Improved strategies for enhanced protein characterization of highly glycosylated molecules

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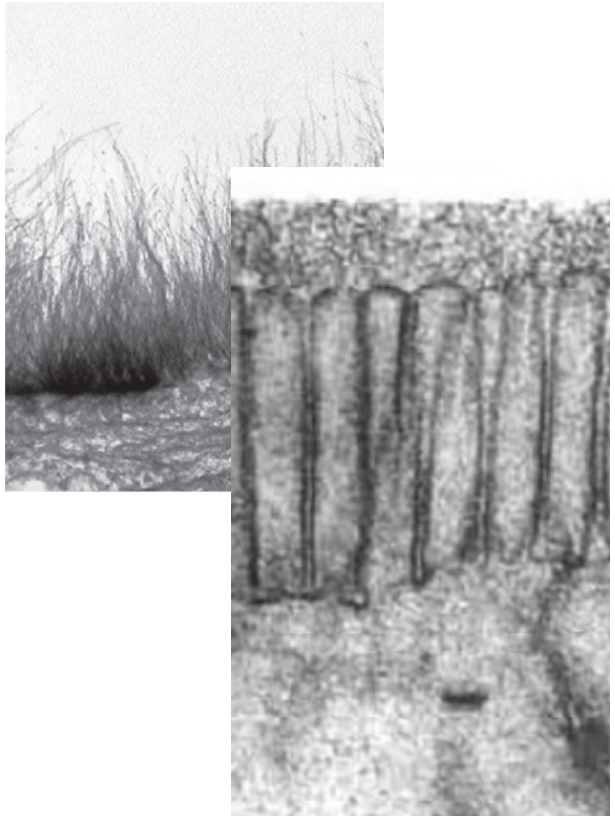

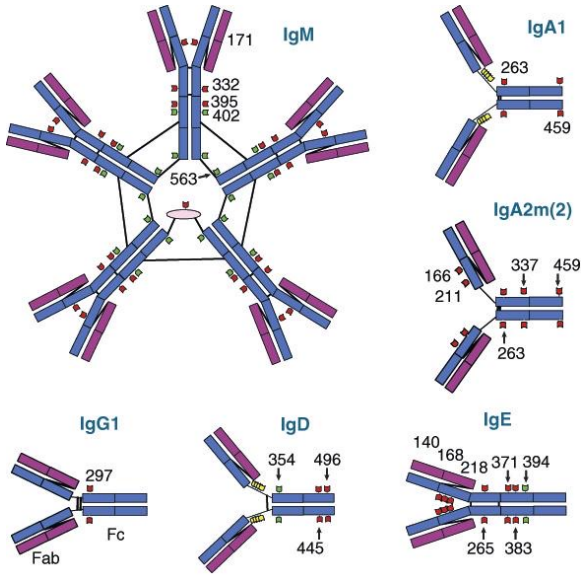

Arnold JN, et al. 2007.
Annu. Rev. Immunol. 25:21–50

Biochimica et Biophysica Acta (BBA) - General Subjects
Volume 1473, Issue 1, 17 December 1999, Pages 4-8

On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database [1]

Rolf Apweiler [a], Henning Hermjakob [a], Nathan Sharon [b]

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https://doi.org/10.1016/S0304-4165(99)00165-8

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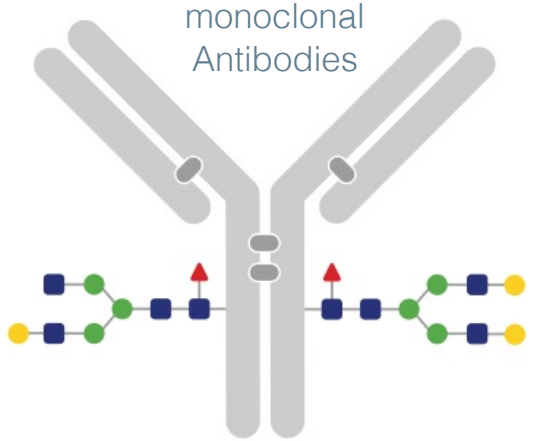
Abstract

The SWISS-PROT protein sequence data bank contains at present nearly 75 000 entries, almost two thirds of which include the potential *N*-glycosylation consensus sequence, or sequon, NXS/T (where X can be any amino acid but proline) and thus may be glycoproteins. The number of proteins filed as glycoproteins is however considerably smaller, 7942, of which 749 have been characterized with respect to the total number of their carbohydrate units and sites of attachment of the latter to the protein, as well as the nature of the carbohydrate-peptide linking group. Of these well characterized glycoproteins, about 90% carry either *N*-linked carbohydrate units alone or both *N*- and *O*-linked ones, attached at 1297 *N*-glycosylation sites (1.9 per glycoprotein molecule) and the rest are *O*-glycosylated only. Since the total number of sequons in the well characterized glycoproteins is 1968, their rate of occupancy is 2/3. Assuming that the same number of *N*-linked units and rate of sequon occupancy occur in all sequon containing proteins and that the proportion of solely *O*-glycosylated proteins (ca. 10%) will also be the same as among the well characterized ones, we conclude that the majority of sequon containing proteins will be found to be glycosylated and that more than half of all proteins are glycoproteins.

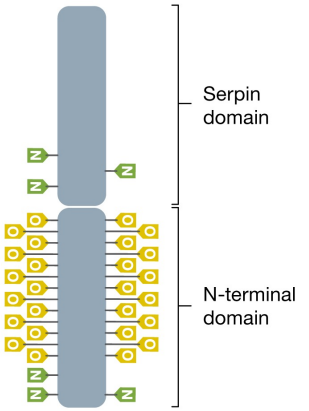
Challenges Associated with Glycans

- Glycans are complex and heterogeneous modifications
- Existence of many isobaric structures
  - Composition: Glucose vs galactose vs mannose → all 162 Da
  - Configuration: α vs β linkage
  - Connectivity: Position of the glycosidic bond (β1-3 vs β1-4 linkage)
- Presence of glycans present challenges in the analysis of other protein modifications
  - Glycan heterogeneity can ‘mask’ other PTMs
  - Removal of glycans can improve characterization of the protein

