Cutting to the chase: Enzymatic workflows for easy characterization of complex glycoprotein charge and size variants on Maurice

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INTRODUCTION

Keeping track and close control of glycosylation in therapeutic monoclonal antibodies and fusion proteins is crucial to ensure safety and efficacy of these important classes of biotherapeutics. The removal of glycans from biotherapeutics pose a challenge when investigating the changes in physicochemical and pharmacological properties. In this work we used the highly specific enzymes SialEXO®, to enable simplified workflows for glycoprofile analysis by desialylation, OglyZOR® for specific hydrolysis of O-glycans, and FabRICATOR® for digestion of antibodies or Fc-fusion proteins. The results demonstrate simplified workflows to quantitate charge and size heterogeneity associated with the removal of glycans and digestion of antibodies, fusion proteins using capillary iso-electric focusing (cIEF) and CE-SDS. The combination of specific enzymatic sample preparation with robust cIEF and CE-SDS has potential to speed up, increase through-put and simplify routine testing of critical quality attributes when developing or manufacturing biotherapeutics.

METHODS

The workflow to characterize complex glycoproteins is illustrated in Figure 1. Samples are treated with various enzymes from Genovis and then analyzed using CE-SDS and icIEF on Maurice.

Enzymatic Digestions

Biotherapeutics (5mg/ml in PBS) were treated with the indicated enzymes at room temperature for 15min (FabRICATOR) and 3h (FabRICATOR, SialEXO, OglyZOR, PNGaseF) resp. All enzymes except for PNGase F were immobilized on agarose beads for easy removal after the reaction.

CE-SDS PLUS method - Maurice CE-SDS PLUS Application Kit (PS-MAK03-S). For the CE-SDS analysis (Figure 5-7), the samples were prepared following the standard Maurice protocol of reduced samples. All the samples were diluted with CE-SDS PLUS Sample Buffer to a concentration of 0.2mg/ml, denatured with β-mercaptoethanol at 70 °C for 10 minutes, cooled on ice for 5 minutes, then vortexed briefly. On Maurice, samples were injected for 20 seconds at 4600 V and separated for 35 minutes at 5750 V.

icIEF methods - Maurice cIEF Method Development Kit (PS-MDK01-C)

USP mAb reference standard

For the USP reference standard (Figure 2), the samples were analyzed following USP 129 icIEF protocol.

Trastuzumab

Trastuzumab samples were diluted into a final mixture of 4% Pharmalytes (3% 8–10.5, 1% 5–8), 4 M urea, 5 mM IDA, 10 mM arginine, and pI markers 5.85, 10.17 prior to pre-focus at 1500 V for 1 minute and then at 3000 V for 10 minutes (Figure 3).

Etanercept

All the controls and treated samples were mixed with an ampholyte mix containing 1% Servalyte (0.5% 2-9, 0.5% 4-7) 6.4 M Urea, 10 mM IDA, 10 mM arginine, 8% SimpleSol, 0.1% Poloxamer 188 and pI markers 4.05 and 9.99. Samples were pre-focused at 1500 V for 1 minute and then at 3000 V for 10 minutes.

icIEF RESULTS

Charge heterogeneity analysis

USP129 reference standard

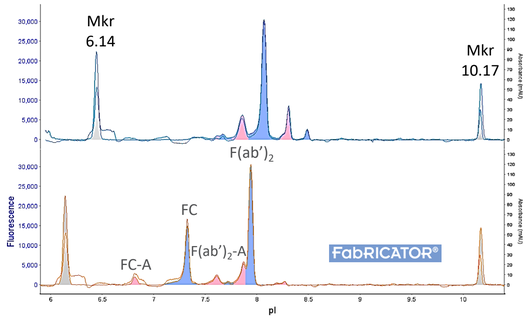

Figure 2. USP129 reference standard, monoclonal IgG, was treated with FabRICATOR, a cysteine protease that targets a specific sequence of IgG below the hinge region producing F(ab')2 and Fc fragments. This treatment allows for localization of the charge variants to the different domain of the antibody.

Trastuzumab

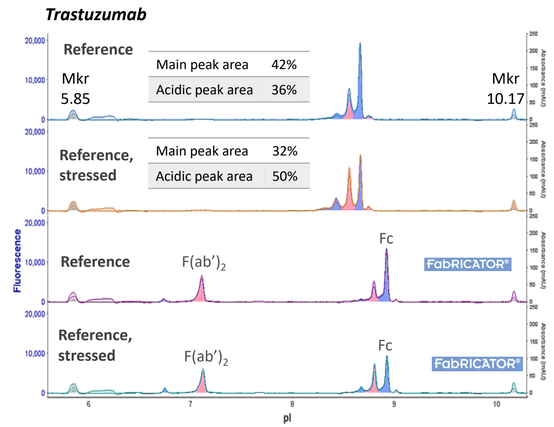

Figure 3. Trastuzumab was analyzed with Maurice icIEF for charge heterogeneity. In the stressed sample (7d at 37°C), there is an increase in the acidic peak area compared to the control. Digesting with FabRICATOR leads to the generation of F(ab')2 and Fc fragments and allows to pinpoint the increase in acidic variants to the Fc region while the Fab remains largely unchanged.

