Non-consensus glycosylation of ScFv × FAB bispecific antibody

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BEAT® is a proprietary bispecific antibody platform of Glenmark Pharmaceuticals, offering unique advantages for development of bispecific antibodies. such as high level of heavy chain heterodimerisation by bio-mimicry, compatibility with standard antibody production processes, built-in purification technology, versatile ScFv × FAB format without need for common light or heavy chain, easy identification of heterodimers due to unique molecular weight. During development of one of the BEAT molecules – ScFv × FAB format – presence of unexpected shoulder on the main peak in non-reducing CE-SDS analysis and new peak in reducing CE-SDS were reported. This unknown species was subsequently enriched by SEC and CEX, affinity purification, and characterised by mass spectrometry.

Materials and Methods

All experiments were performed on BEAT bispecific antibody produced in CHO cell line and purified by Protein A and CEX at Glenmark Pharmaceuticals SA. All CE-SDS analyses were performed using PA800 or PA800plus pharmaceutical analysis systems using IgG Purity and Heterogeneity kit, all from Sciex. SEC analysis and fractionation was performed using TSKgel G3000swxl column connected to Acquity Arc system equipped with PDA detector and fraction manager. CEX analysis and fractionation was performed by salt gradient method using ProPac WCX-10 column connected to Alliance 2695 HPLC system equipped with PDA detector and fraction manager. Affinity purification on immobilized targets was performed using AminoLink™ Plus Coupling Resin and following manufacturer protocols. Intact and reduced MS analysis was performed using Reprosil Gold C4 column on Ultimate 3000 LC system and 5600+ TripleTOF mass spectrometer. Peptide mapping was performed using Waters Acquity UPLC BEH C18 column on Waters Acquity UPLC H-Class system and Waters Synapt G2-Si Q-Tof mass spectrometer. Glycan analysis was performed on Sciex 5800 MALDI-TOF/TOF mass spectrometer.

Enrichment by SEC

Size Exclusion Chromatography was not capable of separating the shoulder observed on CE-SDS. Instead, main peak (monomer) was split into three sub-fractions and each of them was analysed separately on both non-reducing and reducing CE-SDS. This demonstrated enrichment of unknown variant in early eluting part of the main peak (F1), indicating its higher hydrodynamic radius. At the same time, enrichment of ~100 kDa fragment was observed in later eluting part of the of the peak (F3).

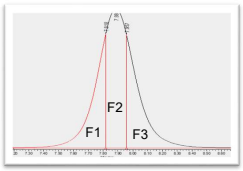


Fig.1. SEC fractions

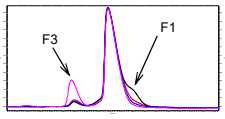


Fig. 2. nrCE-SDS of SEC fractions

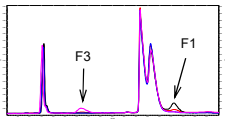


Fig. 3. rCE-SDS of SEC fractions

Enrichment level was further increased by narrowing down collection window and re-injecting enriched samples, to generate samples with up to 80% level of variant, comparing to initial ~3%. Enriched samples were used for MS analysis.

Enrichment by CEX

Fractionation by CEX was performed in order to determine if this variant can be associated with any of the observed charge variants. CEX charge variant profile was separated into seven arbitrary fractions as shown on Figure 4. Collected fractions were analysed by non-reducing (Fig. 5) and reducing (Fig. 6) CE-SDS. CEX fractionation was successful in associating this variant with acidic species (Fraction 2 and 3).

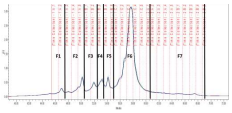


Fig. 4. CEX fractions

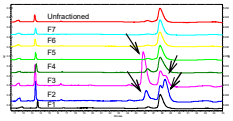


Fig. 5. nrCE-SDS of CEX fractions

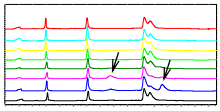
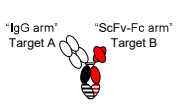


Fig. 6. rCE-SDS of CEX fractions

Affinity purification

CE-SDS results of elutate (binding fractions) from affinity purification on immobilised target molecules is shown on Figure 7 and 8. Variant shoulder (nrCE-SDS) and peak (rCE-SDS) are present in eluate from target A ("IgG arm" of the molecule) but absent on eluate from target B ("ScFv-Fc arm"), indicating a change only in antigen binding region of ScFv. (During the same purification, level of ~100 kDa fragment was reduced, indicating that one of its constituents lacks binding to target B, but not target A.)



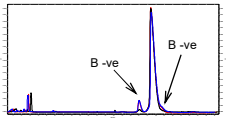


Fig. 7. nrCE-SDS of bound fractions

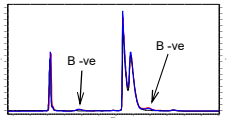


Fig. 8. rCE-SDS of bound fractions

Change in binding to targets was also confirmed by testing SEC enriched fractions by SPR and binding ELISA. Both of those methods have shown reduction in binding only to target of "ScFv-Fc arm" ("target B") following level of enrichment in the variant, while binding to target of "IgG arm" ("target A") remained unaffected.

