Supporting Information

Native-MS Analysis of Monoclonal Antibody Conjugates by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Biotinylation of mAbs on lysine and cysteine residues

For lysine conjugation (Scheme 1a) an Amgen standard human monoclonal antibody (hIgG1) at a concentration of 2 mg/mL in 50 mM sodium phosphate buffer, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5 was alkylated with 5 and 10-equivalents of EZ-Link sulfo-NHS-LC-Biotin (Peirce) for 60 min at RT. For cysteine conjugation (Scheme 1b) the mAb was partially reduced with 5 and 10equivalents of *tris*(2-carboxyethyl)phosphine (TCEP) for 90 min at RT. Ten equivalents of EZ-Link iodoacetyl-LC-Biotin (Pierce) was then added to the reaction mixture which was shaken overnight. Excess TCEP and unreacted LC-biotin was removed through repeated buffer exchange with phosphate buffer saline (PBS) in Amicon ultra-0.5 mL 30 kDa. Deglycosylation was achieved by the addition of PNGaseF (10 uL of 25 U/mg; QA-bio) to the biotinylated products, and the reaction mixtures were incubated overnight at 37 °C. The final products for MS analysis (both native and denatured) were obtained from size exclusion purification (Superdex 200 10/150 GL, 20mM Tris pH 7.5, 150 mM NaCl operated at 0.7 mL/min) where the mAb biotin conjugate molecules were subsequently separated from any unreacted biotin compound. The samples eluting from the SEC are typically collected in 0.5 mL aliquots, over a volume of approximately 2.5 mL, which is then pooled and concentrated over a Centricon 50 kDa spin filter (Centricon, 50 kDa MWCO).

Native Mass Spectrometry

All native-MS experiments were performed using the SolariX 15 Tesla FT-ICR-MS instrument (Bruker Daltonics). The nESI capillary voltage was set to 0.6 to 0.8 kV, positive ionization mode. The temperature of drying gas was 100 ºC and the flow rate was 2.5 L/min. The RF amplitude of the ion-funnels was 300 V peak-to-peak, and the applied voltages were 210 V and 6 V for funnels 1 and 2, respectively. The voltage of skimmer 1 was 50 to 75 V and the skimmer 2 voltage was kept at 20 V. The lowest values of RF frequencies were used in all ion-transmission regions: multipole 1 (2 MHz), quadrupole (1.4 MHz), and transfer hexapole (1MHz). Ions were accumulated for 500 ms in the hexapole collision cell before being transmitted to the infinity ICR cell. The time-of-flight of 2.5 ms was used. Vacuum pressures for different regions were: source 2 mbar; quadrupole 2×10^{-6} mbar; ultra-high vacuum chamber 2.6×10^{-9} mbar. Mild collision induced dissociation (CID) was performed in the hexapole collision cell by collision with SF_6 at a voltage of 20 to 30 V. The mass spectrometer was externally calibrated with

cesium iodide over the *m/z* range 100 to 20,000. 100 scans were averaged for each spectrum and recorded at 512 k data points (transient length 0.17 s) in magnitude mode. MS Control software was Compass solariXcontrol, version 1.5.0, build 103. Prior to native-MS analysis, ADC samples were buffer exchanged into 200 mM ammonium acetate using a BioRad P6 column. Typical native-MS working solutions were 10 µM and were introduced in to the MS using nESI gold coated glass needles (Long Thin Wall, M956232AD1-S; Waters Corporation).

All LC-MS data was acquired on an Agilent 6230 TOF LC/MS system with a 1290 Infinity LC system. Chromatographic separation was achieved using a Zorbax SB300-C8 3.5 µm 2.1 x 50 mm column operated at a temperature of 75 °C. The solvents used were as follows: mobile phase A was water containing 0.1% v/v TFA. Mobile phase B was 90% n-propanol containing 0.1% v/v TFA. Initial gradients conditions were 20% mobile phase B from 0.0 to 1.0 minutes; 1.0 to 9.0 minutes, 20-70% mobile phase B; 9.0-10.0 minutes, 70-100% mobile phase B, where it remains at 100% for 1 further minute. The flow rate was 0.2 mL/min. Approximately 5 ug of IgG1-biotin conjugate was loaded on to the LC-MS system for each analyses.

