A Middle-Level Approach to Simplify Sample Preparation and Data Analysis for Characterization of the Fusion Protein Blinatumomab

Protein Metrics

Antony Harvey[1], Lucy Fernandes[1], Andreas Nägeli[2], Magdalena Widgren Sandberg[2]
[1]Protein Metrics, Boston, MA; [2]Genovis, Kävlinge, Sweden.

Summary

Aim: Middle-level analysis of a fusion protein using targeted linker digestion and automated data analysis tools

Next-generation biopharmaceutical modalities often contain domains with multiple GS linkers to resist proteolysis

The GlySERIAS protease was used to digest the glycine rich linkers of the fusion protein Blinatumomab and the resulting fragments were analyzed by LC-MSMS

The intact workflow in Byos® was used to automatically deconvolute and identify fragment masses. Middle-level sequencing was utilized to confirm the site of cleavage

Introduction and Strategy

Fusion proteins combine multiple protein domains to address specific therapeutic challenges.

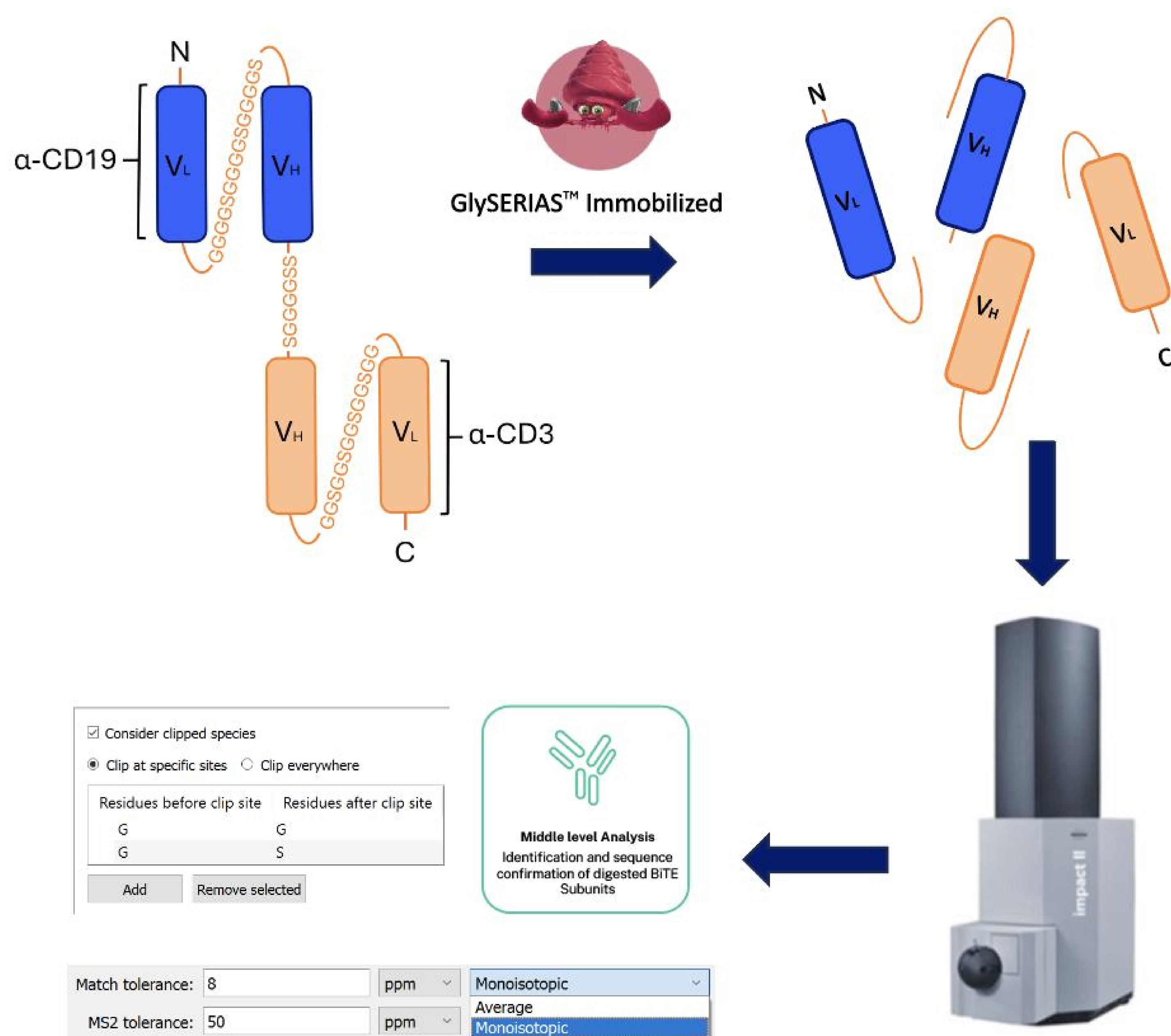
Often these domains are joined using linkers containing multiple glycine residues or glycine (G) interspersed with serine (GS).

