Novel Enzymatic Tools for Middle-level Analysis of Therapeutic mAbs with Hinge Mutations and Other New Modalities

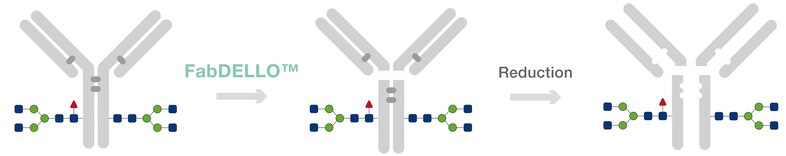
John Lindsay[1], Magdalena Widgren Sandberg[2], Andreas Nägeli[2], Andrea Persson[2] and Helén Nyhlén[2] | [1] Genovis Inc., Cambridge, MA, USA, [2] Genovis AB, Lund, Sweden.

ABSTRACT

The development and manufacturing of therapeutic mAbs require analysis of many different critical quality attributes (CQAs). Recent advances in mass spectrometry have led to sufficiently reliable and easy-to-use instruments to monitor multiple attributes in one single analysis. Middle-level analysis of mAb subunits has become a popular strategy for implementation of such multi-attribute monitoring (MAM) assays since it requires only minimal sample preparation and offers shorter analysis times and less complex data analysis than alternative peptide mapping-based strategies. Middle-level analysis entails antibody digestion at one specific site below the hinge by the FabRICATOR® protease resulting in homogeneous subunits ideally suited for acquisition of high-quality mass spectra.

Mutated hinge regions are common modifications of therapeutic antibody candidates to reduce effector functions. Hinge region mutations have a negative effect on the activity of the FabRICATOR protease because of the high specificity of the enzyme and middle-level LC-MS analysis of these antibodies has therefore not been feasible.

Here we present FabDELLO™, a novel enzymatic tool for digestion of human IgG1 (hIgG1). The protease digests specifically at an exposed lysine residue above the hinge and provides complete IgG digestion in 2 hours at 37°C under native reaction conditions. The enzyme is not inhibited by common hinge mutations and therefore allows for middle-level MAM assays of engineered mAbs. As a specific case, FabDELLO was used to study chain mispairing of a research grade bispecific IgG1.



SUMMARY

- Aim: Middle-level LC-MS analysis of hinge-mutated antibodies and bispecifics
- FabDELLO digests specifically at the exposed lysine residues above the hinge of hIgG1
- The enzyme digests hIgG1 under native, non-reducing conditions at 37°C in 2 hours
- FabDELLO digestion enables attribute monitoring of hinge-mutated hIgG1 by middle-level analysis
- Digestion with FabDELLO without subsequent disulfide bond reduction enabled the study of Fc-glycan pairing of hinge-mutated hIgG1
- Chain pairing of a bispecific IgG in the development phase were studied with improved resolution using FabDELLO
- Fragments from bispecific antibodies generated by FabDELLO can be analyzed at the subunit level for PTMs

RESULTS

Middle-level Analysis Improves MS Spectra Quality

Analysis of antibodies by LC-MS at the intact level provides a fast and simple way to get an overview of product quality. However, although mass spectrometry (MS) instrumentation has improved significantly in recent years, the entire 150 kDa molecule with all its heterogeneity remains a challenging analyte, limiting the amount of detail obtainable by such analyses (*Fig. 1*). Furthermore, as all modifications are measured on the same molecule, the potential for overlapping peaks and ambiguous annotations is high. It is therefore a large risk that some CQAs will be missed during intact MS analysis of mAbs.

Middle-level analysis offers a rapid way to improve the quality of the mass spectra. By digesting the mAb with the FabRICATOR protease at one single site below the hinge followed by disulfide bond reduction, homogenous antibody subunits sizing 23-25 kDa are generated (*Fig. 2*, top). These subunits are small enough to enable the acquisition of high-quality mass spectra on most common MS instrumentation. This allows for the monitoring of many different product quality attributes simultaneously (*Fig. 2*, bottom) and significantly increases the level of analytical detail compared to intact analysis while also providing domain specific information about the mAb.