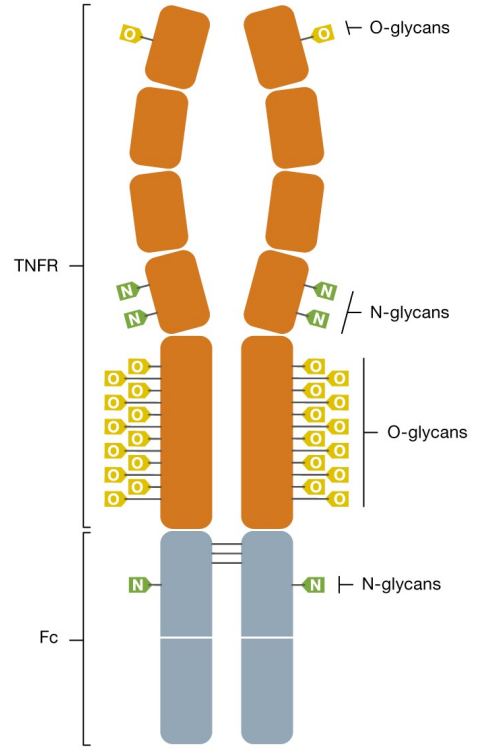
monoclonal Antibodies



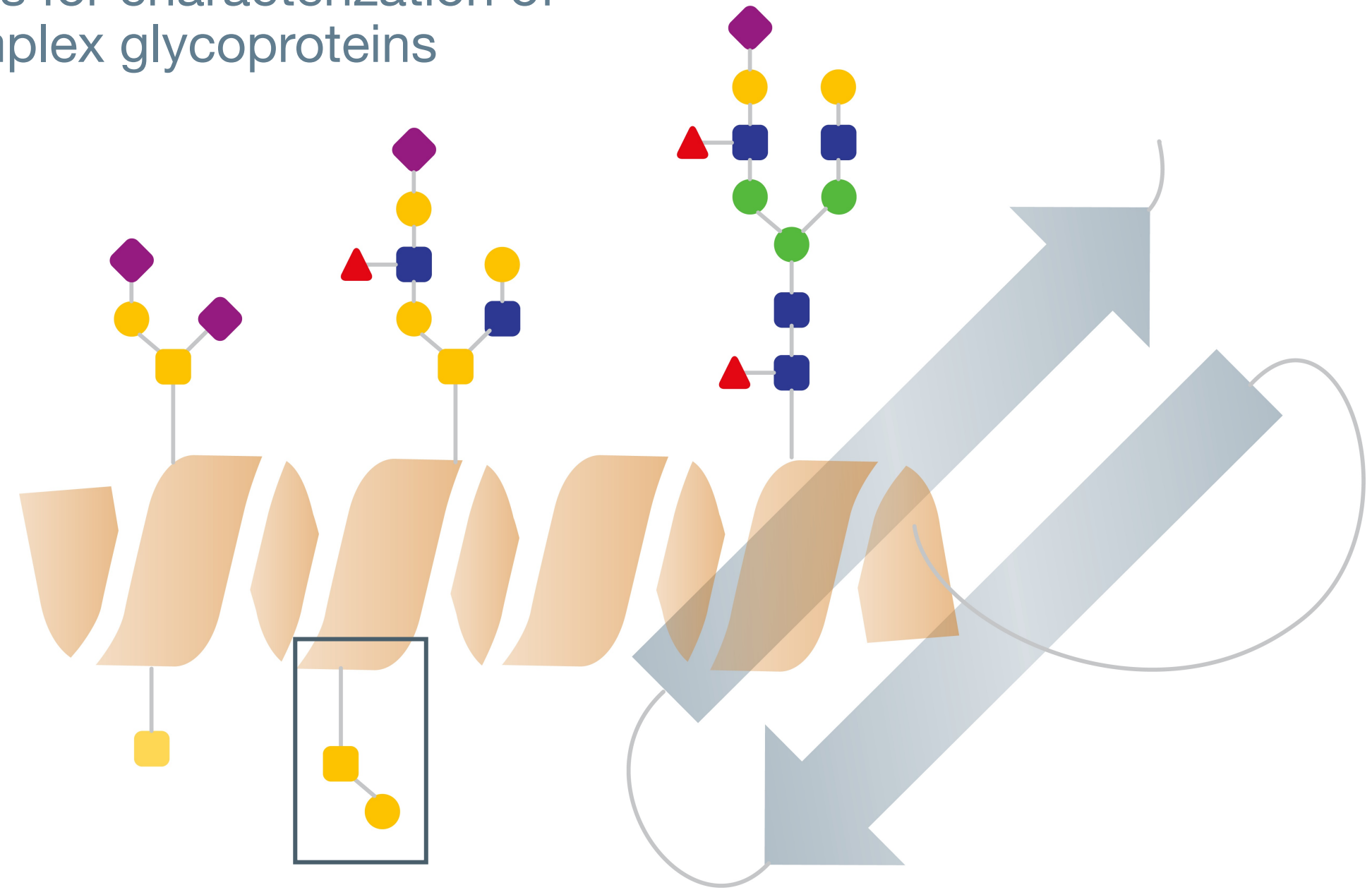
Human C1 inhibitor



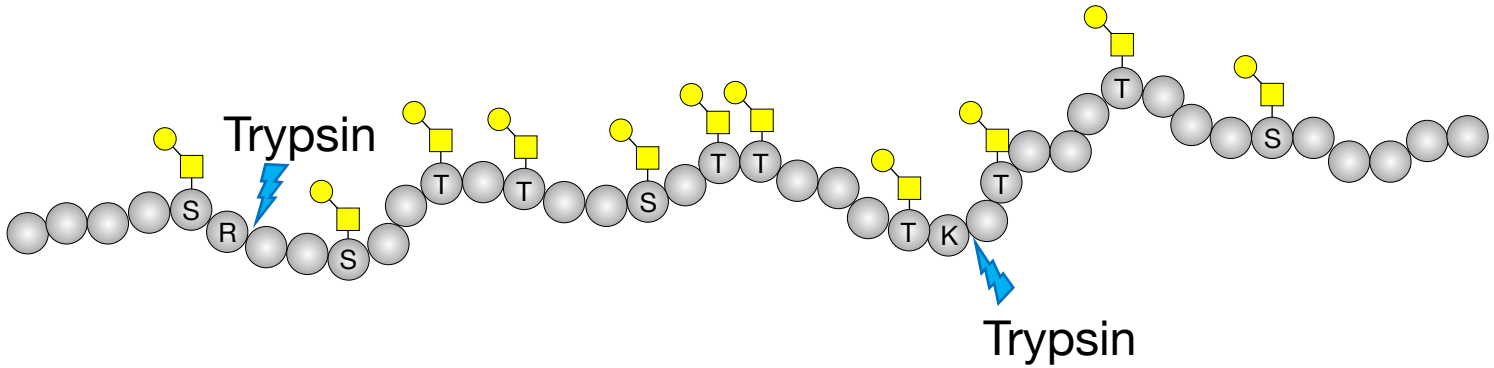
Etanercept (Enbrel®)



Tools for characterization of complex glycoproteins



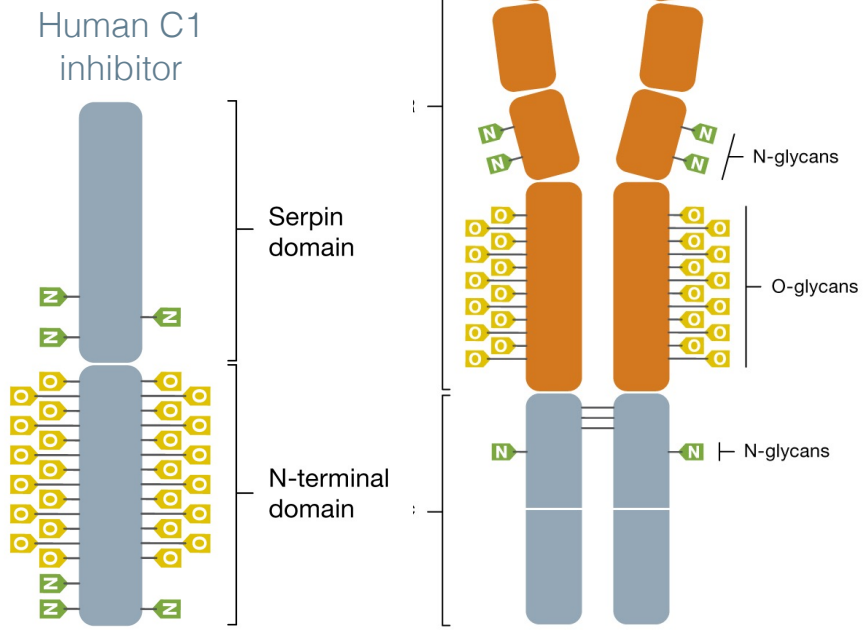
Difficulties with *O*-glycosylation analysis



- Standard digestion (trypsin, Lys-C) difficult for heavy *O*-glycosylation
- *O*-glycans are complex and variable
- Analytical toolbox is limited:

  Few *O*-glycan specific enzymes

  Chemical methods are laborious



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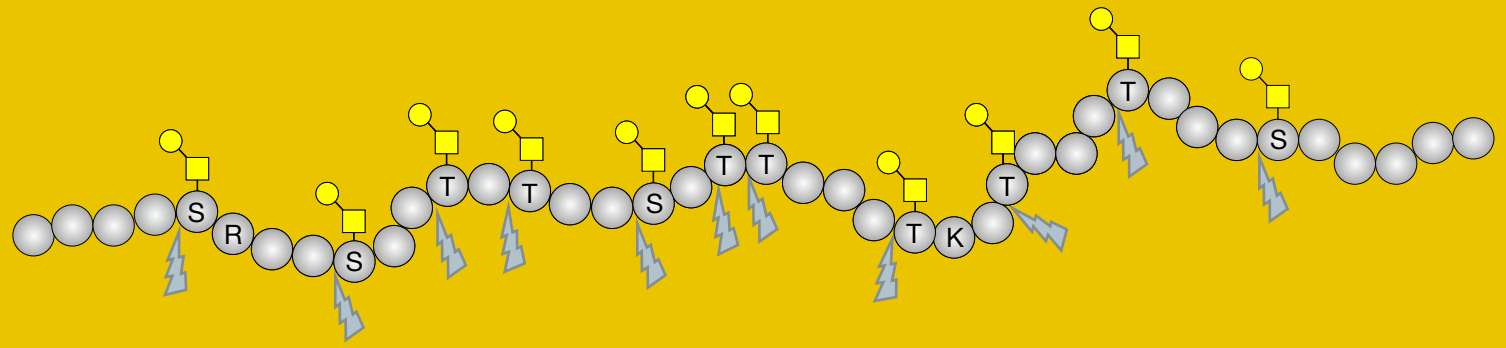
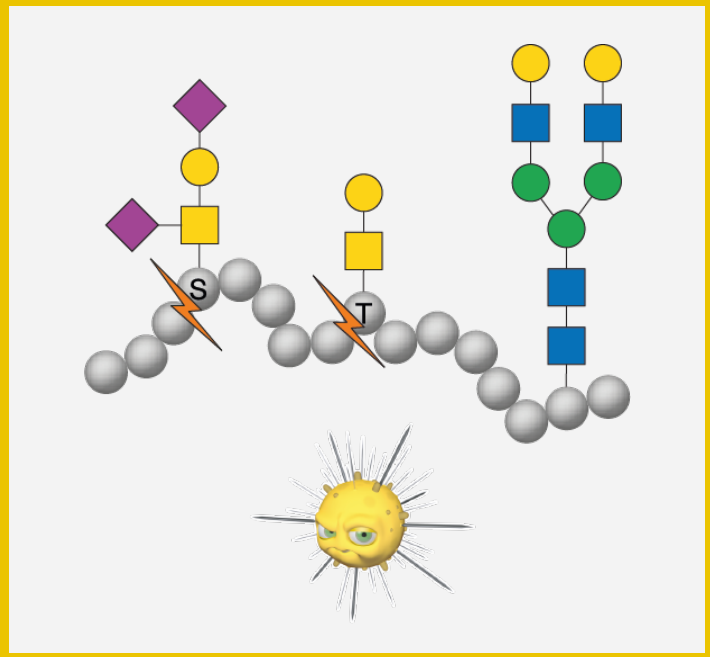
O-glycan-specific protein digestion



- *O*-glycoprotein specific protease
- Hydrolyzes glycoproteins N-terminally to the *O*-glycosylated serine or threonine residues
- Significantly reduced activity on sialylated *O*-glycosylation sites



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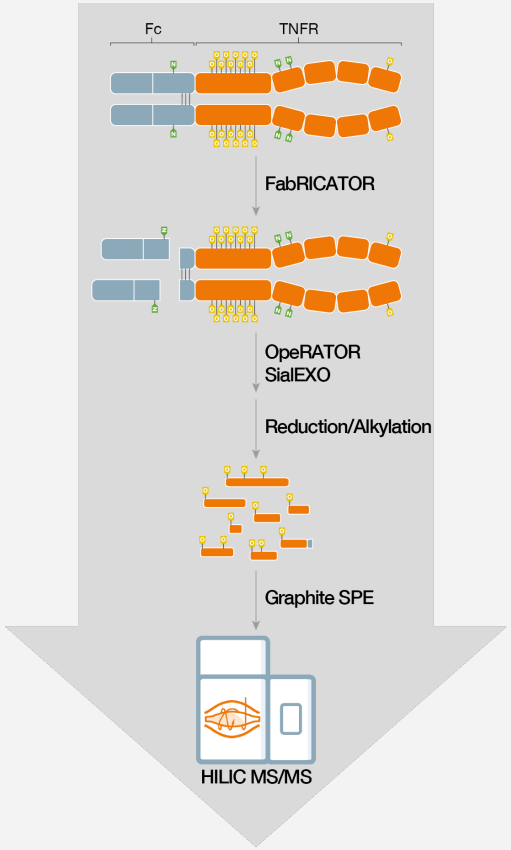
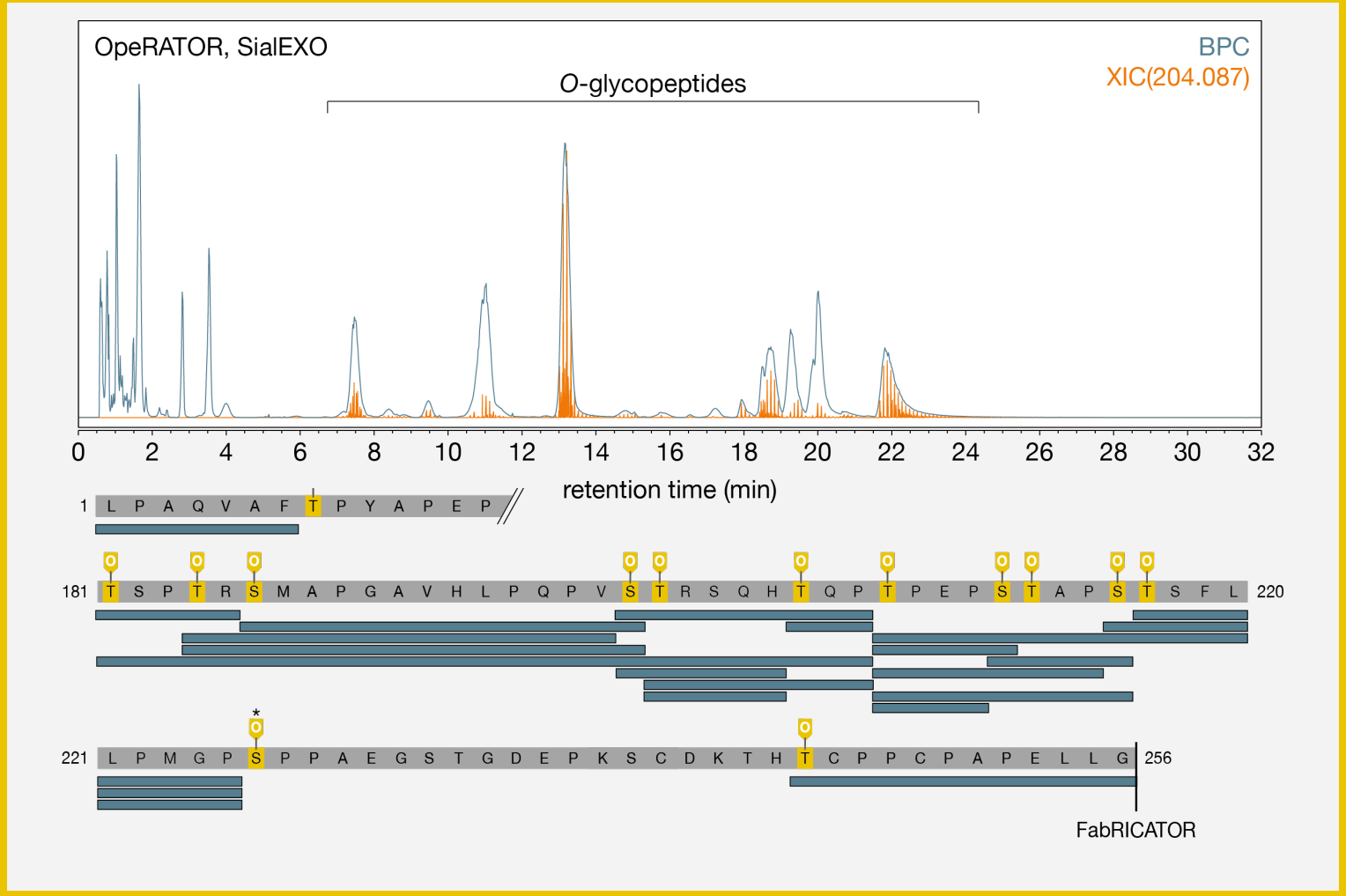
Mapping *O*-glycosylation sites in etanercept





- All *O*-glycosylation sites of etanercept could be identified
- No enrichment needed
- Site-specific mapping without ETD fragmentation