Data was acquired over the *m/z* range 1000-7000. The source fragmentor, skimmer and octapole 1 RF values were: 460 V, 95 V and 800 V (peak-to-peak) respectively. The ESI capillary voltage was 5.9 kV. Gas temperature was 340 °C. Drying gas was 13 L/min. Nebuliser was 25 psig. Oa-ToF calibration was performed using the Agilent Tune Mix using the automated calibration procedure implemented through MassHunter Data Acquisition version B.06.01, Build 6.01.6157.

Data processing (deconvolution) was performed using the PMI Intact Protein processing module [1]. Deconvolution parameters were: Mass Range: 135,000 to 160,000; *m/z* range 1000 to 4000 (denatured oa-ToF) or 4500 to 6500 (native; FT-ICR-MS). Min Difference Between Mass Peaks (Da) 1; Max Number of Mass Peaks 20; Peak Sharpening Disabled; Baseline Radius (*m/z*) 15; Smoothing Sigma (*m/z*) 0.02; Spacing (*m/z*) 0.02; Mass Smoothing Sigma 5; Mass Spacing 2.0; Iteration Max 50; Charge Range 10 to 100 (denatured) or 10 to 50 (native). All MW were determined from the Show Centroided Peaks option.

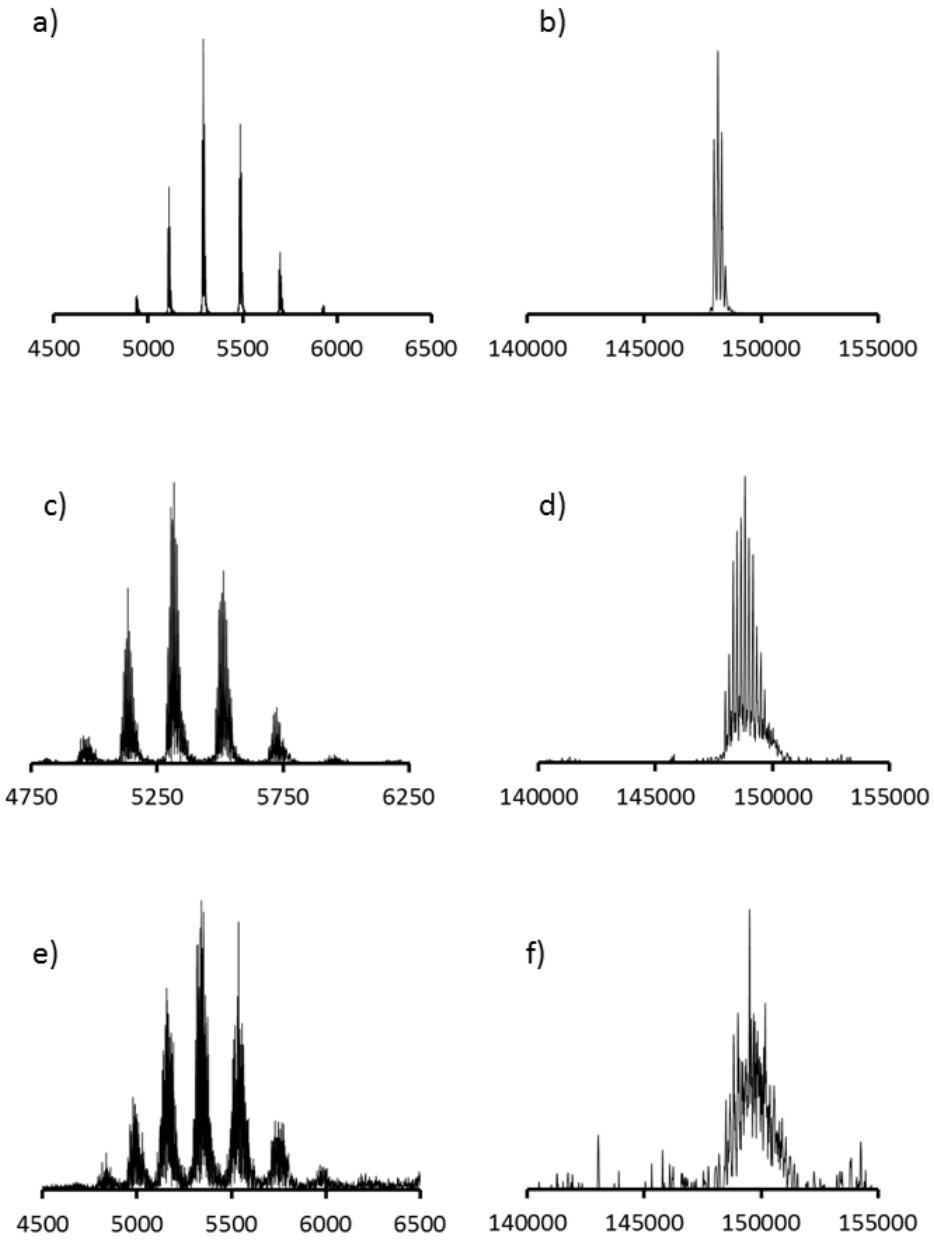

Figure S1. Native nESI FT-ICR MS data for the glycosylated mAb conjugate (lysine-biotin) molecules, displayed in magnitude mode, using an asymmetric Hann apodisation function: a) 0-molar equivalent; b) 0-molar equivalent deconvoluted; c) 5-molar; d) 5-molar equivalent deconvoluted; e) 10-molar equivalent; d) 10-molar equivalent deconvoluted. Samples were buffer exchanged in to 200mM ammonium acetate using the BioRad P6 spin column. Zero charge deconvoluted (PMI [1]) spectra are also displayed.