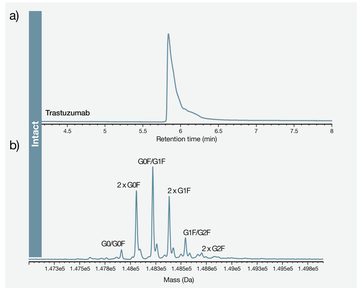


Figure 1. Intact antibody analysis. a) UV chromatogram (280 nm). **b)** Deconvoluted mass spectra of intact trastuzumab, analyzed by reversed-phase on a Waters™ BioAccord™ LC-MS system equipped with a Waters™ BioResolve™ RP mAb column (2.1 x 50 mm).

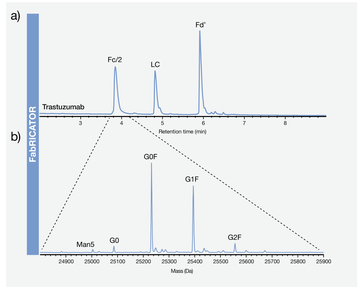


Figure 2. Middle-level analysis using FabRICATOR. a) UV chromatogram (280 nm) from trastuzumab digested with FabRICATOR followed by disulfide bond reduction. **b)** Deconvoluted mass spectrum of the Fc/2 fragment of trastuzumab. The LC-MS analysis was performed as described in *Fig. 1*.

Middle-level analysis of hinge-mutated mAbs

Depending on a mAb's mode-of-action, its potential to elicit immune effector functions is either desired or problematic and drug manufacturers are looking at ways to engineer mAbs to silence or boost specific immune effector functions. One way of achieving this is to introduce specific mutations in the primary sequence such as L234A and L235A – the so-called LALA mutation – which diminishes a mAb's effector functions and has become popular. We tested a middle-level approach using FabRICATOR on risankizumab, a commercially available IgG1 mAb with a LALA mutation, but due to the high specificity towards IgG, FabRICATOR is inefficient in digesting such molecules (*Fig. 3*).

The protease FabDELLO acts specifically at one exposed lysine residue above the hinge of human IgG1 (*Fig. 4a*). This enzyme tolerates hinge mutations and thereby enables middle-level analysis of such antibodies. FabDELLO yields either intact, disulfide-bonded Fab and Fc fragments (50 kDa in size) or, after reduction of interchain disulfides, 23-25 kDa subunits very similar to the ones generated from wild-type mAbs using FabRICATOR. Digesting risankizumab with FabDELLO followed by reduction resulted in homogenous Fc/2, LC and Fd fragments (*Fig. 4a*). By deconvolution of the subunit spectra, the different Fc glycoforms could be studied with high resolution as well as the pyroglutamination of the Fd fragment and glycation of both the LC and Fd fragments (*Fig. 4b-d*). Having a digestion site above the hinge also allowed for middle-level analysis without reduction of disulfides, leaving Fab and Fc fragments intact. This could be used for the characterization of Fc glycan pairing in mAbs or analysis of the intact monovalent Fabs (*Fig. 5*).

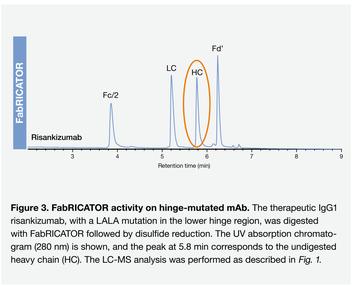


Figure 3. FabRICATOR activity on hinge-mutated mAb. The therapeutic IgG1 risankizumab, with a LALA mutation in the lower hinge region, was digested with FabRICATOR followed by disulfide reduction. The UV absorption chromatogram (280 nm) is shown, and the peak at 5.8 min corresponds to the undigested heavy chain (HC). The LC-MS analysis was performed as described in *Fig. 1*.

