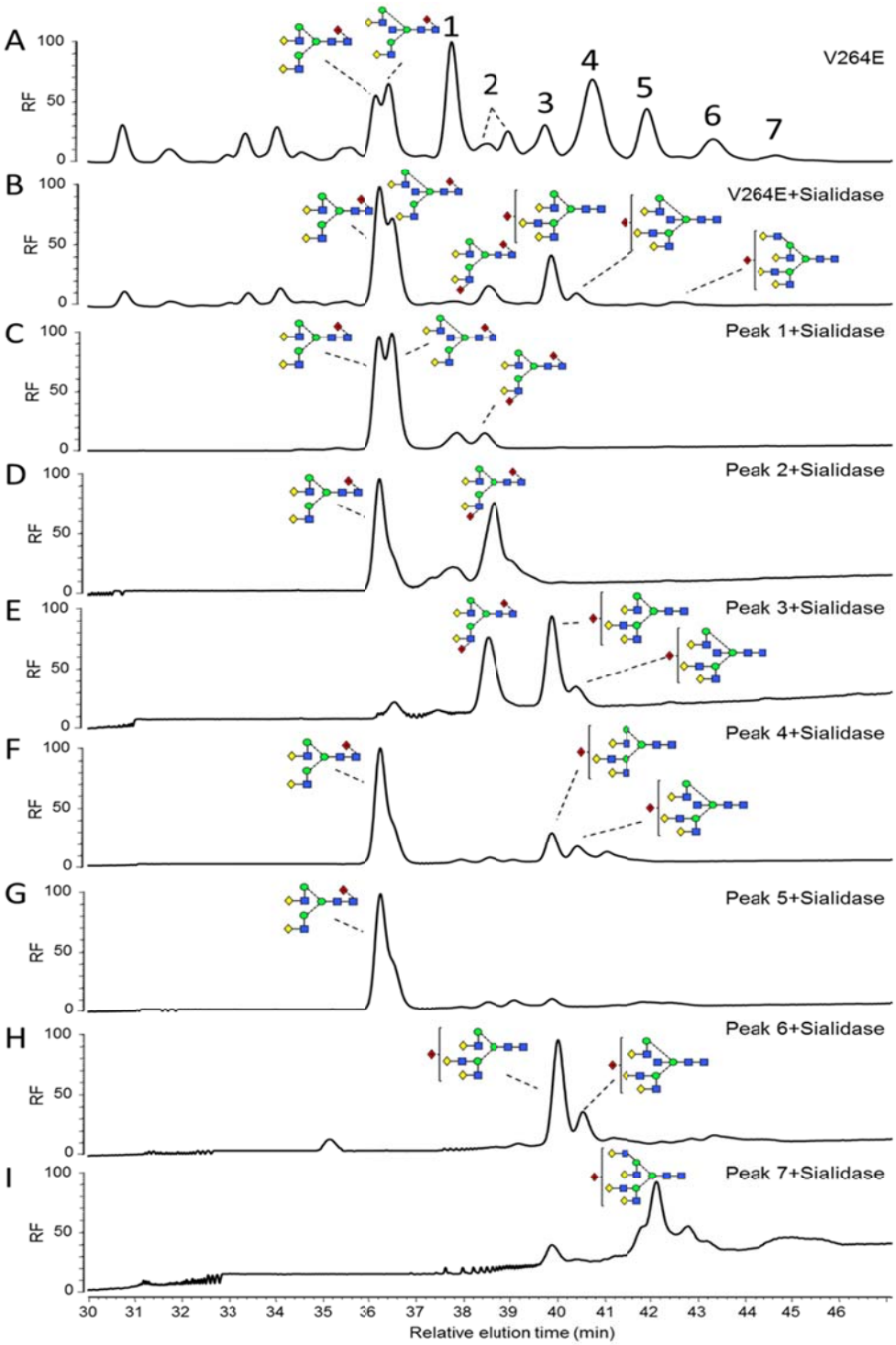


Figure S5. HPLC analysis of sialylated structures of mutant V264E. Individual HPLC sialylated peaks were collected, neuraminidase-treated, and re-analyzed by HPLC. (A) Complete HPLC profile of V264E expressed in HEK 293T. (B) HPLC profile of neuraminidase-digested V264E. (C) HPLC spectrum of desialylated peak 1. (D) HPLC spectrum of desialylated peak 2. (E) HPLC spectrum of desialylated peak 3. (F) HPLC spectrum of desialylated peak 4. (G) HPLC spectrum of desialylated peak 5. (H) HPLC spectrum of desialylated peak 6. (I) HPLC spectrum of desialylated peak 7.