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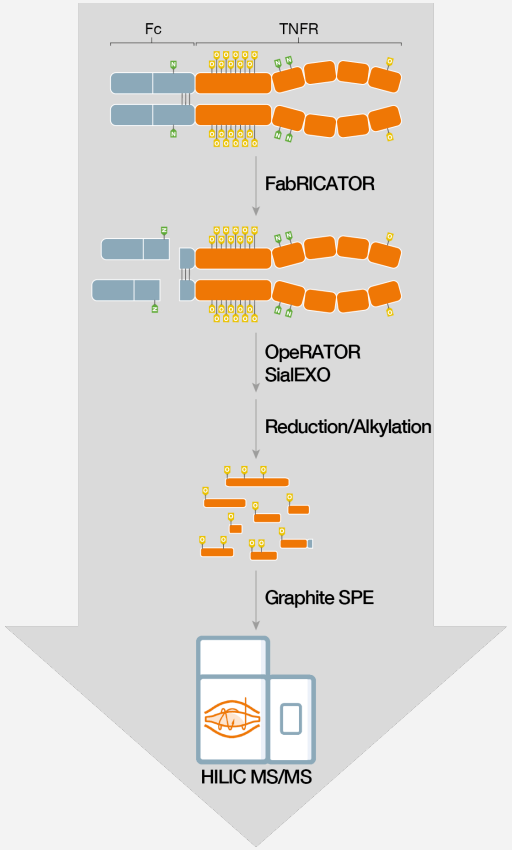
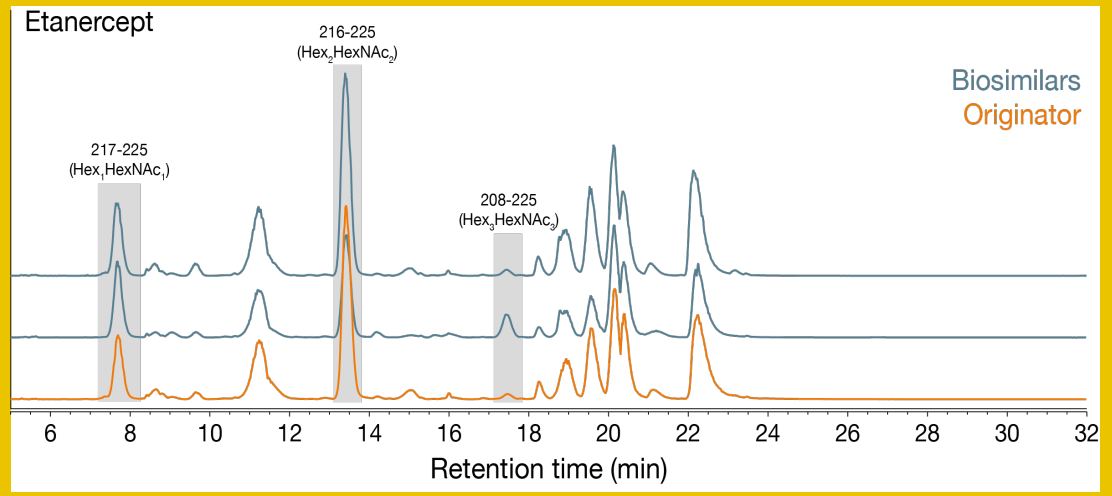
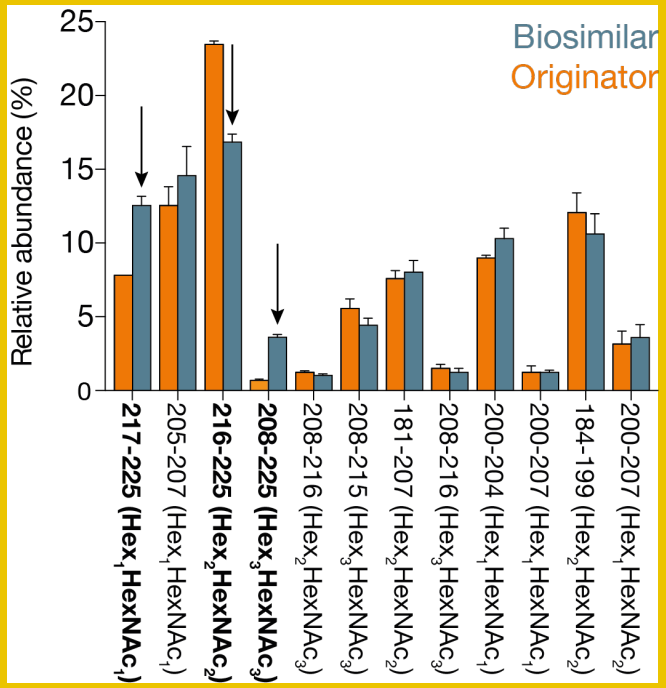


Quantitative comparison of *O*-glycosylation patterns in etanercept

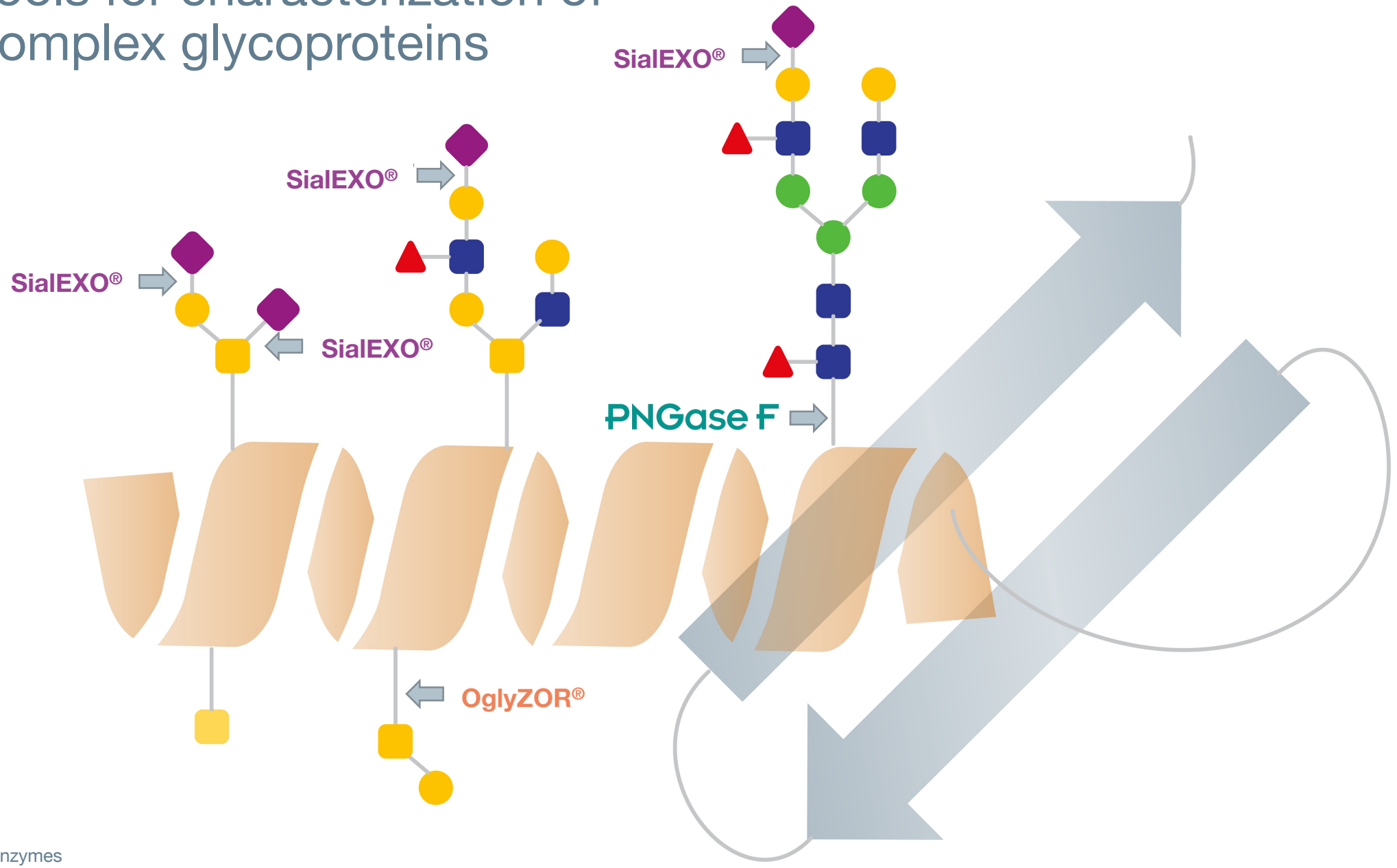
Comparison between originator and biosimilars

- All three peptides stem from the same region and suggest underglycosylation of one residue (216) in the biosimilar
- Quantitative differences mapped site-specifically