These linkers are designed to resist protease degradation, which makes them challenging to analyze by traditional LC-MS approaches.

Middle-level Strategy:

GlySERIAS was used to hydrolyze flexible linkers in the fusion protein Blinatumomab. This strategy was implemented using an immobilized form of the enzyme for more complete linker digestion.

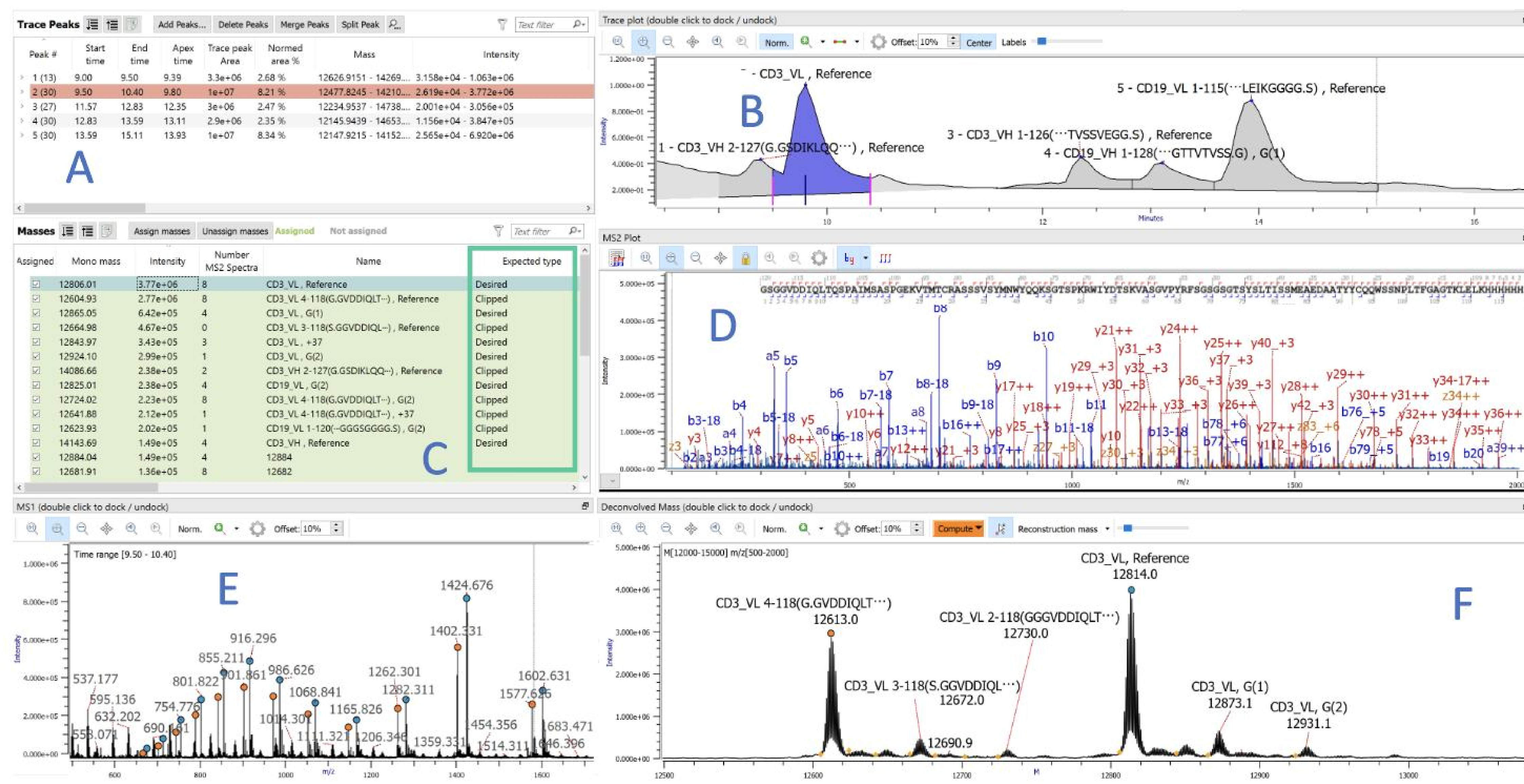
Samples were analyzed using a Bruker Impact II QTOF for LC-MSMS analysis. Raw data files were processed in Protein Metrics Byos (Vendor neutral software), using the intact workflow which was adapted for middle-level/subunit analysis.