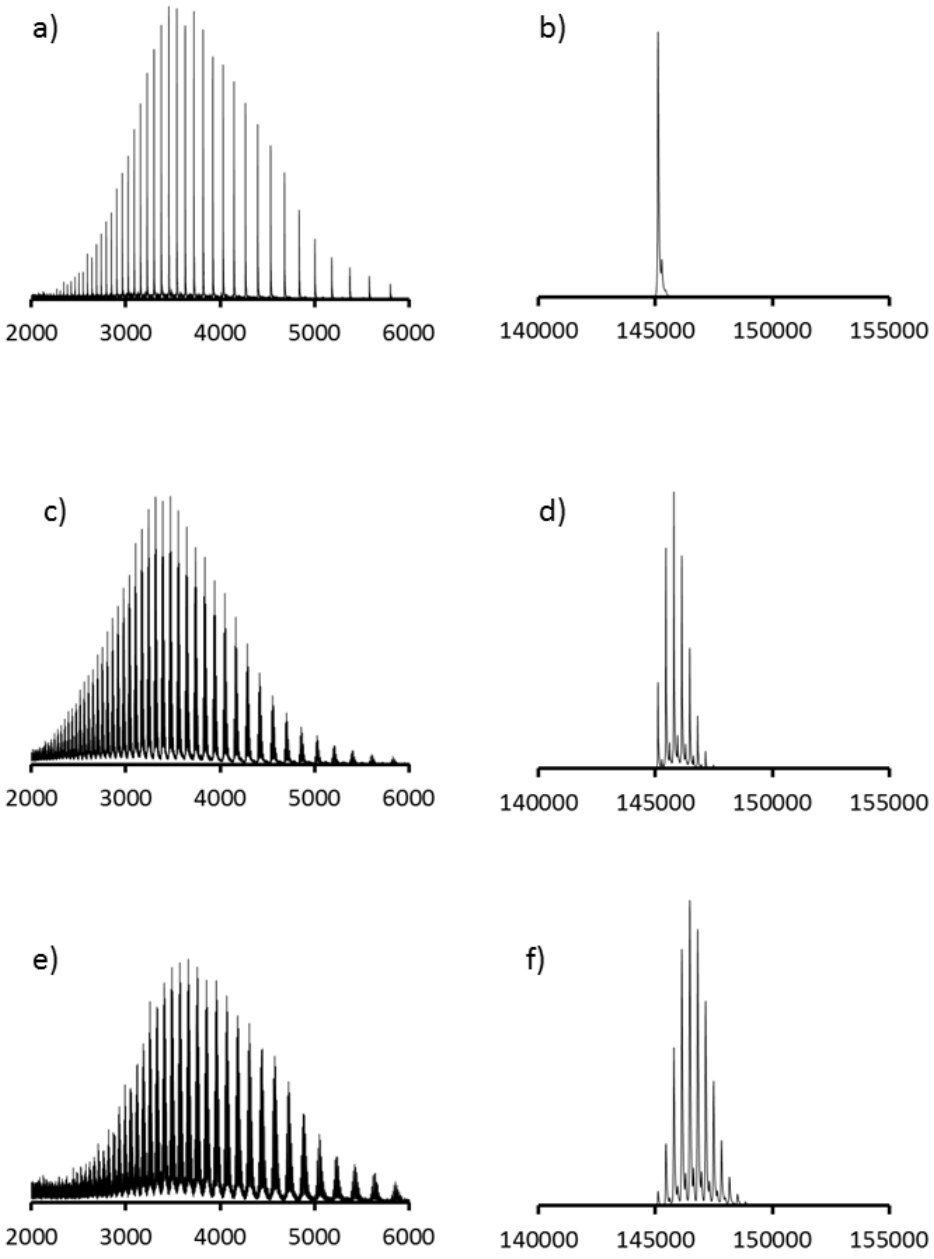

Figure S2. Unprocessed and deconvoluted oa-ToF data for the deglycosylated (PNGaseF treated) mAb conjugate (lysine-bioton) molecules analysed by LC-MS: a) 0-molar equivalent; b) 0-molar equivalent deconvoluted; c) 5-molar equivalent; d) 5-molar equivalent deconvoluted; e) 10-molar equivalent; f) 10-molar equivalent deconvoluted. 5 µg of each conjugate was injected for each LC-MS analysis. Zero charge deconvoluted (PMI [1]) spectra are also displayed.