Etanercept

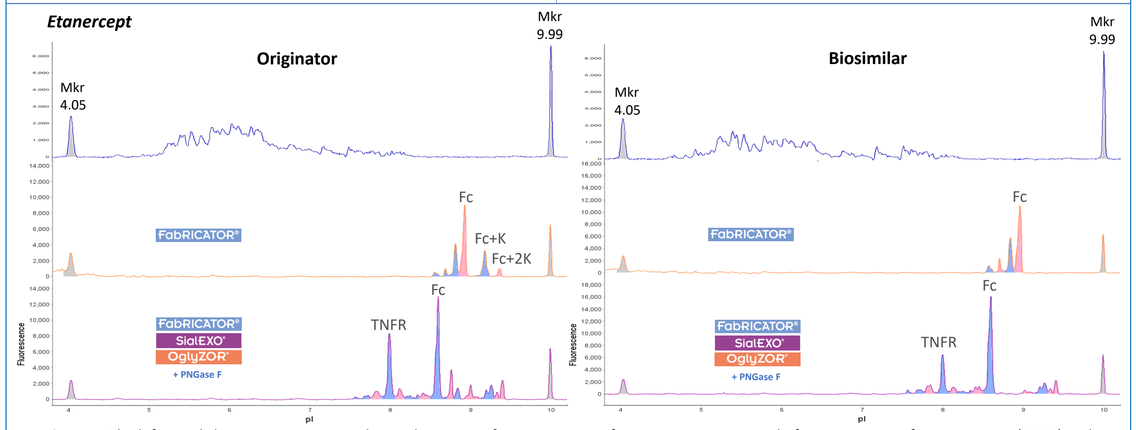

Figure 4. The left panel shows representative electropherograms for etanercept, a fusion protein composed of tumor necrosis factor receptor (TNFR) and Fc part of human IgG1, and the right panel represents a biosimilar. Due to its complex glycosylation, the molecule is too heterogeneous for the charge variants to be resolved on the intact molecule (top panel). Treating the fusion protein with FabRICATOR separated the Fc domain from the heavily glycosylated TNFR domain. The charge variants of the Fc fragments are nicely resolved and can be compared between the originator and the biosimilar etanercept (middle panels). Due to the varying degree of sialylation of its *N*- and *O*- glycans, the charge variants of the TNFR domain are still not resolvable. By treating the samples with a mixture of enzymes for the complete removal of all *N*- and *O*- glycans, this heterogeneity is removed and the underlying charge variants of the protein itself can be analyzed (bottom panel).

CE-SDS RESULTS

Size heterogeneity analysis

USP129 reference standard

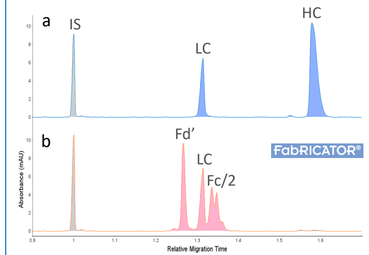

Figure 5. USP129 mAb was analyzed after reduction (a) or treated with FabRICATOR and then reduced (b). This subsequent reduction results in the separation of the F(ab')2 fragment into smaller LC and Fd' subunits.

Trastuzumab

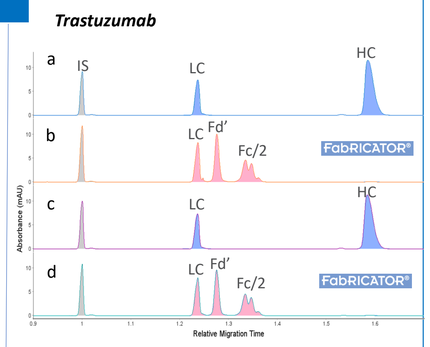

Figure 6. Relative migration time of Trastuzumab fragments after reduction (a) or treatment with FabRICATOR followed by reduction (b). A stressed sample of trastuzumab (7d at 37°C) was analyzed in the same way (c and d).

Etanercept

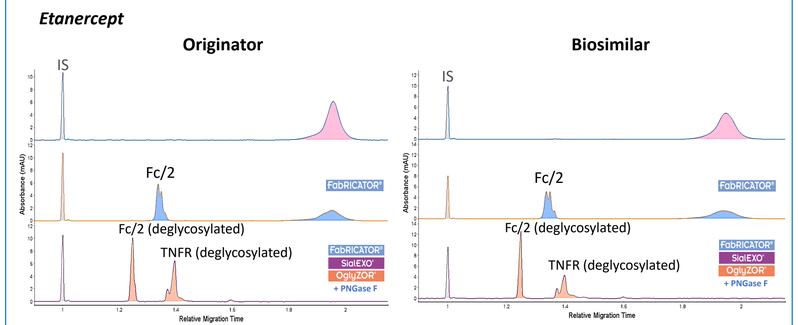

Figure 7. Representative CE-SDS electropherograms for etanercept originator and biosimilar. In both cases, the control shows only one broad peak at RMT = 1.95. When treated with FabRICATOR (middle panels) or a combination of enzymes for complete deglycosylation (bottom panels) the individual subunits (Fc/2 and TNFR) are resolved and can be compared between the two etanercept samples.

CONCLUSIONS

The combined workflow described herein highlights:

- Maurice as a dual-mode analytical tool, capable of icIEF and CE-SDS analysis of complex proteins.
- Benefits of using enzymatic sample preparation with SmartEnzymes™ to simplify workflows, increase throughput and enable deeper characterization of biotherapeutics.

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