Identification and Sequence Confirmation of Digested Fragments

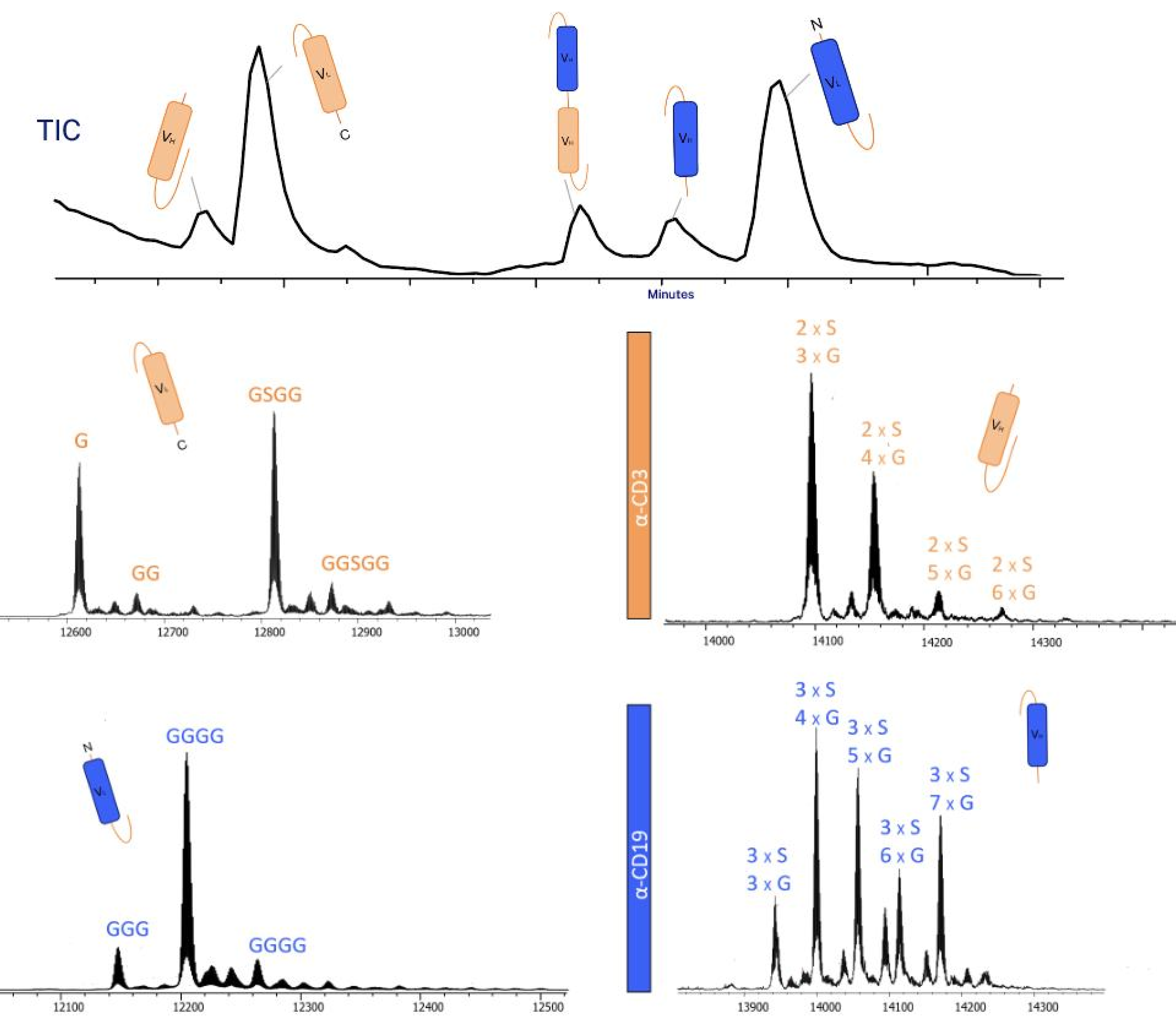
Figure 1. Byos Intact workflow (inspection view). This workflow enabled the efficient identification and validation of the major proteolytic fragments separated by LC-MS. Deconvoluted masses were validated by direct comparison with the raw data (E and F).

Clipped versions of VL and VH subunits were also identified using the built in clip function. Cleavage sites were confirmed through annotation of MS2 spectra (D).



a. Trace Peaks table – Details of the four integrated TIC peaks.
b. Trace plot – TIC showing manually editable peak boundaries.
c. Masses table – Deconvoluted masses found in peak 1. Masses identified based on theoretical monoisotopic masses and clipped species.
d. MS2 Plot – Annotated MS2 spectrum of subunit CD3_VL, confirming sequence and location of cleavage.
e. MS1 spectrum – Colored dots in raw spectrum link multiply charged masses to the deconvoluted masses in F.
f. Deconvoluted Mass spectrum – Isotopic resolution preserved on deconvolution. Monoisotopic masses labeled with yellow diamonds.

Identification Summary



Heterogeneous mixtures of subunits resulting from GlySERIAS digestion were primarily identified through **monoisotopic mass assignments** of deconvoluted MS1 data. This process was facilitated by utilizing the **clip function** in the Byos intact workflow, which automatically detected cleavages between GG, GS and SG residues.

The sequencing capability in this workflow, operating at middle-level, enabled the confirmation of sequences from all major subunits by annotating MS2 data. This, in turn, provided a more confident assignment of the cleavage sites.

By employing this middle-level strategy with GlySERIAS, protein fragments became more amenable to analysis by MS2 compared to full top-down approach using the intact protein.

Conflict of interest statement

Some of the authors are employees and/or shareholders of Genovis which has commercialized the enzyme described here.