Tools for characterization of complex glycoproteins



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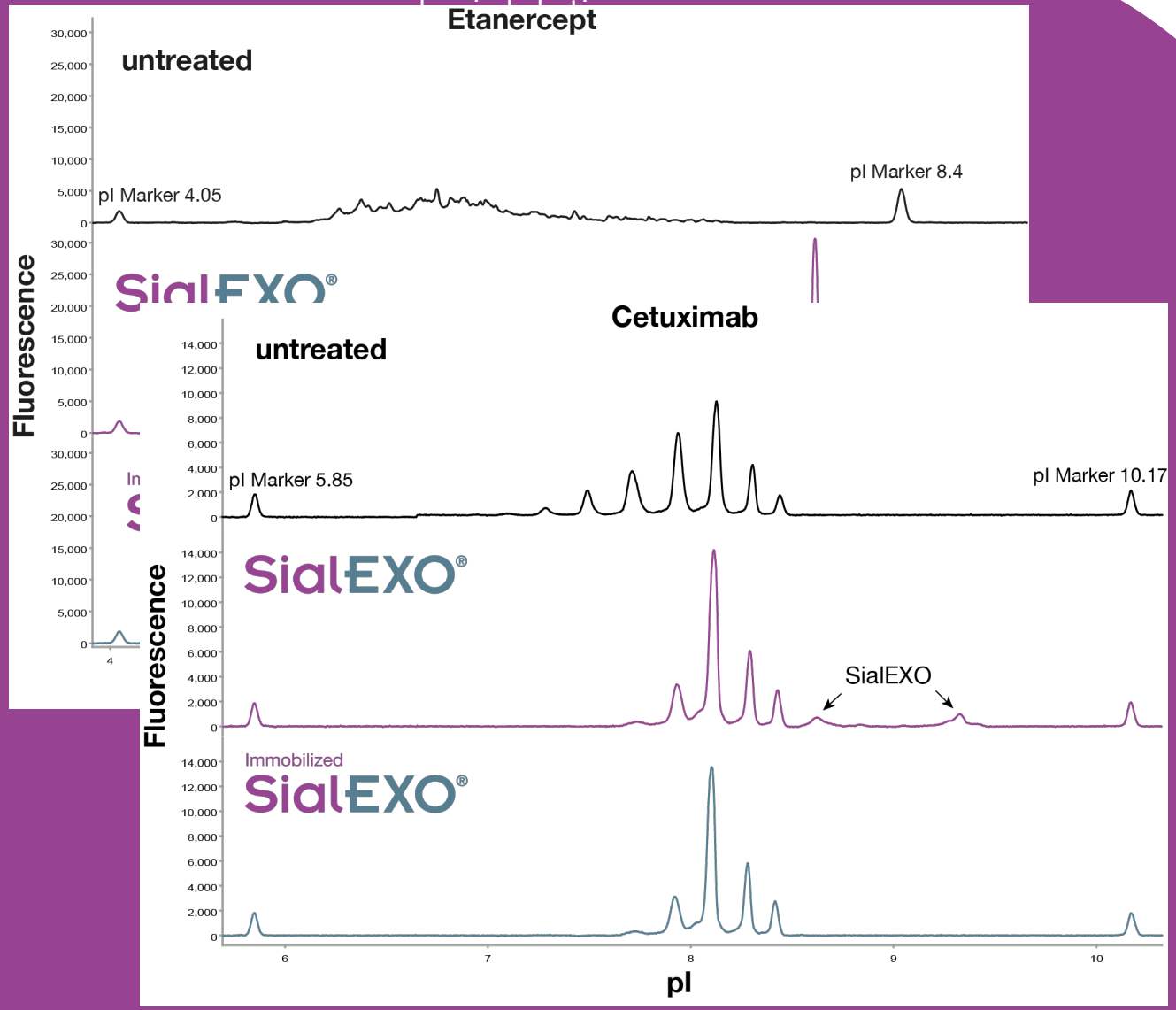
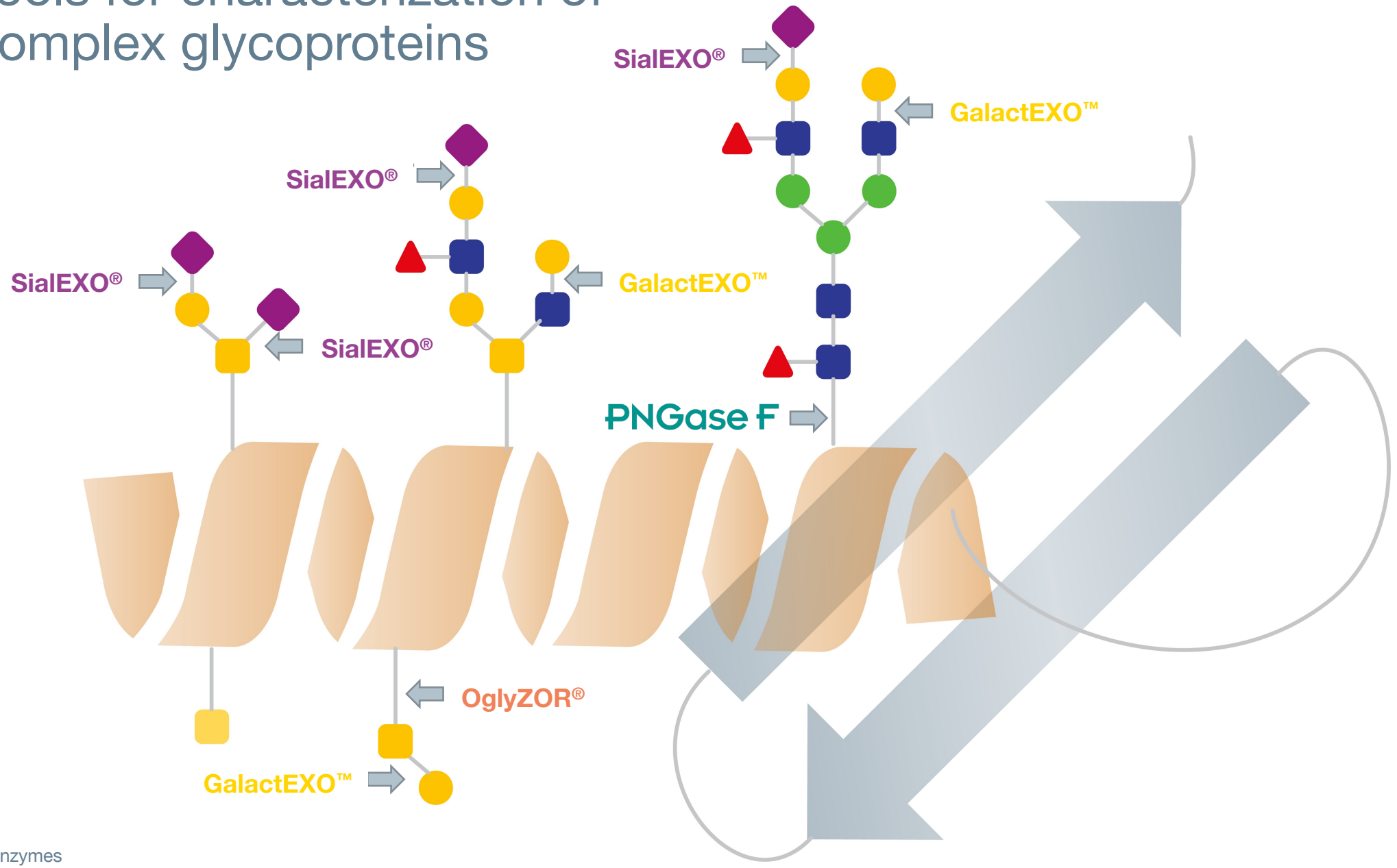
- SialEXO is a broad acting sialidase
- Immobilized SialEXO available for on column desialylation
  - No enzyme in resulting samples
- Improved separation in icIEF