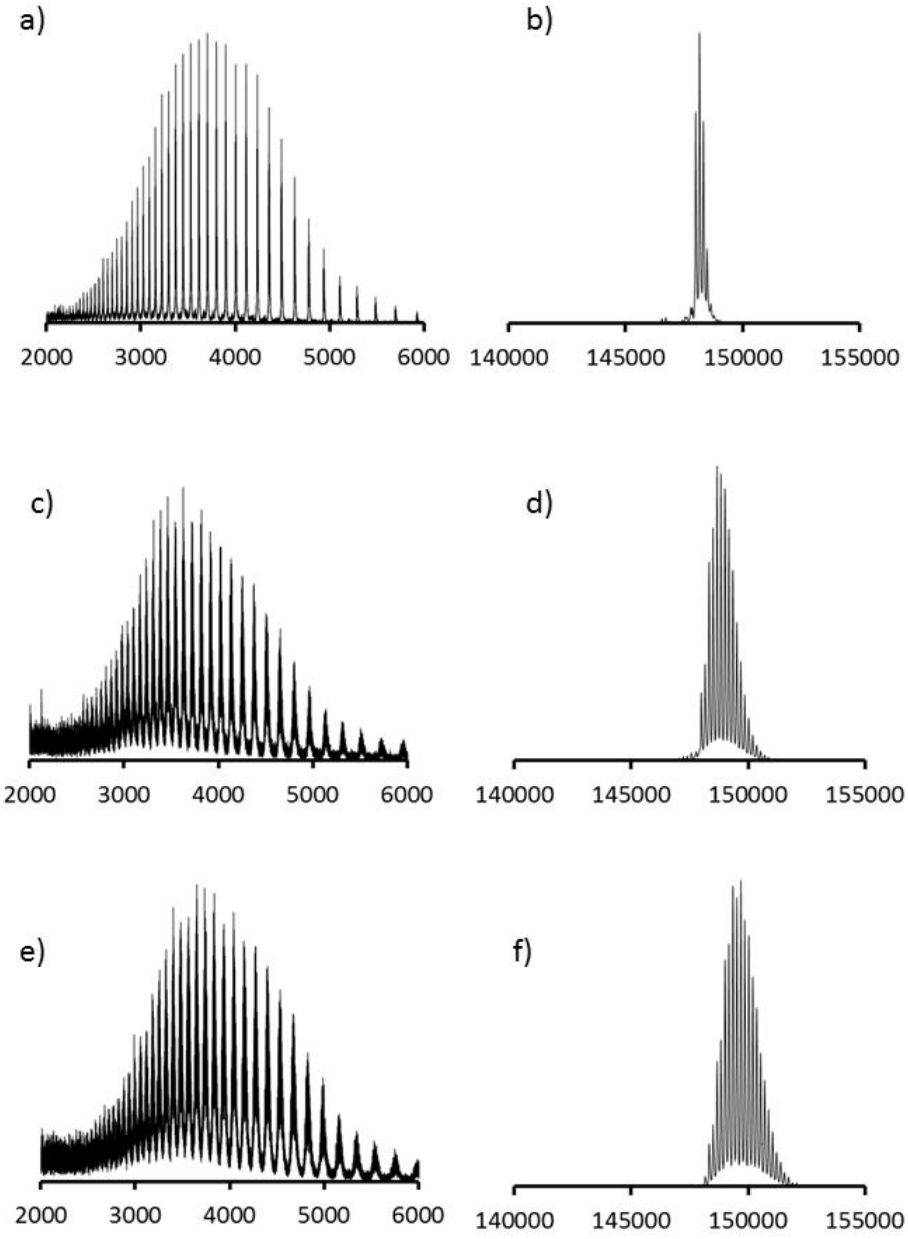

Figure S3. Unprocessed and deconvoluted oa-ToF data for the glycosylated mAb conjugate (lysine-biotin) molecules analysed by LC-MS: a) 0-molar equivalent; b) 0-molar equivalent deconvoluted; c) 5-molar equivalent; d) 5-molar equivalent deconvoluted; e) 10-molar equivalent; f) 10-molar equivalent deconvoluted. 5 µg of each conjugate was injected for each LC-MS analysis. Zero charge deconvoluted (PMI [1]) spectra are also displayed.

Cys-Conjugate 5-molar eq. TCEP Native MW (Da)	**Cys-Conjugate 10-molar eq. TCEP** Native MW (Da)	**DAR Assignment**
	72,946	1LC-1HC 1Drug
73,328	73,314	1LC-1HC 2Drug
	73,709	1LC-1HC 3Drug
74,057	74,090	1LC-1HC 4Drug
122,125	-	1LC-2HC 1Drug
122,513	-	1LC-2HC 2Drug*
122,895	122,925	1LC-2HC 3Drug
123,655	123,660	1LC-2HC 5Drug
145,480	-	DAR1
145,861	145,860	DAR2
146,244	146,245	DAR3
146,630	146,625	DAR4
-	147,010	DAR5
147,395	147,395	DAR6
-	147,787	DAR7
148,080	-	DAR8

Table S1. Deconvoluted MWs detected from the native-MS analysis of the deglycosylated mAb conjugate (cysteine-biotin; 5 and 10-molar equivalents of TCEP) molecules. Deconvolution performed using the PMI deconvolution algorithm [1]. *Indicates that the ion signal was too low for reliable deconvolution, therefore MW determination was performed using Bruker Compass Data Analysis, Deconvolution with Charge State Ruler option. Interestingly, the partially formed cysteine conjugate with a measured MW 122,513 Da was not detected under denaturing LC-MS conditions (Table S2), therefore most likely representing a non-covalently assembled

molecule where the interchain disulphide bond has been biotinylated, but the resulting complex (1LC-2HC 2Drug) remains noncovalently associated in the gas-phase. DAR assignment nomenclature reproduced from Valliere-Douglass [2].