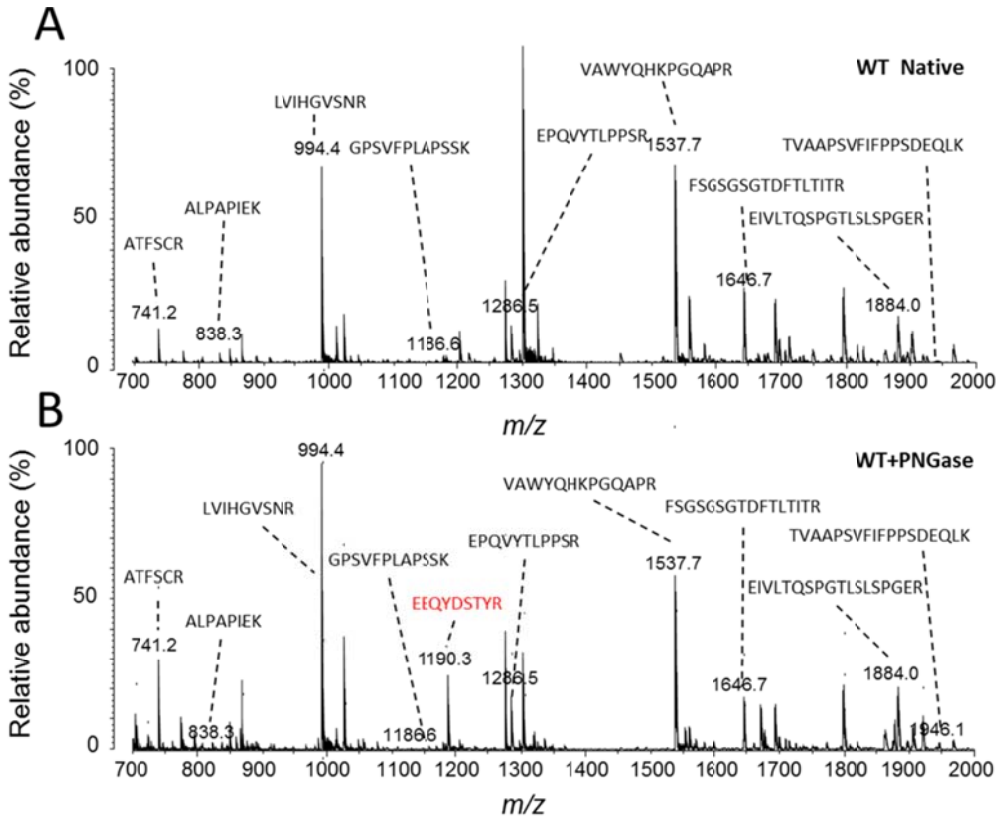


Figure S6. Mass spectra of trypsin-digested, native and deglycosylated IgG. IgG variants were PNGase-treated, then reduced, alkylated and digested with trypsin for 6 hours at 37°C, before analysis by MALDI-TOF MS. The tryptic peptide mass of the native and deglycosylated IgG were calculated online using the PeptideMass program at the ExPASy SIB Bioinformatics Resource Portal. (A) Mass spectrum of trypsin-digested native IgG. (B) Mass spectrum of trypsin-digested, deglycosylated IgG. The deglycosylated peptide containing the conserved N-glycosylation site, Asn297, is highlighted in red.