Charge variant analysis after rapid



Tools for characterization of complex glycoproteins



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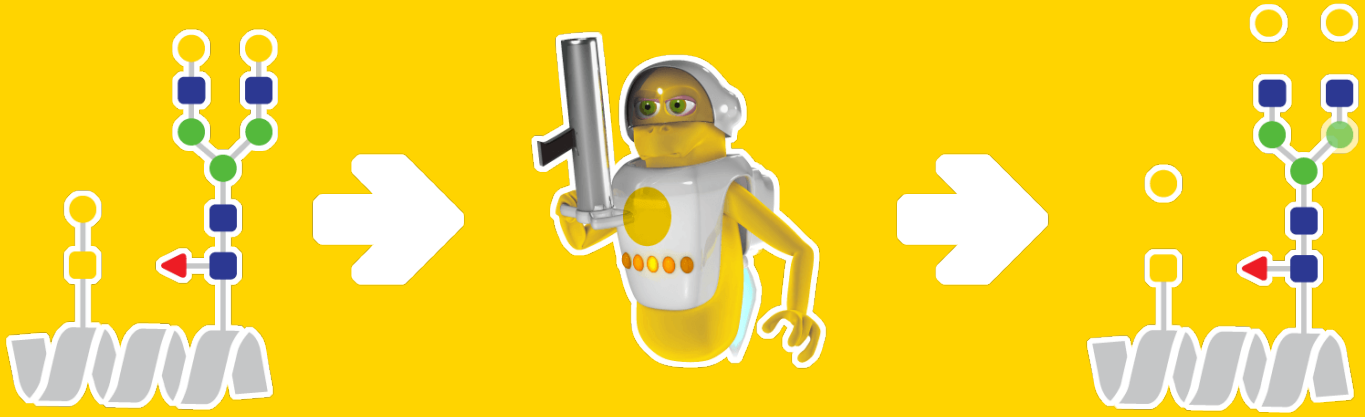
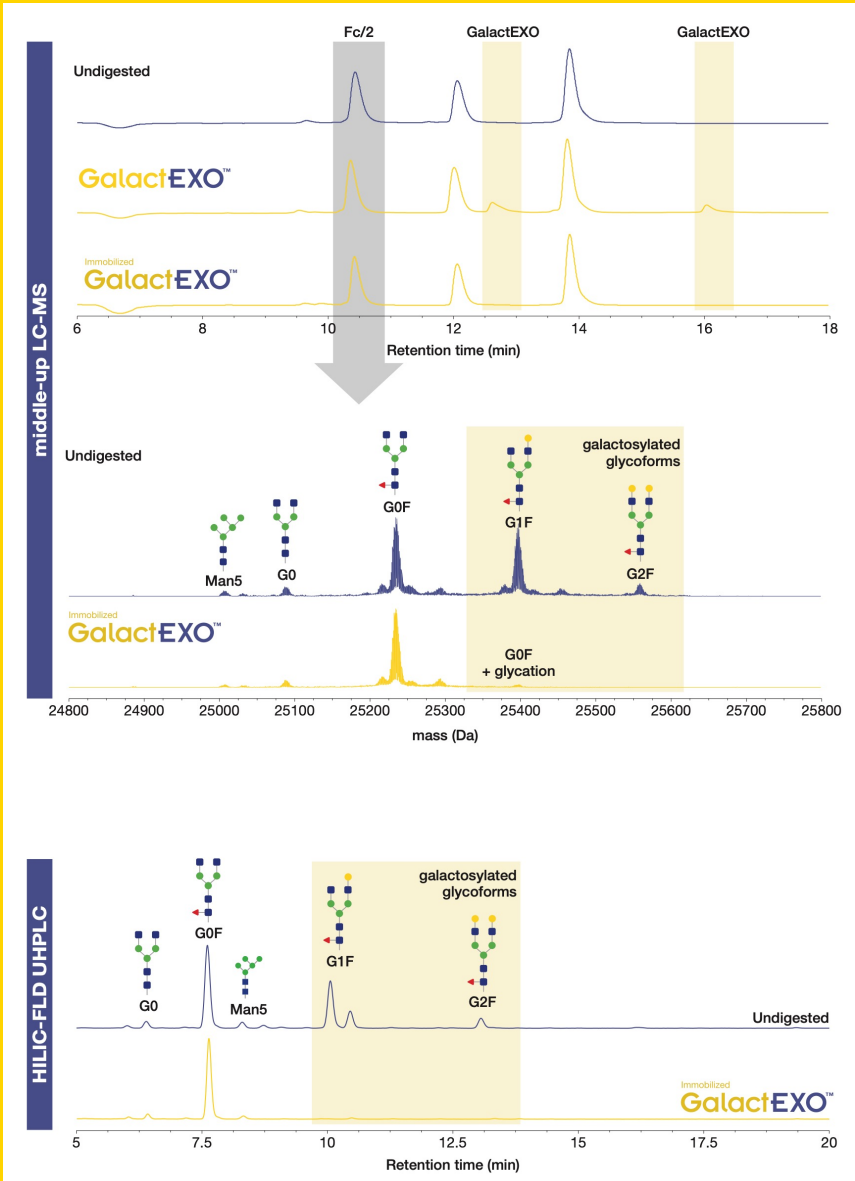
Homogenous G0F glycoforms



- LC-MS of Fc/2 shows hydrolysis of galactose
- Released N-glycan analysis confirms complete removal of galactose
- Detection of underlying modifications - glycation



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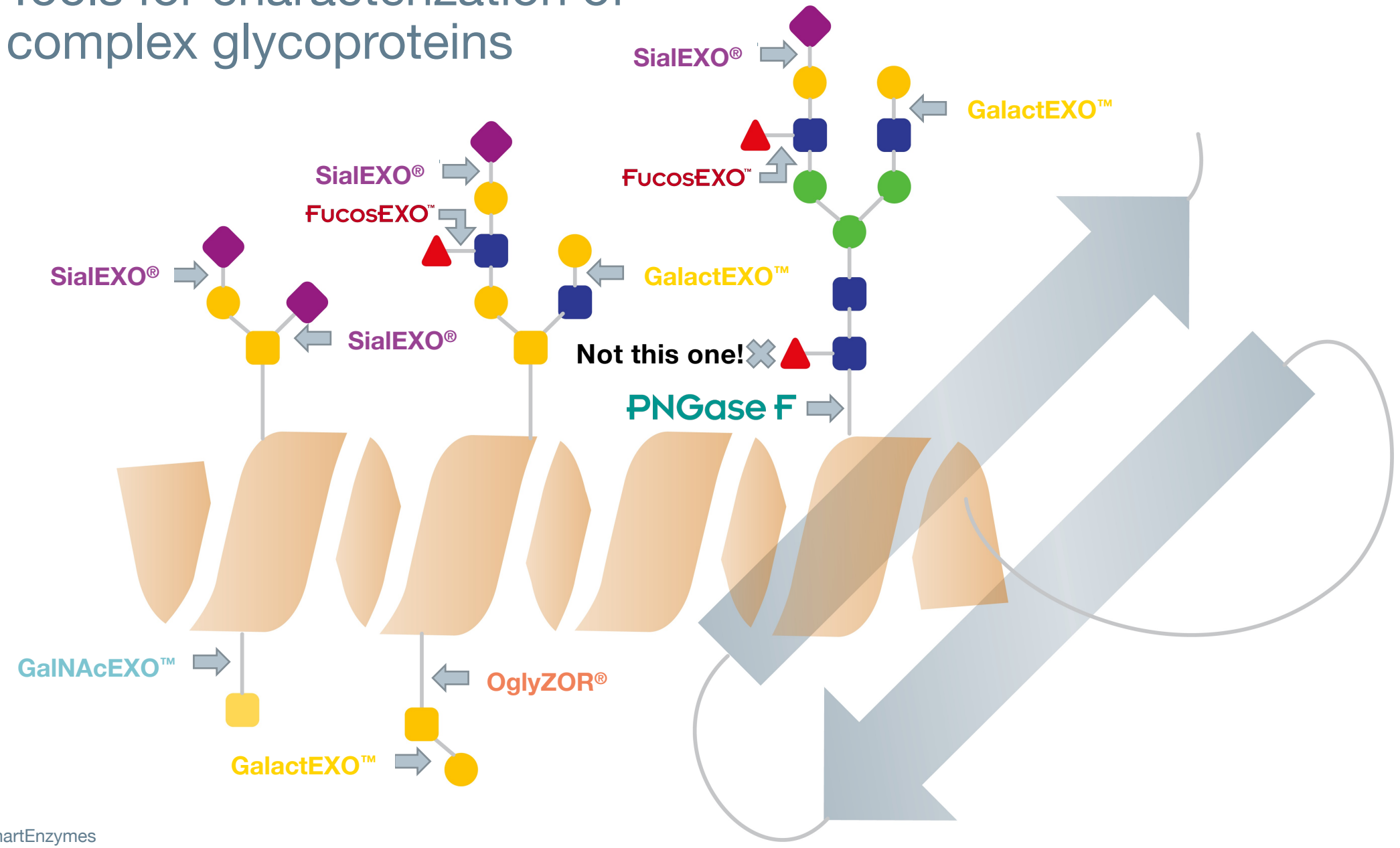
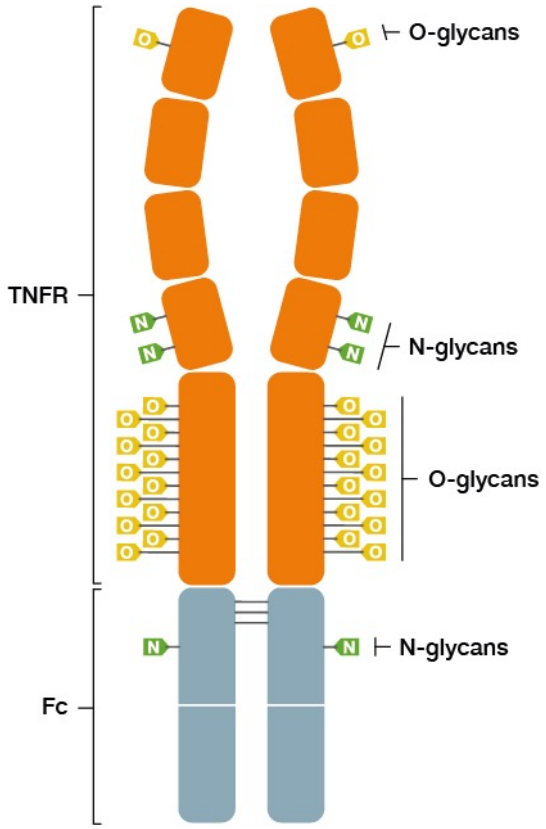
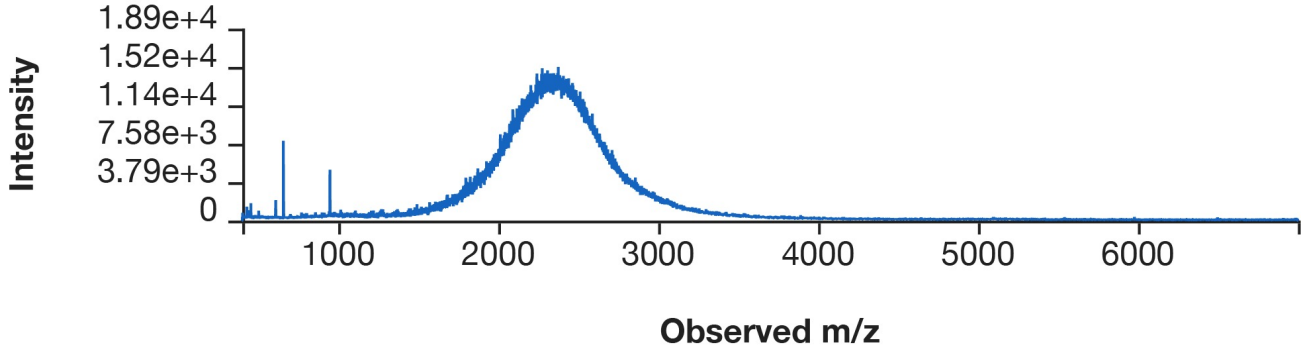