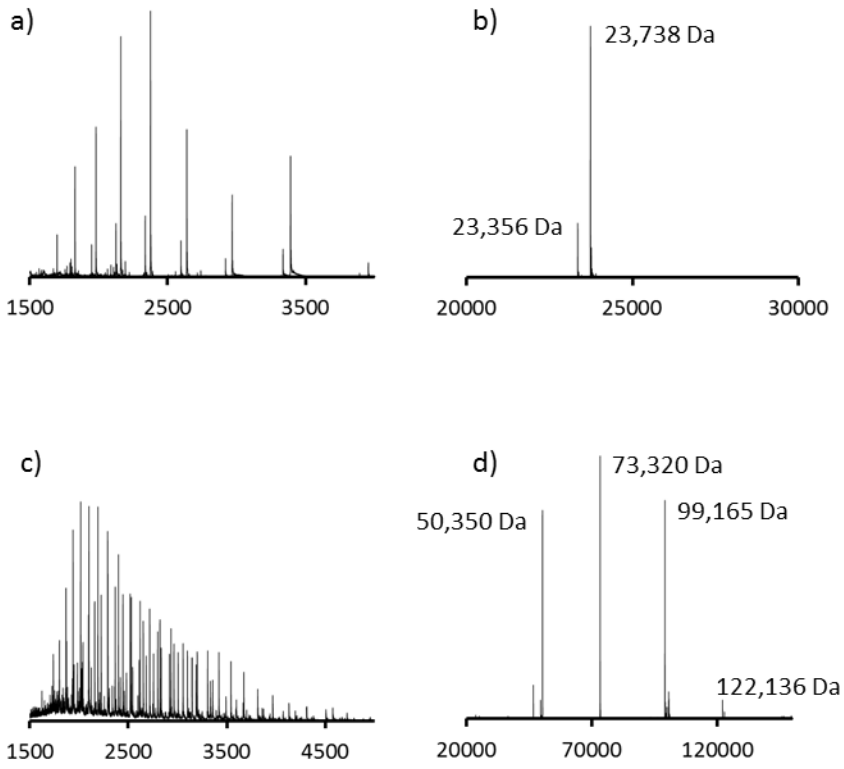

Figure S4. Unprocessed and deconvoluted oa-ToF data for the 5-molar equivalent TCEP deglycosylated mAb conjugate (cyteine-biotin) molecules analysed by LC-MS: a) LC; b) LC deconvoluted; c) heavy chain; d) heavy chain deconvoluted. 5 µg of each conjugate was injected for each LC-MS analysis. Reverse phase LC-separation of the light chain and heavy chain was achieved. Zero charge deconvoluted spectra are also displayed. Deconvolution was performed using Maximum Entropy through MassHunter Qualitative Analysis, Version B.06.00, Build 6.0.633.0.

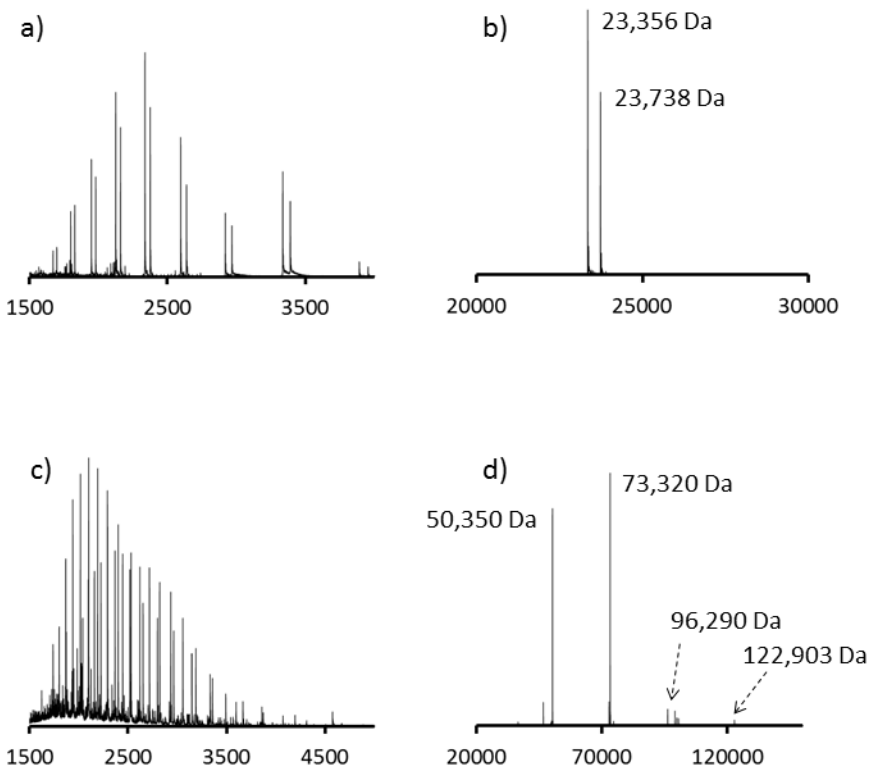

Figure S5. Unprocessed and deconvoluted oa-ToF data for the 10-molar equivalent TCEP deglycosylated mAb conjugate (cysteine-biotin) molecules analysed by LC-MS: a) light chain; b) light chain deconvoluted; c) heavy chain; d) heavy chain deconvoluted. 5 µg of each conjugate was injected for each LC-MS analysis. Reverse phase LC-separation of the LC and HC was achieved. Zero charge deconvoluted spectra are also displayed. Deconvolution was performed using Maximum Entropy through MassHunter Qualitative Analysis, Version B.06.00, Build 6.0.633.0.