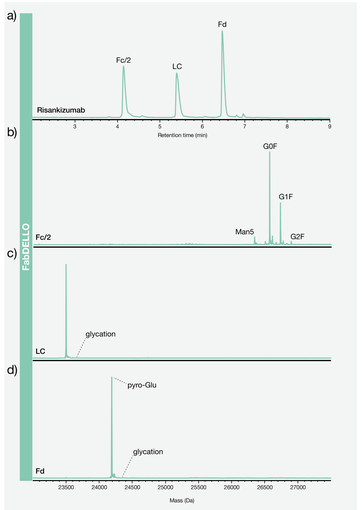


Figure 4. FabDELLO activity on hinge-mutated mAb. Risankizumab was digested with FabDELLO for 2 h in TBS, pH 7.6, and 37°C in the presence of calcium ions followed by disulfide reduction and LC-MS analysis as described in *Fig. 1*. **a)** UV chromatogram (280 nm). **b)** Deconvoluted mass spectrum of the Fc/2 fragment. **c)** Deconvoluted mass spectrum of the light chain. **d)** Deconvoluted mass spectrum of the Fd fragment.

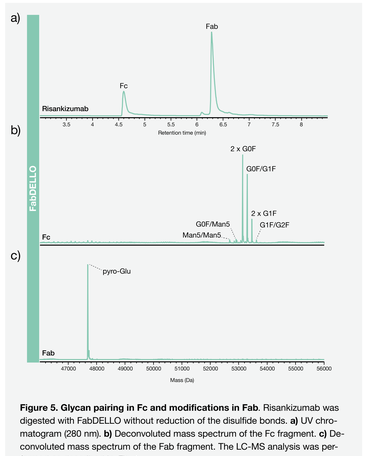


Figure 5. Glycan pairing in Fc and modifications in Fab. Risankizumab was digested with FabDELLO without reduction of the disulfide bonds. **a)** UV chromatogram (280 nm). **b)** Deconvoluted mass spectrum of the Fc fragment. **c)** Deconvoluted mass spectrum of the Fab fragment. The LC-MS analysis was performed as described in *Fig. 1*.

Characterization of Bispecific Antibody Chain Pairing Variants using FabDELLO

Bispecific antibodies (bsAbs) offer an opportunity to extend the therapeutic potential of antibodies but also pose new analytical challenges in their characterization. Production of bispecific IgGs by co-expression of the two different heavy chains and light chains in a single host cell line provides an efficient expression workflow. However, in addition to the desired bispecific IgG, mispaired variants of the antibody may be produced where one or both light chains associate with the wrong heavy chain. This leads to product-related impurities that need to be characterized and monitored (*Fig. 6*).

Figure 6. Schematic representation of the CD3/CD20 bispecific antibody and its mispaired variants. After digestion with FabDELLO, the individual Fabs can be analyzed with increased confindence to study chain mispairing in more detail.

We analyzed a purified research grade recombinant bispecific human IgG1 antibody with anti-CD20-anti-CD3 specificity, by LC-MS at the intact level. This revealed a cluster of different variants of the IgG (*Fig. 7*) including half-antibodies with only one heavy chain and one light chain as well as light chain mispaired variants. However, analysis at the intact level is prone to ambiguous annotations as variants with both light chains switched yield the same mass as the correctly assembled intact antibody.

By digesting the bsAb with FabDELLO, intact, disulfide-bonded Fabs are generated that allow for analysis of chain mispairing with a higher level of detail (*Fig. 6, 8*). As all the different chain mismatched variants yield unique Fab masses at the subunit level, there is no risk for misannotations. Besides the expected Fd + LC Fab combinations, the analysis revealed a high degree of CD3 Fd + CD20 LC and a lower degree of CD20 Fd + CD3 LC mismatched variants. While this was in line with the data from the intact analysis, the latter mismatch could only be detected at the subunit level, illustrating the power of the middle-level approach for such analyses.