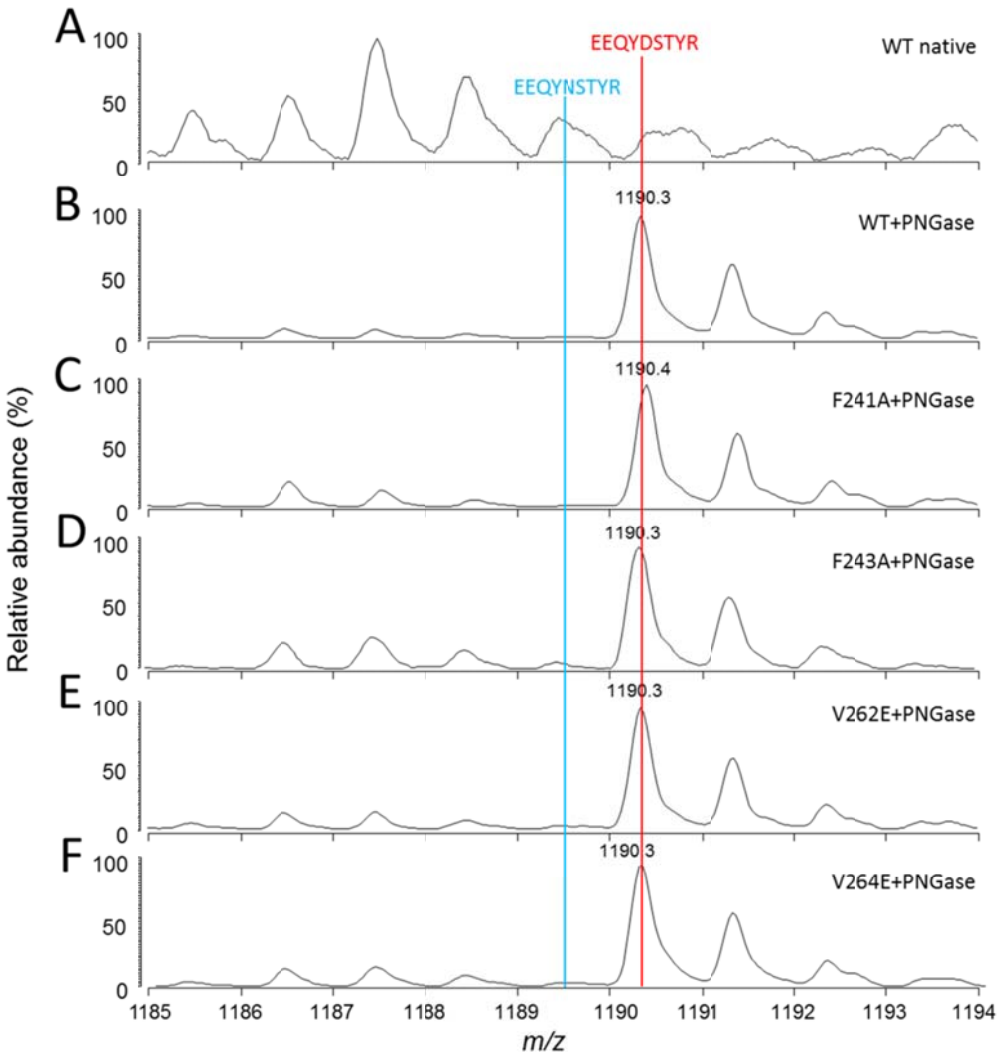


Figure S7. Stacked plot of mass spectra of trypsin digested, deglycosylated native IgG and deglycosylated hydrophobic mutants. IgG deglycosylation was achieved by PNGase F-digestion overnight. For trypsin digestion, IgG variants were reduced, alkylated and digested with trypsin for 6 hours before analysis by MALDI-TOF MS. The tryptic peptide mass of the native and deglycosylated IgG were calculated online using the PeptideMass program at the ExPASy SIB Bioinformatics Resource Portal. (A) Native glycosylated IgG. (B) Deglycosylated IgG. (C) Deglycosylated F241A IgG variant. (D) Deglycosylated F243A IgG variant. (E) Deglycosylated V262E IgG variant. (F) Deglycosylated V264E IgG variant. The position of peptide peaks corresponding to the native deglycosylated peptide containing the conserved N-glycosylation site, Asn297, is indicated by a red vertical line. The position of the peptide peaks corresponding to non-glycosylated peptide containing the conserved N-glycosylation site, Asn297, is indicated by a blue vertical line.