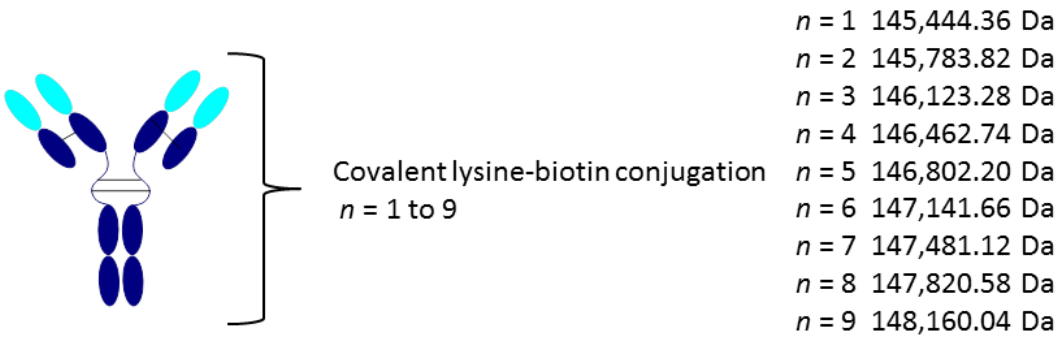

Figure S6. Schematic representation of the lysine conjugated molecule. The calculated MWs of the deglycosylated (PNGaseF treated) mAb conjugated (lysine-biotin) molecules are displayed. Exact locations of the lysine-biotin conjugation were not investigated.