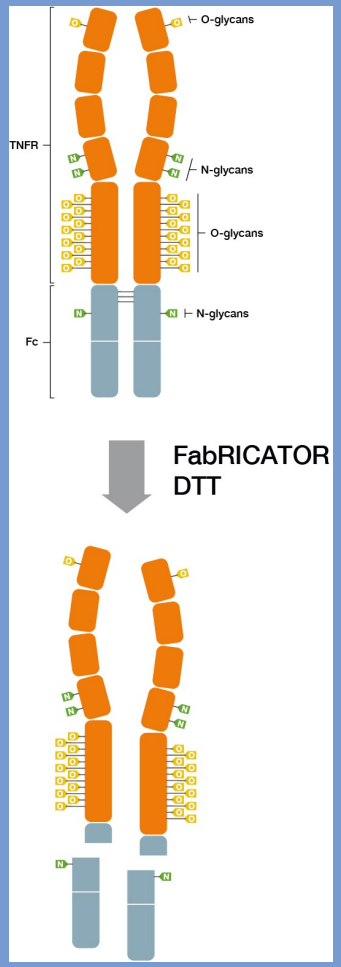
Etanercept case study

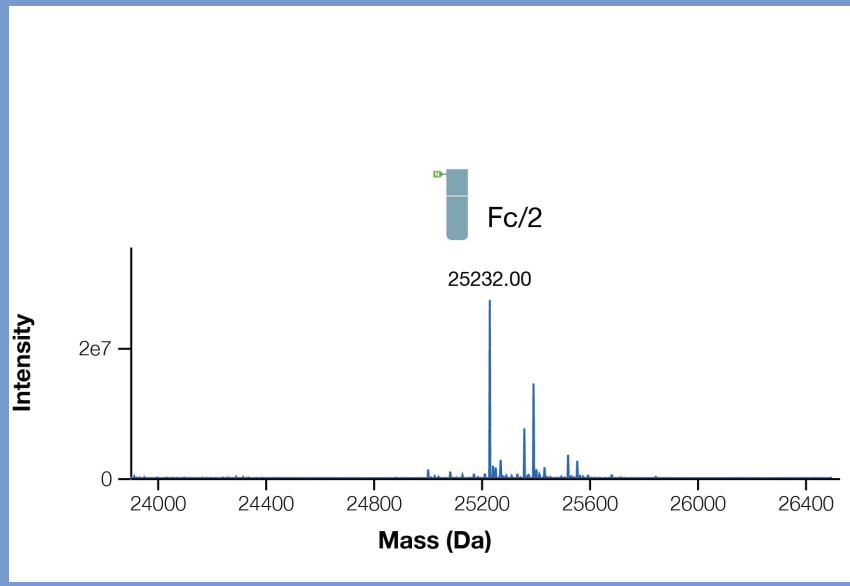


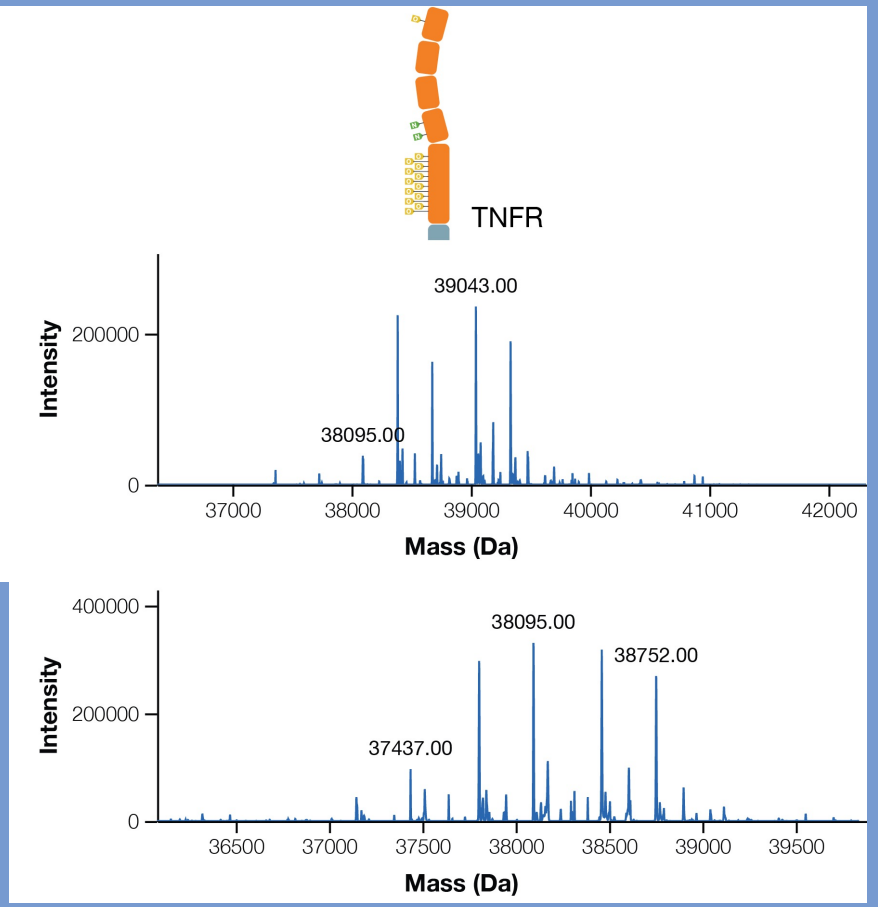
- Fusion protein of TNFR and IgG1 Fc region
- 6 *N*- and up to 24 *O*-glycans
- Heterogeneous and difficult to characterize