Intact and reduced MS analysis

MS analysis of intact BEAT and enriched fraction (Figure 9) has demonstrated that enriched variant has molecular weight higher by approximately 2.5 kDa based on predominant species in deconvoluted spectra. MS analysis of reduced molecule demonstrated that there are no differences between native material and enriched fraction on light chain and heavy chain (not shown), and mass difference observed on intact molecule is limited to ScFv-Fc chain (Figure 10).

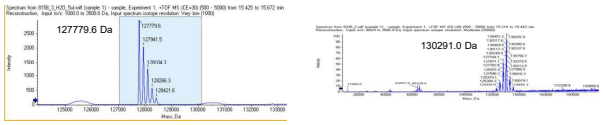


Fig. 9. MS analysis of intact BEAT – native (left), enriched variant (right).

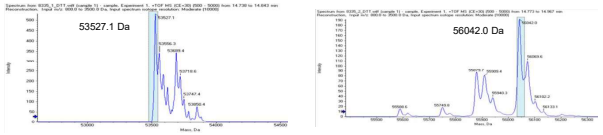
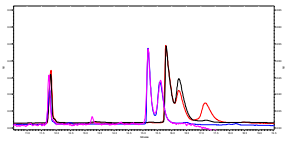


Fig. 10. MS analysis of reduced BEAT – ScFv-Fc chain only – native (left), enriched variant (right).

Digestion with PNGase F

Typically PNGase F is capable of completely removing N-linked glycosylation of Fc region of IgG. Initial experiments performed under native conditions (Fig. 11 – black and blue trace) show that PNGase F does not remove ScFv-Fc variant peak. However, if denaturing conditions are used, PNGase F can completely remove ScFv-Fc variant peak (Fig. 11 – red and pink peak), thus demonstrating it is a glycoform.



Black – native sample
Blue – native sample digested with PNGase F in native conditions
Red – sample spiked with enriched variant
Pink – spiked sample digested with PNGase F in denaturing conditions

Fig. 11. rCE-SDS of PNGase F digested samples.

MALDI analysis of glycans

To perform analysis of only glycans present on ScFv-Fc variant, Fc glycans were digested with EndoS and removed by buffer exchange, then molecule was denatured and ScFv-Fc variant glycans were relased by PNGase F, permethylated, and analysed by MALDI. Detected masses, relative intensities, and proposed compositions and structures are shown on Figure 12.

[M+Na]+ signal (m/z)	Composition				Relative intensity (%)	Selected for MS/MS	Proposed structure	
	Hex	HexNAc	dHex	NeuAc				
1835.90	3	4	1	0	12	-		G0F
2040.00	4	4	1	0	8	-		G1F
2401.17	4	4	1	1	6	-		G1FS
2605.24	5	4	1	1	29	✓		G2FS
2966.39	5	4	1	2	45	✓		G2FS2

Fig. 12. Proposed structures of detected glycans.

MS peptide mapping confirmation of non-consensus glycosylation site

Existing literature on the subject (e.g. Valliere-Douglass et al, J Biol Chem. 2010 May 21;285(21):16012-22) demonstrates possibility of non-consensus, reverse-consensus, and glutamine-linked glycosylation of antibodies. Looking through the sequence of our BEAT molecule, several of such possible sites were detected in ScFv-Fc chain – Figure 13, consensus (green), reverse-consensus (red), and glutamine (turquoise). In order to determine which of those sites was occupied, peptide mapping with MS detection was used. Native and enriched samples were digested with trypsin and PNGase F. Glycosylation sites were detected by looking for deamidated variants of peptides encompassing each potential site above hinge.

Out of four sites, based on deamidation level and comparing to native sample, most likely glycosylation site was determined to be Q117 (QGT motif).

Fig. 13. Location of glycosylation sites in ScFv-Fc sequence

Discussion and conclusions

Separation and fractionation by SEC and CE-SDS demonstrated that this specie is not an assay artefact. MS analysis of enriched fraction has shown mass difference, demonstrating it is not size variant due to e.g. folding or disulphide scrambling. Additionally, even though initial experiments with PNGase F were not successful in removal of that species, mass difference suggested it might be atypical glycosylation (the absence of signal peptide was already excluded in other experiments). Subsequent experiments with PNGase F under denaturing conditions have confirmed this suspicion and lead to further characterisation of glycans by MALDI. Results of this glycan characterisation provide explanation to CEX fractionation results – as most of those glycans were sialylated. Peptide mapping with MS has provided information on potential site of this glycosylation, which is also consistent with affinity purification – glycosylation on Q117 might cause steric hindrance or conformational changes in ScFv, which may in turn affect the binding.