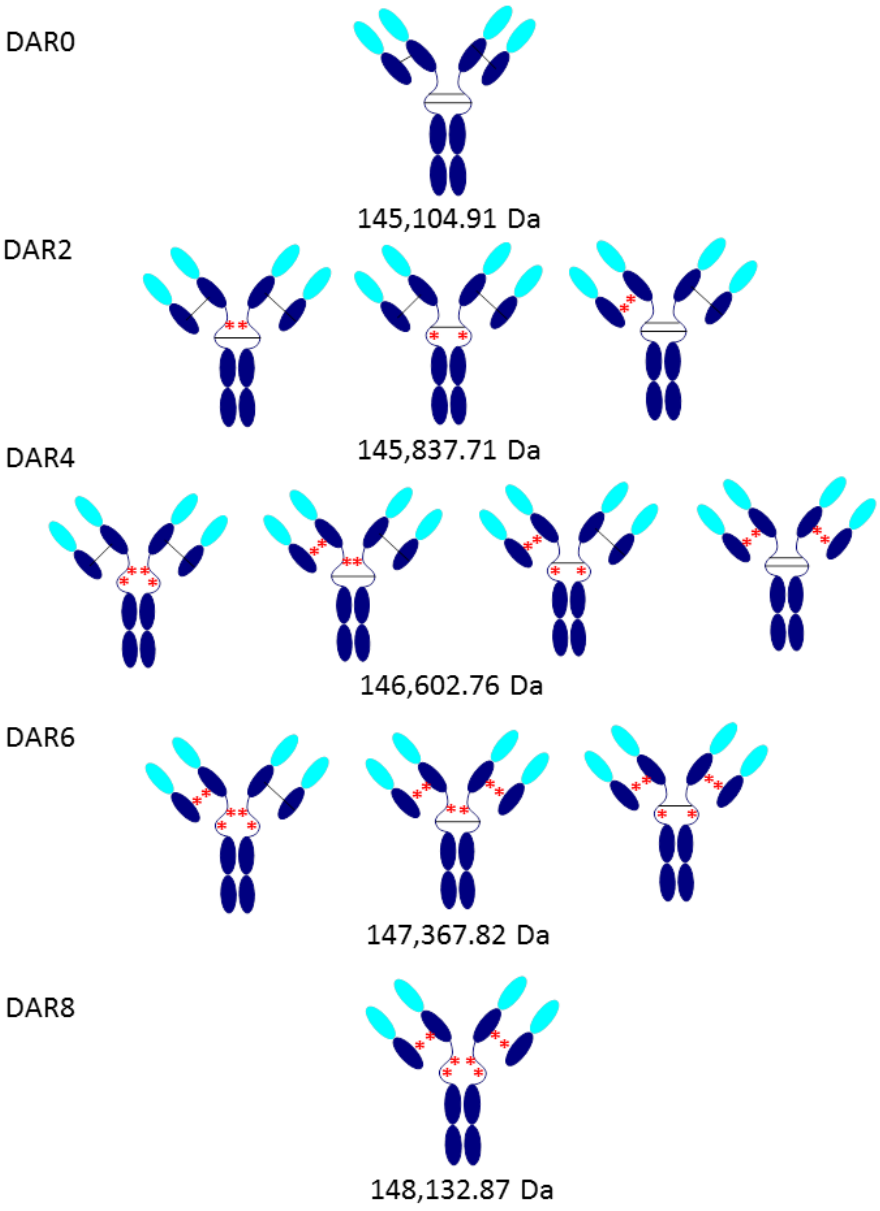

Figure S7. Schematic representation of all the possible isoforms representing the mAb conjugated (cysteine-biotin) molecules. The calculated MWs of the deglycosylated (PNGaseF treated) are also displayed. This figure layout is similar to those already published [3-4]. It is assumed that both cysteine residues from a reduced disulphide bond are covalently modified, thus resulting in an even number of covalent additions (DAR = 2, 4, 6 and 8). However, there are low levels of odd-numbered DAR species (DAR = 1, 3, 5 & 7) suggesting either partial modification of a reduced disulphide bond or non-specific covalent addition to a basic residue, such as lysine or arginine also occurred.

Cys-Conjugate 5-molar eq. Denatured MW (Da)	**Cys-Conjugate 10-molar eq.** Denatured MW (Da)	**DAR Assignment**
23,356	23,356	1LC 0Drug
23,738	23,738	1LC 1Drug
49,585*	49,586*	1HC 1Drug
49,965*	49,968*	1HC 2Drug
50,350	50,350	1HC 3Drug
-	72,937*	1LC -1HC 1Drug
73,320	73,320	1LC-1HC 2Drug
99,165	99,165*	2HC 2Drug
99,932*	99,934*	2HC 4Drug
-	100,698*	2HC 6Drug¶
122,136*	122,143*	1LC-2HC 1Drug
122,903*	122,903*	1LC-2HC 3Drug
-	123,677*	1LC-2HC 5Drug¶

Table S2. Zero-charge deconvoluted molecular weights derived for the 5-molar and 10-molar equivalent deglycosylated IgG1-biotin cysteine conjugated molecules analysed by denaturing LC-MS. Deconvolution was performed using Maximum Entropy through MassHunter Qualitative Analysis, Version B.06.00, Build 6.0.633.0. The numbers in the Drug Assignment column represent the number of biotin covalent additions to either the LC or the HC. The species containing a combination of LC and HC suggest at least one inter-chain disulphide bond has remained intact. *Indicates low level detection. ¶Indicates that nonspecific biotinylation is likely occurring on basic residues.