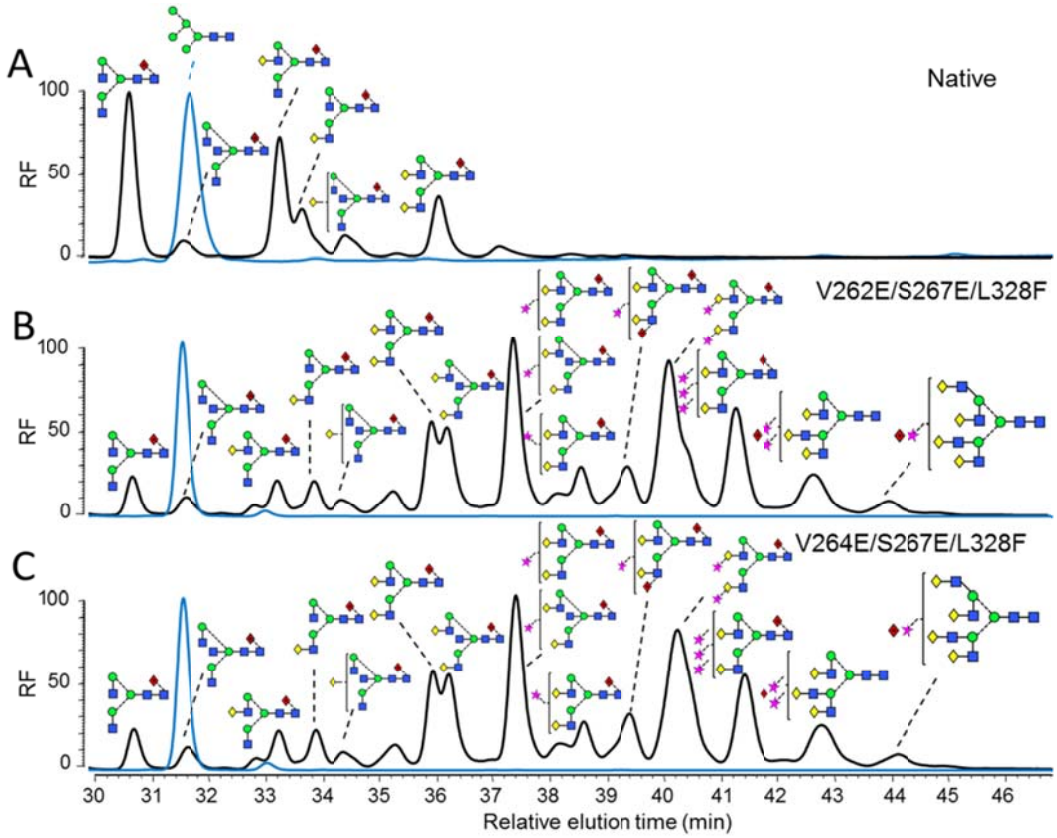
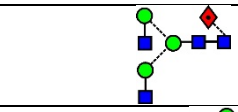
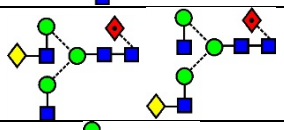
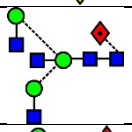
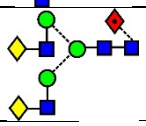
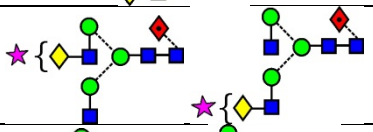
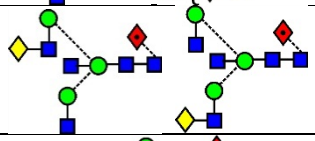
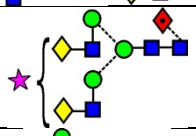
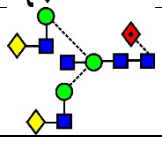
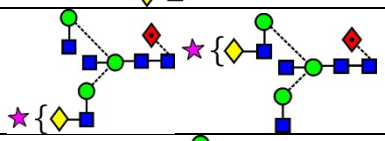
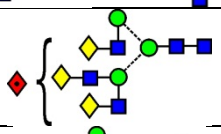
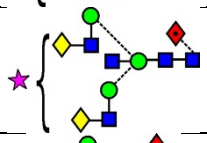
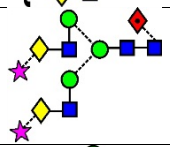
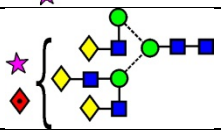


Figure S8. HPLC analysis of 2AA-labelled N-linked glycans from IgG b12 mutants expressed in HEK 293T and HEK 293S cells. Normal-phase HPLC analysis of 2-AA-labelled N-linked glycans, released from target antibody glycoforms by in-gel PNGase F digestion. Glycan profile of IgG expressed in HEK 293T (black) and HEK 293S (blue) for the following variants: (A) Wild type. (B) V262E/S267E/L328F. (C) V264E/S267E/L328F. Symbolic representation of glycan structures follows that of Harvey *et al.*(5): ★ = NeuNAc, ◆ = Gal, ■ = GlcNAc, ● = Man, ◆ = Fuc. The linkage position is shown by the angle of the lines linking the sugar residues (vertical line = 2-link, forward slash = 3-link, horizontal line = 4-link, back slash = 6-link). Anomericity is indicated by full lines for β-bonds and broken lines for α-bonds.

