LC/MS Charge Variant Characterization of an ECD-Fc Fusion Protein using OFFGEL and SAX Fractionation Workflows

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Abstract
Receptor extracellular domain fusion proteins represent a growing class of biopharmaceuticals. Since these fusion proteins are highly glycosylated they are ideal substrates for charge variant characterization. In today's biotechnology industry there is an increasing need to structurally characterize the charge heterogeneity of these variants by methods other than electrophoresis. In this study we have developed rigorous analytical workflows to structurally characterize an ECD-Fc fusion protein using both OFFGEL separation/OFFGEL fractionation and SAX chromatography to characterize the protein charge heterogeneity upstream. We found that the glycosylation in the ECD region is the major contributor to the overall charge heterogeneities. These glycosylated species have been fractionated and structurally characterized using OFFGEL fractionation and Strong Anion Exchange (SAX) chromatography followed by LC/MS.

Introduction
Fc fusion proteins, a growing class of biological therapeutics, are challenging to characterize because of their heterogeneity, especially for highly glycosylated ECD-Fc fusion proteins. Charge heterogeneity of highly glycosylated biological therapeutics is being maintained more frequently using strategies such as OFFGEL (heat mediated desialylation) and enrichment/depletion chromatography to fractionate the protein charge variants. OFFGEL fractionation is an isoelectric focusing technique that has been specifically designed by Illumagen Technologies so that proteins or peptides can be resolved in a very unique manner using LCMS characterization. In this study the OFFGEL fractionation is employed as the tool for the Fc based workflow where we have fractionated the ECD subunit and characterized the charge variants downstream by LCMS.

Methods
OFFGEL Fractionation
A chromatography column (FPLC Amersham Pharmacia Biotech) was used along with the pH 7-8 Bronsted Coated Ion Column from GE healthcare to fractionate Fc of protein per lane in a 3x2 well format. Follow the fractionation, adjacent wells were combined in 12 fractions.

Strong Anion Exchange Chromatography
A stainless steel analytical column (MonoQ 7/300 SuperQ) from GE Healthcare was used to fractionate 150 µg of the protein per lane in a 0.5x2 well format on a quaternary amine stationary phase. The sample was loaded on the column and eluted with a pH 9.5 (titrate with HCl), B: A at pH 3.7, Flow rate: 1.0 ml/min, Detection: UV 280 nm, 0.05%TFA and 0.01%methylopiperazine.

OFFGEL Fractionation Characterization

Figure 1. General structure of an ECD-Fc fusion protein. A construct/sequence of a therapeutic protein construct is shown which has an active extracellular domain to the Fc domain of a human IgG. A construct/sequence is currently developing for several glycoprotein sites. There are two fucosid glycosylation sites on each monomer of the ECD and one fucose per monomer on the Fc domain. There are also up to 6 N-linked glycosylation sites or each monomer in the ECD region.

Figure 2. Characterization Workflow
Characterization of the protein charge heterogeneity in this study is based on two fractionation workflows with LCMS analysis. One workflow uses the OFFGEL technology for the initial enrichment of charge variants in solution after the charged variants are fully loaded. The second is strong ion exchange (SAX) chromatography with offline fraction collection.

Figure 3. Charge distribution profile by IEF pH and EIE
Both techniques separate charge variants according to their pI values and are critical for monitoring both to variability. The capillary technique free advantages of nanoliter LCMS analysis of highly charged proteins such as peptides, Cys and His. The SAX chromatography is more difficult to adapt into further characterization workflows for structural identification.

Figure 4. pI profiles of OFFGEL fractions
There are small shifts (0.2-0.6 units) in each of the OFFGEL fractions showing a small resolution of the charge variants. The data also shows that the earlier fractions closest to the anode have lower pI values, as we would expect. These shifts are probably due to high sialylation. The fractions furthest from the anode (fractions 5 and 6) more closely resemble the deasialylated control.

Figure 5. Peptide map analysis of OFFGEL fractions
The two Fc sites of the uncharged variant are similar in glycan structure. The majority of the peptides are occupied with tertbutylammonium fully sialylated glycans. As the pI of the fraction increase, the complexity of the glycans decrease. The majority of fraction 7 is occupied with high monosaccharides at both sites.

Figure 6. SAX Chromatography of the ECD fusion protein
Strong Anion Exchange (SAX) is used here to separate charge variants using a pH based mobile phase on a quaternary amine stationary phase. We can see in the top trace that the early eluting species at about 20 minutes is consistent with the deasialylated peak.

Figure 7. Middle down LCMS analysis of the Fc subunit from OFFGEL fractions
The ECD subunit masses reveals that the lower pI fractions the glycan structure are more complex and have higher order branching structures with more sialic acid.

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Figure 9. Peptide map analysis of the Fc subunit from OFFGEL fractions
The lower pI fractions at both sites have glycosylations that have higher order branching and a greater amount of sialic acid. The majority of the higher pI fractions have glycans that are occupied with less complex branched and high monosaccharides structures.

Figure 10. Middle down LCMS analysis of the Fc domain from the SAX fractions
This data is also showing that SAX chromatography has little effect on the Fc glycan distribution and the overall structure of the Fc domain.

Figure 11. Middle down LCMS analysis of the O-linked glycosylation from SAX fractions
The lower pI fractions at both sites have glycosylations that have higher order branching and a greater amount of sialic acid. The majority of the higher pI fractions have glycans that are occupied with less complex branched and high monosaccharides structures.

Conclusions
1. Both of these fractionation techniques can be successfully incorporated into workflow platforms to structurally identify charge variants of highly complex glycoproteins.
2. Both workflows show that at the lower pH there is an increasing complexity into workflow platforms to structurally identify charge variants of highly complex glycoproteins.
3. The subunit analysis of the Fc shows that there is little effect in the ratios of the Fc glycan in the separate fractions.
4. The O-linked glycosylations of the ECD domain and the O-link glycosylations in the hinge region are the main contributors to the charge heterogeneity.

SAX Fraction Characterization

Figure 12. SAX Chromatography of the ECD domain and O-linked glycosylations from SAX fractions
The SAX chromatography has little effect on the Fc glycan distribution and the overall structure of the Fc domain.