	Native-MS DAR based on intact MW(ave)	SD	*z*	Native-MS DAR based on individual charge state	SD
mAb lysine conjugate molecule (5-eq biotin)	**1.56**	**0.02**	30 29 28 27 26	1.65 1.57 1.54 1.53 1.62 **Ave DAR: 1.58**	0.04 0.01 0.01 0.02 0.03 **0.04**
mAb lysine conjugate molecule (10-eq biotin)	**3.99**	**0.09**	30 29 28 27 26	3.97 3.83 3.74 3.86 3.87 **Ave DAR: 3.84**	0.17 0.09 0.02 0.03 0.04 **0.04**

Table S3. A comparison of the DAR values derived from the PMI deconvoluted MWs against DAR values calculated for each charge state. The average DAR values are calculated for each charge state, made from three native-MS measurements (vertical). The Average DAR value is also calculated by averaging the DAR values for each charge state (horizontal); The DAR value is not weighted for charge state intensity. All DAR value calculated from MS intensity values made from triplicate native-MS measurements (n=3). As can be observed, excellent consistency is achieved for the DAR value calculated from the intact deconvoluted MW and charge state comparison.

1. Bern, M. W.;Kil, Y. J.;Carlson, E.;Kletter, D.;Tang, W.;Becker, C., *American Society for Mass Spectrometery Annual Conference, 2016, San Antonio, Texas* 2016.

2. Valliere-Douglass, J. F.;McFee, W. A.;Salas-Solano, O., *Anal Chem* 2012, *84*. 2843-2849.

3. Birdsall, R. E.;Shion, H.;Kotch, F. W.;Xu, A.;Porter, T. J.;Chen, W., *MAbs* 2015, *7*. 1036-1044.

4. Guo, J.;Kumar, S.;Chipley, M.;Marcq, O.;Gupta, D.;Jin, Z.;Tomar, D. S.;Swabowski, C.;Smith, J.;Starkey, J. A.;Singh, S. K., *Bioconjug Chem* 2016, *27*. 604-615.