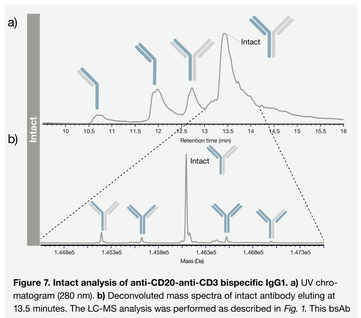


Figure 7. Intact analysis of anti-CD20-anti-CD3 bispecific IgG1. a) UV chromatogram (280 nm). **b)** Deconvoluted mass spectra of intact antibody eluting at 13.5 minutes. The LC-MS analysis was performed as described in *Fig. 1*. This bsAb does not contain any Fc glycosylation.

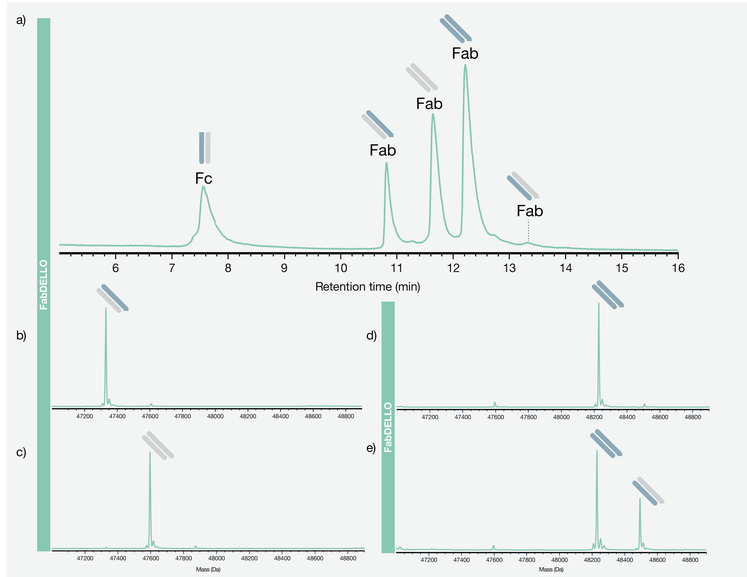


Figure 8. FabDELLO digested anti-CD20-anti-CD3 bispecific IgG. a) UV chromatogram (280 nm). Deconvoluted mass spectrum of the Fab fragments consisting of **b)** [CD3 Fd + CD20 LC], **c)** [CD20 Fd + CD20 LC], **d)** [CD3 Fd + CD3 LC] and **e)** [CD20 Fd + CD3 LC]. LC-MS analysis was performed as described in *Fig. 1*.

Subunit Analysis of a Bispecific mAb

Apart from the chain pairing variants, other CQAs of a bispecific mAb also need to be analyzed. By a simple reduction step, the intact Fab and Fc fragments yielded by FabDELLO digestion were separated into Fc/2, light chain and Fd subunits that could all be separated by reversed-phase LC and analyzed by mass spectrometry in a single run for a more complete picture of product quality of the CD3/CD20 bispecifc antibody (*Fig. 9*).

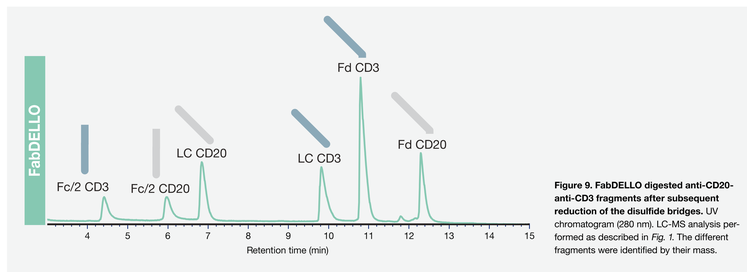


Figure 9. FabDELLO digested anti-CD20-anti-CD3 fragments after subsequent reduction of the disulfide bridges. UV chromatogram (280 nm). LC-MS analysis performed as described in *Fig. 1*. The different fragments were identified by their mass.

Contact email: john.lindsay@genovis.com