Subunit analysis of an Fc fusion protein

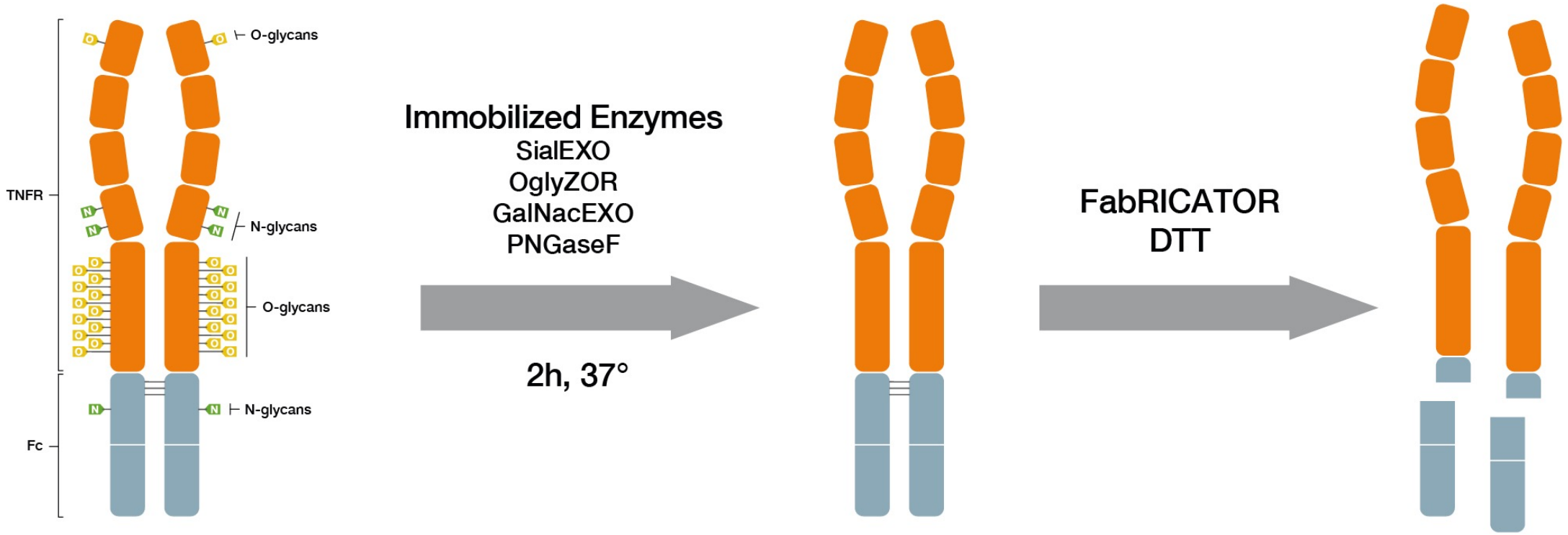






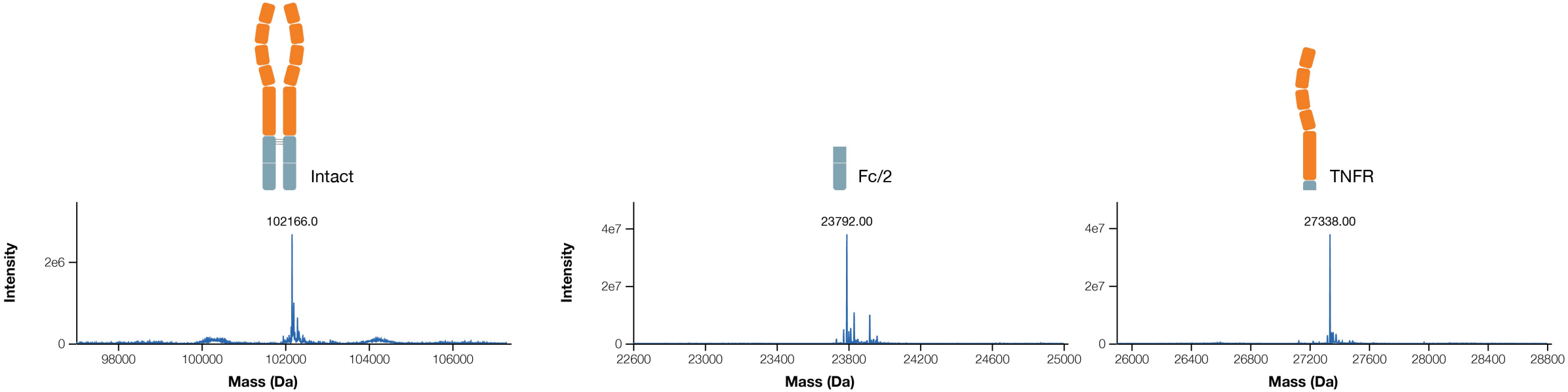
- Separate TNFR from Fc region by FabRICATOR digestion
- Manageable size and complexity

Total Deglycosylation of Etanercept



- Remove glycan heterogeneity for accurate mass determination and analysis of underlying variants
- Fast and simple one-step sample preparation using immobilized enzymes

Total Deglycosylation of Etanercept



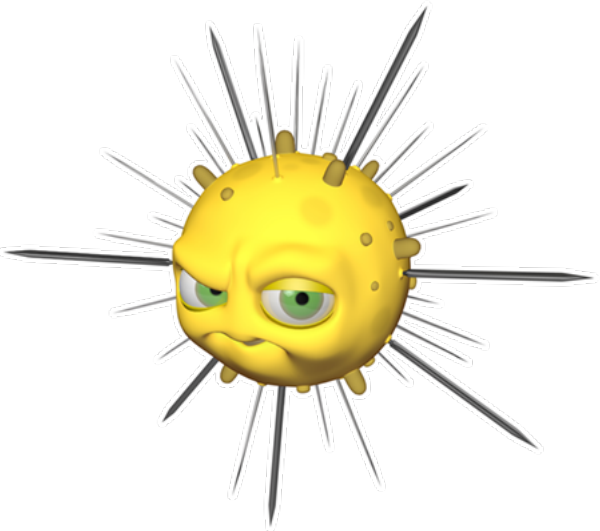
- Complete deglycosylation
- Comparison of originator and biosimilar etanercept
- Highlights the benefit of subunit analysis

Summary

- A variety of Genovis SmartEnzymes available for N- and *O*-glycan processing
  - Glycan trimming
  - Deglycosylation
  - *O*-glycan mapping

- Simplified characterization of protein modifications
  - Removal of heterogeneous glycans enhances ability to observe and analyse some PTMs
  - Allows batch-to-batch comparability
  - Originator vs biosimilar

- Versatile and application specific analysis
  - Sample preparation tools to support a range of analyses
  - SmartEnzymes can be used in combination



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- Fredrik Leo
- Emil Åberg
- Pearse Hall

- Maria Ekemohn
- Jonathan Sjögren
- Fredrik Olsson

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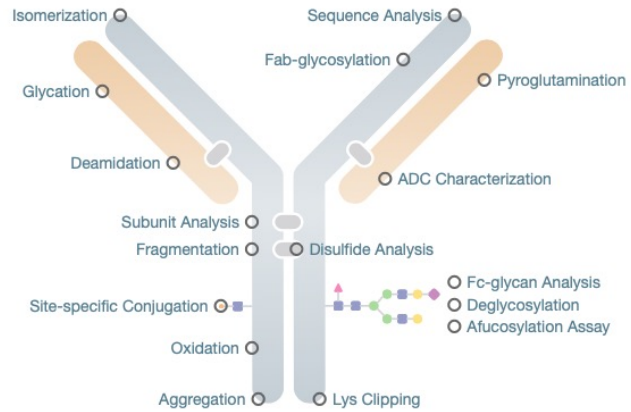
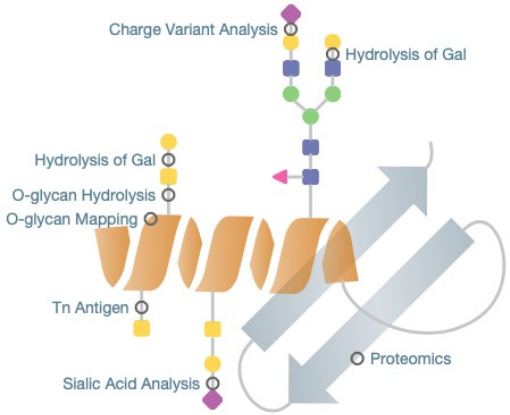
SmartEnzymes Applications



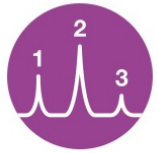


Mass Spectrometric Analysis

Subunit Analysis

Oxidation

Fc-glycan Analysis



Sialic Acid Linkage Analysis

Unmasking Charge Variants

Tn Antigen

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