Table S1. List of masses, compositions and structures of the N-glycans derived from electrospray mass spectrometry and then converted into singly and doubly charged ions using ion mobility extraction.

m/z	Ion	Composition				Structure
		Hex	GlcNAc	Fuc	Neu5Ac	
1559.5	$[M + H_2PO_4]^-$	3	4	1	0	
1721.6	$[M + H_2PO_4]^-$	4	4	1	0	
1762.6	$[M + H_2PO_4]^-$	3	5	1	0	
1883.6	$[M + H_2PO_4]^-$	5	4	1	0	
990.3	$[M+(H_2PO_4)_2]^{2-}$					
1914.7	$[M-H]^-$	4	4	1	1	
1924.6	$[M + H_2PO_4]^-$	4	5	1	0	
1010.8	$[M+(H_2PO_4)_2]^{2-}$					
2076.7	$[M-H]^-$	5	4	1	1	
1086.6	$[M-H+H_2PO_4]^{2-}$					
2086.7	$[M + H_2PO_4]^-$	5	5	1	0	
1091.8	$[M+(H_2PO_4)_2]^{2-}$					
2117.8	$[M-H]^-$	4	5	1	1	
2248.8	$[M + H_2PO_4]^-$	6	5	1	0	
2279.9	$[M-H]^-$	5	5	1	1	
1188.4	$[M-H+H_2PO_4]^{2-}$					
1183.4	$[M-H_2]^{2-}$	5	4	1	2	
2389.8	$[M-H_2+Na]^-$				2	
2441.9	$[M-H]^-$	6	5	1	1	
1269.4	$[M-H+H_2PO_4]^{2-}$					

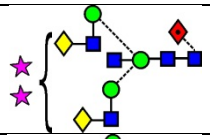
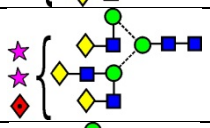
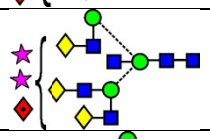
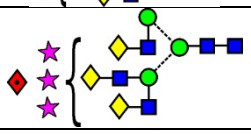
1285.0	$[M-H_2]^{2-}$	5	5	1	2	
1366.0	$[M-H_2]^{2-}$	6	5	1	2	
1467.5	$[M-H_2]^{2-}$	6	6	1	2	
1511.5	$[M-H_2]^{2-}$	6	5	1	3	
1522.5	$[M-H_3+Na]^{2-}$	6	5	1	3	

Table S2. Summary of single IgG b12 mutants' affinity for FcγRs relative to the native (black) or $Man_5GlcNAc_2$ (blue) wild type glycoforms. Each value represents the apparent affinity of a particular mutant glycoform over the same wild type glycoform (i.e. value <1 indicates weaker binding than the same wild type glycoform and vice versa). All data points represent the calculated mean of two independent measurements from a total of at least two experiments.

FcγR	Glycoform	WT	F241A	F243A	V262E	V264E
R1A	Man5	1	1.00	0.40	0.40	0.17
	Native	1	1.00	0.67	0.40	0.40
R2A	Man5	1	0.02	0.75	0.15	0.02
	Native	1	0.10	0.25	0.05	n.b
R2B	Man5	1	0.83	1.00	1.00	0.95
	Native	1	0.25	0.37	0.31	0.46
R3A	Man5	1	0.01	1.17	0.09	n.b
	Native	1	0.02	0.26	0.05	0.01
R3B	Man5	1	0.09	0.37	0.23	0.07
	Native	1	0.19	0.31	0.25	0.41

Table S3. Summary of double and triple IgG b12 mutants' affinity for FcγRs relative to the native (black) or $Man_5GlcNAc_2$ (blue) wild type glycoforms. Each value represents the apparent affinity of a particular mutant glycoform over the same wild type glycoform (i.e. value <1 indicates weaker binding than the same wild type glycoform and vice versa). All data points represent the calculated mean of two independent measurements from a total of at least two experiments.

FcγR	Glycoform	WT	S267E L328F	V262E S267E L328F	V264E S267E L328F
R1A	Man5	1	3.33	0.23	0.01
	Native	1	2	0.15	0.04
R2A	Man5	1	0.3	n.b	n.b
	Native	1	0.03	0.02	n.b
R2B	Man5	1	77.8	2.57	2.57
	Native	1	357.1	4.29	2.5
R3A	Man5	1	0.003	n.b	n.b
	Native	1	0.02	n.b	n.b
R3B	Man5	1	0.2	n.b	0.04
	Native	1	0.57	0.38	0.11